GSH-DA INTERACTIONS: RELEVANCE TO CELL VULNERABLITY IN PARKINSON'S DISEASE

by

ALPA H. GAUTAM, B.A.

A Dissertation submitted to the

Graduate School of Biomedical Sciences,

University of Medicine and Dentistry of New Jersey

and

Graduate School-New Brunswick

Rutgers, The State University of New Jersey

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

Graduate Program in Neuroscience

written under the direction of

Dr. Gail D. Zeevalk

and approved by

_____

_____

_____

New Brunswick, New Jersey

October, 2010

ABSTRACT OF DISSERTAION

GSH-Dopamine interactions: Relevance

to Cell Vulnerability in Parkinson's disease.

By ALPA H. GAUTAM

Dissertation Advisor

Gail D. Zeevalk, Ph.D.

Loss of dopaminergic neurons, oxidative stress, deficient bioenergetics and decreased levels of reduced glutathione (GSH) in the substantia nigra are biochemical hallmarks of idiopathic Parkinson's disease (PD). The intent of this study was to test the hypothesis that disturbances in GSH and dopamine (DA) homeostasis are detrimental to dopaminergic neurons possibly leading to cell damage. Moreover GSH-DA interactions are essential for dopaminergic neuronal survival. Reduced levels of bioenergetics could lead to loss of DA sequestration leading to extra vesicular DA and DA metabolites which could affect cellular function. We observed uptake of reduced DA into intact mitochondria. Oxidation products of dopamine (DAQ) and its major metabolite 3, 4-dihydrophenylacetic acid (DOPAC-Q) inhibited mitochondrial electron transport chain (ETC) activities, specifically complexes I and III in both lysed and intact mitochondria. Substrate activated complex I was irreversibly inhibited while only GSH and not other antioxidants attenuated complex I inhibition, suggesting that inhibition was mediated via oxidized DA rather than ROS production. Reduced levels of GSH in PD brains are unexplained, however it is possible that oxidized products of excess extra vesicular DA

and DOPAC could form adducts with GSH thus removing GSH from the cytosolic and mitochondrial pool.

Glutaredoxin (Grx) is a GSH related enzyme that specifically uses GSH as reductant to deglutathiolate during recovery from an oxidative stress episode. The current study characterized regional cytoplasmic Grx (Grx-1) activity, protein and mRNA expression as well as regional mitochondrial Grx (Grx-2) mRNA from rat brain. The striatum had the lowest Grx-1 activity, protein and message, while Grx-2 message was higher in this region than in other brain regions. Two peaks of Grx-1 activity and protein were observed during development, one within the first post natal week and a second increase in older animals (7-18 months). Since many neurodegenerative and neuropsychiatric diseases are gender biased, Grx-1 activity was measured in female and male rats. No gender differences were observed between males and proestrous females. Grx-1 and Grx-2 mRNA was also measured in cultured mesencephalic rat brain neurons and astrocytes. Neurons expressed 4-fold greater Grx-1 and 14-fold higher Grx-2 than astrocytes. These findings show distinct age, region and cellular differences in Grx activity that may have relevance to neuropathological conditions.

Overall, these studies demonstrated that a loss in DA homeostasis could lead to loss of ETC function and that GSH serves to protect mitochondria from loss of function due to DA exposure. A deficit in GSH, low Grx-1 activity and/or altered DA biodisturbution would likely render regions intrinsically high in oxidative stress, such as the SNpc and striatum, particularly susceptible to oxidative damage thus leading to disease pathology.

ACKNOWLEDGEMENTS

I am eternally indebted and thankful to my committee chair, Dr. Gail Zeevalk. Her expert scientific knowledge, guidance and support from the initial to the final level enabled me to develop an understanding of the subject. Dr. Zeevalk's endless patience and understanding were instrumental in helping me through very difficult times in my research and in completion of this dissertation.

I am heartily grateful to my doctoral committee members for their invaluable encouragement and direction. I am especially thankful to Dr. Pat Sonsalla for helping me decipher how to pen a discussion. I am extremely appreciative to Laura Bernard for her priceless technical assistance and compassionate friendship. I give special thanks to Larry Manzino first and foremost for his remarkable sense of humor and friendship and for his much needed help with the rats. Moreover, I give thanks to Diane Pagano for being such a good listener, friend and provider of scrumptious chocolates.

The road to the completion of my dissertation has been long but rewarding. However, it would have not been possible without the support and love of my husband. His unyielding belief and faith in me has kept me on my journey. I thank you Clift for being so kind and loving and for all that you do for me. I would also like to thank my family and friends who sat through numerous painful presentation practices but still managed to offer constructive criticisms.

Finally, I dedicate this dissertation to my parents for without their loving and constant support, I would not have embarked on this road. I also dedicate my work to Dr. Martin Gluck, whose positive encouragement inspired me to pursue graduate school. Thank you Marty for all that you have taught me.

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

ABBREVIATIONS

3-NT	**3 nitortyrosine**
5-S-Cys-DA	**5'-S cysteinyldopamine**
AD	**Alzheimer's disease**
ALS	**Amyotrophic lateral sclerosis**
BSA	**Bovine serum albumin**
Cat	**Catalase**
DA	**Dopamine**
DAH	**Reduced dopamine**
DAQ	**Oxidized dopamine**
DAT	**Dopamine transporter**
DCPIP	**2,6-dichlorophenolindophenol**
DOPAC	**3, 4-dihydrophenylacetic acid**
DOPAC-H	**Reduced DOPAC**
DOPAC-Q	**Oxidized DOPAC**
ETC	**Electron transport chain**
GR	**Gluthione reductase**
Grx	**Glutaredoxin**
Grx-1	**Cytosolic glutaredoxin**
Grx-2	**Mitochondrial glutaredoxin**
GSH	**Reduced glutathione**
GSH-DA	**Glutathione-Dopamine adduct**
GSSG	**Oxidized glutathione**

H_2O_2	**Hydrogen peroxide**
HC	**Hippocampus**
HD	**Huntington's Disease**
ILBD	**Incidental Lewy Body Disease**
KCN	**Potassium cyanide**
LB	**Lewy Body**
LC	**Locus coeruleus**
MAO	**Monamine oxidase**
MTP	**Mitochondria transition pore**
MPP^+	**1-methyl-4-phenylpyridinium**
MPTP	**1-methyl 4-phenyl 1, 2, 3, 6-tetrahydropyridine**
NADPH	**Nicotinamide adenine dinucleotide phosphatate**
PD	**Parkinson's disease**
Pr-SSG	**Protein-glutathione-mixed disulfides**
RCR	**Respiratory control rates**
ROS	**Reactive oxygen species**
SNpc	**Substantia nigra *pars compacta***
SOD	**Superoxide dismutase**
Trx	**Thioredoxin**
UCHL-1	**Ubiquitin C-terminal hydrolase L1**
UPS	**Ubiquitin-proteasome system**
VMAT2	**Vesicular monoamine transporter 2**

Chapter I: INTRODUCTION

Parkinson's disease (PD) is one of the most common progressive neurodegenerative diseases in the United States. It affects approximately 0.1% of the population older than age 40, 1.0% of the population older than 65 with a mean age onset at 55 (Dauer and Przedborski, 2003, Dawson and Dawson, 2003). The cost of treatment in the Unites States between1999-2003 was estimated at $23.7 billion (Andersen, 2006). These are staggering numbers not only in terms of dollars but also in terms of human cost. PD is a very debilitating disease and decreases quality of life dramatically.

James Parkinson first described PD in 1817 as the "shaking palsy" perhaps due to the characteristic symptom of tremors at rest. In addition to tremors, there are a variety of symptoms that characterize PD and what is now termed as 'parkinsonism syndrome'. This is a disease that includes striatal and nigral dopamine deficiency and direct damage to nigrostratal dopamine neurons. Some of the symptoms are stiffness due to increased resistance to passive movement, bradykinesia: slowness of movement, hyperkinesias: reduced movement amplitude, and akinesia: absence of normal unconscious movement such as arm swing in walking (Dauer and Przedborski 2003).

PD is mainly the result of the loss of dopamine (DA) neurons in the substantia nigra *pars compacta* (SNpc) (Dawson and Dawson, 2003). The dopaminergic cell bodies in the SNpc project primarily to the putamen. These cells normally have large amounts of neuromelanin, which affords the substantia nigra its name because of the resulting

pigmentation. The loss of the SNpc cells parallels a decrease in pigmentation, a hallmark of PD (Dauer and Przedborski 2003). There is a consequent decrease in the level of DA transporter (DAT) mRNA and depletion of DA is mostly in the dorsolateral putamen: where most of the nigrostriatal axons terminate. DA neurons also synapse in the caudate; however, these cell bodies are adjacent to the SNpc in the ventral tegmental area (VTA) are not as affected in PD as the SNpc neurons. Hence the DA depletion in the caudate is much less (Price et. al., 1978). Due to the degeneration of the nigrostriatal pathway and the resulting decrease of DA in the striatum, activity in the internal pallidal segment is increased. This results in increased inhibition of thalamocortical and midbrain tegmental neurons. This causes the hypokinetic symptoms of PD (Dauer and Przedborski 2003).

The loss of DA neurons in the SNpc is only a part of the neuropathology of PD (Dawson and Dawson, 2003). Additional pathological markers of PD are the presence of neuronal proteinaceous cytoplasmic inclusions termed Lewy Bodies (LBs) and dystrophic neurites, (Lewy neurites) (Dauer and Przedborski 2003). Lewy bodies are comprised of proteins such as α-synuclein, parkin, ubiquitin and neurofilaments (Dauer and Przedborski 2003). LBs are usually present in the SNpc and locus coeruleus (LC) of PD patients. The role of Lewy bodies in disease etiology is unclear and has been postulated to either contribute or protect from cell damage (Harrower et. al., 2005).

The neurodegeneration seen in PD is not only limited to dopaminergic cells; it also extends to noradrenergic cells in the LC, serotonergic cells in the, cholinergic cells in the nucleus basalis of Meynert and dorsal motor nucleus of vagus. Neurodegeneration is also seen in the cerebral cortex, specifically in cingulate and entorhinal cortices.

Degeneration of hippocampal (HC) and cholinergic structures result in dementia experienced by PD patients (reviewed by Hornykiewicz and Kish, 1987).

Familial PD

Although the majority of PD cases are idiopathic, approximately 5-10% of the Parkinson population is genetic in origin and represented as familial PD. A growing number of gene mutations have been identified in the familial PD population. The discovery of PD genes is important as it may provide the opportunity for the generation of novel PD models that can help uncover the biochemical pathways leading to PD neurodegeneration in both the idiopathic and familial forms of the disease. Toxin based animal models of PD have yet to yield clear biochemical pathways leading to PD neurodegeneration. Pheonotypic similarities between familial and idiopathic PD indicate shared pathogenic pathways, therefore genetic knowledge would allow focused research on specific pathways.

To date several gene mutations have been found to be linked to familial forms of PD. These include alpha-synuclein (α-synuclein), parkin, ubiquitin C-terminal hydrolase L1 (UCHL-1), DJ-1, PINK1 and LRRK2. The parkin and UCHL-1 genes appear to participate in the ubiquitin-proteosome pathway a compelling discovery since Lewy body formation is one of the hallmarks of PD. PINK1 (Valente et al., 2004; Beilina et al., 2005; Silvestri et al., 2005) and mutant DJ-1(Bonifati et.al., 2003; Zhang et.al., 2005) genes localize to the mitochondria which is intriguing since mitochondrial dysfunction is another hallmark of PD.

Alpha-synuclein

In inherited PD, pathogenic mutations may cause abnormal and possibly toxic protein misfolding thus causing the disease (Dauer and Przedborski, 2003). These mutations may also be interfering with processes that normally recognize and process misfolded proteins. The discovery of missense mutations in the α-synuclein gene is thought to cause an autosomal dominantly inherited form of PD (Dawson and Dawson, 2003). A mutation resulting from a G to A transition at position 209, resulting in an alanine to threonine substitution at position 53 (Ala53Thr) in the α-synuclein protein was found in an Italian-American family and an unrelated Greek family (Polymeropolous et. al., 1997). In addition, a G to C transition at position 88 was found in a small German family (Dawson and Dawson, 2003). Over expression of α-synuclein due to a genomic triplication of wild type α-synuclein causes familial PD found in an Iowa family (Dawson and Dawson, 2003). More recently, a third mutation, E46K, was discovered in a Spanish family in which a glutamic acid was substituted with lysine in the α-synuclein protein (Zarranz et. al., 2004). The E46K mutation resulted in widespread brain pathology and early onset Parkinson symptoms. Although mutations in α-synuclein in familial PD are rare, its presence in LBs points to a more involved role in familial as well as sporadic PD (Dawson and Dawson, 2003).

Alpha-synuclein is widely expressed in the presynaptic nerve terminals in close association with synaptic vesicles (Maroteaux et al., 1988). In dopaminergic terminals it is thought to participate in modulation of synaptic function (Abeliovich et al., 2000). Wild type α-synuclein is a soluble and unfolded protein. Due to a central hydrophobic

region in the protein, it has a tendency to aggregate and initially form an intermediate structure called a protofibril that is an insoluble polymer or fibril (Giasson et. al., 2002). LBs contain an abundance of protofibrils. Misfolding and/or aggregation of α-synuclein may cause toxicity by permeablizing synaptic vesicles (Volles et. al., 2001) allowing DA leakage into the cytoplasm with subsequent ROS and/or DA quinones adding to the pathogensis of PD (reviewed below). Overexpression of mutated α-synuclein in human mesencephalic cell lines leads to an impaired storage and secretion of DA, causing an increase in cytosolic DA and enhanced oxidative stress (Lotharius and Brundin, 2002a; Lotharius and Brundin, 2002b). Moreover, the selective degeneration of dopaminergic neurons may be due to the ability of dopamine to stabilize the alpha-synuclein protofibrils by forming dopamine–alpha-synuclein adducts (Conway et. al., 2001).

Parkin

Homozyote inheritance of parkin mutation is thought to lead to dopaminergic neuronal loss in the SNpc, without the presence of LBs (Mizuno et. al., 2001). Conversely, LBs are present in patients with compound heterozygous parkin mutations (Mata et. al., 2004). This suggests that parkin plays an important role in LB formation. On the other hand, clinical parkinsonism and nigral dopaminergic cell loss can occur in the absence of LB pathology. It is unclear how loss of parkin leads to dopaminergic cell loss, however clues from its function are emerging. Parkin is an E3 ubiquitin ligase (Zhang et. al., 2000; Shimura et al., 2000) which is a component of the ubiquitin proteosome system that specifically targets misfolded proteins and tags them for degradation with polyubiquitination (reviewed by Sherman and Goldberg, 2001). Many parkin substrates

have been identified including synphilin-1, O-glycosylated alpha-synuclein, Pael-R, CHIP, cdc-Rel1A, cyclin E, synaptotagmin X1 (reviewed by Cookson, 2005). Loss of parkin activity could lead to accumulation of misfolded parkin substrates that may be responsible for SNpc dopaminergic neurodegeneration. Overexpression of parkin substrate Pael-R produced dopaminergic cell death *in vitro* that was rescued by parkin overexpression (Yang et. al., 2003). In spite of *in vitro* findings, *in vivo* animal models have been difficult to produce where mice with parkin mutation do not show nigral neuronal loss (Goldberg et. al., 2003).

UCHL-1

Ubiquitin C-terminal hydrolas L1 (UCHL-1) helps the recycling of polyubiquitin chains back to monomeric ubiquitin. UCHL-1 gene has been implicated in causing autosomal dominant PD (Leroy et.al., 1998). A single UCHL-1 mutation has only been reported in two siblings with PD and has not been reported with other families . Therefore, genetic evidence for the role of UCHL-1 in PD is not as strong as for α-synuclein and parkin . The mutation leads to decreased hydrolase activity, which may disrupt the ubiquitin-proteasome system (UPS). Unwanted proteins may not get degraded and accumulate to toxic levels that damage or kill nerve cells in the brain. A mouse model demonstrates axonal dystrophy, sensory and motor ataxia with accumulation of beta amyloid and ubiquitin deposits, but without nigrostriatal neuronal loss (Saigoh et. al., 1999).

There is growing evidence for the importance of UPS in the pathogenesis of idiopathic PD. Post mortem brain tissue from idiopathic PD patients demonstrate functional deficits

in the 20s proteosomal activity (Chung et. al., 2001). Rodents exposed to synthetic and natural UPS inhibitors for 2 weeks developed selective nigral cell loss and LB like formations along with clinical features of PD such as bradykinesia, rigidity and tremors (McNaught et.al, 2004). However, it should be noted that independent confirmation of this has not been demonstrated as yet. Despite this, protein misfolding is likely to play an important role in the etiology of PD.

DJ-1

DJ-1 mutations were identified in two pedigrees with autosomal recessive PD (Bonifati et.al., 2003). One family carried a deletion thought to abolish protein function, while the other had a missense mutation (LI66P) that resulted in the insertion of proline into an alpha-helical region. Expression of the LI66P mutant appears to lead to its accumulation in the mitochondria (Bonifati et.al., 2003; Zhang et.al., 2005), which may interfere with mitochondrial function. DJ-1 is expressed in the brain (Nagakubo et.al., 1997) with greater DJ-1 mRNA expression in subcortical regions (Bonifati et.al., 2003) suggesting an important role in basal ganglia function. DJ-1 protein has many functions including ras-mediated signaling pathway (Nagakubo et.al., 1997); transcription regulation (Takahashi et.al., 2001), apoptosis (Xu et.al., 2005) and alpha-synuclein aggregation (Shendelman et.al., 2004). DJ-1 is also reported to be a cellular monitor of oxidative stress (Mitsumoto and Nakagawa, 2001) and is thought to be neuroprotective from oxidative stress (Abou-Sleiman et.al., 2003) which may be its most important role with regards to PD. In both typical PD and in cases with PD with DJ-1 polymorphisim, mutant DJ-1 localized to only a few nigral LBs, indicating that DJ-1 protein may not be an

essential component of LB formation in PD (Bandopahyay et. al., 2004).

PINK1

PINK1 is a PTEN-inducible kinase, suggesting that it may play a role in cell cycle regulation (Unoki and Nakamura, 2001). Exposure to taxol rendered cells that had a transient knockdown of PINK1 susceptible to apoptosis (MacKeigan et.al., 2005). Therefore, the neuroprotective function of PINK1 may actually lie in the direct regulation of a programmed cell death pathway. Mutations in PINK1 gene cause autosomal recessive PD and were described in three pedigrees (Valente et.al.,2004). Patients with PINK1 mutations do not have typical autosomal recessive juvenile parkinsonism (AR-JP) phenotype but respond well to levadopa treatment (Healey et. al., 2004). PINK1 is located in the cytoplasm (Weihofen et.al., 2007) as well as in the mitochondria in human brain tissue (Valente et al., 2004; Beilina et al., 2005; Silvestri et al., 2005). PINK1 protected mitochondria and cells against stress (Valente et.al., 2004), which makes it of particular interest for research into mitochondrial dysfunction observed in PD.

LRRK2

Mutations in the *leucine-rich repeat kinase 2* (*LRRK2*) gene have been estimated to be involved in up to 13% of autosomal dominant familial PD and in up to 3% of sporadic PD cases (Berg et. al., 2005; Taylor et. al, 2006). LRRK2 gene is expressed in human and rodent brain (Paisán-Ruiz et al. 2004) and has a kinase domain (Korr et.al., 2006). LRRK2 mutation pathologies include nigral cell loss, the presence of Lewy bodies and other proteinaceous inclusions (Shen, 2004). In cell cultures, LRRK2 protein has been

found to interact with Parkin (Smith et al., 2005) which is known to cause early onset autosomal recessive parkinsonism (Kitada et. al., 1998). Recently, Li et. al., (2009) created LRRK2 transgenic mice that display motor behavioral, histopathological and neuropathological features of PD. Downregulation of LRRK2 in expanded or differentiated human mesencephalic neural progenitor cells resulted in increased cell cycle- and cell death-related markers and LRRK2 downregulation in human mesencephalic neural progenitor cells, following differentiation, yielded reduced number of DA neurons (Milosevic et. al..,2009). Studies by Milosevic et. al., (2009) showed that LRRK2 protein may have a role in differentiation and/or survival of dopaminergic neurons.

Idiopathic PD

The etiology of idiopathic PD is presently unknown; however, the pathogenesis of idiopathic PD is believed to be multi-factorial, involving an interaction between genetic and environmental factors (Gorell et. al., 1997, Menegon et. al., 1998; Olanow and Tatton 1999; Dauer and Przedborski, 2003). Several herbicides (paraquat, heptachol dieldrin), pesticides (rotenone), synthetic drug products (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and metals (manganese, iron, copper) may cause misfolding or aggregation of α-synuclein that form Lewy bodies characteristic of PD (Dauer and Przedborski, 2003). Studies also show that rotenone and MPTP inhibit mitochondrial complex I (Dawson and Dawson 2003), leading to cell damage and eventual death particularly in the SNpc specific to PD.

Studies of risk factors for PD development associated with the consumption of

well water, rural environment and occupational exposure to these chemicals have proven to be inconclusive (Koller et. al., 1990). Consequently, endogenous events such as oxidative stress (Cohen et. al., 1999), mitochondrial dysfunction (Fahn and Cohen, 1992; Wallace et. al., 1997), improper dopamine (DA) sequestration (Forno, 1996) and loss of glutathione (GSH) specifically in the SNpc of PD post mortem brains (Perry et al., 1982) have been implicated in the pathogenesis of idiopathic PD.

Oxidative stress and PD

Oxidative stress is caused by reactive oxygen species (ROS) which include free radical molecules. Free radicals are products of normal cell processes, such as respiration. ROS consist of an oxygen atom with an unpaired electron making it a very strong oxidizer (Beal, 2003). Thus, it will take electrons from anywhere it can, such as proteins, lipids or DNA causing major disruptions in the cell. There are many different markers for oxidative stress, such as, carbonyls in proteins, fatty acid oxidation products and oxidized DNA bases (Ischiropoulos and Beckman, 2003).

Stress due to reactive species does not necessarily have to involve ROS; reactive nitrogen species (RNS) which are also strong oxidizers due to an unpaired electron on the nitrogen atom (Ischiropoulos and Beckman, 2003) can also be harmful. RNSs can nitrosylate tyrosine residues on proteins thus creating a marker for nitrosative stress, 3-nitrotyrosine (3-NT). In fact 3-NT is one of the earliest markers found in Alzheimer's disease (AD), and in degenerating upper and lower motor neurons in amyotrophic lateral sclerosis (ALS) patients (Ischiropoulos and Beckman, 2003). In the midbrains of PD

patients there is evidence of accumulation of oxidized products from lipid (Yoritaka et al, 1996), protein (Alam et al., 1997) and nucleic acids (Kikuchi et al., 1999), enhanced superoxide dismutase activity (SOD) (Saggu et al., 1989) and decreased levels of the antioxidant reduced glutathione (GSH) (Perry et al, 1982) without a concomitant increase in oxidized glutathione (GSSG) (Sofic et al, 1992). Levels of GSH synthesizing enzymes are also not affected. In fact there is increased activity of γ glutamyl transpeptidase (γ-GT), an ecto enzyme associated with the hydrolysis of extracellular GSH to gamma-glutamate and cysteinyl glycine, (Sian et al, 1994b). PD brain samples have been reported to have increased levels of nigral iron (Dexter et al., 1989). Iron and other transition metal may produce free radicals (Olanow and Taton., 1999) leading to oxidative stress damage.

The first indication of oxidative stress involvement in PD comes from studies of postmortem basal ganglia tissue of PD patients revealing pathological and biochemical alterations related to oxidative stress (Jenner et. al., 1992). However, this is not proof *per se* for a causal role for oxidative stress. It merely implicates oxidative stress involvement. It is difficult to find a direct connection between oxidative stress and PD from post mortem tissue because the markers for oxidative stress, i.e. ROS have a very short half life making their measurement difficult and possibly inaccurate (Koutsilieri et.al., 2002).

Hence, researchers have turned to in vitro and in vivo models. These models take advantage of neurotoxins that induce specific dopaminergic neurodegeneration (Dauer and Przedborski, 2003). Toxins such as 6-hydroxydopamine (6-ODHA), paraquat,

rotenone and MPTP (Dauer and Przedborski, 2003) are most frequently used to model PD. These toxins produce ROS. Moreover, rotenone and MPTP inhibit complex I, which may also produce ROS and subsequent oxidative stress related damage.

6-ODHA:

6-Hydroxydopamine (6-ODHA) is a hydroxylated analogue of dopamine and is one of most common neurotoxins used to create *in vivo* and *in vitro* degeneration models of central catecholaminergic projections, including the nigrostriatal system, (Blum et.al., 2001). 6-ODHA induced toxicity is quite selective for monoamingergic neurons, resulting from preferential uptake by DA and noradrenergic transporters (Luthman et.al., 1989). 6-ODHA accumulates in the cytosol and inactivates biological macromolecules by generating quinones that attack nucleophilic groups (reviewed by Cohen and Werner, 1994). Sachs and Jonsson (1975) have reported that 6-ODHA mediated neuron degeneration in the presence of iron involves hydroxyl radicals, suggesting oxidative stress involvement. It has been shown that 6-ODHA treatment reduces striatal levels of the antioxidant reduced glutathione (GSH) and antioxidant enzyme superoxide dismutase (SOD) activity (Perumal et al. 1992). Moreover, 6-ODHA can damage mitochondrial complex I (Betarbet et al. 2002) and lead to the formation of superoxide radicals (Hasegawa et al. 1990). As in PD, the ventral midbrain and SNpc dopaminergic neurons show a sensitivity to 6-ODHA, while tuberoinfundibula neurons are almost completely resistant ((Dauer and Przedborski 2003). 6-ODHA induced pathology, however, differs from PD, as there is the lack of LB formation. Moreover, 6-ODHA does not affect other brain areas that are also involved in PD, such as in anterior olfactory structures, lower

brain stem areas or the locus coeruleus (Betarbet et. al., 2002).However, 6-ODHA is still used to model PD because at least in mice, rats, cats and primates, 6-OHDA is a highly effective toxin for dopaminergic neurons (Beal, 2001). Additionally, 6-ODHA unilaterally injected animals show asymmetric circling behavior, the magnitude of which depends on the degree of the nigrostriatal lesion. Direction of circling is opposite to that of the lesion (Dauer and Przedborski 2003).The unilateral lesion can be quantitatively assayed, making this model a useful tool because of the possibility to assess anti-PD properties of new drugs and therapies.

Taken together it can be said that 6-ODHA inhibits mitochondrial respiration and produces oxidative stress which may lead to neuronal damage and/or death. It should be noted that it is unclear whether the mechanism by which 6-ODHA kills dopamenergic neurons is the same as in PD (Dauer and Przedborski 2003).

Paraquat:

Exposure to the herbicide paraquat may increase the risk of PD (Liou et. al., 1997). Although paraquat does not penetrate the blood brain barrier easily, Shimizu et al., (2001) demonstrated that paraquat can be taken up into the brain by neutral amino acid transporters in the blood-brain barrier and subsequently transported into cells in a sodium-dependent manner. Since paraquat is structurally similar to the active MPTP metabolite, 1-methyl-4-phenylpyridinium (MPP+), it has been hypothesized that the mechanism of paraquat-mediated neurotoxicity is also similar (Shimizu et al., 2001). However, recently, Richardson et. al., (2005) have shown that paraquat is a weak

inhibitor of complex I and is not a substrate for the DA transporter (DAT), indicating that the mechanism of paraqaut mediated dopaminergic cell death is different from MPP+. Oxidative stress followed by caspace-3 mediated cell death is attributed to paraquat mediated toxicity (Ramachandiran et. al., 2007). Castello et. al., (2007) have demonstrated paraquat uptake into rat brain mitiochondria under non- respiring and respiring conditions. The study found that complex III of rat brain mitochondria was involved in paraquat induced reactive oxygen species (ROS) generation, which could add to an oxidative stressed environment leading to cell damage and/or death. Paraquat is used to produce toxin based animal PD models because systemic exposure of rodents to paraquat has been shown to reproduce many of the pathological features of PD such as specific loss of SNpc dopaminergic neurons and upregulation and formation of α-synuclein aggregation in mice and in α-synuclein expressing cells (Manning-Bog et al., 2002; McCormack et al., 2002**;** Ischiropoulos and Beckman, 2003).

Rotenone:

Rotenone is used as an insecticide and fish poison. Exposure to rotenone, as with paraquat, could increase risk of PD. Although rotenone treated rats demonstrated systemic uniform complex I inhibition throughout the brain they had many characteristics of PD including, selective nigrostriatal dopaminergic degeneration, α-synuclein positive inclusions, and motor deficits (Betarbet et. al., 2000). Rotenone mediated inhibition of complex I is thought to cause oxidative damage leading to degeneration of dopaminergic neurons (Sherer et. al., 2003b). Rotenone is highly lipophilic, hence can readily enter all organs and cells (Dauer and Przedborski, 2003; Zeevalk and Bernard, 2005), therefore

the relatively selective degeneration of nigrostriatal neurons suggests that dopaminergic neurons may be sensitive to complex I inhibition.

In spite of these findings, rotenone may have limited uses as a PD model because of diminished toxicity when administered orally (Betarbet et.al., 2000) and inconsistencies in findings from different laboratories. For example, in contrast to Betarbet et. al., (2000), Ferrante et. al., (1997) have shown sparing of dopaminergic neurons after acute exposure to rotenone. Moreover, in some laboratories, rats chronically exposed to rotenone showed significant loss of neuronal groups other than striatal neurons (Hoglinger et. al., 2003), which challenges the selectivity of dopamine neurons to rotenone toxicity.

MPTP:

Drug users in 1982, in Santa Clara County, CA were diagnosed with Parkinsonism after having used 1-methyl-4-phenyl-4-propionoxypiperidine (MPPP), a street analog of the drug Demerol (Langston et. al., 1983). It was later discovered that the MPPP was contaminated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), the responsible toxicant. MPTP produces irreversible parkinsonism syndrome within days of drug use. It causes all the characteristics of PD including tremor, rigidity, slowness of movement, and freezing. Both humans and primates exposed to MPTP respond positively to levodopa treatments as do PD patients. The pathophysiological similarities between PD and MPTP cases in monkeys include preferential degeneration of putamen vs. caudate dopaminergic nerve terminals (Moratalla et al. 1992). Dopaminergic pathways

were damaged in a similar pattern as in PD, with a greater loss in SNpc than the VTA (Sirinathsinghji et.al., 1992) and neuromelanin containing DA neurons were more susceptible to MPTP induced degeneration (Herrero et.al., 1993). The regional pattern of dopaminergic neuronal degeneration was also similar in MPTP treated mice (Seniuk et. al., 1990; Muthane et. al., 1994). In spite of these similiarities, MPTP models lack some PD characteristics such as the absence of monaminergic neuronal loss in other brain regions typical in PD such as locus coeruleus (Forno, 1996). Acute exposure to MPTP of humans and monkeys failed to form LBs, however recently a mouse model was developed in which chronic administration of MPTP and probenecid (MPTP/p), an adjuvant that blocks the rapid clearance of the toxin and its metabolites, produced dopamine loss, motor dysfunction and formation of lipofuscins. In PD, lipofuscins are associated with LB formation (Meredith et. al., 2008). Differences notwithstanding, knowledge of the toxic pathways of MPTP has allowed investigations of the molecular pathways involved in dopaminergic neuronal death.

Mechanism of MPTP toxicity

MPTP can be administered systemically because it is highly lipophillic and can cross the blood brain barrier (BBB) easily (Markey et. al., 1984). MPTP is oxidized to 1-methyl-4-phenyl-2,3-dihydropyridinium (MPDP+) by monoamine oxidase-B (MAO-B) in astrocytes and serotonergic neurons. Afterwhich it is converted to 1-methyl-4-phenylpyridinium (MPP+), the active toxic metabolite, and is released by an unknown mechanism into the extracellular space. MPP+ is then actively taken up by dopamine transporters (DAT), serotonin and norepinepherine transporters (Javitch et. al., 1985;

Mayer et. al., 1986). MPP+ toxicity was established with the following observations: 1-pretreatment with MAO-B inhibitors such as deprenyl prevented MPTP metabolism and attenuated toxicity (Heikkila et. al., 1984), 2-pretreatment with DAT inhibitors such as mazindol or genetic deletion of DAT prevented MPP+ entry into DA neurons and blocked dopaminergic toxicity in mice (Javitch et. al., 1985) and 3- dopaminergic neuronal degeneration correlated with brain MPP+ concentration (Giovanni et.al., 1991). The specificity of MPP+ toxicity to SNpc dopaminergic neurons is not entirely explained by uptake by DAT, since MPP+ also accumulates in other monoaminergic neurons (Speciale et. al., 1998) but degeneration occurs only in dopaminergic neurons. For example the rate of MPP+ uptake was higher in striatal synaptosomes than other brain regions studied (Chiba et. al., 1985). Therefore it seems that dopaminergic neurons are particularly vulnerable to MPP+ mediated toxicity.

Once inside the neurons, MPP+ is further electrophoretically accumulated into synaptic mitochondria (Nicklas et. al., 1985). This accumulation is energy dependent and is driven by the electrical potential of the mitochondrial membrane (Ramsay and Singer, 1986). MPP+ is concentrated within the mitochondria and penetrates into the hydrophobic rotenone binding site of NADH-ubiquinone oxidoreductase enzyme (complex I) of the mitochondrial electron transport chain (ETC) (Ramsay et. al., 1987). MPP+ binds at or near the highest potential Fe-S cluster of NADH dehydrogenase and coenzyme Q, the same site as for rotenone, barbiturates and piericidin A (Ramsay et. al., 1987; Ramsay et. al., 1991). MPP+ binding to complex I sites inhibit the electron flow from NADH dehydrogenase to coenzyme Q with consequent energy impairment (Nicklas et. al., 1985;

Ramsay et. al., 1987) possibly leading to neurodegeneration. Moreover, inhibition of complex I increases ROS superoxide production, which may form toxic superoxide radicals or react with nitric oxide to form peroxynitrite.

Mitochondrial dysfunction and dopamine neurons:

Evidence for mitochondrial impairment in PD comes from studies of post-mortem PD brains. PD brain mitochondria showed a 25-30% deficient in activity of complex I of the mitochondrial ETC specifically in the SNpc (Schapira et. al., 1991). However, decreased levels of complex I activity have also been reported in other tissue types of PD patients such as muscle and platelets (Mizuno et. al., 1989; Parker et. al., 1989; Haas et. al., 1995). Additionally, decreased activities of combined complex II and III in platelet mitochondria of PD patients compared to controls have also been reported (Haas et. al., 1995). Nevertheless, regardless of the mitochondrial deficit, the majority of the degeneration occurs in nigrostriatal dopamine neurons. The selective vulnerability of dopamine neurons to energy impairment has been postulated to result in part from loss of dopamine homeostasis. Unsequestered cytosolic dopamine, therefore, has been suspected to be an endogenous toxin and vulnerability factor in PD (Hastings et. al., 1996).

Dopamine toxicity

Under normal physiological conditions DA is actively taken up by vesicular monoamine transporter-2 (VMAT2) and kept sequestered in high millimolar concentrations in synaptic vesicles (Staal et.al., 2004). However, energy impairments could release vesicular DA into the extracellular space, where it can undergo oxidation to form hydrogen peroxide, superoxide, or quinones (Graham, 1978). Oxidative processes that result in ROS formation can also promote catechol quinone formation. Similar pathways exist for DOPA, DOPAC, norepinephrine and epinephrine (Graham, 1978). The DA quinone (DAQ) can cyclize to form aminochrome. The aminochromes then polymerize to form neuromelanin (Graham, 1978). Neuropathological studies have revealed that the loss of dopaminergic neurons in PD is not distributed equally over the substantia nigra, but occurs primarily in those cells with a high concentration of neuromelanin granules (Hirsh et. al., 1991).

DAQs are reactive electrophiles that can form covalent bonds with cellular nucleophiles such as DNA or reduced cysteinyl residues on proteins via 5'cysteinyldopamine forming 5-S-Cys-DA protein adducts (quinoproteins) (Graham, 1978; Hastings and Zigmond 1994). If the cystienly group is in an active site of the protein, then the modification could disrupt normal protein function and harm normal cellular activities leading to the degenerative process in PD (Stokes et al, 1999). Caudle et. al., (2007) reported that disruption of DA storage in mice by reduction of VMAT2 expression led to an age dependent degeneration of nigrostriatal dopaminergic neurons, progressive decrease of tyrosine hydroxylase positive neurons, accumulation of α-synuclein and degeneration in

substantia nigra, thus demonstrating that loss of DA homeostasis can be toxic to cell survival and produce features of PD pathology. More recently, Chen et. al., (2008) demonstrated that transgenic mice in which striatal non dopaminergic neurons were engineered to uptake extracellular DA into their cytosol that was released from dopaminergic terminals developed motor dysfunction and striatal neurodegeneration. These two studies illustrated that unsequestered cytosolic DA *in vivo* can lead to dopamine cell degeneration.

DA oxidation and mitochondria

The mechanism of DA toxicity is not yet known, however, particular interest has been focused on modification of cysteinyl residues by oxidized dopamine, i.e. dopamine quinone (DAQ). DA mediated damage, following intrastriatal injections of exogenous DA in rat brains, was attenuated with the addition of the antioxidant ascorbate or quinone scavenger reduced glutathione (GSH) and directly correlated to the amount of quinone protein modification, suggesting DAQ formation (Hastings et. al., 1996). Another focus of study has been the effects of DA oxidation on mitochondrial function since mitochondrial proteins are extremely sensitive to inhibition by sulfhydryl modifying agents (Gutman et al., 1970). Our laboratory and others (Gluck et. al., 2002; Gluck and Zeevalk, 2004; Berman and Hastings, 1999) have shown that in intact, well coupled rat brain mitochondria, exposure to dopamine resulted in impaired oxidative phosphorylation, suggesting that the mitochondria can be a downstream target of dopamine oxidation products. Gluck and Zeevalk (2004) reported that inhibition of mitochondrial respiration was increasingly MAO independent with increasing DA

concentrations whereas at lower concentrations of DA the inhibition was MAO dependent. These results suggest that at high concentrations of DA, as might occur with loss of DA homeostasis, mitochondrial respiration inhibition may be catechol mediated while at lower concentrations inhibition of mitochondrial respiration may be due to factors produced from MAO-mediated DA metabolism, such as DOPAC or H_2O_2 suggesting a potential inhibitory role for DA metabolites. To address this, Gluck and Zeevalk (2004) assayed for inhibitory potency of closely related phenyethylamine congeners of DA and found that only DOPAC was capable of inhibiting respiration at comparable concentrations with DA.

In addition to inhibition of mitochondrial respiration, exposure of intact, isolated mitochondria to DAQ resulted in mitochondrial swelling that was completely prevented in the presence of cyclosporine A, signifying the opening of permeability transition pores. The addition of GSH was also able to block DAQ induced mitochondrial swelling (Berman and Hastings 1999). Since GSH can act as a quinone scavenger; this would have removed DAQ and prevented its interaction with the permeability pore. Dopamine oxidation and quinonoprotein formation have also been correlated with inhibition of mitochondrial ETC activities (Gluck and Zeevalk, 2004; Khan et. al., 2005, Jana et. al., 2007).

DAQ mediated inhibition of mitochondrial respiration and ETC activities may be due to covalent quinone binding to critical cyteine residues forming quinoprotiens on mitochondrial protein subunits. Significant amount of quinoprotein adduct formation has

been reported in mitochondria after incubation with DA (Khan et. al., 2001; Jana et. al., 2007). Loss of free cysteinyl groups on mitochondrial proteins has been reported following exposure of intact, well coupled rat brain mitochondria to DAQ, including the 75 kDa subunit of NADH dehydrogenase of complex I, mitochondrial creatine kinase, mortalin/GRP75, mitofilin, voltage-dependent anion channel 2 (VDAC2) as well as several other proteins (Van Laar et al. 2008a). This loss was not ubiquitous, suggesting that subsets of mitochondrial proteins are more susceptible to quinone modification. Specific rat brain mitochondrial proteins that were modified by DAQ analyzed by autoradiography, mass spectrometry, and peptide mass fingerprinting have been identified (Van Laar et. al., 2008b). The study found DAQ binding to mitochondrial creatine kinase, isocitrate dehydrogenase and succinate Co A ligase. More relevant to this project was the discovery of DAQ modified protein subunits of Complex I (75 kDa and 30 kDa) and complex III (ubiquinol-cytochrome c reductase core protein 1). This modification could affect complexes I and III activities. In addition to mitochondrial protein modifications, the Van Laar et. al., (2008b) study also found DAQ modified PD associated genes DJ-1 and UCH-L1 in DA-exposed SH-SY5Y cells in isolated brain mitochondria. DAQ modified PD associated genes, α-synuclein and parkin have also been reported (Conway et. al., 2000; LaVoie et. al., 2005). DAQ mediated modification was postulated to contribute to the accumulation of α-synuclein and other proteins (Martinez-Vicente et. al., 2008).

These studies indicate that protein modification by oxidized DA metabolites and ensuing protein dysfunction could be one possible reason for the selective neurodegeneration of

nigrostriatal dopaminergic neurons in PD. It should be noted that DAQ mediated inhibition of mitochondrial respiration was not attenuated with coincubation with the antioxidants catalase and superoxide dismutase to eliminate ROS, whereas coincubation with the quinone scavenger, GSH, completely prevented the inhibition and mitochondrial swelling (Berman and Hastings, 1999), suggesting that prevention of quinone formation at the level of the mitochondria is protective. In view of the above discussion, mitochondria and DA interactions may be important to the selective vulnerability of nigrostriatal neurons in PD. To date, however, there have been few studies to characterize the site of action of reactive quinones on mitochondrial electron transport chain function. Studies to address this are the subject of Aims 1 and 2 of this thesis.

GSH, DA and mitochondrial dysfunction

Interestingly, another PD hallmark is decreased levels of reduced GSH specifically in the subtantia nigra (Perry et. al., 1982; Riederer et al.,1989; Sian et al., 1994a,b) without the concomitant increase in oxidized GSH (GSSG) (Riederer et. al., 1989). The loss of GSH could be a contributing factor to DAQ mediated toxicity.In addition to cysteinyl residues on proteins (Fig 1a) and other cellular nucleophiles such as DNA, oxidized DA can covalently bind to the thiol antioxidant glutathione (GSH) (Stokes et al., 1999, Bindoli et al., 1992) forming 5-S-glutathionyl-DA (5-S-G-DAor GSH-DA) (Fig 1b).

FIGURE 1

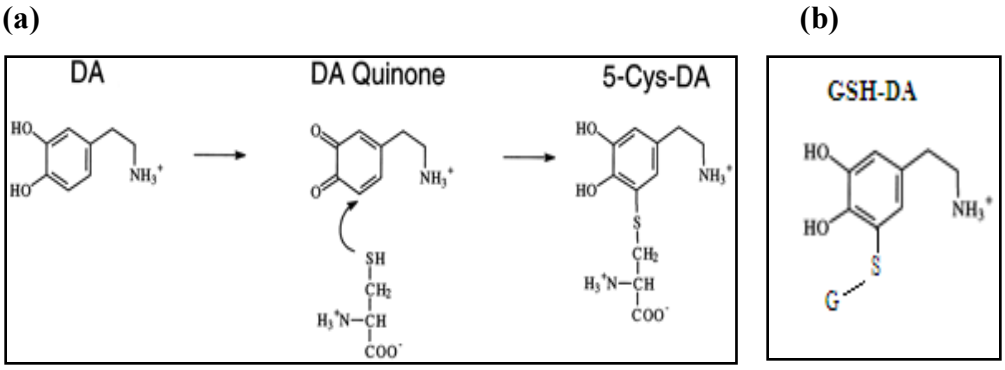


Fig 1: (a) Oxidation of DA to DA Quinone and subsequent formation of 5-Cys-DA (LaVoie and Hastings, 1999) (b) 5-S-glutathionyl-DA (5-S-G-DA, or GSH-DA).

In a cell free system the GSH-DA adduct rate formation is three orders of magnitude faster than intracyclization or *o*-quinone reduction by ascorbate (Tse et al., 1976). Thus GSH-DA adduct formation may be the most prominent fate for DA in an environment rich with ROS, DA and GSH. Tse et. al., (1976), also reported that the adduction of cysteine is even faster than GSH. This is potentially important because cysteine is reported to be the rate limiting amino acid in GSH synthesis (Dringen and Hirrlinger 2003). GSH depletion in the presence of an oxidizing environment and catecholamines was greatly attenuated with the addition of SOD. This is of interest because PD brains show an increase in SOD activity (Saggu et al., 1989), perhaps to counteract the GSH loss or in response to an increase in oxygen radical production. In biological systems it has been reported that thiol adducts form mostly with free cysteine, GSH, or protein bound cysteine (Graham, 1978; Montine et al., 1997). Glutathionyl and cysteinyl catechol thiol adducts derived from DA and DOPAC have been identified as the

major non-protein-bound catechol thiol adducts in the striatum and midbrain of humans and other mammals (Fornstedt et al., 1990). These studies suggest that in an oxidizing environment, cysteine adduction to catechol quinones could lower available cysteine levels which could abate GSH synthesis. This may be one of the reasons for the observed low levels of GSH in PD brains (Sofic et al., 1992; Sian et al., 1994a).

Studies have revealed that mitochondrial damage was an important consequence of GSH deficiency in many cell types (Meister, 1995) including neurons (Bolanos et al., 1995). Treatment with the GSH synthesis inhibitor, buthionine sulfoximine (BSO), in combination with MPTP and MPP^+ in preweaning mice, enhanced the toxic effects of the drugs resulting in increased loss of dopaminergic neurons (Wullner et al., 1996). GSH depletion induced death of rat embryonic mesencephalic neurons in culture and produced profound morphological effects, including decreased catecholamine fluorescence, increased levels of lipid peroxidation, lipofuscin accumulation and increased numbers of dystrophic axons (Grasbon-Frodl et al., 1996). GSH depletion in rat mesencephalic cultures potentiated damage to DA and GABA neurons exposed to the mitochondrial complex II inhibitor malonate (Zeevalk et al., 1998). These results indicate that GSH depletion in a subset of neurons makes them more susceptible to oxidative damage. Several independent laboratories, (Perry et. al., 1982; Riederer et al., 1989; Sian et al., 1994a,b) have reported that the SNpc of rodents, primates, and humans contain lower levels of GSH that other brain regions. Taking into account that the substantia nigra of PD brains have ~50% less GSH than those of matched control subjects (Riederer et al., 1989) it is possible that catechol-GSH and/or cysteine adduction contributes to

dopaminergic neurodegeneration by depleting GSH (Hastings et al., 1996; Shen and Dryhurst, 1996; Spencer et al., 1998).

Data gathered from PD post mortem brain tissues are representative of late-stage disease components that, therefore, do not shed light on events that lead to PD. To circumvent this limitation, Dexter et al.,(1994) examined brain samples from people with Incidental Lewy Body Disease (ILBD) a disease thought to be representative of pre-symptomatic PD since ILBD brain tissue contains Lewy bodies in the SNpc and shows modest nigral cell degeneration but individuals are asymptomtic (Dexter et al., 1994). The study found that levels of GSH were decreased by 35% in ILBD brain tissue compared to control subjects. This suggests that loss of GSH may occur early in PD and compromise the nigral redox state rendering it vulnerable to oxidative stress damage. It is interesting to note that although the Dexter et al., (1994) study found a decrease in reduced GSH levels in ILBD brain tissue, complex I activity was not significantly decreased. This implies that GSH depletion is an early event which could lead to downstream mitochondrial dysfunction. Jha et al., (2000) found that depletion of GSH in dopaminergic PC12 cells resulted in selective inhibition of mitochondrial complex I activity. Recent work by Chinta and Anderson (2006) employed chronic GSH depletion in DA neurons as well as acute depletion. This is important since neurodegeneration occurs over time. They found that chronic depletion of GSH has similar effects on mitochondrial complex I activity as those observed following acute depletion. Zeevalk et al (1998) found that decreasing endogenous levels of GSH greatly potentiated toxicity in mesencephalic neurons treated with a mitochondrial inhibitor malonate. The study also

showed that GSH plays an important neuroprotective role during energy stress. DAQ formation occurs more readily in an oxidizing environment such as the SNpc. Thus, a tenable sequence of events in PD may be loss of DA homeostasis with DA oxidation which could lead to GSH-DAQ adduct formation, thereby decreasing free GSH levels and leading to mitochondrial dysfunction. Mitochondrial dysfunction could occur through loss of GSH and/or DAQ mitochondrial interactions.

Glutathione, glutaredoxin and mitochondrial dysfunction

Glutathione consists of the amino acids glycine, cysteine and glutamate. In the brain GSH concentrations can range from 1 mM to 10 mM (Meister and Anderson, 1983). GSH has many important cellular functions including amino acid transport, maintaining ascorbic acid in its reduced form and in the formation of deoxyribonucleotides (Andersen, 1998). GSH is an electron donor in hydrogen peroxide reduction by glutathione peroxidase (GPx) (Griffith and Meister, 1985). The product of this reduction is glutathione disulfide (oxidized glutathione, GSSG).

A high intracellular concentration of GSH protects against radicals such as superoxide radical anion, nitiric oxide or hydroxyl radical in non-enzymatic reactions (Sáez et al., 1989). During episodes of oxidative/nitrosative stress, thiols in cysteine residues within proteins can undergo reversible and irreversible redox alterations due to ROS/RNS formation. Since many thiol groups are found at active sites of proteins and protein motifs that function in protein regulation and trafficking, cellular signaling and control of gene expression, irreversible thiol modification could lead to protein dysfunction and

interfere with normal cellular function (Dalle-Donne et. al., 2007). Oxidative/nitrosative stress can induce the formation of protein-glutathione-mixed disulfides (Pr-SSG). Pr-SSG formation is a reversible formation of mixed disulfides between protein cysteines and GSH and is termed S-glutathiolation or S-glutathionylation (Ziegler, 1985) (Fig 2). HPLC analysis of cellular mixed disulfides demonstrated that most of the protein-bound thiol (> 85%) is accounted for by GSH (Chai et. al., 1994; Ravindranath and Reed 1990; Schuppe-Koistinen et. al., 1994). The normal physiological intracellular environment is a reducing environment with a GSH/GSSG ratio kept at or greater than 100. The GSH/GSSG ratio serves as cytosolic redox buffer (Dalle-Donne et. al., 2007) therefore; oxidation of a small amount GSH could promote S-glutathiolation and shift the equilibrium toward mixed disulfide formation (Dalle-Donne et. al., 2007). In most non-CNS tissues, cells, and organelles, undergoing oxidative stress, GSH is oxidized to GSSG which can either be extruded from the cell or reduced to regenerate GSH by the actions of glutathione reductase (GR) at the expense of nicotinamide adenine dinucleotide phosphatate (NADPH) (Griffith and Meister, 1985). In neurons, (Shivakumar et. al., 1995; Ehrhart and Zeevalk, 2001) and brain mitochondria (Ravindranath and Reed, 1990), oxidative stress results in formation of Pr-SSG at available cysteine residues. Pr-SSG formation can occur via several pathways 1) interaction between a thiyl radical and GSH, 2) thiol exchange reaction between protein thiols and GSSG 3) direct interaction between glutathione thiyl radicals and protein thiols thought to be mediated via glutaredoxin (Fig 2) (Dalle-Donne et. al., 2007). Although protein S-glutathiolation is seen in cells of non-neural origin (Schuppe-Koistinen et al., 1994), it is more marked in brain, most likely because extrusion of GSSG is not seen in mitochondria in brain

(Ravindranath and Reed, 1990). Pr-SSG formation has been implicated in buffering of oxidative stress, stabilization of extracellular proteins, protection of proteins against irreversible oxidation of critical cysteine residues, and regulation of enzyme activity (Ziegler et al., 1985; Thomas et al, 1995, Ehrhart and Zeevalk, 2001, Ehrhart and Zeevalk, 2003;Dalle-Donne et. al., 2007).

FIGURE 2

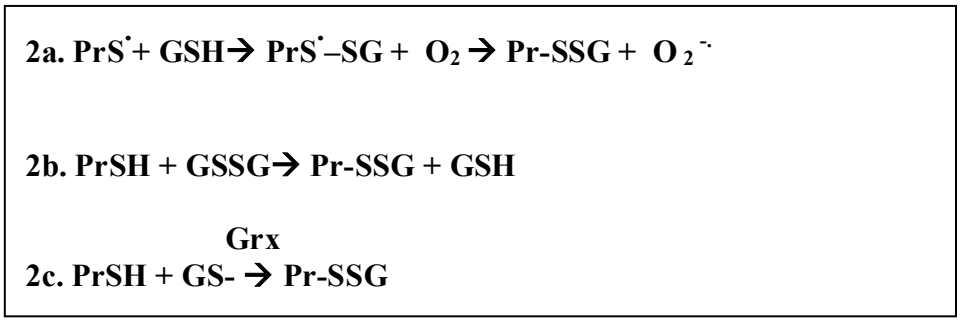


Fig 2: Oxidation of a protein thiol can yield a thiyl radical ($PrS^{\cdot}$), which can then react with a glutathionylate anion (GS-) to form a radical mixed disulphide ($Pr\text{-}SG^{-\cdot}$), which will lose an electron to oxygen to form superoxide radical (O_2^{-}), leaving a mixed disulphide (Pr-SSG) (Equation 2a). Alternatively, a protein thiol can react with GSSG to form a mixed disulfide. (Equation 2b). Additionally, the thiyl radical of GSH, generated by reactions with hydroxyl radicals, can also from Pr-SSGs with protein thiols (Equation 2c). This reaction is thought to be mediated by Grx but remains controversial.

S-glutathiolation of proteins during oxidative stress has been suggested to either contribute to toxicity or protect vulnerable protein thiols. Cohen and Kesler (1999) found that incubation of intact rat brain mitochondria with tyramine, a mixed monoamine

oxidase-A/B substrate, resulted in suppression of both state 3 and state 5 mitochondrial respiration, accompanied by a rise in mitochondrial PrSSG. Their study concluded that inhibition of mitochondrial respiration by monoamine oxidase (MAO) was due to the formation of PrSSG, inhibiting complex I and other thiol-dependent enzymes of the inner membrane. Bernard and Balasubramanian (1995) reported loss of activity of mitochondrial enzymes such as succinic dehydrogenase, isocitrate dehydrogenase, total ATPase and NADH dehydogenase after exposure to 1mM of GSSG which increased Pr-SSG formation 15-fold. They associated the inhibition to the increase in Pr-SSG. In contrast, Ehrhart and Zeevalk et al., (2001) attributed protection of mixed cell cultures from malonate toxicity to a three-fold increase in Pr-SSG formation following exposure to malonate plus ascorbate. These studies provided evidence that formation of Pr-SSG during metabolic insult may be a protective mechanism due to prevention of irreversible oxidation of vulnerable sulfhydryl groups from irreversible oxidation by free radicals.

Under reducing conditions, i.e. once the GSH/GSSG ratio has returned to normal, S-glutathiolation is a reversible reaction catalyzed by glutaredoxin (Dalle-Donne et. al., 2007). Under oxidizing conditions, however, the thiolation may be maintained indefinitely which could affect protein function (Borges et. al., 2002; Dalle-Donne et. al., 2007; Zeevalk et. al., 2003). Additionally, persistent glutathiolation could also lead to depletion of cytosolic GSH, thereby changing the GSH/GSSG ratio and adding to the oxidative environment. The removal of the GSH moiety (deglutathiolation) from protein mixed disulufides is, therefore an important reaction in maintaining the cellular redox status. Dethiolation of S-glutathiolated proteins can be achieved by either non-enzymatic

reduction by direct thiol/disulfide exchange reactions with GSH once the GSH/GSSG ratio has reached resting values or more efficiently, by enzymatic cleavage of the disulfide bond involving the action of thioredoxin (Trx), glutaredoxin (Grx-1) and/or protein disulfide isomerase (PDI) (Jung et al., 1996). Although Trx and PDI can also dethiolate protein mixed disulfides with GSH, Grx-1 is considered the principle enzyme involved in this reaction because it is 10-fold more efficacious (Jung et. al., 1996). Grx can catalyse the reduction of protein disulfide bonds in proteins using GSH as the obligatory reductant and forming GSSG. GSSG is in turn recycled to GSH by a NADPH dependent reaction utilizing GR. During the reaction cycle it is thought that a cysteine pair in the active site of glutaredoxin is converted to a disulfide (Fig 3a). Glutaredoxin is particularly important for deglutathiolation of Pr-SSGs. In this reaction only a single GSH molecule is required (Fig 3b). It should be noted that the direction of the glutaredoxin-catalyzed cycle depends on the relative concentrations of GSH and GSSG. High concentrations in the cell of GSSG relative to GSH will drive glutathioylation or the oxidation of protein thiols to disulfides (Dalle-Donne et. al., 2007).

Cytosolic Grx (Grx-1) protein and message has been identified in rat brain neurons and the enzyme purified from whole brain cytosol (Balijepalli et al., 2000). The gene for a mitochondrial Grx (Grx-2) has also been reported (Lundberg et al., 2001; Gladyshev et al., 2001). Ehrhart et al., (2002) identified Grx activity localized to rat brain mitochondria, thus providing proof for mitochondrial location for Grx in brain. There is a growing list of functions assigned to protein thiolation in mammals ranging from involvement in cell differentiation/proliferation (Takashima et al. 1999), signal

transduction (Bandyopadhyay et al. 1998, Hirota et al. 2000, Daily et al. 2001) and apoptosis (Chrestensen et al. 2000, Daily et al. 2001).

FIGURE 3

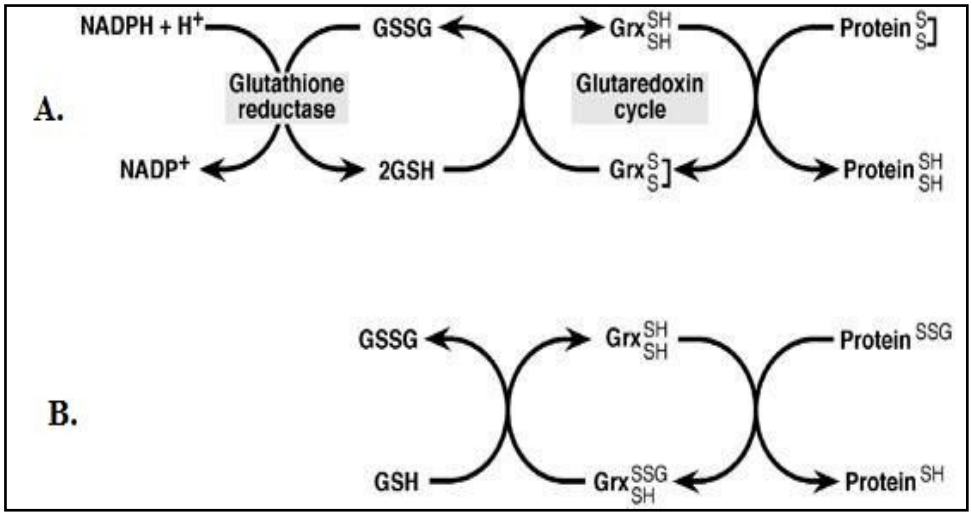


Fig 3: A. Reduction of protein disulfide bonds catalyzed by glutaredoxin (Grx) using two reduced glutathione (GSH) molecules. The resulting oxidized gluathione (GSSG) is reduced to GSH by the actions of glutathione reductase (GR) at the expense of NADPH **B**. Reduction of protein mixed disulfides (Pr-SSGs) via Grx requiring only a single GSH molecule http://www2.imbf.ku.dk/proteinbiol/projects/glutaredoxin.html).

Little is known regarding Grx-1 distribution and activity in brain. Ballijepalli et al., (1999) reported elevations in hippocampus and lowered activity in striatum and cerebellum relative to whole brain in rat. Grx-1 message was detected in rat brain neurons (Ballijepalli et. al., 1999, Takagi et. al., 1999), but not in astrocytes or microglia (Takagi et. al, 1999). This has not been confirmed, but is interesting in view of the fact

that glia have approximately 2-fold more GSH than neurons (Slivka et al., 1987; Pileblad et al., 1991; Rice and Russo-Menna, 1998) and are more resistant to oxidative stress.

The evidence presented above argues for an important role for Grx-1 in physiological and pathophysiological events. While a decrease in GSH can compromise the many functions served by GSH, low Grx-1 activity may result in a lessened ability to reverse protein thiolation which can result in altered protein function. Since S-glutathiolation is one of the main events in response to oxidative stress in brain and brain mitochondria, it is likely that Grx-1 and Grx-2 play an important role during neuronal recovery from oxidative stress by restoring protein thiol homeostasis. A better understanding of Grx in brain with specific attention to brain regions and risk factors (age and gender) associated with Parkinson's disease will be the subject of Aim 3 of this thesis.

Specific Aims

The working hypothesis in the laboratory proposes that GSH-DA interactions are important to the survival of dopaminergic neurons; moreover, disturbances in GSH or DA homeostasis are detrimental to dopaminergic neurons possibly leading to cell damage and/or death through mitochondrial dysfunction. This thesis proposal will encompass 3 specific aims described below.

Aim 1 hypothesizes that the MAO independent inhibition of mitochondrial respiration by DA is mediated via oxidized dopamine (quinone) acting on ETC complexes. This aim will expand on previous data and characterize the effects of reduced and oxidized DA (DAH, DAQ respectively) . Since our laboratory demonstrated that the major metabolite of DA, i.e. DOPAC was equipotent with DA in inhibiting mitochondrial respiration (Gluck and Zeevalk, 2004), reduced and oxidized DOPAC (DOPAC-H and DOPAC-Q, respectively) will be examined on individual ETC complexes. Subaim 1a will investigate the effects of reduced and oxidized DA on complexes I, II, III, and IV of the mitochondrial ETC. Subaim 1b will examine whether reduced and oxidized DOPAC has similar inhibitory effects and potency as DA on individual complexes of the mitochondrial ETC. Subaim 1c will determine the effects of various antioxidants and metal and /or radical chelators on inhibition of mitochondrial function by reduced and/or oxidized DA and DOPAC. Subaim 1d will determine the reversibility of any inhibitory effects of reduced or oxidized dopamine on ETC function as this would provide indirect evidence for an association of DAQ with ETC components.

Aim 2 addresses the question of whether ETC function in intact mitochondria is inhibited by extramitochondrial DA or DOPAC. Characterization studies in Aim 1 are conducted in freeze thawed mitochondria, thus it is unclear whether DA or DOPAC have any effect on intact mitochondrial ETC functions. Subaim 2a will determine whether DA or DOPAC can access the mitochondrial matrix in intact, well coupled mitochondria. Subaim 2b will determine whether extra-mitochondrial DAH or DOPAC-H irreversibly inhibits ETC complexes. Subaim 2c will determine if radiolabeled DAQ binds to lysed

mitochondrial membranes containing the ETC complexes and if this binding can be prevented by chelation of the quinones with GSH.

Aim 3 proposes to characterize Grx-1 message, expression and activity in rat brain in an effort to provide insight into a possible role for Grx-1 in oxidative stress induced neuronal damage. Abbot et al. (1990) demonstrated that the relative variations in levels of GSH in different mouse brain regions are cortex>cerebellum>hippocampus>striatum>SN and Chen et al., (1989) found that brain GSH profiles vary throughout lifespan, namely, high values during growth dropping to a maturation plateau and then decreasing 30% during aging. Since glutaredoxin (Grx-1) specifically uses GSH as the reductant to reverse protein modifications by glutathiolation and little is known regarding Grx-1 distribution, it would be important to profile Grx-1 activity, protein concentration and message by region, age and gender to see if it is similar to the GSH profile. Furthermore, Grx-1 expression or activity changes with region, gender or age could predispose the stratum or midbrain to oxidative damage. Subaim 3a will measure regional differences in activity and protein for Grx-1 and message for Grx-1 and Grx-2. Subaim 3b will determine differences in Grx-1 specific activity and protein with respect to age and gender. Since the only studies to indicate the presence or absence of Grx-1 expression in neurons and glia used semi-quantitative techniques, Subaim 3c will determine Grx-1 mRNA in mesencephalic neurons vs. glia using quantitative RtPCR.

Chapter II: MATERIALS AND METHODS

Materials:

All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.)

Animals:

All experiments were conducted in Sprague Dawley rats from Charles River Laboratories (Wilmington, MA, USA) in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The local Animal Care Committee approved all procedures. Rats were housed in pairs at 20-22°C on a 12 hr light-dark cycle with food and water available *ad libitum*.

Mitochondria Isolation: (Aims 1 and 2; Chapters 3 and 4)

Mitochondrial isolation followed the procedure of Clark and Nicklas, 1970. Briefly, brains were homogenized in a glass homogenizer in 20 ml of ice cold isolation buffer containing: 5.0mM MOPS, 75mM sucrose, 1.0mM EGTA, 225mM d-mannitol, pH 7.4. The crude homogenate was centrifuged at 1800 g for 6 min, the pellet (P1) discarded and the supernatant (S1) centrifuged at 12200 g for 8 min. The resulting pellet (P2) was resuspended in Ficoll and centrifuged further at 12200g for 15 min (Gluck et al., 2002). The supernatant was discarded and the pellet was resuspended in 1:1 volume of ice cold incubation buffer containing: 0.005M KH_2PO_4 , 0.095 M KCl, 0.075M mannitol, 0.025M sucrose, 0.02M Tris-HCL, 0.001 M EGTA. pH 7.4. The mitochondria were aliquoted and stored at -80 C.

DAH/DAQ and DOPAC-H/DOPAC-Q solutions: (Aim 1; Chapter 3)

DAH and DOPAC-H solutions were made in ice cold HPLC water and kept on ice. The solutions were monitored at 480nm and 400nm, respectively, to verify any quinone formation (Graham 1978, Sugumaran et al., 1992). DAQ and DOPAC-Q solutions were made in phosphate buffer pH 8.0 and kept at room temperature. A standard curve made of [25U] tyrosinase-mediated-fully oxidized DA was used to quantify the desired DAQ concentrations. The standard curve for DOPAC-Q was made of [250U] tyrosinase-mediated-fully oxidized DOPAC-Q to quantify the desired DOPAC-Q concentrations. DA used in ETC assays was air oxidized rather than oxidized by tyrosinase due to interactions between tyrosinase mediated DAQ formation and NADH oxidation. The DAQ concentration was monitored at 480nm until the desired absorbance for corresponding DAQ concentration on the standard curve was reached. For example, 50uM DAQ had an absorbance of 0.16, whereas 100um DAQ had an absorbance of 0.31. Oxidized DOPAC used in the ETC assays was oxidized by tyrosinase due to the low auto-oxidation of DOPAC. Moreover, tyrosinase-mediated-DOPAC-Q formation did not interfere with NADH oxidation. The DOPAC-Q concentration was monitored at 400nm until the desired absorbance for the corresponding DOPAC-Q concentration on the standard curve was reached.

NADH-Oxidase: (Aim 1; Chapter 3)

The oxidation of NADH was measured by following the decrease in absorbance at 340nm. Activity was expressed as nmoles of NADH oxidation/min/mg protein (ϵ= 6.81mM-1 . cm-1). NADH-Oxidase assay was modified from Ragan et. al., (1988).

Briefly, the assay medium contained K2HPO4 (20mM) pH 7.4, and EDTA (0.5mM). A parallel negative control with rotenone (10uM) was also run. NADH-Oxidase activity was inhibited by ≥ 90 % by rotenone. NADH oxidation reaction was the rotenone sensitive activity. Reaction cuvettes with assay medium and DAH/DAQ or DOPAC-H/DOPAC-Q were monitored at 480nm and 400nm, respectively, to verify quinone presence just prior to use. Immediately following the quantification of quinone, NADH (0.13mM) was added and the absorbance change was recorded for 2 min. Mitochondria (50 ug/ml) were then added, and enzyme-catalyzed NADH oxidation was measured for 3-5 min. NADH-Oxidase activity was measured in mitochondria either with or without a 30 min preincubation with DAH/DOPAC-H or DAQ. Control reactions with NAD+ plus DAH or DOPAC-H were run to determine if the catechols could reduce NAD+ back to NADH potentially mimicking inhibition, however, neither catechol was shown to interfere with the reaction.

Complex I: NADH:ubiquinone oxidoreductase: (Aims 1 and 2; Chapters 3 and 4)

Complex I specific activity was measured spectrophotometrically by following the decrease in absorbance due to the oxidation of NADH at 340nm. Activity was expressed as nmoles of NADH oxidation/min/mg protein (ϵ= 6.81mM-1 . cm-1). Complex I assay was modified from Birch-Machin et al., 1994. Briefly, the assay medium contained K2HPO4(20mM) pH 7.4, EDTA(0.5mM), potassium cyanide (KCN) (2mM), antimycin A(0.002mg/ml), and Coenzyme Q1(0.065mM). The reaction was initiated by the addition of NADH (0.13mM) and the absorbance change was recorded for 2 min. Mitochondria (50ug/ml) were then added and enzyme-catalyzed NADH oxidation was measured for 3-

5 min. A parallel negative control with rotenone (10uM) was also run. Complex I activity was the rotenone sensitive NADH:ubiquinone oxidoreductase activity. Rotenone inhibited complex I activity by ≥ 95%. Reaction cuvettes with assay medium and DAH/DAQ or DOPAC-H/DOPAC-Q were monitored for quinone content as stated above. Neither DAH nor DOPAC-H were found to non-enzymatically interfere with the complex I assay. Complex I activity was measured in mitochondria either with or without a 30 min preincubation with DAH/DOPAC-H or DAQ.

Complex II: succinate:ubiquinone oxidoreductase: (Aim 1; Chapter 3)

Complex II specific activity was measured by following the reduction of 2,6-dichlorophenolindophenol (DCPIP) at 600nm. Activity was expressed as nmoles of DCPIP reduction/min/mg protein (ϵ= 19.1mM-1. cm-1). Complex II assay was modified from Birch-Machin et al., (1994). Briefly, the assay medium contained K2HPO4 (25mM) pH 7.4, EDTA(0.1mM), KCN (2mM), rotenone (10uM), antimycin(0.002mg/ml), succinate (25mM) and mitochondria (50ug/ml) and incubated for 15 min at room temperature. DCPIP (0.050mM) was added and absorbance change was recorded for 2 min. This was the non-enzymatic reduction rate of DCPIP and was subtracted from the enzymatic rate. Coenzyme Q1 (0.065mM) was then added to initiate the reaction and the enzyme-catalyzed reduction of DCPIP was measured for 3-5 min. A parallel negative control with malonate (10mM) and mitochondria was also run. Complex II activity was the malonate sensitive activity. Malonate inhibited complex II activity by ≥ 90-95%. In control studies, without mitochondria, DAH and DOPAC-H were found to reduce DCPIP. Since complex II assay measures the reduction of DCPIP this added to the

forward reaction and masked inhibition. In order to correct for the artifact, a parallel assay with malonate (10mM) plus DAH or DOPAC-H was run and the non-enzymatic rate was subtracted from samples with DAH or DOPAC-H and mitochondria. DAQ and DOPAC-Q did not create an artifactual interference however, in order to maintain consistency of assay conditions parallel controls with inhibitors and DAQ/DOPAC-Q were also run. Complex II activity with DAQ/DOPAC-Q was the malonate sensitive activity.

Complex III: ubiquinol: ferricytochrome C oxidoreductase: (Aims 1 and 2; Chapters 3 and 4)

Complex III specific activity was measured by monitoring at 550nm the reduction of oxidized cytochrome C (III) to reduced cytochrome C (II). Activity was expressed as nmoles of cytochrome C reduction/min/mg protein (ϵ= 19mM-1. cm-1). Complex III assay was modified from Krahenbuhl et al., 1994. Briefly, cytochrome C was oxidized by exposure to O_2 gas. The reaction medium contained K2HPO4 (25mM) pH 7.4, MgCl2(5.0mM), KCN(2.0mM), bovine serum albumin (BSA, 2.5mg/ml), dodecyl maltoside(0.44mg/ml), rotenone(10uM), decylubiquinol (0.15uM/ml), and cytochrome C (III) (80uM). Mitochondria (10ug/ml) were added to initiate the reaction and the enzymatic reduction of cytochrome C (III) was measured for 3-5 min. A parallel negative control with complex III inhibitors myxothiazol (60uM) and antimycin (0.002mg/ml) was also run. Enzyme activity was inhibited by $\geq$ 95%. Complex III activity was the inhibitor sensitive activity. Reaction cuvettes with assay medium and DAH/DAQ or DOPAC-H/DOPAC-Q were monitored for quinone formation as stated above. In control studies,

in the absence of mitochondria DAH or DOPAC-H but not their oxidized counterparts were found to pass electrons to oxidized cytochrome C (III) to produce reduced cytochrome C (II). This added to the forward reaction and created an artifact that masked inhibition by DAH or DOPAC-H since complex III assay measures the production of reduced cytochrome C (II). Corrections to the rate made by subtraction of the nonenzymatic rate could not be made as reaction rates were variable and inconsistent (see Results). DAQ and DOPAC-Q did not react with cytochrome C, however, for consistency and to control for any reduced catechols present when DAQ or DOPAC-Q was added to mitochondria, rates were run in the presence or absence of myxothiazol and antimycin and the non-inhibited rate was subtracted from the overall rate to obtain the corrected complex III inhibitable rate. For the complex III assay, mitochondria (10 ug/ml) were preincubated with DAQ and DOPAC-Q for 15 min at room temperature. Decylubiquinol was synthesized according to Krahenbuhl et al., (1991) by reduction of decylubiquinone (10 μmol) with sodium borohydride (NaBH4) in 2 ml of a 1:1 ethanol:H2O mixture (v/v, pH 2). The decylubiquinol formed was extracted twice with 1 ml of diethylether: cyclohexane 2:1 (v/v). The combined organic phases were washed with 2 ml of 2M NaCl and evaporated to dryness at room temperature under a stream of nitrogen. The residue was dissolved in 1ml of ethanol pH 2.0, and the resulting light yellow solution was acidified by the addition of 10 μl of 0.1 M HCl. This solution was stored at -20 °C under light protection.

Complex I/III: NADH:cytochrome C–oxidoreductase: (Aim 1; Chapter 3)

Complex I/III specific activity was measured by monitoring at 550nm the reduction of cytochrome C. Activity was expressed as nmoles of cytochrome C reduction/min/mg protein (ϵ= 19mM-1. cm-1). The assay was modified from Birch-Machin et. al., 1994. Briefly, the assay medium contained K2HPO4 (20mM) pH 8.0, EDTA (0.5mM), KCN (2mM), NADH (25uM), and cytochrome C (50uM). Mitochondria (50ug of protein) were added to initiate the reaction and the enzymatic reduction of cytochrome C was measured for 3-5min. Complex I/III activity was inhibited by $\geq$ 95% by the complex I inhibitor rotenone. Complex I/III activity was the rotenone (10uM) sensitive activity.

Complex II/III: succinate:cytochrome C–oxidoreductase: (Aim 1; Chapter 3)

Complex II/III specific activity was measured by monitoring at 550nm the reduction of cytochrome C. Activity was expressed as nmoles of cytochrome C reduction/min/mg protein (ϵ= 19mM-1. cm-1). Reaction cuvettes with assay medium and DAH/DAQ or DOPAC-H/DOPAC-Q were monitored for quinone formation as stated above. Complex II/III assay was modified from Ragan et al., 1988. Briefly, the assay medium contained K2HPO4 (20mM) pH 7.4, EDTA (0.5mM), KCN (2mM), succinate (pH 7.4) (20mM), and cytochrome C (50uM). Mitochondria (50ug/ml) were preincubated with succinate for 15min at room temperature. Cytochrome C was added to initiate the reaction and the enzymatic reduction of cytochrome C was measured for 3-5 min. A parallel negative control with complex III inhibitors myxothiazol (60uM) and antimycin (.002mg/ml) or complex II inhibitor malonate (10mM) was also run. Complex II/III activity was inhibited by $\geq$ 90-95% by inhibitors. Complex II/III activity was the inhibitor sensitive

activity. In the absence of mitochondria in control studies, DAH and DOPAC-H were found to reduce oxidized cytochrome C. This reaction added to the forward reaction since complex II/III assay measures the reduction of cytochrome C. To adjust for this artifactual interference during the reaction, a parallel control with DAH/DOPAC-H plus inhibitors and mitochondria was also monitored. This rate was subsequently subtracted from samples with DAH/ DOPAC-H. In order to maintain consistency and control for any reduced catechols present when DAQ or DOPAC-Q was added to mitochondria, the assay was run in the presence or absence of inhibitors myxothiazol and anitmycin A and the non-enzymatic rate subtracted from the overall rate to obtain the corrected complex II/III inhibitable rate.

Complex IV:ferrocytochrome C oxidase:oxygen oxidoreductase: (Aim 1; Chapter 3)

Complex IV specific activity was measured by following the oxidation of reduced cytochrome C (II) to oxidized cytochrome C (III) at 550nm. Activity was expressed as nmoles of cytochrome C oxidized/min/mg protein (ϵ= 19mM-1. cm-1). Complex IV assay was modified from Birch-Machin et. al., 1994. Briefly, the assay medium contained K2HPO4 (25mM) pH 7.4, cytochrome C (II) (80uM) and dodecyl maltoside (0.44mg/ml). Mitochondria (50ug/ml) were added to initiate the reaction and the enzymatic oxidation of cytochrome C (II) was measured for 3-5 min. A parallel negative control with KCN (2mM) was also run. KCN inhibits complex IV activity $\geq$ 95-100%. Complex IV activity was the KCN sensitive cytochrome C oxidase activity. Reaction cuvettes with assay medium and DAH/DOPAC-H or DAQ/DOPAC-Q were monitored for quinone formation as stated above. In the absence of mitochondria, DAH or DOPAC-

H reduced oxidized cytochrome C back to reduced cytochrome C This can create a competing back reaction since complex IV assay measures the oxidation of reduced cytochrome C to oxidized cytochrome C producing an effect that mimicks inhibition(see Results). Mitochondria (50ug/ml) were pre-incubated with DAQ or DOPAC-Q for 15 min at room temperature. prior to assay in the complex IV reaction. Rates reported are the KCN inhibitable rates. Reduced cytochrome C (4.0 mM) was prepared in 20mM potassium phosphate buffer, pH 7.0, and reduced using 7.8 mM L-ascorbic acid. This mixture was dialyzed for 16 hours at 4° C using 20 mM potassium phosphate, pH 7.0, as dialysis buffer, aliquoted and stored frozen at -80°C.

Mitochondrial O_2 Consumption: (Aim 2; Chapter 4)

Oxygen consumption was carried out at 30°C in a closed chamber containing a Clark type O_2 electrode connected to a YSI model 5300 oxygen monitor and XY chart recorder. Mitochondria (0.3 mg/ml) were suspended in 1.0 ml incubation buffer containing 0.095M KCL, 0.075M mannitol, 0.025M sucrose, 0.005M $KH2PO_4$, 0.02M Tris-HCL, 0.001M EGTA, pH 7.4. Initial base line respiration was measured by adding NAD-linked substrates glutamate and malate (10.0mM). O_2 consumption under resting state (no adenosine diphosphate (ADP), state 2) was measured for 2 min followed by the addition of ADP (0.25mM) and O_2 consumption was measured for 2 min. Following the complete use of added ADP, the non-active respiration rate (state 4) was measured for 2 min. The mean respiratory control ratio (RCR) (sate 3 rate/state 4 rates) was 8.3 ± 1.5 (±SEM) derived from four individual experiments.

Mitochondria plus DAH, DOPAC-H samples (Aim 2; Chapter 4):

Intact mitochondria (0.3 mg/ml) were incubated with DAH or DOPAC-H (1mM, 7.5 mM, 15.0mM final concentrations), pargyline and clorgyline (10.0uM) to prevent H2O2 production, and ascorbate (100.0uM) to prevent catecholamine oxidation in isolation buffer for 10m at 30°C then immediately placed on ice to stop the reaction. The samples were then centrifuged in a table top centrifuge (15,000 RPM) for 10 minutes at 4°C. The supernatant was discarded and the remaining mitochondria pellet was resuspended in isolation buffer (1.0 ml, pH 7.4). This was repeated once more and the resulting pellet was resuspended in isolation buffer (1.0 ml, pH 7.4). The samples were freeze thawed 5 times to lyse the mitochondria and stored at -80°C.

Dopamine Uptake by Intact Mitochondria: (Aim 2; Chapter 4)

Isolated, intact rat brain mitochondria (approximately 0.4mg protein) were pre-incubated at either 0°C or 30°C for 5 min in 0.9 ml of mitochondrial incubation buffer (see above), containing 4 uM each clorgyline and pargyline (MAO A and B inhibitors, respectively), 100 uM mazindol (to block dopamine uptake by the dopamine transporter), 10 uM Ro4-1284 (to block uptake by the vesicular monoamine transporter , kind gift of Hoffman-La Roche, Nutley, N.J. USA), and 200 uM ascorbate (to prevent dopamine oxidation). 3H-labeled dopamine (specific activity 28Ci/mmol) supplemented with cold dopamine to final concentrations of 1-15 mM was added and incubations continued for another 10 min. At the end of incubation, uptake was stopped by the addition of 5 ml of ice cold incubation buffer. Mitochondria were washed extensively with incubation buffer using a Brandel Cell Harvester Model M24R (Gaitherburg, MD). Mitochondria collected on

GF/F Whatman filters presoaked with 0.5% polyethylamine, were extracted with 0.2N perchloric acid. Radioactivity from the acid extracted matrix was quantified by scintillation counting. Nonspecific radioactivity was eliminated by subtracting counts obtained at 0°C from those at 30°C. Results were reported as nmol dopamine/mg mitochondrial protein.

Grx-1 Activity: (Aim 3; Chapter 5)

Glutaredoxin activity was measured using cysteinyl glutathione disulfide (Toronto Research Chemicals, Inc. Toronto, Canada) as the substrate as described by (Balijepalli et.al., 2000). The reaction buffer (1ml), 0.137 mM potassium phosphate buffer (pH 7.6) with 0.1% Triton-X, contained 0.35 mM NADPH, 0.5 mM GSH, and 1.0 U yeast glutathione reductase (Sigma). Substrate, 0.1 mM cysteinyl-glutathione disulfide was added and the background reaction rate was monitored for 2 min by following the decrease in absorbance at 340nm. Post-mitochondrial cytosol (0.15 mg/ml) was then added to the same cuvette and the rate of the reaction followed for another 2 min. Rates were corrected for background and the amount of product formed (NADPH oxidized) per min per mg protein at 25°C was calculated using an extinction coefficient of 6200 M^{-1} $cm^{-1.}$

Isolation of post mitochondrial cytosol: (Aim 3; Chapter 5)

The cytosol was isolated from rat brain following the procedure of Clark and Nicklas, 1970. Briefly, brains were homogenized in a glass homogenizer in 20 ml of ice cold isolation buffer containing: 5.0mM MOPS, 75mM sucrose, 1.0mM EGTA, and 225mM

D-mannitol, pH 7.4. The crude homogenate was centrifuged (Sovall RC-5B Refrigerated Centrifuge) at 1800 g for 6 min, the pellet (P1) discarded and the supernatant (S1) centrifuged at 12200 g for 8 min. The resulting pellet (P2) was resuspended in Ficoll and centrifuged further at 12200 g for 15 min (Gluck et. al., 2002) This pellet, containing mitochondria was frozen at -80 C and used in other studies. The supernatant (S2) was aliquoted in Ultra Centrifuge tubes and centrifuged at 50000 g for 90min (DuPont Beckman L7 Ultracentrifuge). The resulting pellet was discarded and the supernatant (S3) aliquoted into ice-cold sterile test tubes and stored at -20°C prior to measurement of Grx activity or protein.

Regional Study Brain Dissections: (Aim 3; Chapter 5)

Cytosol was isolated from 90d Sprague Dawley male rats obtained from Charles River Laboratories. Rats were euthanized by guillotine and the brain was removed. The brain was placed on its ventral surface and the cerebellum was separated first, then the pons and medulla region was cut as one piece representing the brain stem. The remaining brain was turned on to its dorsal surface and a diagonal cut was made caudal to the hippocampus representing midbrain. It was then turned over to its ventral surface and separated medially into two sagittal pieces. Hippocampii from both halves were separated from the neocortex. This was followed by separating the striatum from both halves. The remaining neocortices from both halves were combined. Dissected regions were immediately placed in 20 ml of ice-cold isolation buffer and homogenized in a glass homogenizer. An n of one comprised pooled regions from 6 rat brains. A total n of three was used for independent experiments for this study. The dissection of all six brains was

completed in 35 min at room temperature. Cytosol was collected from the dissected brain regions as described above.

Gender Study Brain Dissections: (Aim 3; Chapter 5)

For the pro-estrous female vs. male study, Sprague dawley rats (200-250 g) were used. The females presented at least four regular 4-day estrous cycles, as determined by daily vaginal smears every morning between 9:00 a.m. and 10 a.m. Unstained material was observed under a light microscope at 100 and 400 X magnification. The slides were viewed again after drying and stained with Papanicolaou (PAP) stain and stained according to Hubscher et. al.,(2005). Animals were euthanized by guillotine and the brains were removed. A total n of 3 was used for independent experiments for this study. In a second study, mixed estrous females were used for comparison with males to replicate what had been done in the mouse (Kenchappa et. al., 2004).

Age Study Brain Dissections: (Aim 3; Chapter 5)

The age points for post mitochondrial cytosol collection were embryonic day 20 (E20), post natal day 3 (P3), post natal day 7 (P7), post natal day 14 (P14), post natal day 30 (P30), post natal day 90 (P90), 7 months (7M), 12 months (12M), and 18 months (18M). Grx-1 activity was measured in male rat cytosol for age groups P30-18M, however both male and female brains were used for the E20 to P14 age groups. Values were means for three individual experiments using pooled brains from 3 animals for all age groups from P30 to 18M. For age E20 one litter of pups represented one sample. Three litters from different mothers were used. For ages P3, P7, and P14 the litter was divided into groups

of three pups representing one sample. For post natal day 7 to 18 months ages, rats were euthanized by guillotine and the brain was removed.

Neuronal enriched cell culture: (Aim 3; Chapter 5)

Mesencephalon from fetal day 15 Sprague–Dawley rats were dissected, pooled, mechanically dissociated and centrifuged at 1000 g for 10 min. Dissociated cells (3.6 x 106 cells/cm2) were plated into 6-well plates previously coated with polyornithine and fetal bovine serum and incubated at 37 C, in a 5% CO_2 incubator. Approximately 4 hr after cells were plated, serum-containing DMEM was replaced with serum-free N_2 supplemented medium (Invitrogen, Eugene, OR), as described in Wood et. al.,(2003). N_2 supplement (8.6 uM insulin, 1 mM human transferrin, 2 uM progesterone, 10 mM putrescine, 3 uM selenium) is a serum-free supplement that promotes the growth of post-mitotic neurons (Invitrogen, Eugene, OR).

Glia enriched cell cultures: (Aim 3; Chapter 5)

Glia enriched cells were prepared from the mesencephalon from fetal day 15 Sprague–Dawley rats. The mesencephalon was dissected, pooled, mechanically dissociated and centrifuged at 1000 g for 10 min. and cultured in polylysine coated flasks containing minimum essential medium (Invitrogen, Eugene OR), supplemented with 30mM glucose, 100U/ml penicillin 100U/ml streptomyacin (Pen-Strep), 2mM glutamine, and 18% fetal bovine serum (FBS, Gibco) designated NM-15 medium. After 1 week in NM-15 medium, flasks were agitated on a shaker at 450 rpm at 37°C for 15 min, then incubated for 2 hr and the shaking repeated once more. On the next day the loose non-glial cells

were removed and the adhering cells were supplemented with warm NM-15 medium. After 4 days the remaining cells were trypsinized and replated in 6-well dishes, at a density of 104 cells/cm^2.

Western: (Aim 3; Chapter 5)

Protein (25μg) for each fraction was prepared in sample buffer containing 6mM Tris, 1.7% SDS, 5% glycerol, 0.1 M DTT and 2% bromophenol blue and loaded onto a 15% polyacrylamide gel. Proteins were transferred to nitrocellulose using a Panther Semidry Electroblotter (Owl Separation Systems). The nitrocellulose was divided along the 25kD marker. The nitrocellulose above the 25kD piece was exposed to mouse anti-actin (Amersham Life Sciences) (1:10,000) overnight and the piece below the 25kD mark was exposed to rabbit anti-human glutaredoxin-1 antibody (1:1000) (American Diagnostics, Greenwich, CT) overnight. The actin blot was incubated with anti-mouse horseradish peroxidase conjugated immunoglobulin (Amersham Life Sciences, Arlington Heights, IL) for 1 hr and the Grx-1 blot was incubated with anti-rabbit horseradish peroxidase conjugated immunoglobulin (Amersham Life Sciences, Arlington Heights, IL) for 1 hr. Visualization was by chemiluminescence using a Western Lightning Kit (Perkin Elmer Life Sciences) and exposure to X-ray film.

Grx Real-time quantitative reverse transcriptase-PCR: (Aim 3; Chapter 5)

Extraction of mRNA was performed as described by the manufacturer (QIAGEN, Valencia, CA). RNA was eluted into 50 μl of RNase-free water, and concentrations measured using a spectrophotometer (Eppendorf Biophotometer; wavelength, 260/280

nm; Eppendorf North America, New York, NY). cDNA was generated using 1 µg of RNA, 10 µl of 10x random primers, and MultiScribe Reverse Transcriptase (Applied Biosystems, Foster City, CA). The solutions were brought to 50 µl with RNase-free water and incubated at 25°C (10 min) and at 37°C (2 hr). Samples were stored at –20°C. Real-time PCR was performed using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Bedford, MA). Reactions were performed in a total volume of 25 µl using SyBr Green Master Mix (Applied Biosystems); 2 µl of cDNA template synthesized as described above per sample was used, with 10 µM forward and reverse primers. The final concentration of template was 40 ng/reaction. The target amplifications were performed in duplicate. Thermal cycling conditions included 2 min at 50°C, 10 min at 95°C, followed by 40 cycles at 95°C for 15 s and 1 min at the annealing temperature. Primers for Grx-1 were as follows: forward primer, 5'-atgctaatggccagtgccc; reverse primer, 5'-ctcagccccatggttagagg. Primers for Grx-2 were as follows 5'-aaacaccaccttggtttcttcg; reverse primer, 5'tgatgtgaaagatggaccgga. Primers for GAPDH were the following: forward primer, 5'-tatcggacgcctggttacca; reverse primer, 5'-ctcagccttgactgtgccg. Primers for GFAP were: forward primer, 5'-gagctcaaccagcttcgacg; reverse primer, 5' tgccgaggtcctgtgtgag. The amount of Grx-1 message in the individual regional brain samples and enriched neuronal and astrocytes samples were normalized to GAPDH and were defined by the C_t method (Livak and Schmittgen, 2001). Primer sets yielded a single PCR product as monitored using melting curves in each reaction well.

Statistical analysis:

Statistical analysis was calculated using ANOVA one-way test with Tukey post hoc test for comparison or an unpaired t test using GraphPad Software, version 3.0.

Chapter III: CHARACTERIZATION OF REDUCED AND OXIDIZED DOPAMINE AND 3, 4-DIHYDROPHENYLACETIC ACID, ON BRAIN MITOCHONDRIAL ELECTRON TRANSPORT CHAIN ACTIVITIES

A. Introduction

Parkinson's disease (PD) is an age related neurodegenerative movement disorder. Genetic mutations can cause PD, (Bonifati 2007) however; the majority of PD cases are idiopathic (Vila and Przedborski, 2004). A major hallmark of the disease is the chronic progressive loss of dopaminergic cells in the SNpc (Hornykiewicz and Kish 1986). The etiology of PD is unknown; however loss of GSH specifically in the SNpc of PD post mortem brains (Perry et al., 1982, Reiderer et al., 1989, Jenner et al., 1993); oxidative stress (Cohen et al., 1988); DA sequestration (LaVoie and Hastings 1999a); increased synthesis and turnover of DA (Hornykiewicz and Kish, 1986; Forno, 1996) and mitochondrial dysfunction (Fahn and Cohen, 1992; Wallace et al., 1997) have been implicated in the pathogenesis of idiopathic PD. Specific to mitochondrial dysfunction, mitochondrial deficits have been reported in the SNpc of brains, blood platelets and muscle biopsies from PD patients (Parker et. al., 1989; Schapira et. al., 1991; Schapira, 1994; Penn et al., 1995; Haas et al., 1995). MPP^+, the active metabolite of MPTP inhibits complex I and leads to Parkinsonian like symptoms in humans (Langston et al., 1983; Nicklas et al., 1985), whereas the pesticide rotenone, also a complex I inhibitor can model PD in rodents (Betarbet et al., 2000; Alam and Schmidt, 2002; Sherer et al., 2003b), further supporting a role for mitochondria in the disease process. DA neurons have an intrinsic oxidatively stressed environment due to high iron content, low antioxidant activity and the presence of dopamine and its metabolites. DA metabolism by

monoamine oxidase (MAO) results in the formation of the DA metabolite 3,4-dihydroxyphenylacetic acid (DOPAC), ammonia, and hydrogen peroxide (H_2O_2). In the presence of free metal ions such as Fe^{2+}, H_2O_2 can undergo Fenton chemistry and form hydroxyl radicals, which can cause oxidative stress damage and subsequent cell death (Halliwell and Gutteridge, 1989). Another mechanism thought to cause oxidative stress and subsequent damage is DA oxidation, which forms a quinone and other downstream-oxidized products in the presence of Fe^{2+}, and/or enzymes such as prostaglandin H synthase (Stokes et al, 1999, Hastings, 1995, Xu et al, 1998). DA can also oxidize to form hydroquinones and semiquinone radical intermediates (Graham, 1978; Zhang and Dryhurst, 1993). DA derived o-quinones in general are highly reactive electrophiles that may readily bind covalently to cellular nucleophiles such as DNA, GSH and reduced sulfhydryl groups contained in protein cysteinyl residues via 5-S-Cys-DA, forming protein adducts (Montine et al., 1997). 5-S-Cys-DA has been found in human substantia nigra (Rosengren et al, 1985). In rodents exposed to exogenous DA, DA and DOPAC cysteinyl adducts have been observed in stratial proteins in vivo (Hastings et al., 1996). Adduction to cysteinyl residues on proteins could result in protein dysfunction leading to cytotoxicity.

One potential downstream target site for the increase in cytosolic DA and its oxidation products is the mitochondrion. Exposure to DA inhibited mitochondrial ETC complexes I and II activities (Gluck et. al., 2002). Additionally, DOPAC, a major DA

metabolite, was shown to inhibit mitochondrial O_2 consumption similarly to DA (Gluck and Zeevalk, 2004). DA oxidation products have also been shown to affect mitochondrial function (Hastings et. al., 1996). Studies, however, of DA toxicity on mitochondrial function have been incomplete and report contradictory results that range from no effect of DA on mitochondrial function (Morikawa et al., 1996) to low IC_{50s} of 8-12 uM (Ben-Sachaar et. al., 1995, 2004). Inconsistencies among these studies have raised questions regarding the DA species i.e. reduced or oxidized (DAH, DAQ respectively) that is responsible for inhibition, the sites within the ETC that are affected and if the major DA metabolite, DOPAC, reduced and oxidized (DOPAC-H, DOPAC-Q), similarly affects mitochondrial function. The current study has characterized DAH, DAQ, DOPAC-H, and DOPAC-Q effects on individual mitochondrial ETC activities while monitoring quinone levels. The study also determines the reversibility of any inhibitory effects of reduced or oxidized dopamine on ETC function. We report that DAQ and DOPAC-Q inhibit complexes I and III activities of the mitochondrial electron transport chain (ETC) while DAH causes inhibition of complex II but not the other ETC complexes. Moreover, we observe that DAQ irreversibly inhibits complex I only when exposure occurs during active electron flow.

B. Results

Quantification of dopamine and dopac quinones

Prior studies of the effects of DA on mitochondrial function have not clearly differentiated reduced DA from oxidized DA and have reported conflicting results (Ben-Sachaar et al., 1995; Morikawa et al., 1996; Khan et al., 2005). In the current study, DA was monitored at 480 nm (Graham, 1978) and a standard curve made of tyrosinase-mediated fully oxidized DA was used to quantify the desired DAQ concentration at the time of exposure (Fig 4A: pg 65). DOPAC quinone formation was monitored at 400 nm (Fig 4B: pg 65) (Sugumaran et al., 1992). When the desired quinone concentration was reached, it was immediately used in the reactions. In addition to lack of differentiation between reduced and oxidized DA, past literature has also been inconsistent regarding the amount of exposure time of mitochondria to DA. In our study, NADH-Oxidase and complex I activities were measured with and without 30min preincubation periods at room temperature. No differences were found with pre-incubation versus no pre-incubation when care was taken to ensure that DA and DOPAC were fully oxidized or reduced. All remaining studies were measured with a 15 min pre-incubation at room temperature.

Effects of Reduced Catechols DA and DOPAC On Mitochondrial ETC Complex Activities.

NADH-Oxidase activity was not attenuated by DAH or DOPAC-H.

NADH-Oxidase measures complex I, III and IV. Hence, the electron flow from complex I is not inhibited as it moves down the ETC to complex III and complex IV. Complex II, the succinate dehydrogenase complex, is not a part of the NADH pathway; therefore, its activity is not measured in this assay. NADH-Oxidase was not inhibited by DAH or DOPAC-H at 1 mM with a 30 min pre-incubation (Fig 5A and B: pg 66) or without a pre-incubation, (data not shown). The results demonstrate that complex I, III or IV are not inhibited by reduced DA or DOPAC and that time of exposure to the catechols was not a factor when the catechols remained fully reduced.

Complex I activity was not altered by DAH or DOPAC-H.

The effects of DAH and DOPAC-H on individual ETC activities were assayed to confirm the results of the NADH Oxidase assay since the NADH-Oxidase assay measures the linked activities of complex I, III and IV. Consistent with the NADH-Oxidase assay, complex I activity was not affected by DAH or by DOPAC-H with a 30 min pre-incubation (Figs 5C and D: pg 67) or with no pre-incubation period (data not shown). The results show that exposure to the reduced catechols did not affect complex I activity.

Complex II activity was inhibited by DAH but not DOPAC-H.

In control studies, it was found that DAH and DOPAC-H reduced DCPIP in the absence of mitochondria. Since the complex II assay measures the reduction of DCPIP this

created an artifactual interference masking potential inhibition. In order to correct for this, parallel reactions with DAH or DOPAC-H plus mitochondria and the complex II inhibitor malonate were run. Complex II activities of samples with DAH or DOPAC-H and mitochondria were the inhibitor sensitive activities. Corrected complex II activities showed that DAH inhibited complex II activity by 32.7 ± 2.0 and 41.9 ± 5.5 % (± SEM) with 500 uM and 1 mM DAH, respectively,(Fig 6A: pg 68). Complex II activity was not significantly affected by DOPAC-H (Fig 6B,:pg 68 although there was a trend towards lower activity). Reduced DA can undergo oxidation to form ROS such as superoxide and hydrogen peroxide (Hastings and Zigmond, 1994). To assay whether inhibition of complex II in the presence of DAH was due to ROS, we studied the effects of superoxide scavenger superoxide dismutase (SOD) (1U) and hydrogen peroxide scavenger catalase (1U) (Cat) on DAH mediated inhibition of complex II. It was found that SOD protected from DAH mediated inhibition by 52.6 % while catalase failed to protect from inhibition indicating that ROS generation was contributing to the inhibition of complex II by DAH (data not shown).

The effect of DAH and DOPAC-H on complex III activity

Complex III activity could not be independently assessed because in control studies in the absence of mitochondria, DAH and DOPAC-H reduced oxidized cytochrome C and added to the forward reaction masking potential inhibition. This produced an artifactual interference that resulted in inconsistent data that could not be corrected by subtraction in the presence of inhibitors. Although the complex II/III assay also measures the reduction of cytochrome C, unlike the complex III assay that required oxidized cytochrome C, the

complex II/III assay called for a mixture of reduced and oxidized cytochrome C. This mixture did not react with DAH and DOPAC-H to the same extent as oxidized cytochrome C and inhibitor corrected rates could be used to determine the artifactual interference. Complex II/III activities with DAH or DOPAC-H and mitochondria were sensitive to inhibitors of both complex II and III. DAH did not significantly attenuate complex II/III activity up to 250 uM, however it was inhibited to a similar extent as was observed with complex II with the higher concentrations of DAH (Fig. 6C: pg 69). Similar to what was found for DOPAC-H and complex II, DOPAC-H did not significantly inhibit complex II/III up to 500 uM (Fig 6D: pg 69). These findings suggest that inhibition of complex II/III in the presence of DAH was due to complex II inhibition. Additionally, since NADH-Oxidase activity, which measures complex I, III and IV activities, was not affected by 1 mM DAH or DOPAC-H (Fig 5A, 5B: pg 66) this further supports that complex III was not affected by DAH or DOPAC-H.

The effects of reduced DA and DOPAC on complex IV activity.

Complex IV activity was not affected by 1mM DOPAC-H (Fig 7B: pg 70) however; it was significantly inhibited by 1mM DAH (Fig 7A: pg 70). In control studies, in the absence of mitochondria, it was found the DAH and DOPAC-H reduced oxidized cytochrome C. Complex IV activity monitors the oxidation of reduced cytochrome C, therefore, the presence of DAH in the assay created a competing back reaction mimicking inhibition. The inhibition observed in the presence of DAH was not prevented by: (2U) SOD plus (2U) Cat, MAO A or B inhibitors (10 uM) pargyline and (10 uM) clorgyline, respectively or dithioreitol (DTT) (50 uM) (data not shown). In the absence of

DAH, these reagents did not affect complex IV activity (data not shown). In addition, NADH oxidase activity, which includes complex IV, was not inhibited by DAH or DOPAC-H (Fig 5A, 5B: pg 65), although the complex IV inhibitor KCN attenuated NADH oxidase activity ≥ 95-100%. Given these findings, it was concluded that DAH does not inhibit complex IV and the inhibition observed by DAH in the complex IV activity assay is an artifact due to an interaction between DAH and the product of the complex IV reaction, i.e., oxidized cytochrome C.

Effects of Oxidized Catechols DA and DOPAC on Mitochondrial ETC Complex Activities.

The quinones did not produce internal artifacts with any of the components of the ETC assays. However, to maintain consistencies in assay methodology, for the reduced and oxidized catechols, activities were measured in the presence or absence of the appropriate inhibitors. As with the reduced catechols, activities for NADH Oxidase and complex I were measured in mitochondria that were pre-incubated for 30 min or received no pre-incubation, while all other assays were conducted with a 15 min pre-incubation at room temperature.

NADH-Oxidase was dose dependently inhibited by DAQ and DOPAC-Q.

NADH-oxidase was dose dependently inhibited by DAQ and DOPAC-Q without a pre-incubation period with IC_{50s} of 158.5 ± 0.01 and 177.8 ± 0.10 (mean uM ± SEM) , respectively (Fig 8A, 8B: pg 71). With a 30 min preincubation at room temperature with DAQ, the IC_{50} was 150 ± 3.7 um (data not shown) indicating that once DAQ was formed,

time was not a factor. The results demonstrate the potential involvement of complex I, III or IV in the inhibition associated with DAQ and DOPAC-Q.

Complex I activity was inhibited by DAQ and DOPAC-Q in a dose dependent manner.

The NADH-Oxidase assay showed the potential vulnerability of complexes I, III and IV activities to DAQ and DOPAC-Q. In order to determine the site of DAQ, DOPAC-Q inhibitory action, we studied activities of the individual complexes. It was found that complex I activity was inhibited in a dose dependent manner by DAQ and DOPAC-Q when there was no pre-incubation with IC_{50s} of 50.1 ± 0.036 and 181.9 ± 0.01 (mean uM ± SEM), respectively (Fig 9A, 9B: pg 72). The IC_{50} for DAQ with a 30 min pre-incubation was 50.0 ± 1.4 uM (data not shown). The results suggest that inhibition of complex I is quinone mediated and as with NADH-Oxidase. Once the quinone is formed time was not a factor.

Complex I inhibition by DAQ was not attenuated by the antioxidants SOD and catalase and required activation for inhibition by DAQ.

SOD and catalase failed to prevent the inhibition of complex I by DAQ (Fig 9C: pg 73). This supports that complex I activity was inhibited by oxidized DA rather than ROS products formed during quinone formation. To examine if inhibition was reversible and if activation of complex I was necessary for DAQ mediated inhibition, mitochondria were pre-incubated with DAQ at room temperature for 30 min in the absence of substrate (Fig 9D: pg 74). Thereafter, in some incubations, the enzyme was activated by addition of

NADH and activity was measured. Mitochondria were then pelleted, the supernatant was removed and the pellet resuspended. Complex I activity was again measured in the absence of any added DAQ. Non-activated complex I mitochondria were pre-incubated with DAQ for 30 min at room temperature, pelleted, resuspended and then complex I activity was measured. In this preparation, complex I activity was not measured prior to pelleting; hence the enzyme was not activated during exposure to DAQ. Complex I activity was only inhibited when DAQ exposure occurred during the active oxidation of NADH by complex I. This result suggests that activated complex I was irreversibly modified by DAQ.

Complex II activity was not affected by oxidized DA or DOPAC.

In contrast with the reduced catechols, the results show that oxidized DA (Fig 10A: pg 75) or DOPAC (Fig 10B: pg 75) did not inhibit complex II activity. DAQ and DOPAC-Q did not create an artifactual interference with DCPIP, however to maintain consistency of assay conditions, parallel controls with the complex II inhibitor, malonate, and DAQ or DOPAC-Q were also run. The non-enzymatic reaction rates of samples with the oxidized catechols and mitochondria were subtracted from samples with DAQ or DOPAC-Q and mitochondria. The lack of effect of DAQ on complex II activity further supports that ROS generation during redox chemistry with DAH contributed to the inhibition of complex II by the reduced catechol as observed in Fig 6A, pg 68.

Complex III activity was inhibited by both DAQ and DOPAC-Q in a dose dependent manner.

DAQ and DOPAC-Q inhibited complex III activity in a dose dependent manner with IC_{50s} of 151.4 ± 0.086 uM and 109.6 ± 0.065 uM, respectively (Figs 11 A, B: pg 76). Reversibility of complex III inhibition by DAQ was assayed; however the results were inconsistent and unreliable due to loss of complex III activity after pelleting and re-suspension of the mitochondria for both the control and DAQ treated samples.

GSH protects against complex I/III inhibition by DAQ.

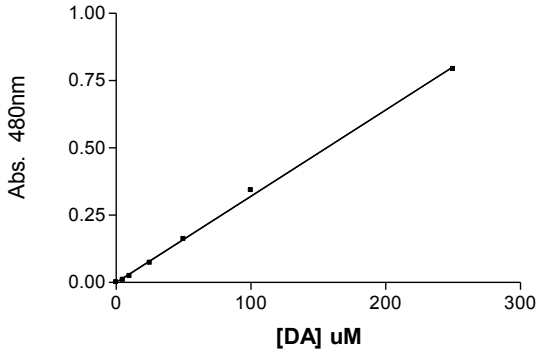
Since DAQ and DOPAC-Q inhibited complexes I and III activities, we studied the effects of antioxidants and a metal chelator on DAQ mediated inhibition of combined complex I/III. We assayed complex I/III activity with (200 uM) DA plus tyrosinase (25U) , and one of the following: catalase (1U) (Cat), to remove H_2O_2, superoxide dismutase (SOD) (1U) to remove superoxide plus (1U) Cat, the metal chelator desferoxamine (1mM), antioxidant trolox (1mM), and the quinone scavenger GSH (3 mM). Only GSH provided significant and nearly complete protection from DAQ mediated inhibition while catalase, catalase/SOD, desferoxamine and trolox were without effect (Fig 11C: pg 77). When added in the absence of DAQ, the antioxidants and metal chelator did not effect complex I/III activity (data not shown). The results suggest a role for catechol quinones and/or oxidized DA products in mitochondrial ETC inhibition rather than ROS since of the agents tested, only GSH can prevent and/or sequester quinone formation.

Oxidized DA and DOPAC do not attenuate complex IV activity.

DAQ (up to 250 uM) and DOPAC-Q (400 uM) did not inhibit complex IV activity (Fig 12A, 12B: pg 78). Higher concentrations were not run since the IC_{50} for overall inhibition of NADH-Oxidase activity, which includes complex IV was 158.5 ± 0.01 uM for DAQ, and 177.8 ± 0.16 uM for DOPAC-Q (Fig 8A, 8B: pg 71 and Table 1).

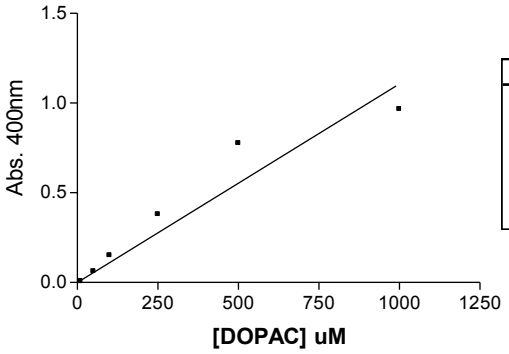
FIGURE 4

A.



[DA] uM	Abs
0.00	0.000
5.00	0.010
10.00	0.024
25.00	0.073
50.00	0.161
100.00	0.343
250.00	0.793

B.



[DOPAC] uM	Abs
10.000	0.01
50.000	0.06
100.000	0.15
250.000	0.38
500.000	0.78
1000.000	0.97

Fig 4: Examples of standard curves made prior to each experiment. Various concentrations of DA (A), fully oxidized by 25 U tyrosinase and DOPAC (B), fully oxidized by 250 U tyrosinase were made in buffer pH 8.0 and absorbance was monitored at 480 nm and 400 nm respectively. DA and DOPAC were considered fully oxidized once the absorbance ceased to increase. The standard curves were used to determine the DAQ and DOPAC-Q concentrations in the reaction experiments immediately prior to measurement of ETC activities. DAH and DOPAC-H solutions were made in ice cold HPLC water, kept on ice and monitored prior to each assay.

FIGURE 5

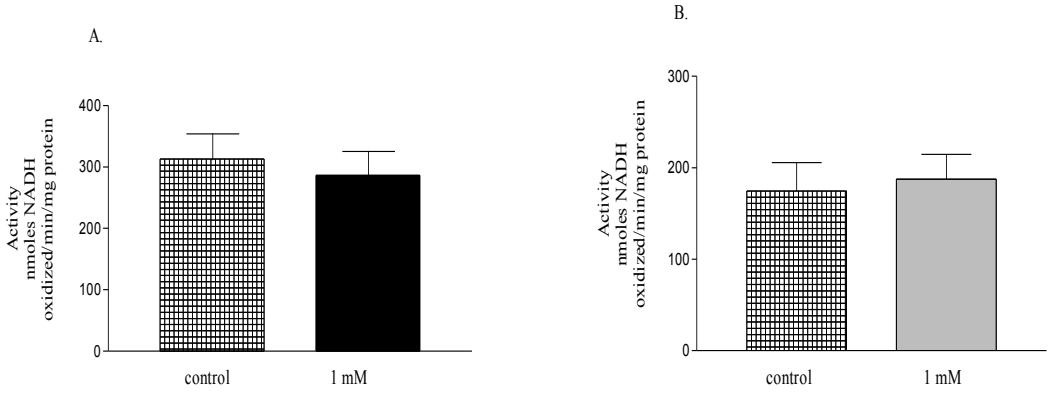


Fig 5 A,B: NADH oxidation was measured by following the decrease in absorbance at 340 nm, as described in Methods. Freeze thawed rat brain mitochondria were exposed to 1 mM DAH and DOPAC-H for 30 min at room temperature prior to assay (A, B). Values for DAH and DOPAC-H effects were expressed as nmoles of NADH oxidized/min/mg protein, ± SEM. Each value is the mean of three individual experiments.

FIGURE 5

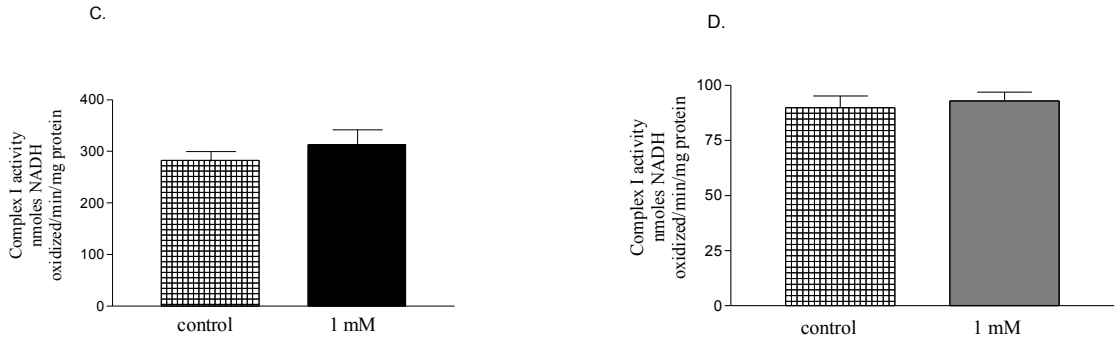


Fig 5 C,D: (C, D) Complex I specific activity was measured by following the decrease in absorbance due to NADH oxidation at 340 nm, as described in Methods**.** Freeze thawed rat brain mitochondria were exposed to 1 mM DAH (C) and DOPAC-H (D) for 30 min at room temperature prior to assay. Each value is the rotenone sensitive activity mean ± SEM of three individual experiments and is expressed as nmoles of NADH oxidation/min/mg protein. NADH-Oxidase and complex I activities were not attenuated by either of the reduced catechols.

FIGURE 6

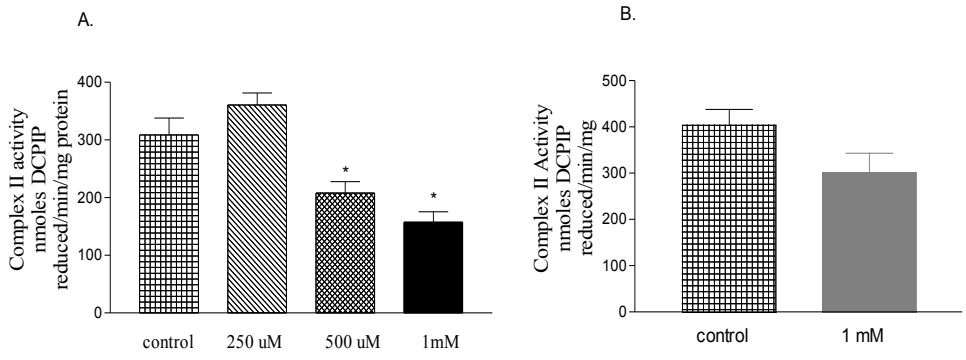


Fig 6 A,B: Complex II activity was measured by following the decrease in absorbance due to DCPIP reduction at 600 nm, as described in Methods. Freeze thawed rat brain mitochondria were exposed to various concentrations of DAH (A) or 1mM DOPAC-H (B) in the reaction medium with a 15 min pre-incubation at room temperature. Each value is the malonate sensitive activity mean ±SEM and is expressed as nmoles of DCPIP reduction/min/mg protein of three separate experiments. Complex II activity was not attenuated by DOPAC-H but was inhibited 32.7 ± 2.0 and 41.9 ± 5.5 % by 500 uM and 1 mM DAH, respectively.

FIGURE 6

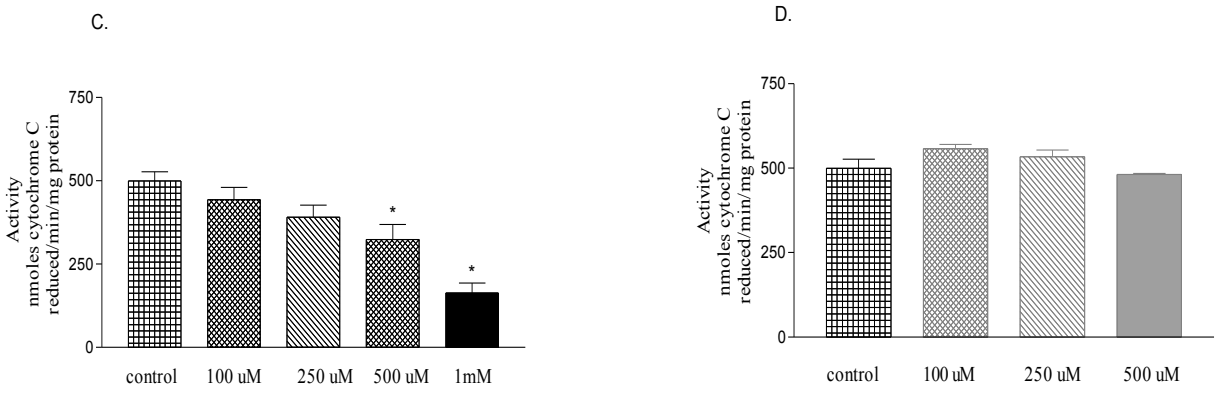


Fig 6 C,D: (C, D) Complex II/III specific activity was measured by following the decrease in absorbance due to cytochrome C reduction at 550 nm, as described in Methods. Freeze thawed rat brain mitochondria were exposed to various concentrations of DAH (C) or DOPAC-H (D) in reaction medium with a 15 min pre-incubation period at room temperature. Values are the mean ± SEM of three separate experiments and are expressed as nmoles of cytochrome C reduction/min/mg protein (*$p < 0.05$, different from control).

FIGURE 7

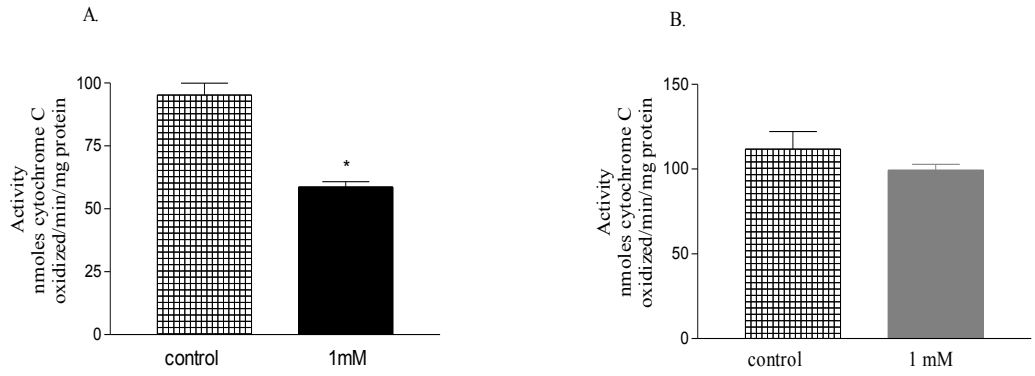


Fig 7: Complex IV specific activity was measured by following the decrease in absorbance due to cytochrome C reduction at 550 nm, as described in Methods. Freeze thawed rat brain mitochondria were exposed to 1 mM DAH (A) and 1 mM DOPAC-H (B) in reaction medium with a 15 min pre-incubation period at room temperature. Activity is expressed as nmoles of cytochrome C reduction/min/mg protein. Values are the mean ± SEM of three separate experiments. ($^{*}p<0.05$ different from control). Complex IV was significantly inhibited by DAH however, this inhibition was concluded to be due to an internal artifact and not a real affect (see results). Complex IV was not significantly affected by DOPAC-H.

FIGURE 8

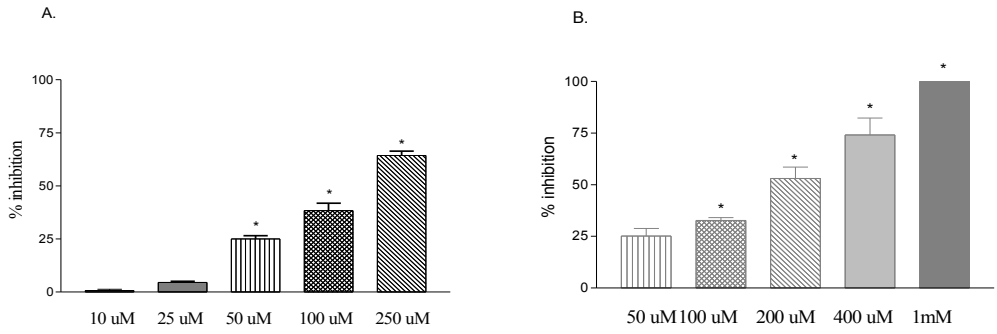


Fig 8: NADH oxidation activity was measured in freeze thawed rat brain mitochondria immediately following exposure to various concentrations of DAQ and DOPAC-Q (A, B) in the reaction medium without a pre-incubation period. Values are the mean ±SEM of three separate experiments and were expressed as % inhibition of control (* $p < 0.05$ different from control). Enzyme activity was dose dependently inhibited by DAQ and DOPAC-Q.

FIGURE 9

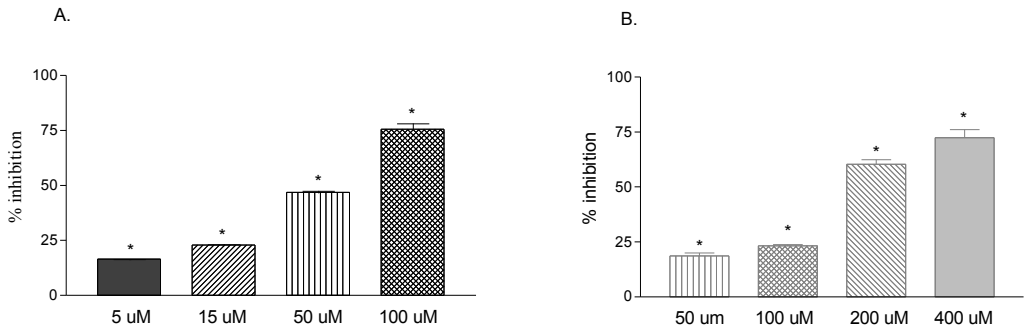


Fig 9 A,B: Complex I specific activity was measured in freeze thawed rat brain mitochondria immediately following exposure to various concentrations of DAQ or DOPAC-Q (A, B). DAQ and DOPAC-Q effects were expressed as mean % inhibition of control. Values are the mean ±SEM of three separate experiments (*$p<0.05$ different from control). Complex I was dose dependently inhibited by DAQ and DOPAC-Q.

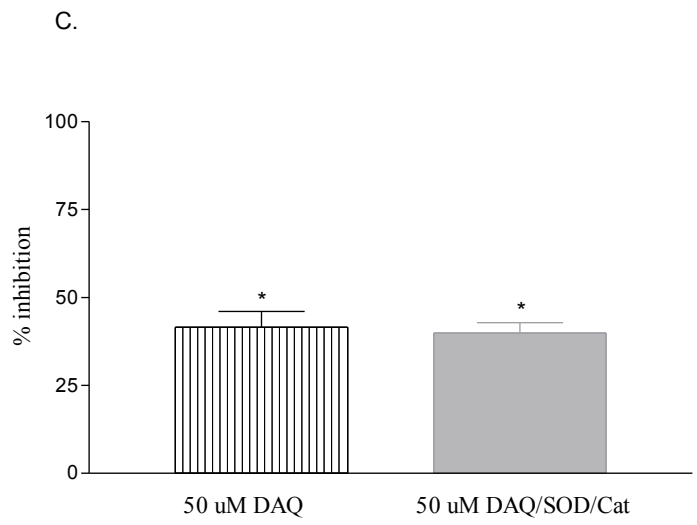


Fig 9C: (C) Freeze thawed mitochondria were exposed to 50 uM DAQ and 50 uM DAQ plus superoxide dismutase (SOD) [2U] and catalase (Cat) [1U]. Activity is expressed as the mean % inhibition of control. Values are the mean ±SEM of three separate experiments. SOD and catalase did not prevent DAQ mediated inhibition. (*$p<0.05$ different from control)

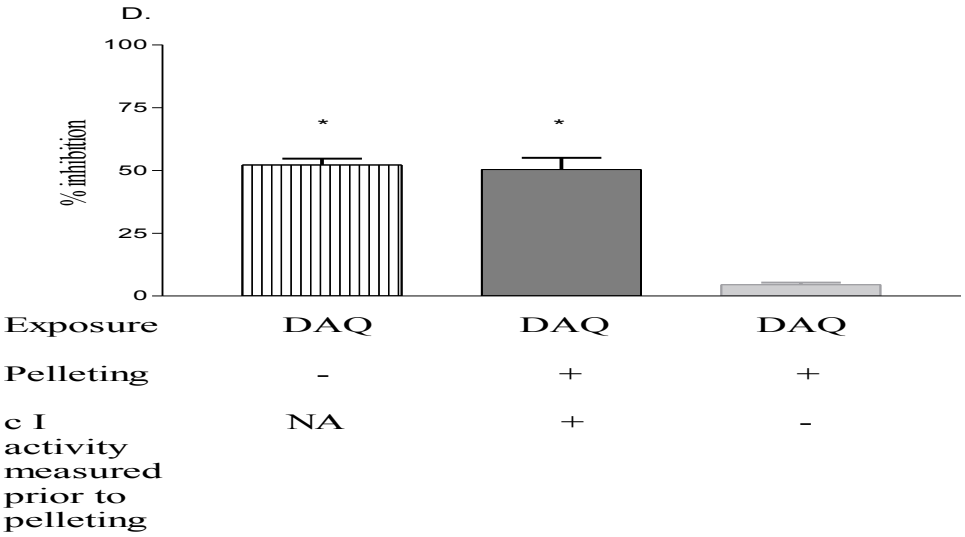


Fig 9D: (D) To examine the reversibility of inhibition, mitochondria were pre-incubated with 50 uM DAQ at room temperature for 30 min, thereafter the enzyme was activated by measuring activity (first bar on graph). The mitochondria from this reaction were then pelleted and the supernatant was removed. Mitochondria were resuspended and complex I activity was re-measured (second bar on graph). As a control, non-activated mitochondria were pre-incubated with DAQ for 30 min at room temperature, pelleted first and then complex I activity was measured. In this preparation complex I activity was not measured prior to pelleting, hence the enzyme was not activated (third bar on graph). Values are the mean ±SEM of three separate experiments (*$p<0.05$ different from control). Only activated complex I was irreversibly inhibited by DAQ.

FIGURE 10

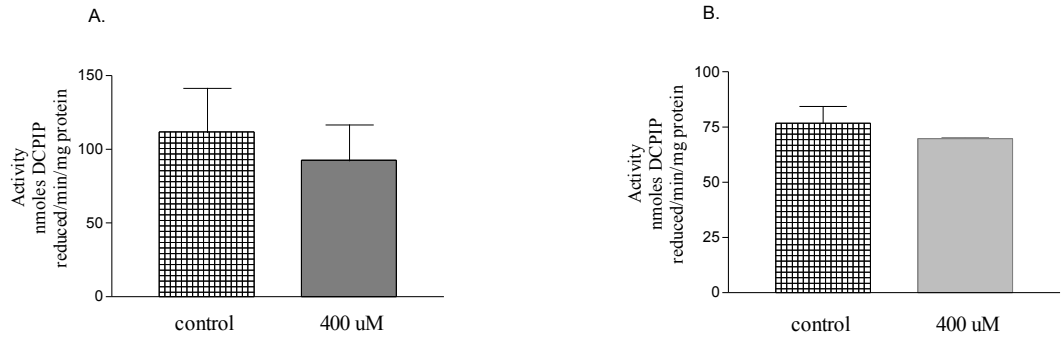


Fig 10: Complex II activity was measured in freeze thawed rat brain mitochondria following exposure to [400 uM] of DAQ (A) or [400 uM] DOPAC-Q (B) for 15 min at room temperature in reaction medium. Each value is the malonate sensitive activity mean ±SEM and is expressed as nmoles of DCPIP reduction/min/mg protein of three separate experiments. Oxidized DA or DOPAC did not attenuate complex II activity.

FIGURE 11

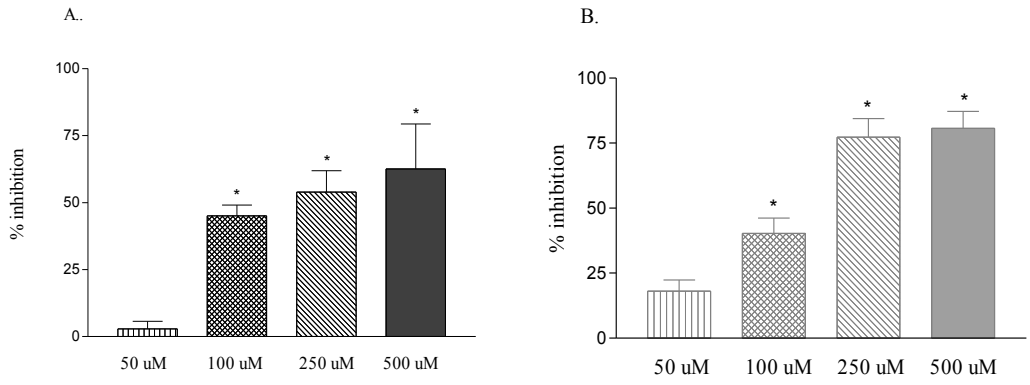


Fig 11 A,B: Complex III specific activity was measured by following the increase in absorbance due to cytochrome C reduction at 550 nm, as described in Methods. Freeze thawed rat brain mitochondria were exposed to different concentrations of DAQ (A) or DOPAC-Q (B) in the reaction medium with a 15 min pre-incubation period at room temperature. Activity is expressed as nmoles of cytochrome C reduction/min/mg protein. Values are the mean ± SEM of three separate experiments (*p<0.05different from control). Complex III activity was dose dependently inhibited by DAQ and DOPAC-Q.

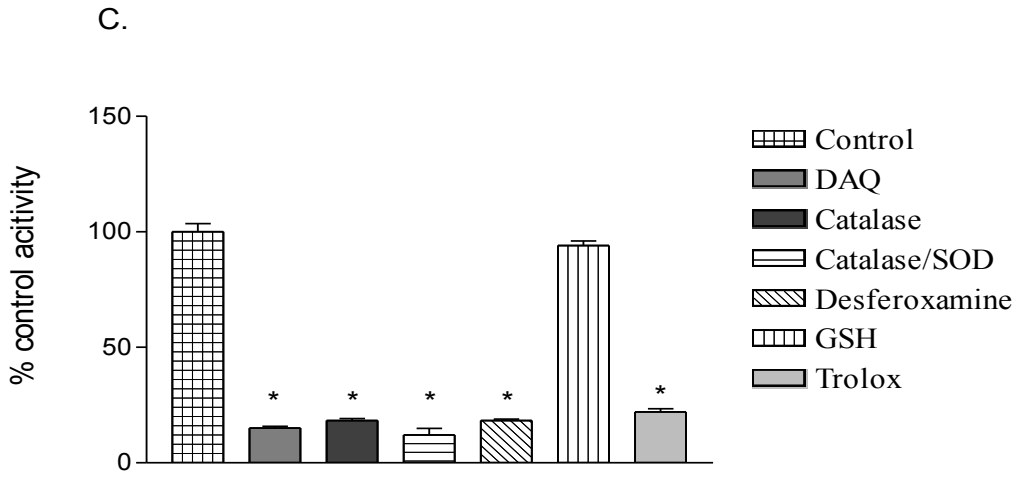


Fig 11C: (C) Complex I/III activity was measured by following the decrease in absorbance due to cytochrome C reduction at 550 nm, as described in Methods. Freeze thawed rat brain mitochondria were incubated with final concentrations of 200 uM DA plus [25U] tyrosinase and with one of the following: 1U catalase, 1U catalase +1U SOD, 1 mM desferoxamine, 3 mM GSH, or 1 mM trolox. Activity is expressed as the mean % of control. Values are the mean ± SEM of three separate experiments (*$p<0.05$different from control). Only GSH provided protection from DAQ mediated inhibition of complexes I/III activity.

FIGURE 12

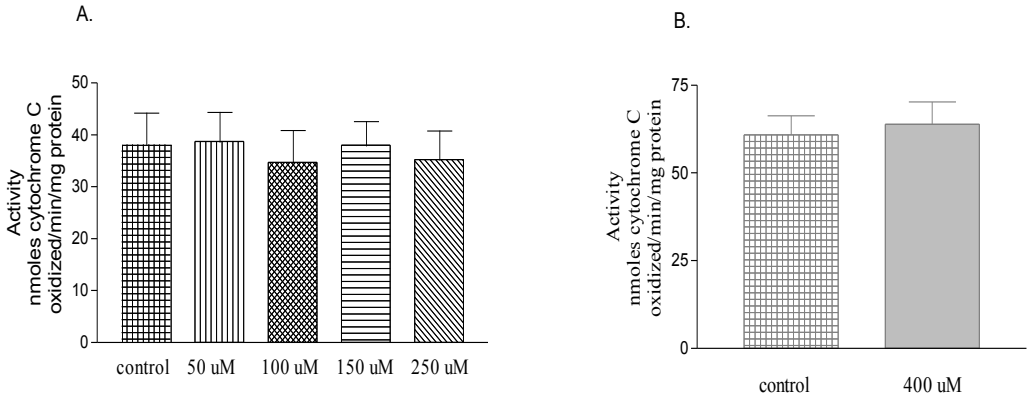


Fig 12: Complex IV specific activity was measured in freeze thawed rat brain mitochondria directly following exposure to various concentrations of DAQ (A) or 400 uM DOPAC-Q (B) in the reaction medium with a 15 min pre-incubation period at room temperature. Activity is expressed as nmoles of cytochrome C reduction/min/mg protein. Values are the mean ± SEM of three separate experiments. Complex IV activity was not attenuated by DAQ or DOPAC-Q.

Table 1: Effects of Reduced and Oxidized DA and DOPAC on ETC Complex Activities:

Mitochondrial ETC complex	DAH	DOPAC-H	IC_{50} Values for DAQ ± SEM	IC_{50} Values for DOPAC-Q ± SEM
NADH-Oxidase (I, III, IV)	No effect at 1mM	No effect at 1mM	158.5 uM ± 0.1	177.8 uM ± 0.1
Complex I	No effect at 1mM	No effect at 1mM	50.1 uM ± 0.036	181.9 uM ± 0.017
Complex II	Significantly inhibited at 500 uM and 1mM	No effect at 1mM	No effect up to 400 uM	No effect up to 400 uM
Complex III	No effect at 1mM	No effect at 500 uM	151.4 uM ± 0.086	109.6 uM ± 0.065
Complex IV	Inhibited at 1mM due to artifactual competing back reaction between oxidized cytochrome C and reduced dopamine	No effect at 1mM	No effect up to 250 uM	No effect up to 400 uM

Table 1: Summary of results for inhibition of mitochondrial electron tansport chain (ETC) activities by reduced dopamine (DAH) and DOPAC (DOPAC-H) and oxidized dopamine (DAQ) and DOPAC (DOPAC-Q). IC_{50} values represent the means ±SEM of three separate experiments for each assay.

C. Discussion

The present study examined the effects of both reduced and completely oxidized DA and DOPAC on ETC enzymes. The study is the first to differentiate between reduced and oxidized DA and DOPAC and measure their effects on each individual ETC complex activity. Three major findings arose from these experiments: 1) reduced DA (DAH) only inhibited complex II activity of the ETC, while reduced DOPAC (DOPAC-H) did not affect any ETC activities 2) oxidized DA and DOPAC (DAQ and DOPAC-Q respectively) inhibited complexes I and III of the ETC and 3) DAQ irreversibly inhibited activated complex I suggesting adduction of the quinone to nucleophilic groups within the active site of complex I leading to inhibition.

In order to confirm the oxidation status of DA and DOPAC at the time of assay both catechols were monitored spectrophotometrically (see methods) prior to measuring activities. Moreover, the time of exposure to DA and DOPAC were carefully assessed since the duration of exposure may contribute to a changing oxidation status and thus differing effects on activities. The results showed that when kept reduced, DA and DOPAC did not inhibit NADH-Oxidase activity with or without a pre-incubation period of up to 30 min. In contrast, no pre-incubation was necessary to fully inhibit NADH-Oxidase when exposure was carried out with the completely oxidized catechols. Thus time of exposure is a factor as DA becomes oxidized. At its extremes, no inhibition of NADH-linked electron flow occurs in the presence of reduced DA or DOPAC, while robust inhibition is observed with the oxidized catechols.

Since the NADH-Oxidase assay measures the combined activities of complexes I, III and IV the effects of the catechols were determined on individual ETC complex activities. Complex I was not inhibited by either DAH or DOPAC-H up to 1 mM with and without a 30 min pre-incubation. This is consistent with Morikawa et. al., (1996) who reported no inhibition of complex I in mouse brain mitochondria by 10 mM DA or 5mM DOPAC. However, time of exposure was not indicated. Khan et al., (2005) also found no inhibition of complex I with DA in rat brain mitochondria with a 15 min pre-incubation. In contrast Ben-Sachaar et al., reported inhibition of complex I in rat brain mitochondria by DA and DOPAC with IC_{50s} of 8.12 ± 0 .75 uM (Ben-Sachaar et al., 1995) and 61.2 ± 8.08 uM (Ben-Sachaar et al., 2004) respectively, with a 1 min pre-incubation. A possible reason for the low IC_{50} for DA mediated inhibition in these studies, may be that they pretreated the mitochondria with 2% digitonin for 1 min prior to assay. Digitonin is used as a detergent to solubilize lipids, and as such, it may have altered the mitochondrial membranes exposing sites sensitive to inhibition that may not have been available in their normal state. In addition, none of the studies mentioned above quantified for quinone formation, leaving open the possibility that the degree to which DA was oxidized in the preparation contributed to the different results.

In the current study, NADH-Oxidase activity was dose dependently inhibited by DAQ and DOPAC-Q with IC_{50s} of 158.5 uM and 177.8 uM, respectively, and complex I inhibited by DAQ with an IC_{50} of 50.1 uM. Ben-Sachaar and colleagues (2004) reported that desferoxamine partially attenuated the DA mediated inhibition of complex I activity. Since DA oxidation would be prevented by desferoxamine, the findings from this study

would be consistent with a quinone mediated inhibition. Our results of quinone mediated inhibition of complex I are also consistent with Khan et al. (2005) and Jana et al. (2007) who reported inhibition of complex I by 400 uM DA after an incubation period of 2 hrs Khan et. al. (2005) measured quinone production spectrophotometrically after the 2 hr incubation and found an increase in absorbance, which was interpreted as quinone formation. Moreover, similar to our findings, Khan et al., (2005) found that 5 mM GSH prevented DA mediated inhibition. GSH can keep DA in its reduced form and prevent quinone formation or chelate quinones already formed, thus further supporting a toxic role for quinones. Although Khan et al (2005) monitored for general quinone production, it was not quantified making the concentration of quinone required for inhibition uncertain.

Complex I was also shown to be dose dependently inhibited by DOPAC-Q with an IC_{50} of 181.9 uM. This result is at variance with Jana et al. (2007), where only a marginal but statistically significant inhibition of complex I activity was observed in the presence of 400 uM DOPAC after 2 hrs incubation. They did not monitor for DOPAC quinone formation, however they measured quinoprotein adduct formation and did not observe any with DOPAC exposure. It was concluded that DOPAC was far less toxic to ETC activities than DA. It should be noted that in their study they air oxidized both DA and DOPAC. In the current study, DOPAC had to be oxidized via tyrosinase because it did not air oxidize as did DA. Therefore, taking into account the lack of DOPAC mediated quinoprotein formation and method of oxidation in the Jana et al., (2007) study; it leaves to question whether DOPAC had oxidized in their assay conditions. Our findings

would indicate that in the oxidized state, DOPAC is similar in potency to DA in inhibiting complex I. This is consistent with previous findings from the laboratory on the effects of DA and DOPAC on mitochondrial O2 consumption where DA and DOPAC were equipotent in inhibiting respiration. (Gluck and Zeevalk, 2004).

The present study is the only study to investigate the effects of reduced and oxidized DA and DOPAC on the individual activities of complexes II and III. Malonate sensitive complex II activity was significantly inhibited by 1 mM DAH but not by DOPAC-H or oxidized DA and DOPAC. Monitoring of the malonate sensitive rate its important as DAH can pass electrons to DCPIP and mask inhibition. The superoxide chelator SOD significantly protected from DAH mediated inhibition, suggesting that ROS generation contributed to the inhibition. In the current study the effects of the reduced catechols on complex III activity could not be measured due to an internal artifact created by an interaction between DAH, DOPAC-H and reduced cytochrome C that was not amenable to correction (see Methods and Results for details). Therefore, the effects of DAH and DOPAC-H were assayed on complex II/III activity. Complex II/III activity was inhibited by DAH comparable in magnitude to the inhibition observed with complex II. Further, DOPAC-H did not inhibit complex II or complex II/III activites. Considering that NADH-Oxidase activity, which includes complex III activity, was not inhibited by either reduced catechol and that complex II was inhibited by DAH but not by DOPAC-H, the evidence would suggest that complex III activity was not affected by the reduced catechols. Both oxidized catechols inhibited complex III activity in a dose dependent manner with IC50s of 151.4 uM and 109.6 uM, respectively.

Ben-Sachaar et al., (2004) reported a dose dependent inhibition of complex I/III by DA, however since they reported inhibition of complex I; it is unclear whether complex III was also inhibited by DA. Also in question was the oxidation state of DA in this study.

Complex IV activity was significantly inhibited by 1 mM DAH. This inhibition, however, was concluded to be an internal artifact created by the interaction between DAH and reduced cytochrome C mimicking inhibition (see Results for details), since NADH-Oxidase activity, which includes complex IV activity, was not attenuated by DAH. Ben-Sachaar et al., (2004) also reported a lack of inhibition of complex IV by DA up to 1mM with a 1 min incubation. In the present study, oxidized DA up to 250 uM did not inhibit complex IV activity. In contrast with our findings of no inhibitory effect of DAQ on complex IV, Khan et al., (2005) and Jana et al., (2007) reported 62.8 % and 57 % inhibition of complex IV by 400 uM DA following 2 hrs of incubation. Since the IC_{50} for DAQ inhibition in the NADH-Oxidase assay in our study was 158.5 uM, we did not test for the effects of DAQ at concentrations greater than 250 uM. It is possible; therefore, that higher concentrations of DAQ may have inhibited complex IV activity. On the other hand, DAQ concentration was not quantified in the Khan et al., (2005) or Jana et al., (2007) studies where 400 uM DA was used. Consequently, there may have been residual DAH still present in the assay medium at 2hr. As we have observed, DAH can interact with the product of the complex IV assay, i.e. oxidized cytochrome C producing a competing back reaction that would mimic inhibition. Thus, the studies of Khan et al. (2005), and Jana et al., (2007) may have observed a competing back reaction between DAH and cytochrome c and interpreted this as inhibition. Complex IV activity

was not affected by reduced or oxidized DOPAC with a 15 min pre-incubation, which is in accordance with Jana et al., (2007) who reported only marginal effects on complex IV activity by 400 uM DOPAC in rat brain mitochondria after a 2 hr pre-incubation.

DA oxidation produces o-quinones that are highly reactive electrophiles that can covalently bind to cellular nucleophiles such as DNA and reduced sulfhydryl groups contained in protein cysteinyl residues via 5'cysteinyldopamine forming 5-Cys-DA protein adducts (Stokes et al., 1999; Bindoli et al., 1992). If the cysteinyl residue that DAQ covalently adducts to is found at the active site of a protein, it may inhibit protein function and possibly lead to cellular damage and /or cell death. Although the formation of quinoproteins was not investigated in this study, there are several studies that report the formation of quinoproteins in mitochondrial proteins after exposure to DA (Khan et al 2001; Jana et al., 2007). Quinoprotein formation indicates covalent modification of cysteinyl residues on proteins by quinone forming catechols such as DAQ. Using 2D difference in gel electrophoresis, Van Laar et al., (2008a) showed that in intact rat brain mitochondria exposed to DA plus tyrosinase (to form DAQ), several mitochondrial proteins including the key complex I 75-kDa subunit, demonstrated loss of cyteine reactive residues indicating quinone adduction. LaVoie and Hastings (1999a) reported toxicity to DA terminals in the striatum of methamphetamine (METH) treated animals which was accompanied by a threefold increase in protein cysteinyl-DA adducts. DAQ induced toxicity has also been shown in respiring mitochondria. Berman and Hastings (1999) reported that dopamine quinone caused a large increase in isolated intact mitochondrial resting state 4 respiration and caused a significant increase in swelling of

rat brain mitochondria, suggesting that DA and/or DAQ gains entry into intact mitochondria and modifies critical proteins. A recent study by Van Laar et al., (2008b) supports this as evidence is provided to show that ^{14}C-DAQ covalently binds to multiple subunits of Complex I (75kDa and 30kDa subunits) and Complex III (ubiquinol-cytochrome *c* reductase core protein 1 and Rieske Fe–S protein) in intact rat brain mitochondria. Our results compliment these finding to show that complexes I and III are inhibited by the quinones of DA and DOPAC and that substrate stimulated complex I is irreversibly inhibited by DAQ. DA oxidation occurs more readily at a pH above 7.4. The mitochondrial matrix pH has been reported to be 7.98 in HeLa cells and 7.91 in rat cardiomyocytes (Llopos et al., 1998). Therefore, if reduced DA or DOPAC were to gain access to the mitochondrial matrix, they would likely oxidize and increase the potential to interact with ETC proteins.

The findings reported here suggest that many of the inconsistencies in the literature with regard to the sensitivity of ETC complexes to inhibition by DA or DOPAC are due to the oxidation state of the catechols. With the exception of complex II, the reduced catechols show no inhibitory effect on ETC activities. Further, the presence of the reduced catechols and in particular DAH can produce artifacts that can either mask inhibition (complex II and III) or mimic inhibition (complex IV). Thus, care must be taken to ensure that non-ETC related redox chemistry is taken into account. It was further observed that pre-incubation time is only a factor when the redox status of the catechol changes from reduced to oxidized. In contrast with the reduced catechols, oxidized DA and DOPAC inhibited complexes I and III. Further studies on the effects of DA and

DOPAC on ETC activities in intact mitochondria and characterization of uptake of the catechols are needed.

Chapter IV: EFFECTS OF EXTRA MITOCHONDRIAL DOPAMINE AND DOPAC ON ETC FUNCTION IN INTACT MITOCHONDRIA.

A. Introduction

In dopaminergic neurons, more than 90% of dopamine is sequestered in terminal vesicles and is protected from degradation (Eisnhofer et. al., 2004). When not sequestered in vesicles, cytosolic DA can be toxic. DA has been hypothesized to act as an endogenous neurotoxin contributing to the neurodegeneration seen in Parkinson's disease (Graham, 1978; Hastings et. al., 1996; Stokes et. al., 1999). Studies by Cyr et. al., (2003) have shown that sustained elevation of extracellular DA in DA transporter (DAT) knock-out mice caused striatal neuronal degeneration. Jiang et. al., (2008) reported that exposure of neuroblastoma cell line (SH-SY5Y) to DA transporter antagonist GBR 12909 and DA induced cell toxicity (Jiang et. al., 2008). Incubation with GBR 12909 increases extracellular DA concentrations, therefore Jiang et. al., (2008) reported that DA toxicity was due to extracellular DA oxidation rather than DA receptor overstimulation. Several studies have showed that DA and/or its metabolite, 3,4-dihydroxyphenylacetic acid (DOPAC) inhibited the ETC activities in freeze thawed mitochondria (Gluck et. al., 2004; Khan et. al., 2005; Ben-Sachar et. al., 2004) suggesting that in addition to extracellular targets, mitochondria may be a downstream target of increased cytosolic DA and/or DOPAC. Inconsistencies amongst these studies, however, raised questions regarding which catecholamine species, i.e. reduced or oxidized, was responsible for the inhibition. In Aim 1 of this thesis, it was shown that oxidized DA (DAQ) and DOPAC (DOPAC-Q) were more toxic to ETC activities, specifically complexes I and III than

their reduced counterparts. Moreover, DAQ irreversibly inhibited complex I activity, suggesting that the inhibition was due to covalent binding to complex I proteins.

We previously showed that complex I activity was irreversibly inhibited by oxidized DA and DOPAC (chapter 3). It was hypothesized that the inhibition was due to covalent binding of the oxidized DA quinones to complex I proteins. Complex III activity was also shown to be inhibited by both oxidized catechols, although reversibility of the inhibition could not be assessed. DA oxidation occurs more readily at a pH above 7.4. Llopos et. al. (1998) have shown that the mitochondrial matrix pH is 7.98 in HeLa cells and 7.91 in rat cardiomyocytes. Therefore, if reduced DA or DOPAC were to gain access to the mitochondrial matrix, they should readily possibly oxidize and increase the potential to interact with ETC proteins. Our previous studies were done in freeze thawed rat brain mitochondria and it was unclear whether DA or DOPAC could access the mitochondrial matrix to inhibit ETC function. Therefore, the current study investigated DA and DOPAC uptake into intact rat brain mitochondria and the effects of reduced DA and DOPAC on complex I activity in freshly isolated intact rat brain mitochondria.

B. Results

Dopamine uptake by intact brain mitochondria.

The enzymes of the mitochondrial ETC are embedded in the inner membrane of mitochondria; therefore the effects of DA would only be relevant if DA can gain access to the enzymes. To address this, uptake of various concentrations of DA was measured in isolated intact rat brain mitochondria incubated with ^{3}H-labeled DA as described in Methods and was quantified by scintillation counting. The exposure paradigm used reduced DA since 1) previous work by the laboratory showed that exposure of intact

mitochondria to reduced DA or DOPAC resulted in inibition of O_2 consumption and 2) vesicular release of DA into the cytosol, at least in terms of immediate exposure, would be to DA and DOPAC in their reduced forms. Mitochondrial dopamine concentration after incubation with 1.0mM of DA was 9.2 ± 0.81 nmol/mg protein (±SEM), for 5.0mM and 10.0mM it was 48.2 ± 3.60 and 78.60 ± 6.50 nmol/mg protein (±SEM) respectively, and for 15.0 mM it was 137.0 ± 24.30 nmol/mg protein (±SEM) (Fig 13A: pg 91, Table 2). The DA uptake was energy dependent as only minimal counts were observed at 0˚C and these non-specific counts due to binding were subtracted from counts at 30˚C (data not shown). Uptake was also time dependent, percentage of total DA uptake was measured at various time points. At 1.25 min % of total uptake was 2.10 ± 0.60 (±SEM), at 2.5 min it was 21.80 ± 2.60 (±SEM), at 5.0 min it was 55.0 ± 4.0 (±SEM), and uptake was saturated by 10.0 min 100.00 ± 5.50 (±SEM) (Fig 13B: pg 91, Table 3). DA uptake was independent of DA vesicular uptake, , DAT uptake, metabolism by MAO A or B or free oxidation since uptake was determined in the presence of R041284, mazindol, clorgyline, pargyline and ascorbate, inhibitors of VMAT2 uptake, DAT uptake, MAO A and B and a general antioxidant, respectively.

FIGURE 13

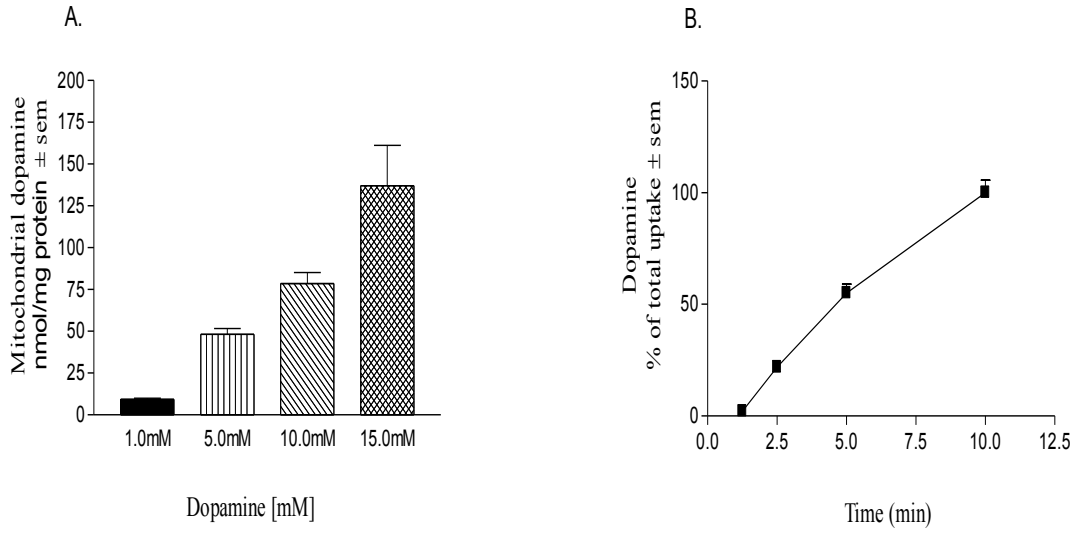


Fig 13: A) Dopamine uptake in isolated intact rat brain mitochondria was determined by incubation for 10 min at 30C with 3H-labeled dopamine as described in Methods. Ascorbate (200μM), Ro4-1284 (10μM), mazindol (100μM), clorgyline (4μM) and pargyline (4 μM) were present to block autoxidation, dopamine vesicular uptake, uptake by the dopamine transporter, and metabolism of dopamine by MAO A or B, respectively. Radiolabeled dopamine extracted from the mitochondrial matrix was quantified by scintillation counting. Uptake was expressed as concentration of mitochondrial DA nmol/mg of protein. DA concentrations used were 1.0mM, 5.0mM, 10.0mM and 15.0mM. B) Time dependence of uptake of dopamine by intact mitochondria. Results were expressed as % of total DA uptake at time points of 1.25, 2.5 min, 5.0 min, and 10.0 min. Values were the mean ±SEM of three separate experiments run in duplicate.

Table 2: Mitochondrial concentration of DA quantified by scintillation counting.

Exposure Concentration	DA, 1.0 mM	DA, 5.0mM	DA, 10.0 mM	DA, 15.0 mM
DA concentration in mitochondria nmol/mg protein ±SEM	9.2 ± 0.81	48.2 ± 3.60	78.60 ± 6.50	137.0 ± 24.30

Table 2: Isolated intact mitochondria were incubated with 3H-labeled dopamine supplemented with cold dopamine to final concentrations of 1-15 mM for 10 min at 30°C. Mitochondrial DA concentrations were measured by scintillation counting. Values are means ±SEM of three individual experiments.

Table 3: Time dependent mitochondrial DA uptake**.**

	Time, min			
% of total uptake ±SEM	1.25	2.50	5.0	10.0
	2.10 ± 0.60	21.80 ± 2.60	55.0 ± 4.0	100.00± 5.50

Table 3: Summary of results for time dependent mitochondrial DA uptake. Isolated mitochondria were incubated with DA at 30°C and uptake was quantified by scintillation counting at various time poins. DA uptake was saturated at 10.0 min. Values are means % of total uptake ±SEM of three separate experiments.

Complex I activity was attenuated by extra mitochondrial DAH or DOPAC-H.

Prior to exposure to the catecholamines, the respiratory control rates (RCR) of the mitochondrial preparations were established. RCRs were determined by calculating the ratio of state 3 (active respiration) to state 4 (resting respiration) respiration rates. State 3 or active respiration is the rate of oxygen consumption following the addition of adenosine diphosphate (ADP) in the presence of mitochondria substrates glutamate + malate. These substrates supply the energy necessary to drive the ETC. The ETC consists of four enzyme complexes that take part in redox reactions in which electrons are transferred from a donor molecule to an acceptor molecule. The energy from the transfer of electrons from donor to acceptor molecules is used to force protons from the mitochondrial matrix to the intermembrane space, creating an electrochemical proton gradient across the mitochondrial inner membrane (Berg et. al., 2007). In the presence of inorganic phosphate, ADP binds ATP synthase, an enzyme complex in the inner mitochondria membrane that synthesizes adenosine triphosphate (ATP) from ADP and inorganic phosphate. ADP binding opens a channel that permits the driving of protons accumulated in the inner membrane via the ETC into the matrix (Berg et. al., 2007). The energy that is released as protons move down their electrochemical gradient is used to produce ATP known as oxidative phosphorylation. As energy in the gradient is removed, the ETC spontaneously speeds up, thus increasing oxygen consumption. The correlation between oxidative phosphorylation and ETC is termed coupling. Therefore, the addition of ADP increases oxygen consumption. In isolated mitochondria, oxygen consumption decreases as ADP levels decrease. This decrease in oxygen consumption due to the absence of ADP is known as inactive or state 4 respiration. Oxygen consumption despite

the absence of ADP is an indication of uncoupling, wherein protons move into the matrix via another passageway other than ATP synthase (Berg et. al., 2007). Uncoupling in isolated mitochondria may be caused by chemical uncouplers or damaged membranes that form a pathway for protons to diffused back into the matrix. RCR rates ranging from 6-12 indicate good mitochondria membrane integrity, i.e. the mitochondria are well coupled. The mean RCR rate for mitochondria used in measuring complexes I and III activities following DA and DOPAC exposure was 8.3 ± 1.5 (±SEM). After extablishment of the RCR, intact mitochondria were pre-incubated with various concentrations of DAH or DOPAC-H for 10 min at 30°C with (see Methods). The samples were then centrifuged and the supernatant was discarded removing any unbound catechols. The resulting pellet was resuspended, washed and centrifuged. The final pellet was freeze thawed and used for complexes I and III activities measurement. Complex I activity was dose dependently inhibited by both DAH and DOPAC-H exposure. (Fig 14: pg 95, Table 4). The dose dependent inhibition of complex I following exposure of intact mitochondria to DAH or DOPAC-H indicates the irreversible nature of the inhibition. These results further support the hypothesis that increased cytosolic DA can access the mitochondrial matrix and through binding of the catechol quinone to complex I proteins results in activity inhibition.

FIGURE 14

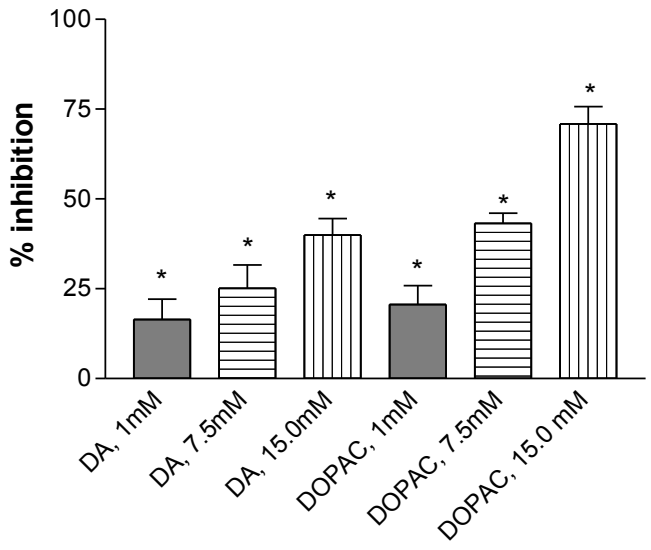


Fig 14: Complex I specific activity was measured in the freeze thawed rat brain mitochondria following incubation of intact rat brain mitochondria with various concentrations of DAH or DOPAC-H after which the samples were pelleted and freeze thawed to lyse. DAH and DOPAC-H effects were expressed as mean % inhibition of control. Values were the mean ±SEM of three separate experiments (*$p<0.5$ or better different from control). Both DAH and DOPAC-H inhibited complex I activity in a dose dependent manner.

Extra mitochondrial DAH, DOPAC-H inhibits complex III activity.

Complex III activity was dose dependently inhibited by 1mM DAH by 29.9% ± 8.7(± SEM), 43.4% ± 6.1 (± SEM) at 7.5mM DAH, 61.2% ± 5.9 (± SEM) at 15.0mM DAH (Fig 15: pg 97, Table 4). DOPAC-H, 1.0mM inhibited complex III activity by 26.1% ± 11.8 (± SEM) at 1.0mM DOPAC-H, 52.8% ± 13.8 (± SEM) at 7.5mM DOPAC-H, 61.9% ± 13.1 (± SEM) at 15.0mM DOPAC-H (Fig 15: pg 97; Table 4). These results suggest that reduced DA and DOPAC gain entry into intact mitochondria and effect ETC activity.

FIGURE 15

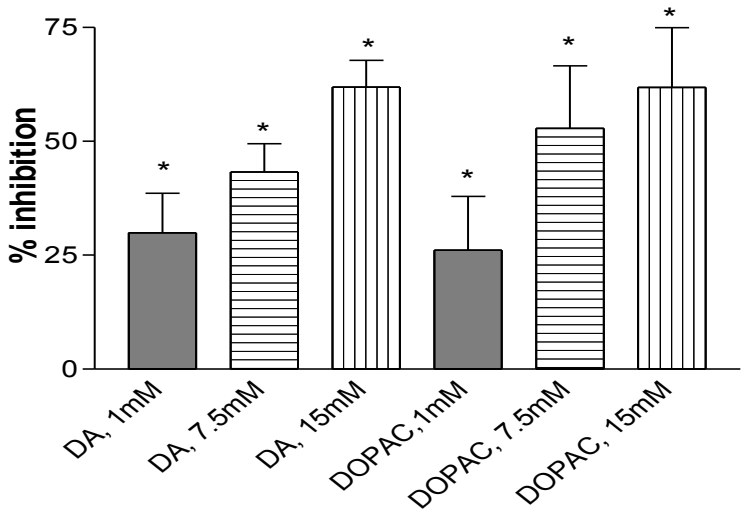


Fig 15: Complex III specific activity was measured by following the increase in absorbance due to cytochrome C reduction at 550 nm, as described in Methods. Intact rat brain mitochondria were pre-incubated with various concentrations of DAH and DOPAC-C, thereafter the samples were pelleted,washed and freeze thawed to lyse the mitochondria. Complex III activity was measured in the freeze thawed mitochondria. Activity was expressed as the mean % inhibition of control. Values were the mean ±SEM of three separate experiments (*$p<0.5$ or better different from control). Complex III activity was dose dependently inhibited by DAH and DOPAC-H.

Table 4: Effects of DA and DOPAC on complexes I and III activities

Mitochondrial ETC complex	Mean % inhibition of control ± SEM					
	DA 1.0 mM	DOPAC 1.0 mM	DA 7.5 mM	DOPAC 7.5 mM	DA 15.0 mM	DOPAC 15.0 mM
I	16.4± 5.7	20.6 ± 5.3	25.1± 6.5	43.2 ± 2.9	39.9± 4.6	70.9± 4.8
III	29.9± 8.7	26.1± 11.8	43.4± 6.1	52.8± 13.8	61.2± 5.9	61.9±13.1

Table 4: Summary of results for inhibition of ETC complexes I and III activities by DA and DOPAC. Values represent the means % inhibition of control ±SEM of three separate experiments for each assay.

C. Discussion

Results from Aim 1 showed that oxidized dopamine (DAQ) and its metabolite DOPAC (DOPAC-Q) inhibit mitochondrial ETC activities, specifically complexes I and III in lysed rat brain mitochondria. Moreover, we have shown that complex I was irreversibly inhibited by DAQ suggesting covalent binding of the quinone to complex I proteins. The effects of DA and DOPAC on ETC activities of lysed mitochondria are relevant only if these products gain entry into intact mitochondria. Hence, the objectives of this study were to investigate whether extra-mitochondrial DA and DOPAC could gain access to inner mitochondrial matrix and if mitochondrial ETC activities, specifically complexes I and III, were affected by extra mitochondrial DA or DOPAC. The third objective of our study was to investigate if radiolabeled DAQ binds to mitochondrial membranes containing the ETC complexes, however this study was not conducted since, recently, Van Laar et al., (2008b) reported that ^{14}C-DAQ covalently binds to multiple subunits of Complex I (75kDa and 30kDa subunits) and Complex III (ubiquinol-

cytochrome *c* reductase core protein 1 and Rieske Fe–S protein) in intact rat brain mitochondria, supporting our earlier findings.

DA, DOPAC and intact mitochondria

Tritiated DA (^{3}H-DA) uptake into intact mitochondria was quantified by scintillation counting and also by measurement by HPLC with electrochemical detection with similar results. We observed active, time dependent DA entry into intact, well coupled rat brain mitochondria. The mechanism of DA uptake into the mitochondria is unknown. In the present study VMAT and DAT inhibitors were included in the study, suggesting that DA accumulation by mitochondria was independent of transporters. These results corroborate a recent study which also reported VMAT and DAT independent DA accumulation into intact mitochondria isolated from the dopamine cell line SH-SY5Y cells (Brenner-Lavie et. al., 2008). In this study, however, uptake was inhibited by MPP+, suggesting that DA accumulation was dependent upon the mitochondrial membrane potential. Indeed, significant dissipation of mitochondrial membrane potential ($\Delta\psi m$) was observed by Brenner-Lavie et. al.,(2008). Alternatively, Berman and Hastings (1999) reported a significant increase in mitochondria swelling after exposure to oxidized DA. The swelling was inhibited with the addition of cyclosporin A, suggesting that DAQ may cause the opening of mitochondrial transition pores (MTP) since cyclosporin A inhibits MTP opening. The findings by Berman and Hastings (1999) suggest that DA entry into mitochondria may be due to compromises in the mitochondrial membranes. MTPs allow passage of solutes smaller than m.w. of 1500

dalton, therefore, mitochondria may become permeable to DA if it is present in too high a concentration in the cytosol.

Complexes I and III activities and extra-mitochondrial DA and DOPAC

DA and DOPAC caused dose dependent significant inhibition of complex I activity in intact rat brain mitochondria that were pre-incubated with various concentrations of the catechols. Berman and Hastings (1999) have reported DAQ mediated toxicity to respiring mitochondria; however their study did not measure direct complex I or III activities as it measured respiration rates. In contrast to our earlier findings of the effects of oxidized DA and DOPAC on complex I activity (chapter 3) wherein oxidized DA was more potent an inhibitor of complex I activity than DOPAC (IC_{50s} of 50.1 ± 0.036 and 181.9 ± 0.01 respectively, mean uM ± SEM), in intact mitochondria, the inhibitory effects of DA and DOPAC at lower concentrations (1.0mM and 7.5mM) were equipotent, however at the higher concentration (15.0mM) DOPAC inhibited complex I activity significantly more than DA. One possible reason for this may be that at higher concentrations more DOPAC gained entry into intact mitochondria as compared to DA. Complex III activity was similarly inhibited by DA and DOPAC at all concentrations tested. This supports our earlier study (chapter 3) in which DA and DOPAC were found to have comparable IC_{50s} for complex III inhibition. Since DA and DOPAC inhibition for complexes I and III differed only at the 15.0mM concentration and only for complex I, this difference might also be due to experimental error and would need to be reexamined before drawing any conclusions. The findings, however, are in

accord with previous work from this laboratory (Gluck and Zeevalk, 2004) to show that DOPAC was equipotent with DA in inhibitory O_2 consumption in intact mitochondria.

The findings from this aim are the first to show that extra-mitochondrial DA and/or DOPAC can affect complexes I and III activities in intact mitochondria. This suggests that the catechols gained entry into the mitochondria. The mitochondria were incubated with DA, DOPAC in the presence of ascorbate which would have prevented catechol oxidation. This supports that reduced DA, DOPAC gained entry into the mitochondria. Mitochondria matrix pH has been reported to be 7.98 in HeLa cells and 7.91 in rat cardiomyocytes, an environment that would promote catechol oxidation (Llopos et al., 1998). Therefore DA or DOPAC could have oxidized once inside the mitochondria. Moreover, the inhibitory affects of DA and DOPAC may be due to covalent binding of oxidized DA or DOPAC to complex I and III proteins since the intact mitochondria were pelleted and washed and the supernatant discarded after pre-incubation with the catechols, which would have removed any unbound DA or DOPAC in the supernatant. Indeed, negligible traces of DA or DOPAC quantified by HPLC were detected in the final supernatant (data not shown). Results from Aim 1 corroborate this hypothesis since the results showed irreversible inhibition of complex I by oxidized DA. Complex III irreversiblility could not be ascertained due to technical difficulties, however results from Aim 2 indicate that inhibition of complex III is also irreversible. Findings by Van Laar et. al., (2008b) further support our results as they reported binding of oxidized DA to complex I and III subunits in intact rat brain mitochondria. Future studies investigating DOPAC uptake into intact mitochondria are still necessary to determine if extra-mitochondrial DOPAC has a similar time and temperature dependence as DA.

Additionally, as was done with oxidized DA by Berman and Hastings (1999), the effects of oxidized DOPAC on mitochondrial membrane integrity should be investigated as this could offer an explanation for the difference between effects of DOPAC and DA on complex I activities at the higher concentration.

Chapter IV: REGION, GENDER, AGE AND CELL SPECIFIC CHARACTERIZATION OF GLUTAREDOXIN IN RAT BRAIN.

A. Introduction

Loss of dopaminergic neurons and mitochondrial dysfunction in the nigrostriatal pathway are some of the hallmarks of PD. Dopamine, therefore, has been thought to be an endogenous toxin related to disease pathology. Deficits in bioenergetics could lead to a loss of DA homeostasis. The first two aims of this dissertation, therefore, investigated the consequences of DA and its metabolites on mitochondrial function. Oxidative stress and decreased levels of GSH are also hallmarks of PD. GSH is part of the major antioxidant system in brain (Meister, 1995). An important component of this system is the enzyme glutaredoxin (Grx) which has recently been identified in whole brain cytosol (Balijepalli et. al., 1999) Grx activity has been localized to mitochondria (Ehrhart et. al., 2002). Glutaredoxin is an important defense against oxidative stress (Ehrhart and Zeevalk, 2003; Zeevalk et. al., 2003) and in maintaining mitochondrial function (Kenchappa and Ravindranath., 2003). The focus of aim 3 is to better characterize Grx in order to provide a foundation for understanding the role of Grx in pathophysiological processes.

Oxidative stress is a common pathological hallmark of various neurological disorders such as Alzheimer's disease (AD), Amyotrophic lateral sclerosis (ALS), Parkinson's disease (PD), Huntington's disease (HD) and neuropsychiatric diseases such as autism and schizophrenia (Bains and Shaw, 1997; Do et. al., 2000; Yao et. al., 2001). The brain is particularly vulnerable to the deleterious effects of oxidative stress since

compared to other tissues; it utilizes the highest amount of oxygen to produce energy and is deficient in antioxidant systems with lower activity of glutathione peroxidase and catalase compared to other organs (Kedar, 2003). A known consequence of oxidative stress is the formation of protein-glutathione-mixed disulfides (Pr-SSGs) between protein cysteines and glutathione and is termed S-glutathiolation or S-glutathionylation (Holmgren, 1989; Prinz et. al., 1997; Ritz et. al., 2000). Protein modification by glutathiolation is now recognized as a means of controlling physiological and pathophysiological pathways in cells (Ghezzi, 2005). During oxidative stress, glutathiolation is thought to protect protein cysteine residues from irreversible oxidation (Ehrhart and Zeevalk, 2003; Zeevalk et. al., 2003). Deglutathiolation can be achieved by enzymatic cleavage of the disulfide bond involving the action of oxidoreductase enzymes thioredoxin (Trx), protein disulfide isomerase (PDI) and glutaredoxin (Grx) (Jung and Thomas, 1996). Both Trx and Grx dethiolate protein-glutathione-mixed disulfides however, Grx is considered the principle enzyme involved in this reaction because it is 10-fold more efficacious in removing glutathione from proteins (Gravina and Mieyal 1993; Chrestensen et.al., 2000).

Glutathione is the major water soluble antioxidant in the brain (Meister, 1991). It exists in its reduced (GSH) and oxidized forms (GSSG). Under normal physiological conditions, the intracellular environment is a reducing environment, with GSH/GSSG ratio at around 100 (Gilbert, 1995). The high GSH/GSSG ratio comprises a major redox buffer in cytosol (Gilbert, 1995). Most S-glutathiolation is transient, and is readily reversed by Grx using GSH as reductant under reducing conditions however, under

oxidizing conditions S-glutathiolation can be maintained indefinitely (Gilbert, 1995). Deglutathiolation by Grx helps replenish GSH levels and helps maintain the normal reducing environment that is critical to cell survival (Schafer and Beuttner, 2001). Glutaredoxins (Grx) are low-molecular-weight proteins (9–12 kDa) (Holmgren and Aslund 1995). Grx enzymes comprise a family of isoforms that are localized to different sub-cellular compartments. Cytosolic Grx (Grx-1) protein and message has been identified in rat brain neurons and purified from whole brain cytosol (Balijepalli et. al., 1999). The gene for a nuclear/ mitochondrial Grx (Grx-2) has also been reported (Lundberg et. al., 2001; Gladyshev et. al., 2001) and brain mitochondria have been shown to contain Grx activity (Ehrhart et. al., 2002). Since, S-glutathiolation of proteins is increased in neuronal cells in response to oxidative stress (Ehrhart and Zeevalk, 2001; Zeevalk et. al., 2003), Grx-1 and Grx-2 may play important roles in brain during cellular recovery from oxidative stress by restoring protein thiol homeostasis by means of its deglutathiolation function.

There have been only a few studies to examine Grx in brain (Balijepalli et. al., 1999; Takagi et. al., 1999; Kenchappa et. al., 2004; Karunakaran et. al., 2007), although none of the studies provide a full characterization. Characterization of Grx in brain could provide insight into the potential contribution of this enzyme to disease processes with regard to gender, age or regional susceptibility. The current study was, therefore, undertaken to examine differences in Grx-1 expression and activity in rat brain at different ages of development, among different brain regions and the influence of gender taking into account stages of the estrous cycle. In addition, semi-quantitative in situ

hybridization and immunocytochemical studies for Grx-1 suggest the absence of Grx-1 in glia (Takagi et. al., 1999, Akterin et. al., 2006). Paradoxically, astrocytes are more resistant to oxidative stress and have a higher glutathione concentration than neurons (Rice and Russo-Menna, 1998; Kitimura et. al., 1999). To examine the cell specific expression of Grx more precisely in neuronal cells, the current work also measured, by quantitative PCR, Grx-1 and Grx-2 message in purified cultures of mesenchephalic neurons and astrocytes.

B. Results

Grx-1 activity and protein in various regions of rat brain. Activity of glutaredoxin (Grx-1) was measured in cytosol prepared from different brain regions of 90 day, male rats including cortex (CX), striatum (ST), hippocampus (HC), brainstem (BS), midbrain (MB), and cerebellum (CB). Grx-1 enzyme activity was lowest for the striatum while the brain stem had the highest Grx-1 activity compared to other brain regions (Fig 16: pg 107). Western blot analysis of Grx-1 protein was measured in the same regions as that for activity. Consistent with Grx-1 activity, the results showed the highest levels of Grx-1 protein in brainstem while the striatum had the lowest amount of Grx-1 protein compared to the other brain regions (Figs 17 A and B: pg 109). Grx-1 protein band was seen at 12kD. The hippocampus also had low Grx-1 protein.

FIGURE 16

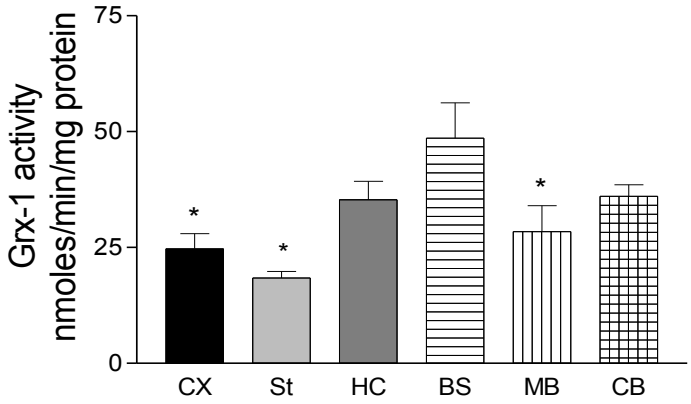
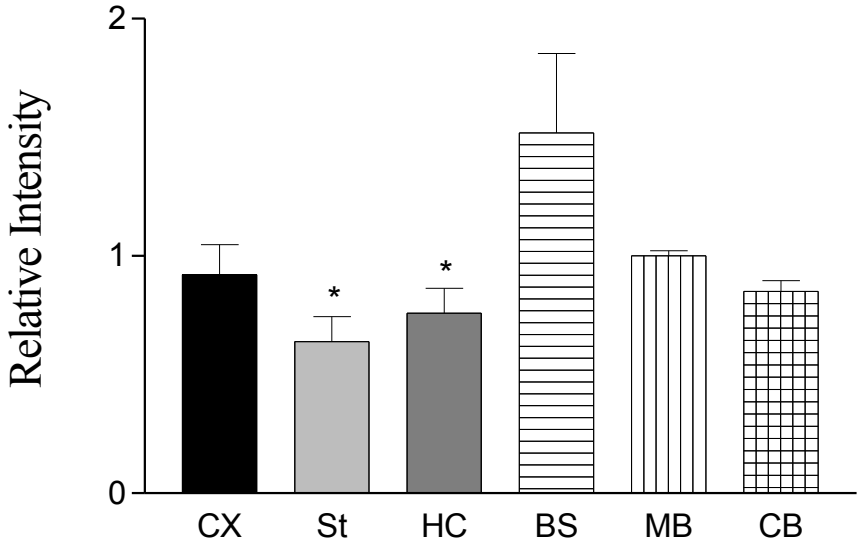


Figure 16: Grx-1 activity was measured in post mitochondrial cytosol of 90d, male rats. Different brain regions were dissected and homogenized and post mitochondrial cytosol was collected (see Methods). Brain regions examined were cortex (CX), striatum (St), hippocampus (HC), brain stem (BS), midbrain (MB), and cerebellum (CB). The rate of Grx-1 activity was determined spectrophotmetrically as described in Methods. Values are the mean ±SEM of 3 individual experiments using pooled brain regions from 6 animals for each experiment (*$p<0.05$ significantly different from brain stem). The striatum had the lowest activity and the brainstem the highest of the regions examined.

Figure 17: (A) Densitometric analyses of the Western blots representing the ratios of relative intensity of the Grx-1 to actin bands for each region are presented. Regions of the brain examined were cortex (CX), striatum (St), hippocampus (HC), brain stem (BS), midbrain (MB), and cerebellum (CB). Values are the mean ±SEM of 3 individual experiments using pooled brain regions from 6 animals for each region (*$p<0.05$, significantly different from brain stem). (B) a representative Western blot for the presence of Grx-1 in rat brain, age 90d is shown. The regions (defined in the legend to Fig 16: pg 107) were homogenized and cytosol for each region was collected and separated by SDS-PAGE followed by Western blotting and immunostaining with rabbit anti-human glutaredoxin-1 antibody. A single band was observed at approximately 12 kDa. The blot above the 25 kDa mark was immunostained with mouse-anti actin. Actin band is seen at approximately 42 kDa. The striatum and hippocampus had significantly lower Grx-1 protein amount than the brain stem.

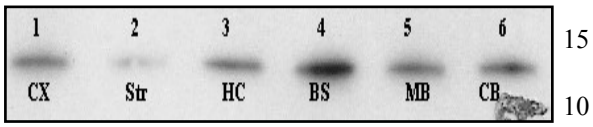
FIGURE 17

A

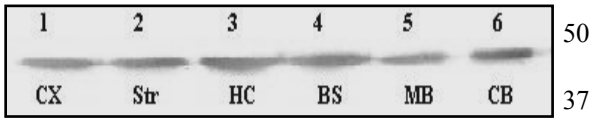


B

1. Grx-1 band seen at ~12kD



2.Actin band seen at 42kD



Regional mRNA analysis of Grx-1 and Grx-2.

Grx-1 and mitochondrial glutaredoxin (Grx-2) mRNA were measured by real time quantitative reverse-transcriptase PCR (Qt-PCR) for various regions of male rat brain. The mRNA amount was normalized to GAPDH. When compared to whole brain Grx-1 message was lowest in the striatum, which was consistent with Grx-1 activity and protein levels. In contrast to activity and protein for Grx-1, hippocampus showed the highest level of message. In comparison to whole brain, regional message for Grx-2 was similar to that for Grx-1, with the exception of striatum where message was found to be significantly higher than whole brain and most other brain regions (Figs 18A and 18B: pg 112).

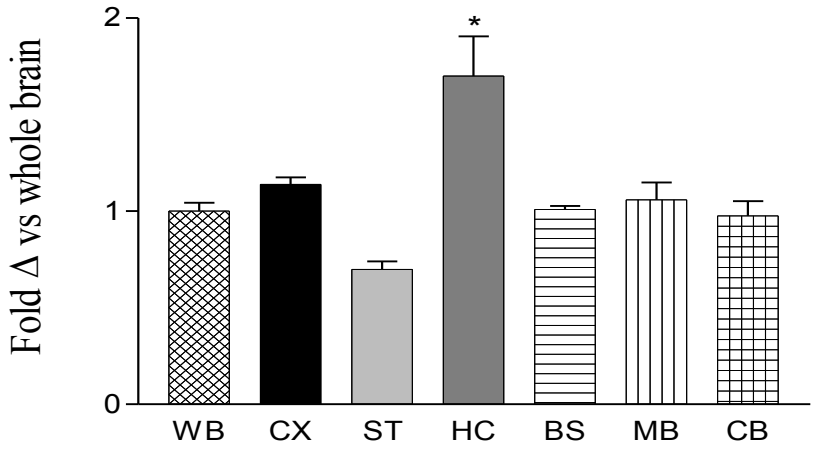
Grx-1 activity in female and male rat brains.

Grx-1 activity was measured in post mitochondrial cytosol prepared from brains of females with mixed estrous cycles and male whole rat brains age 90 days (see Methods) in order to repeat studies done in mouse (Kenchappa et. al., 2004). In a separate experiment, Grx-1 activity was measured in proestrus females since serum estrogen levels are highest in this estrous phase. There was no significant difference between females with different stages of the estrous cycle and proestrus female and male brains (Figs 19A and 19B: pg 114).

Figure 18: Real-time quantitative reverse transcriptase PCR (Qt-PCR) measurements of Grx-1 (a) and Grx-2 (b) mRNA were measured in various regional homogenates from 90d male rat brains. The regions studied were whole brain (WB), cortex (CX), striatum (St), hippocampus (HC), brain stem (BS), midbrain (MB), and cerebellum (CB). The amount for each brain region was normalized to GAPDH and was defined by the C_t method of Livak and Schmittgen (2001). Values are the mean ± SEM of six separate experiments (a)*$p<0.01$, significantly different from all other brain regions; (b) *$p<0.01$, significantly different from all other brain regions except HC. The striatum had the lowest Grx-1 message while the hippocampus had the highest Grx-1 message compared to all other brain regions. Conversely, Grx-2 message was highest in the striatum relative to all other brain regions.

FIGURE 18

A


Fold Δ vs whole brain
2
1
0
*
WB
CX
ST
HC
BS
MB
CB

B.

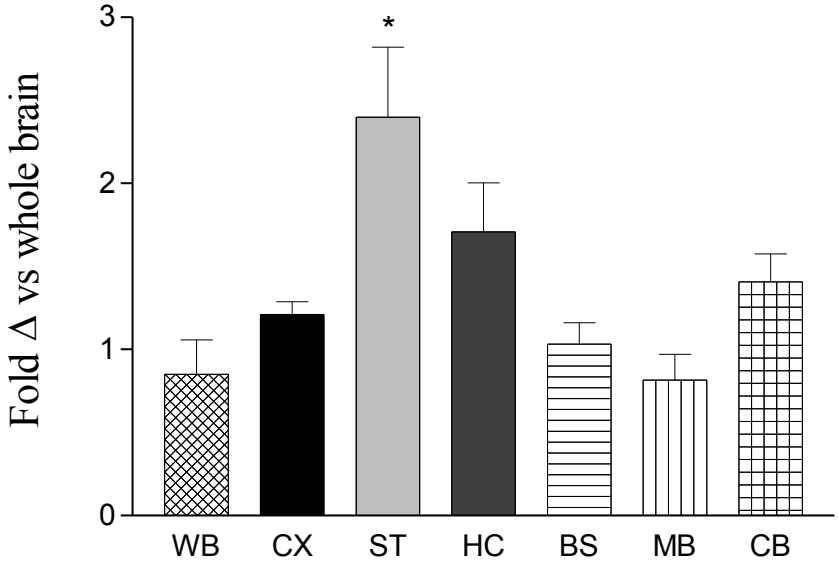
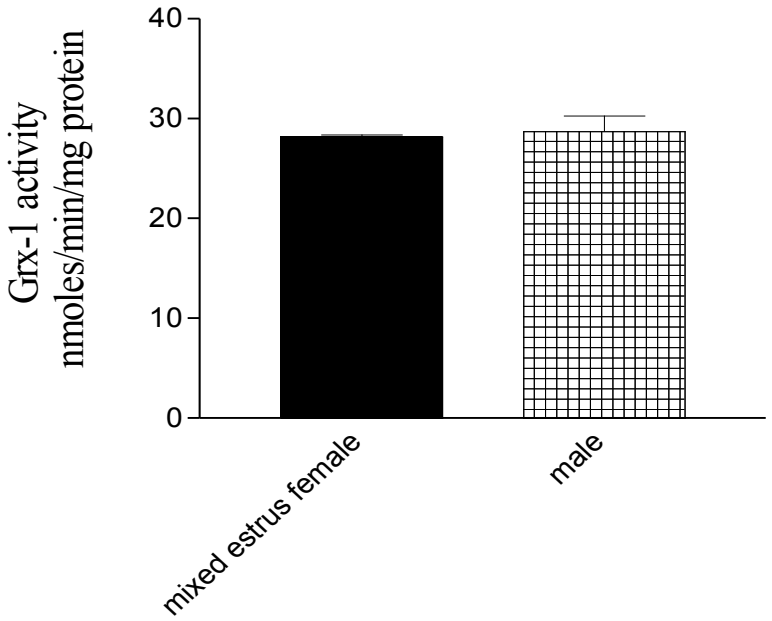

Fold Δ vs whole brain
3
2
1
0
*
WB
CX
ST
HC
BS
MB
CB

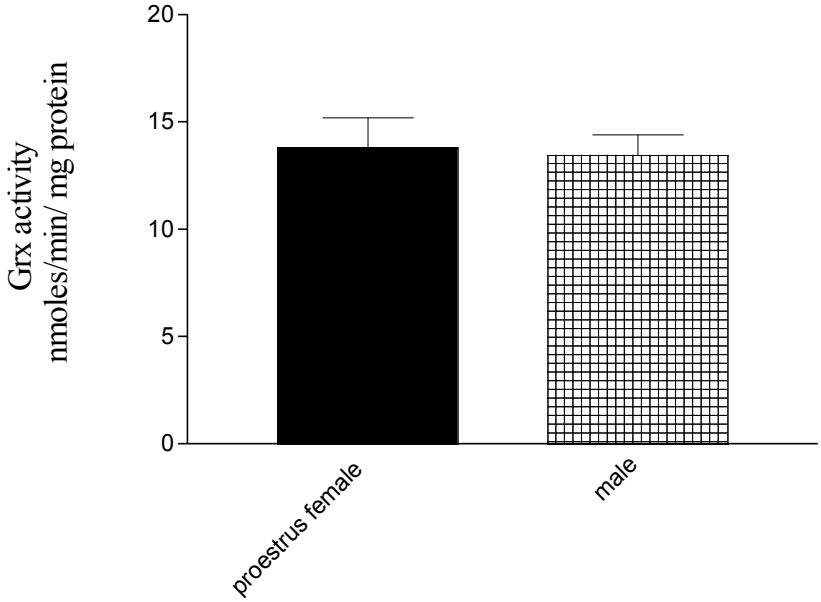
Figure 19: Grx-1 activity was measured in post mitochondrial cytosol of mixed estrous cycle (A) female and male and (B) proestrus female and male 90d rat brains. The rate of Grx-1 activity was determined spectrophotmetrically (see Methods). Values are the mean ± SEM for 3 individual experiments. No significant difference between mixed estrous female and proestrus female and male brain was observed.

FIGURE 19

A.


Grx-1 activity
nmoles/min/mg protein
40
30
20
10
0
mixed estrus female
male

B.


Grx activity
nmoles/min/ mg protein
20
15
10
5
0
proestrus female
male

Grx-1 activity and protein in different age groups of male rats.

Grx-1 activity was measured in cytosol prepared from different age groups of male rats, with the exception of E20 to P14 for which both genders were used. Since the gender study showed no difference between male and female, mixed gender should not affect results. There were two periods throughout the life of the rodent where Grx-1 activity was significantly increased, one in the first week postnatal and a second in older animals (Fig 20: pg 116). Western blot analysis of Grx-1 protein for whole brain from different age groups showed a similar pattern to Grx-1 activity, i.e. an early increase in the postnatal and a second increase in middle age and older animals (Figs 21A and 21B: pg 118).

Grx-1 and Grx-2 mRNA analysis in neurons and astrocytes:

The mRNA for Grx-1 and Grx-2 was measured using Qt-PCR from neuronal enriched and astrocyte enriched mesencephalic cultures from embryonic day 15 rats. GFAP, an astrocytic marker, had negligible presence in the neuronal culture, indicating that the culture lacked astrocytes. In contrast, GFAP was approximately 45-fold higher in astrocyte enriched cultures. As expected GAPDH was similar for both cultures. Grx-1 message was 4 fold higher and Grx-2, 14 fold higher in neurons than astrocytes (Fig 22: pg 119).

FIGURE 20

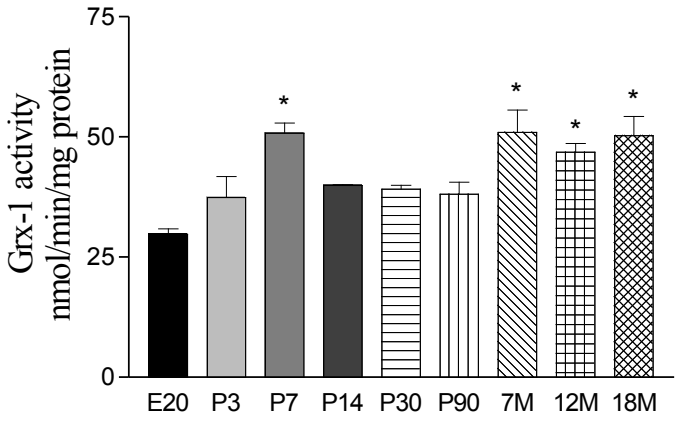
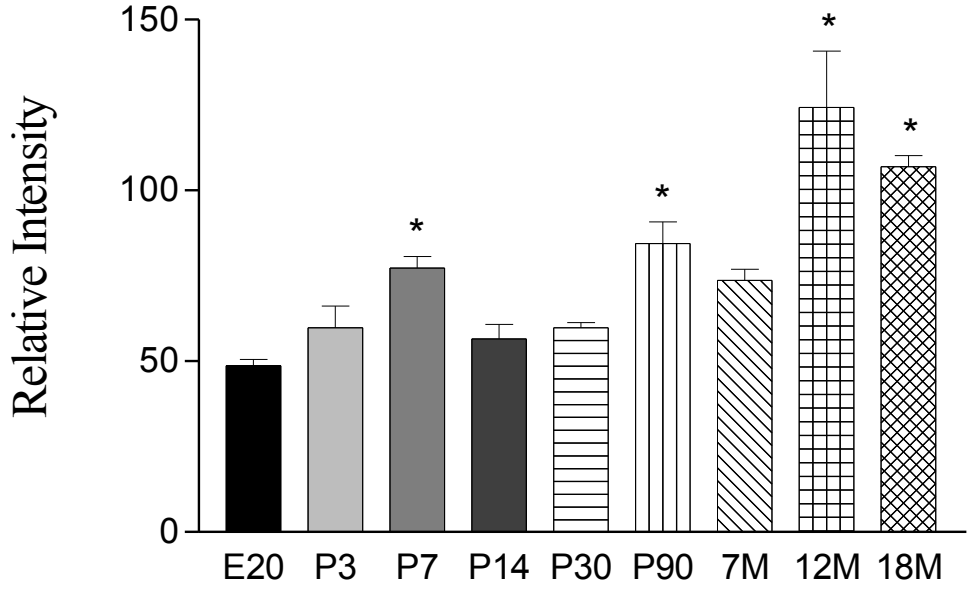


Figure 20: Grx-1 activity in different age groups of rats. Grx-1 activity was measured in post mitochondrial cytosol of male rats of ages, post natal day 30 (P30), 90 (P90), 7 months (7M), 12 months (12M), and 18 months (18M). Brain post-mitochondrial cytosol was also collected from embryonic day 20 (E20), post natal day 3 (P3), 7 (P7), and 14 (P14), however both male and female brains were used. Values are the mean ±SEM for 3 individual experiments using pooled brains from 3 animals for all age groups from P30 to 18M . For age E20 one litter of pups represented one sample. Three litters from different mothers were used for ages P3, P7, and P14 where a litter was divided into groups of three pups representing one sample. (* $p<0.05$, significantly different from E20).

Figure 21: (a) Densitometric analyses of the Western blots representing the ratios of relative intensity of Grx-1 to actin bands for each age are presented graphically. Grx-1 protein was measured in post mitochondrial cytosol of rats brains with age and gender as described in Fig 20, pg 115. Values are the mean ±SEM of three individual experiments using pooled brains from 2 animals for each experiment. (* $p<0.5$ or better, significantly different from E20). (b) Western blots for Grx-1 protein from rat brain. Ages are as described in (Fig 20: pg 115). Grx-1 band is seen at approximately 12 kDa. An equal amount (25 μg/sample) of total protein from rat brain post mitochondria cytosol was separated by SDS-PAGE and transferred by Western blotting. The lower half of the blot was then immunostained with rabbit anti-human glutaredoxin-1 antibody while the upper blot was immunotstained for actin. The actin band is seen at approximately 42 kDa.

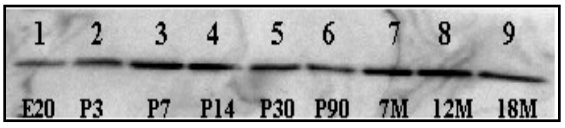
FIGURE 21

A.



B

1.Grx-1 band seen at ~12Kd **kD**



2.Actin band seen at ~47Kd

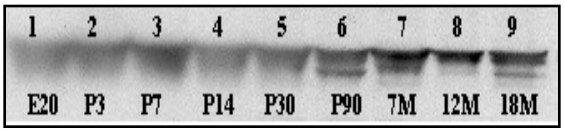


FIGURE 22

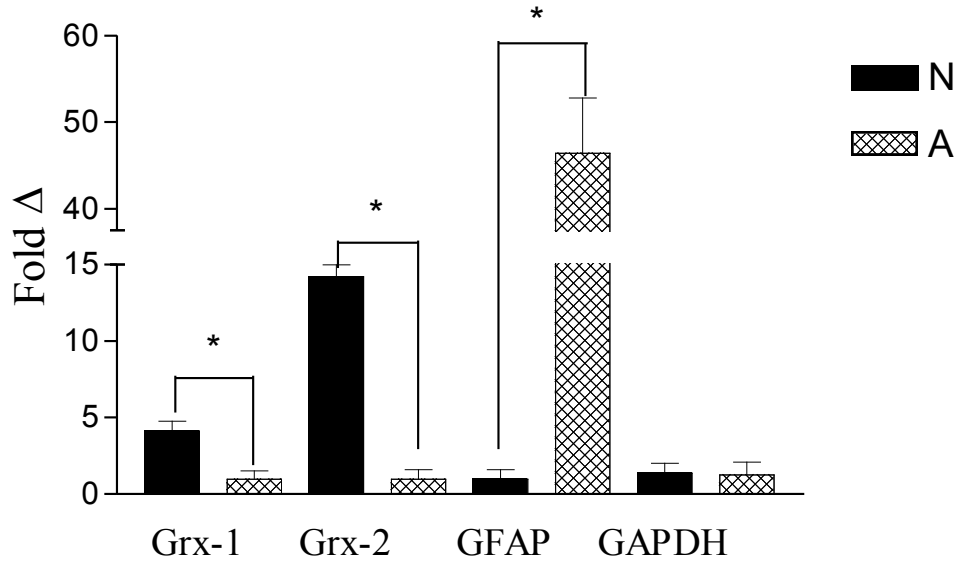


Figure 22: Real-time quantitative reverse transcriptase-PCR (Qt-PCR) mRNA measurements of Grx-1 (first set of bars), Grx-2 (second set of bars), the astroycytic marker, glial fibrillary acidic protein (GFAP) (third set of bars), and the loading control glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (fourth set of bars) were measured in mesenchephalic neuronal enriched and astrocyte enriched cell cultures of embryonic day 15 rat brain. Values are the mean ± SEM of 4 to 5 separate experiments (*$p<0.001$, astrocytes significantly different from neurons). The neuronal enriched cultures had greater amounts of both Grx-1 and Grx-2 message as compared with astrocyte enriched cultures. Message was quantified by the C*t* method of Livak and Schmittgen (2001).

C. Discussion

Grx is part of the thioredoxin superfamily of enzymes first identified in E.coli and later in liver, heart, thymus and placenta cytoplasm from pig, rat, calf and humans (Mieyal et. al., 1995). However, there is little information about Grx activity and

distribution in brain. Grx protein and message has been isolated from whole rat brain cytosol and identified in rat brain neurons (Balijepalli et. al., 1999). Immunoreactivity for Grx has been reported in CA3 neurons in the hippocampus, layer V neurons in the cortex and small and medium neurons in the striatum of rat brain (Takagi et. al., 1999). Grx facilitates the reversible thiolation and dethiolation of cysteinyl groups on proteins with GSH (Gravina and Mieyal, 1993; Chrestensen et. al., 2000). Under normal conditions, thiolation/dethiolation of proteins play a critical role in regulation of enzyme activities (Dalle-Donne et. al., 2007). During oxidative stress in neurons (Shivakumar and Ravindranath, 1993; Shivakumar et. al., 1995, Earhart and Zeevalk, 2001) and brain mitochondria (Ravindranath and Reed, 1990), formation of Pr-SSG at available cysteine residues increases and this increase has been postulated to protect critical cysteine groups from irreversible oxidation (Erhart and Zeevalk, 2001). Persistent S-glutathiolation can affect protein function, hence deglutathiolation is important in maintaining thiol homeostasis (Dalle-Donne et. al., 2007). Therefore, differences in Grx-1 or Grx-2 capacity could contribute to impaired recovery from oxidative stress. This may be of particular relevance for several neurodegenerative disorders such as AD, ALS, HD, PD and neuropsychiatric diseases such as autism and schizophrenia (Andersen, 2004; Yao et. al., 2001) as these disorders have been linked to oxidative stress. Since Grx is the principal thiolating/dethiolating enzyme for mixed disulfides containing GSH in the cell (Jung and Thomas., 1996; Lundstrom-Ljung et. al., 1999; Chrestensen et. al., 2000), knowledge of its regional activity, and changes with age or gender and cell type can provide valuable information regarding its' role in normal or patho-physiological responses to oxidative stress.

Regional analysis of Grx-1 activity, protein and Grx-1 and Grx-2 mRNA message:

At present, there is one report of regional distribution of cytosolic Grx-1 activity in rat brain that shows elevations in hippocampus and lowered activity in striatum and cerebellum relative to whole brain (Balijepalli et. al., 1999). In agreement with these findings, the current study also found significantly lower Grx-1 activity in striatum and midbrian with relatively high levels in hippocampus. In contrast with Balijepalli et. al., (1999), our study found cerebellum to have activity comparable to that observed in hippocampus, whereas Balijepalli et. al., (1999) found lower Grx activity in cerebellum than hippocampus. Brain stem was not monitored in the study by Balijepalli et. al., (1999), but in the current study was found to have the highest activity compared with all other brain regions examined in the present work. Activity levels generally correlated with protein levels as observed by Western with striatum expressing the lowest amount of protein and brain stem the highest. Quantitative PCR for Grx-1 message in striatum was in accord with the low protein and activity as message for Grx-1 in striatum was lower than for other brain regions. Qualitative assessment of Grx-1 message in striatum by in situ hybridization in rats (Balijepalli et. al., 1999) also demonstrated low message for Grx-1 in striatum. Low levels of Grx-1 activity, protein and message in the striatum suggests the possibility of a potential vulnerability of the striatum to oxidative stress since low Grx-1 activity may compromise the ability of striatal elements to reverse protein-glutathione-mixed disulfide formation. Indeed, selectively down-regulating Grx-1 in mouse brain cortex and striatum using antisense oligonucleotides inhibited mitochondrial complex I activity compared to controls (Kenchappa and Ravindranath., 2003). Complex I was not inhibited in the hippocampus where Grx-1 was not down-

regulated. In addition to low levels of Grx in the striatum, relative variations in levels of GSH in different regions of mouse brain have been reported with cortex > cerebellum > hippocampus > striatum > substantia nigra (Abbot et. al., 1990). Since Grx specifically requires GSH to reduce mixed disulfides, low levels of both Grx and GSH in the striatum make this region particularly vulenerable to oxidative stress. This may be of particular relevance to PD and schizophrenia since the substantia nigra of PD (Perry et. al., 1982) and caudate of schizophrenia post-mortem brains show decreased levels of GSH (Yao et. al., 2006). The findings may also have relevance to HD as oxidative stress and mitochondrial dysfunction are thought to contribute to disease processes (Beal, 2004). Low levels of protein and/or activity for Grx-1 may further compromise the ability of the striatum to reverse protein glutathiolation following oxidative stress. The high levels of Grx-1 activity and protein in the BS should protect this region from oxidative damage. In PD, however, the LC and raphe nucleus, both of which are located in the brain stem, are also affected. Consequently this incongruity challenges the hypothesis that diminished Grx-1 levels could be detrimental to cells. One possible explanation for this disconnect in BS between high Grx-1 activity and protein levels and neuronal degeneration could be that there are variations in Grx-1 protein in different regions of the BS. Since whole BS homogenate was used in the current study, regional differences within each sub-region would not be detected. Quantitative PCR showed the highest levels of message for Grx-1 in hippocampus consistent with a high level of activity in this region, whereas message for Grx-1 in brain stem could not explain the high level of Grx-1 protein or activity observed in this region. Such differences could be accounted for by regional differences in expression or protein turnover.

In contrast with message for cytosolic Grx-1, message for the mitochondrial glutaredoxin, Grx-2, was higher in striatum than in all other brain region examined and nearly 3-fold higher than in whole brain. Intense staining for Grx-2 mRNA using fluorescence in situ hybridization was observed in the striatum of mouse brain slices (Karunakaran et. al., 2007). The striatum and mitochondria have an intrinsically oxidative environment (Halliwell and Gutteridge, 1989; Wei et. al., 1996) and therefore, greater levels of Grx-2 may be required to counter the oxidative stress and maintain mitochondrial thiol homeostasis and function. Low levels of mitochondrial Grx-2 messages in the midbrain may have relevance to PD since complex I of the ETC activity is decreased in the substantia nigra of PD post mortem brain (Parker et. al., 1989). This may render mitochondria in this region more vulnerable to oxidative stress. While mitochondria are vulnerable to oxidative stress, deficits in mitochondrial respiration can fuel a further increase in oxidative stress (Hasegawa et. al., 1990, Adams et. al., 1993, Zeevalk et. al., 2005). Studies have shown that Grx-1 and Grx-2 help maintain complex I function (Kenchappa and Ravindranath, 2003). Indeed, there was up-regulation of Grx-2 mRNA and protein 1h following administration of the complex I inhibitor, 1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine (MPTP) in mice (Karunakaran et. al., 2007). This response may be due to the oxidative stress caused by MPTP and complex I inhibition. Overexpression of Grx-2 protected Neuro2a cells from MPTP mediated toxicity, whereas down-regulation of Grx-2 using antisense oligonucleotides in mice resulted in a significant inhibition of complex I activity in the striatum and midbrain (Karunakaran et. al., 2007).

Thus, regional differences in Grx- 1 and 2, as well as GSH may contribute to the

selective vulnerability of neurons to an oxidative challenge.

Grx-1 activity in female and male brains:

Clinical and in vitro studies suggest that estrogen may have neuroprotective effects as an antioxidant (Sugioka et. al., 1987; Subbaiah et. al., 1993; Inestrosa et. al., 1998). Results of gender difference studies in AD vary from no significant differences (Hebert et. al, 2001) to a higher prevalence in women compared to men (Andersen et. al., 1999; Barnes et. al., 2005), however, studies showed a decreased risk for AD in women with estrogen treatment (Paganini-Hill and Henderson, 1994; Paganini-Hill and Henderson, 1996). Epidemiological studies report greater incidences of schizophrenia in men than in women (Aleman et. al., 2003). Men were also at greater risk for developing PD than women (Wooten et. al., 2008).

Kenchappa et. al., (2004) reported higher levels of Grx-1 activity and mRNA in the striatum and midbrain of female mice as compared with males. This same laboratory, Diwakar et. al., (2007) also reported higher levels of Grx-1 activity, protein and mRNA in lumbosacral cords of female mice vs. male and overiectomized mice. Neither study monitored the estrous cycles of the female mice used in measuring Grx-1 activity, protein and mRNA, therefore estrogen levels were unknown. In the current study, one female group from rat was of mixed estrous cycle status to duplicate what had been done in mouse, while a second study was done in proestrus females. Proestrus female rats were used because maximum serum estrogen is reached during the proestrus phase (Becker et. al., 2005). The gender study showed no difference in Grx-1 activity between mixed estrous cycle or proestrus female and male rats. The disparate results for Grx-1 between mice and rats suggest that gender differences in brain Grx-1 activity vary amongst

different species, but further study is needed to confirm this observation. Gender effects on Grx-1 activity in human brain has not yet been studied but would be important as gender differences in Grx activity in humans may contribute to the gender bias observed in several neurodegenerative and neuropsyhiatric conditions.

Grx-1 in brain during aging

The developmental profile of Grx-1 in brain has not been studied previously. Many neurodegenerative diseases are age associated; hence, characterizing Grx-1 activity and protein with respect to age could provide novel information about Grx-1 function during aging. In this study, Grx-1 activity and protein was characterized in rat brain at different ages ranging from embryonic day 20 to 18 months. Grx-1 activity and protein increased in the first postnatal weeks and then in older animals. The early increase in Grx-1 activity in young animals could point towards an increased necessity for Grx-1 activity in response to changes in metabolic processes due to a switch to more aerobic metabolism or in response to neuronal maturation and growth. In older animals, the increase in activity may be due to increased levels of oxidative stress related to aging (Yoshikawa and Hirai 1967; Benzi, 1990; Halliwell, 1992). It would be of interest to know if Grx-1 activity differed between control brain and brains from patients with late-onset neurodegenerative diseases such as AD and PD as a deficiency in Grx-1 activity with aging could contribute to neuronal vulnerability.

Grx-1 and Grx-2 mRNA in neurons and glia:

Glial cells are more resistant than neurons to oxidative stress and play an important role in antioxidant defense mechanisms (Bolanos et. al., 1995; Rice and Russo-Menna 1998; Watts et. al., 2005). Previous studies indicate that in neuron-glial coculture systems, glial cells protect neuronal cells against hydrogen peroxide (H_2O_2) toxicity by maintaining the neuronal glutathione level or by directly scavenging free radicals (Langeveld et. al., 1995; Desagher et. al., 1996). Astrocytes are known to have approximately 2-fold more GSH than neurons (Slivka et. al., 1987; Pileblad et. al., 1991; Rice and Russo-Menna 1998). The present study found 4- fold more Grx-1 mRNA in neurons than in astrocytes. This is consistent with Takagi et. al., (1999) who reported rat astrocytes to have little to no Grx-1 when viewed qualitatively by in situ hybridization. Grx-2 message was 14-fold higher in neurons than in astrocytes.

Karunakaran et. al., (2007) reported constitutive expression of Grx-2 in both neurons and glia in mouse and human brain, although intensity of fluorescence and regional abundance of message were more evident in neurons. The low expression of Grx-1 in astrocytes is somewhat surprising since glia are more resistant to oxidative stress than neurons. Our findings with quantitative PCR confirm evidence to indicate that Grx-1 is predominately if not exclusively neuronal and further suggest that astrocytes may have alternative enzymes for thiolation/dethiolation such as thioredoxin (Trx) or novel proteins that contain a glutaredoxin active site (Bassan et. al., 1999).

Thioredoxin is a oxidoreductase enzyme containing a dithiol-disulfide active site (Holmgren, 1989). As with Grx, there are two isoforms of Trx, cytosolic (Trx-1) and mitochondrial (Trx-2). Thioredoxin plays an important role in many thiol-dependent

cellular reductive processes, antioxidant protection, and signal transduction. Moreover, Trx reduces and maintains the function of many proteins during oxidative stress. Lippoldt et. al., (1995) have observed Trx message by in situ hybridization in the rat brain. Cytosolic Trx (Trx-1) staining was increased in astrocytes in the white matter of AD brain suggesting astrocytes may use Trx to defend against oxidative stress (Asahina et al., 1998). By employing the Trx antioxidant system rather than the Grx system as neurons do, astrocytes avoid competing with neurons for the Grx reductant GSH since the Trx system uses the enzyme thioredoxin reductase to reduce oxidized Trx (Holmgren, 1989).

In summary, the present study demonstrates significant regional, age and cell specific differences in activity, protein and/or message for brain Grx that could provide a foundation for better understanding the role of Grx in normal and pathophysiological processes.

CHAPTER VI: GENERAL DISCUSSION

Parkinson's disease is characterized by progressive selective degeneration of dopaminergic cells of the nigrostriatal pathway and the presence of Lewy bodies. 1-2% of the population over the age of 65 is affected by PD. Genetic mutations in alpha-synuclein (α-synuclein), parkin, ubiquitin C-terminal hydrolase L1 (UCHL-1), DJ-1, PINK1 and LRRK2 have been linked to familial PD , however the majority of PD cases are sporadic. The etiology of sporadic PD remains unidentified; nonetheless, oxidative stress (Cohen, 1999), mitochondrial dysfunction (Fahn and Cohen, 1992; Wallace et. al., 1997), improper dopamine sequestration (Forno, 1996) and loss of glutathione (GSH) (Perry et al., 1982) have been implicated in the pathogenesis of idiopathic PD. Development of a Parkinsonian syndrome in humans following exposure to MPTP provided support to the theory that environmental factors through mitochondrial dysfunction and energy impairment play a role in the neuropathogenesis of PD (Nicklas et. al., 1985). Deficiencies in complex I and II/III of the mitochondrial ETC have been reported in PD post mortem brain (Schapira et. al., 1990) skeletal muscle (Bindoff et. al., 1991; Shoffner et. al., 1991) and platelets (Haas et. al., 1995; Benecke et. al., 1993).

In spite of the systemic deficits in ETC function observed in PD, cell death is predominantly observed in the nigrostriatal dopaminergic neurons. Unregulated cytosolic DA itself has, therefore, been thought to be a toxic factor contributing to the selective degeneration of nigrostriatal DA neurons. DA toxicity has been the subject of intense investigation, yet the mechanism of DA mediated cell damage and/or death remains enigmatic. Several studies, however, have reported mitochondrial dysfunction caused by DA (Hasintgs et. al., 1996; Berman and Hastings, 1999; Gluck et. al., 2002; Gluck and

Zeevalk, 2004). This is relevant to PD as mitochondrial dysfunction has been observed in patients with the disease. It is possible that DA and /or products of DA metabolism may be toxic to mitochondria. Enzymatic metabolism of DA by MAO produces H_2O_2 and DOPAC, the former of which can enter the mitochondria and undergo Fenton chemistry and form $HO^{\cdot}$ which inhibits complex I and II activities (Gluck et. al., 2002). Reduced DA per se can inhibit mitochondrial respiration (Berman and Hastings, 1999; Gluck et. al., 2002; Gluck and Zeevalk, 2004). Furthermore, DOPAC has been shown to be equipotent to DA in inhibiting mitochondrial O_2 consumption (Gluck and Zeevalk, 2004). DA can also oxidize either enzymatically (Hastings, 1995) or non-enzymatically, in the presence of Fe^{2+} and/or an oxidizing environment, to form H_2O_2 and highly reactive electrophilic quinone and semiquinone intermediates (Graham, 1978; Zhang and Dryhurst 1993). Oxidized DA can form covalent bonds with protein thiols and could affect protein function (Graham, 1978; Hastings and Zigmond 1994; Stokes et. al., 1999).

Investigations of the effects of DA and DOPAC on mitochondrial ETC function have yielded varied results from no inhibition of ETC activities such as complex I, by DA or DOPAC in mouse brain (Morikawa et. al., 1996) to low IC_{50s} of DA of 8.12 ± 0 .75 uM (Ben-Sachaar et al., 1995) and 61.2 ± 8.08 uM (Ben-Sachaar et al., 2004) in rat brain mitocondria. The oxidation states of DA in the studies by Ben-Sachaar et al.,(2004) were not monitored. Whereas, Morikawa et. al., (1996) failed to monitor time of exposure. Hence, while both studies assayed for the affects of DA on mitochondrial function, it is unclear whether the DA was in its reduced or oxidized redox state. In Khan et al., (2005) and Jana et. al., (2007), the mitochondria were preincubated with DA or DOPAC for 2hrs prior to measuring complex activities, which would have allowed time for catechol

oxidation. Quinone formation was monitored by Khan et. al., (2005), however the quinone concentration was not quantified, therefore the concentration of quinone required for inhibition was unclear. In contrast to Ben-Sachaar et. al., (2004), aim 1 results from this thesis showed that ETC activities were unaffected by DAH and DOPAC-H, with the exception of complex II, which was in agreement with Morikawa et. al., (1996). Only complexes I and III were inhibited by DAQ, which was in accordance with Khan et. al., (2005) and Jana et. al., (2007). In contrast to Jana et. al., (2007), in our study complexes I and III activities were also inhibited by DOPAC-Q. A possible reason for the dissimilar results may be that in the Jana et. al., (2007) study DOPAC may not have oxidized since they air oxidized DOPAC. In our study DOPAC had to be oxidized via tyrosinase because it did not air oxidize. In accordance with Khan et. al., (2005), only GSH protected from inhibition by DAQ, suggesting that the inhibition was due to oxidized DA products rather than ROS formation. Moreover, quinone mediated inhibition of substrate activated complex I activity was irreversible suggesting quinone adduction to exposed thiols groups on active sites of complex I. The mechanism of quinone mediated inhibition is presently unknown; however quinone adduction to accessible protein thiols could be a possible mechanism.

The findings from this thesis would indicate that inconsistencies in the literature regarding the affects of DA, DOPAC on ETC activities are likely due to differences in assay conditions and the redox states of the catechols. Additionally, the presence of the reduced catechols can create artifacts which can either mask inhibition by pushing the reaction forward by donating electrons to DCPIP as in the case of complex II or oxidizing cytochrome C in the complex III assay. Reduced catechols can also take part in reactions

which can mimic inhibition by pushing the reaction backward by accepting electrons from reduced cytochrome C as in the case of complex IV. Thus, non-ETC related redox chemistry must be taken into account. For example in aim 1 we found that DAH oxidized reduced cytochrome C, which resulted in a decrease in activity since the assay measured the oxidation of reduced cytochrome C thus mimicking inhibition. Contrary to Khan et. al., (2005), aim 1 results show that complex IV was not affected by DAQ. A possible explanation for this discrepancy could be that in the Khan et. al. (2005) study it was uncertain whether the DAQ solution was fully oxidized since the concentration was not quantified; hence there may have been residual DAH in the solution which could have taken part in non-ETC redox chemistry that presented as inhibition. In the studies of the thesis, in aim 1, the oxidation states of DA and DOPAC were differentiated and the quinone concentrations were quantified prior to assaying activities of individual ETC complexes. In addition, corrections for artifactual interferences were made when possible to account for non-ETC reactions. In total, Aim 1 findings present evidence to explain the inconsistencies amongst various studies, and indicate that oxidized DA and DOPAC are more potent inhibitors of ETC activities than the reduced catechols. Furthermore, oxidized DOPAC was equipotently toxic to ETC activities as compared to DA, which was in accordance with the Gluck and Zeevalk., (2004) study wherein DA and DOPAC equally inhibited rat brain intact mitochondrial O_2 consumption. This is significant since most studies look at the effects of DA and overlook DOPAC and the few that have studied DOPAC did not consider the redox state of the catechol (Jana et. al., 2007). The equipotency of DA and DOPAC as ETC inhibitors is relevant since most cytosolic DA is quickly metabolized to DOPAC, therefore high concentrations of cytosolic DA as a result

of compromised bioenergetics may result in high concentrations of DOPAC and add to a toxic environment for mitochondria.

The studies in the first aim were all conducted in freeze thawed mitochondria in which sites vulnerable to DA or DOPAC would be exposed. Hence, it remained unclear whether extra mitochondrial DA or DOPAC could gain entry into the mitochondria and whether the catechols altered mitochondrial ETC activities. In the second aim, studies showed a dose and time dependent active uptake of extra mitochondrial DAH into intact mitochondria. Additionally, aim 2 demonstrated that complexes I and III activities were dose dependently inhibited in intact, well coupled mitochondria that were exposed to extra mitochondrial DAH and DOPAC-H. In general, the catechols were equipotent in inhibiting complexes I and III activities, with the exception of the highest concentration of catechols tested. These results were corroborated by the studies in aim 1 where DAQ and DOPAC-Q had similar IC_{50}'s. Further, Gluck and Zeevalk also reported similar IC_{50}'s for DA and DOPAC in inhibiting intact mitochondrial O_2 consumption. The intact mitochondria and reduced catechols were co-incubated with ascorbate, hence it is likely that reduced DA and DOPAC gained entry into the mitochondria and then oxidized due to the alkaline environment of the mitrochondrial matrix. Based on the findings, it is hypothesized that the inhibition was due to covalent binding to vulnerable protein thiols by oxidized DA or DOPAC products rather than DAH and DOPAC-H since the reduced catechols were shown not to inhibit to complexes I and III activities (aim 1, chapter 3). Following this, the next part of this thesis project was to investigate whether radiolabeled DAQ binds to mitochondrial membranes containing the ETC complexes. However, this study was not conducted since Van Laar et. al., (2008b) have recently shown DAQ

binding to complex I and III subunits of intact rat brain mitochondria, thereby further corroborating the current hypothesis. Aims 1 and 2 results augment the finding of Van Laar et. al., (2008b) which showed that DAQ binds to specific mitochondrial proteins by demonstrating that the binding leads to dysfunction of those susceptible proteins in both lysed and intact mitochondria. In the Van Laar et. al., (2008b) study DA uptake was not quantified, nevertheless since the mitochondrial preparation in their study was incubated with oxidized DA, it is likely that DAQ was taken up into the mitochondria. The mechanism of DAQ uptake into mitochondria is unknown; however Berman and Hastings (1999) reported mitochondrial swelling, following exposure to DAQ. The swelling was prevented with the addition of cyclosporin A, a pharmacological agent that inhibits opening of mitochondrial transition pores (MPT), suggesting that DAQ may cause the opening of MPT. This opening may allow the passage of DA (mw 153.18) or DOPAC (mw 168.14) into the mitochondria as MPTs make mitochondria permeable to solutes smaller than m.w. of 1500 daltons. In aim 2 of this project, the uptake study demonstrated DAH uptake. Additionally, in the second part of aim 2, the mitochondria were incubated with reduced catechols and ascorbate which would keep the catechols in the reduced state, suggesting that the reduced catechol was taken up into the mitochondria. Brenner-Lavie et. al., (2008) showed DA accumulation into mitochondria isolated from SH-SY5Y cells that was dependent upon mitochondiral membrane potential. Hence the observations from these studies suggest that both speicies of DA can gain entry into the mitochondria. The mechanism of DAH uptake into the mitochondria was not investigated in this project, nevertheless since the uptake was energy dependent it is possible that the mechanism of DAH entry is distinct from that of DAQ.

Greater than 90% of DA in dopaminergic neurons is protected from degradation by active sequestration in vesicles in neuronal terminals (Eisenhofer et. al., 2004). Therefore, abnormalities in mitochondrial bioenergetics could result in deregulation of DA sequestration and increase cytosolic or extracellular DA. Since DA has been shown to be taken up by mitochondria in aim 2, it is plausible to theorize that DA overflow could result in accumulation of dopamine within mitochondria. Once inside the mitochondria, DA oxidation may be favored due to the basic mitochondrial matrix environment (Llopis et al., 1998) and lead to ETC dysfunction and cell damage/death (Caudle et. al., 2007; Chen et. al., 2008). The IC_{50} value for DAQ mediated complex I inhibition in aim 1 was 50.0 uM, however this value is only relevant if DA concentrations within mitochondria can reach this level. Vesicular DA concentration in neurons has been reported to be up to 60.0 mM (Giedde, 2003) and although the DA uptake study findings (Aim 2) revealed a nominal percentage of DA uptake, it is possible that mitochondrial DA accumulation over time could reach toxic levels as observed with the progressive accumulation of MPP+ in mitochondria (Singer et.al., 1988). Indeed, DA uptake has similar characteristics to MPP+ uptake as it is also energy (Aim 2) and mitochondrial membrane potential dependent (Brenner-Lavie et. al., 2008).

Increased cytosolic DA could also result in increased cytosolic DOPAC and add to the toxic environment since DOPAC has be shown in aims 1 and 2 to be equipotently toxic as DA to mitochondrial ETC function and O_2 consumption (Gluck and Zeevalk, 2004). In addition to mitochondrial dysfunction caused by covalent binding of DAQ to vulnerable thiols, removal of mitochondrial GSH by forming GSH-DA adducts would augment ROS formation. Both of these pathways could increase oxidative stress, which

could further inhibit mitochondrial function and decrease bioengergetics. Therefore, DAQ and/or DOPAC-Q toxicity may render dopaminergic neurons specifically vulnerable to damage and/or death.

Another hallmark of PD is the loss of GSH in the SNpc (Perry et. al., 1982; Riederer et al.,1989; Sian et al., 1994a,b) without a concomitant increase in GSSG (Riederer et. al., 1989). GSH is the major antioxidant in the brain (Meister and Anderson, 1983; Cooper, 1997) and has been detected in extracellular space and in the cerebrospinal fluid (Dringen and Hirrlinger, 2003) as well in mM concentrations in neurons and glia. A decrease in GSH levels has lead to mitochondrial dysfunction (Meister, 1995; Bolanos et al., 1995), enhanced the toxic effects of MPTP (Wullner et al., 1996), and potentiated damage to DA and GABA neurons in rat mesencephalic cultures exposed to a mitochondrial inhibitor (Zeevalk et. al., 1998). GSH depletion is thought to be an early event which leads to complex I inhibition as ILBD patients have low levels of GSH but no effect in complex I activities (Dexter et al., 1994). The cause of reduced levels of GSH is unknown, however a possible reason could be that GSH may be bound to oxidized catechols forming 5-S-G-DA or GSH-DA (Stokes et al., 1999, Bindoli et al., 1992). DA has been shown to bind to GSH, in a cell free system, at faster rates than intracyclization or o-quinone reduction by ascorbate (Tse et al., 1976). Thus GSH-DA adduct formation may be the most prominent fate for DA in an environment rich with ROS, DA and GSH. Tse et. al. (1976), reported that the adduction of cysteine is even faster than GSH. This is interesting because neurons specifically use cysteine as a precursor to synthesize GSH. Additionally, cysteine is reported to be the rate limiting amino acid in GSH synthesis (Dringen and Hirrlinger 2003). Thus, cysteine adduction to catechol quinones could limit

GSH synthesis. The reduced levels of GSH are also not due to decreased levels of GSH synthesizing enzymes. In fact there is increased activity of γ glutamyl transpeptidase (γ-GT), associated with the hydrolysis of extracellular GSH to L-cysteine (CySH), glutamate and glycine, (Sian et al, 1994b).

Another GSH associated enzyme important to GSH's function in redox control is glutaredoxin (Grx). Little is known, however, about Grx in brain. Gluataredoxin is the principal enzyme involved in the glutathiolation and deglutathiolation of protein thiols using GSH as the reductant (Dalle-Donne et. al., 2007). There are several isoforms of gluatredoxin, one is localized in the cytoplasm (Grx-1) (Balijepalli et. al., 1999) and the second has been identified in the nucleus and mitochondria (Grx-2) in brain (Lundberg et. al., 2001; Gladyshev et. al., 2001; Ehrhart et. al., 2002). During episodes of oxidative stress, exposed protein thiols are glutathiolated to protect against irreversible oxidation. Once the episode has subsided and a normal redox state has been established, the thiol groups are deglutathiolated, a function for which Grx is thought to be particularly important, since prolonged glutathiolation could affect protein function (Fig 23) (Dalle-Donne et. al., 2007). Therefore, descriptive information on regional glutaredoxin specific activity, protein levels and message may lay the foundation to understanding the role of this enzyme during physiological or pathophysiological conditions. In aim 3 of this thesis, Grx-1 activity, protein and message levels were lowest in the rat brain striatum compared to other brain regions, which could compromise the ability of the striatum to reverse glutathiolation following an oxidative stress episode. There is potential relevance to neurodegenerative diseases such as PD (Yoritaka et al, 1996; Alam et al., 1997; Kikuchi et al., 1999; Saggu et al., 1989), HD and neurophyciatric disease such as

scizophrenia (Ischiropoulos and Beckman, 2003) since oxidative stress has been implicated in disease pathology. Correlations between Grx activity and brain regions vulnerable to neurodegeneration were not always observed for example, BS was found to have highest levels of Grx-1 activity and protein compared to other brain regions, which could render this region resistant to oxidative stress damage. But in PD, neurodegeneration is also observed in brainstem regions, LC and raphe nucleus, thereby contesting the premise that high Grx levels could be neuroprotective. Potential reasons for this disparity could be that there are differences in Grx activity and protein levels in various microenvironments in the brainstem. Moreover, the afferents from the LC and raphe nucleus have extensive distributions to several different efferent targets; therefore the terminals are exposed to different environments. As a result the LC and raphe nucleus degeneration observed in PD may be due to retrograde effects of toxicity specific to a region outside of the brainstem nuclei. An example for this is the SNpc neuronal death in sporadic PD which is postulated to follow loss of synaptic terminals in the striatum (Bernheimer et. al., 1973). Overall the data suggest that no one factor is responsible for neurodegeneration but rather numerous factors can contribute to the vulnerability or resistance of a region to damage.

Grx-2 message, in contrast to Grx-1, was higher in striatum compared to other brain regions. The striatum and mitochondria have an intrinsically high oxidizing environment (Halliwell and Gutteridge, 1989; Wei et. al., 1996) and therefore, higher levels of Grx-2 may be required to counter the oxidative stress and maintain mitochondrial thiol homeostasis and function. Low levels of Grx-2 message in the midbrain may render mitochondria in this region more vulnerable to oxidative stress.

This may be relevant to PD as mitochondrial dysfunction has been identified in PD, specifically in the SNpc (Parker et. al., 1989). Additionally, the striatum and SNpc have been reported to have lower levels of GSH than other brain regions in rat (Abbot et. al., 1990); therefore, low levels of both Grx and GSH in the striatum make this region particularly vulnerable to oxidative stress since Grx specifically uses GSH to deglutathiolate Pr-SSGs formed during an oxidative stress episode. Furthermore, low levels of Grx could retard cytosolic GSH restoration via deglutathiolation, thereby decreasing the GSH/GSSG ratio potentially promoting further S-glutathiolation (fig 24) leading to protein dysfunction. The findings reported in aim 3 suggest that regional difference in Grx-1 and 2 could contribute to the selective vulnerability of the nigrostriatal neurons.

FIGURE 23

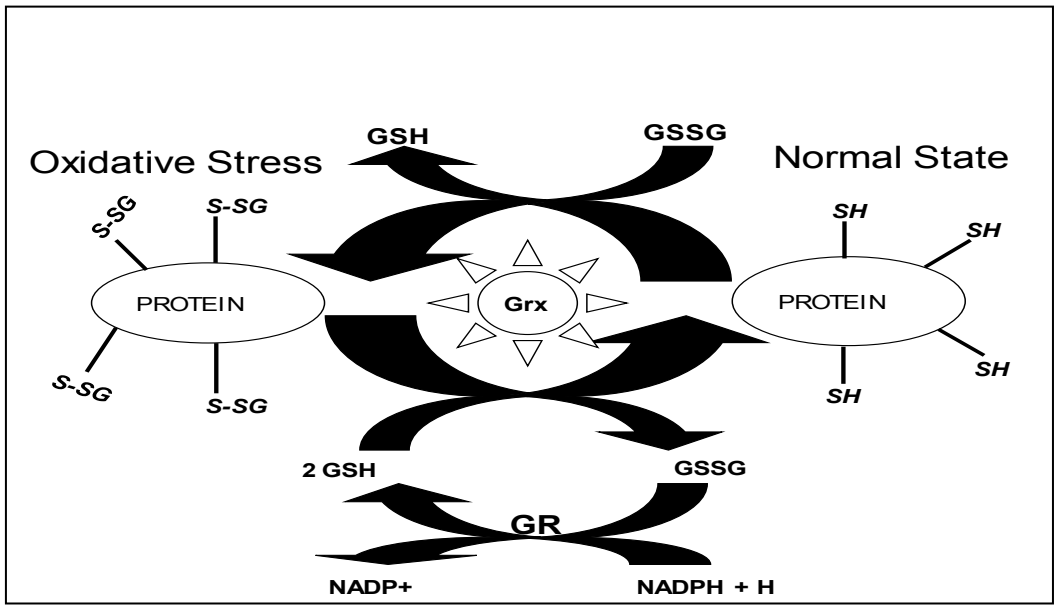


Fig 23: Schematic of one possible mechanism of gluathiolation of exposed protein thiols (Pr-SH) during an oxidative stress episode or decrease in GSH/GSSG ratio, forming protein mixed disulfides (Pr-SSG). Gluathiolation is the result of a thiol/disulfide exchange between GSSG and protein thiols. Once the reducing intracellular redox balance has been restored to normal or an increase in the GSH/GSSG ratio, gluatathiolation is reversed (degluathiolation) via Grx. Deglutathiolation via Grx specifically uses reduced glutathione (GSH) as the reductant, forming oxidized glutathione (GSSG). Reduced glutathione is regenerated enzymatically via gluatredoxin reductase (GR), consuming NADPH in the process. The direction of the glutaredoxin-catalyzed cycle depends on the GSH/GSSG ratio. High cellular concentrations of GSH relative to GSSG will drive deglutathioylation or the reduction of protein thiols (Dalle-Donne et. al., 2007).

Gender biased studies of Grx-1 activity and protein levels in mice have shown greater activity and protein levels in females than males (Kenchappa et. al., 2004; Diwakar et. al., 2007). Conversely, there was no gender difference in Grx-1 activity in rats in the findings reported in aim 3 (chapter 5). The differences in the gender studies between mice and rats suggest that Grx-1 activities vary amongst species. Epidemiology studies of several neurodegenerative and neuropsyciatric diseases such as PD and schizophrenia revealed a higher prevalence of men than women (Wooten et. al., 2008; Aleman et. al., 2003) while gender studies of AD patients varied from no differences (Hebert et. al, 2001) to a higher prevalence in women compared to men (Andersen et. al., 1999; Barnes et. al., 2005). Gender biased studies of Grx activity in humans have not been conducted yet, however they would be worthwhile since differences in Grx activity in men and women may shed light on to understanding the observed gender bias in disease predisposition.

Many neurodegenerative diseases are age related; therefore knowledge of Grx-1 activity and protein during aging could provide insight into disease pathology. In this study, Grx-1 activity and protein levels were studied in rat brains of age groups ranging from embryonic stage to animals of 18 months. In rat, there were two stages in which Grx-1 activity and protein levels increased, first during the early stages from embryonic day 20 to postnatal day 14 and a second increase in older animals. Increased Grx-1 activity and protein in older aged animals may be in response to greater oxidative stress associated with aging; therefore disruptions in antioxidant activities such as glutaredoxin could enhance vulnerability due to aging.

Astrocytes contain much higher amounts of GSH than neurons (Slivka et al., 1987; Pileblad et al., 1991) and play an important role in antioxidant defense mechanisms (Bolanos et. al., 1995; Rice and Russo-Menna 1998; Watts et. al., 2005). Despite this, Takagi et. al., (1999) reported a lack of Grx-1 in astrocytes. The finding is counterintuitive since astrocytes are more resistant to oxidative stress than neurons. To better clarify this, the present study examined the presence of Grx-1 and Grx-2 message in mesenchephalic astrocytes and neurons cultures obtained from rat brains. The results corroborated Takagi et. al., (1999), as astrocytes were found to have little to no Grx-1 or Grx-2 as compared to neurons. These findings suggest that astrocytes may have other means for thiolation/dethiolation than gluatredoxins such as the thioredoxin antioxidant system. By not utilizing the Grx system, astrocytes can afford to secrete GSH and cysteine that neurons specifically use to synthesise neuronal GSH (Dringen and Hirrlinger, 2003).

Summary and Future Directions

Dopamine toxicity has been considered one of the factors contributing to the nigrostriatal cell death observed in Parkinson's disease, although the mechanism of action is still unknown. Most of the DA, is sequestered within vesicles to protect from DA toxicity. Since the sequestration is energy dependent, compromises in bioenergetics, one of the biochemical hallmarks of Parkinson's disease, could affect DA sequestration leading to increased cytosolic DA levels resulting in increased DA metabolites such as DOPAC, quinones and hydrogen peroxide. The studies presented in this thesis have demonstrated that oxidized products of DA and its metabolite DOPAC irreversibly inhibit mitochondrial ETC function, specifically complexes I and III. We also show that

reduced DA can gain access to mitochondrial matrix and subsequently affect ETC function. The findings put to rest many of the inconsistencies in literature regarding DA and DOPAC toxicity and the responsible species. We propose that the mechanism by which oxidized DA and DOPAC attenuated ETC activities is by covalently binding to exposed thiols since the inhibition observed in aim 1 was irreversible and attenuated only by GSH and not other antioxidants. Our results were corroborated by Van Laar and colleagues (2008b) as they observed DAQ binding to complex I and III subunits and by Khan et. al. (2005) as they reported protection from DA toxicity by GSH. Oxidation of extra cytosolic DA could also occur in the interstitial space since DA readily oxidizes at normal pH. Additionally, due to the presence of high levels of iron in the SNpc, DA auto-oxidation may be even further favored, rendering this region specifically vulnerable to mitochondrial dysfunction and subsequent cell damage/death.

The striatum was reported to have a greater loss of dopaminergic terminals than neuronal cell death in the SNpc of PD post mortem brains (Bernheimer et al.,1973). This suggests that dopaminergic nerve terminal loss may precede cell death in the SNpc. Herkenham et. al. (1991) observed striatal terminal loss prior to neuronal death in the SNpc of MPTP treated mice, moreover, protection of the terminals prevented cell death (Wu et. al., 2003). The mechanism for terminal loss is unclear; however products of DA metabolism, i.e. quinones, DOPAC and oxidative stress caused by hydrogen peroxide metabolism could be factors that create a toxic environment. GSH is one of the main antioxidants and quinone chelators in the brain. Additionally, GSH forms reversible Pr-SSGs on vulnerable protein thiols during episodes of oxidative stress to protect them from irreversible oxidation. Therefore disturbances in GSH or components of the GSH

system could render the striatum susceptible to toxicity. In fact, low levels of GSH in the striatum and SNpc of mice compared to other brain regions has been observed (Abbot et. al., 1990). Deglutathiolation of Pr-SSGs is important since it would re-establish the protein to its normal state and restore the normal cytosolic GSH levels. Glutaredoxin is the enzyme responsible for thiolation and dethiolation of Pr-SSG's using GSH as the reductant. Rat brain striatum was found to have low levels of Gr activity, protein and messeage, compared to all other brain regions studied (aim 3, Balijepalli et. al., 1999). Our study also found lower levels of Grx activity and protein in the midbrain compared to other brain regions. This suggests that striatum and midbrain may be at a greater risk of damage due to DA metabolites than other brain regions. This is relevant to PD as low levels of GSH have been observed in the SNpc of PD brains (Perry et. al., 1982). The current project is the first to analyze regional distribution of mitochondrial Grx (Grx 2). In contrast to Grx message levels, we observed highest levels of Grx 2 message in the striatum. This leads to question why there is a persistent dopaminnergic terminal loss in the striatum, which may also be an upstream event to nigral cell death, despite this heightened antioxidant system in the striatal mitochondria. One possible explanation may be that since mitochondria are dependent upon uptake of cytosolic GSH to maintain GSH levels, decreased levels of cytosolic GSH could lead to reduced levels of mitochondrial GSH. This could render the mitochondria vulnerable to toxicity notwithstanding high levels of Grx 2. We also examined Grx activity and protein levels in rat brain with respect to age and found there are increases in Grx activity and protein during the early prenatal and postnatal periods and then in older age animals from 14 to 18 months. The increase in Grx in older animals may be a compensatory mechanism to counter increases

in oxidative stress due to aging. Gender based studies showed no differences between male and female rats.One study used females of mixed estrous cycle and a second females used in the proestrous phase since this is the phase in which serum estrogen is at its highest levels. Our findings in rats on gender differences in Grx differed from those in mice and should therefore be more thoroughly investigated and expanded to include human brain.

There has been only one report using qualitative assessment, which states that glia have negligible amounts of Grx when compared to neurons. In the present work using quantitative PCR, astrocytes were shown to contain 4 fold less Grx 1 and 14 fold less Grx 2 message than neurons. This is intriguing since astrocytes generally contain more GSH than neurons and are more resistant to oxidative stress than neurons. In fact an increase in number of astrocytes was observed in PD and other neurodegenerative disorders (Forno et. al., 1992). Astrocytes may have other means of deglutathiolation and no information at preent is known requiring mixed disulphide formation in astrocytes or its reversal. This would be a fruitful area for future investigation. The studies from this thesis support the hypothesis that GSH-DA interactions are important to the survival of dopaminergic neurons; moreover, disturbances in GSH or DA homeostasis are detrimental to dopaminergic neurons possibly leading to cell damage and/or death through mitochondrial dysfunction as shown schematically in Fig 24 below.

Given that DOPAC was an equipotent inhibitor with DA on ETC function, future studies of DOPAC uptake into mitochondria should be investigated. Additionally, since the nigrostriatal pathway is specifically affected in PD, regional differences in DA and

DOPAC uptake into mitochondria should also be assayed. The affects of reduced and oxidized DA and DOPAC on ETC functions assayed in this thesis project were in mitochondria from whole brain homogenates, a regional study may reveal specific regional sensitivities to the catecholamines, as was shown in our Grx study that demonstrated less activity in the striatum than other brain regions. Additionally, regional study of Grx activities with respect to age should also be undertaken since the age study was also performed in whole brain homogenates. Therefore, it is unknown whether there were differences in Grx activities in specific brain regions as the animal aged. Grx activity in neurons and astrocytes should be examined since mRNA alone may not necessarily indicate activity levels. Finally, other means of dethiolation by astrocytes, such as the thioredoxin system should also be examined.

FIGURE 24

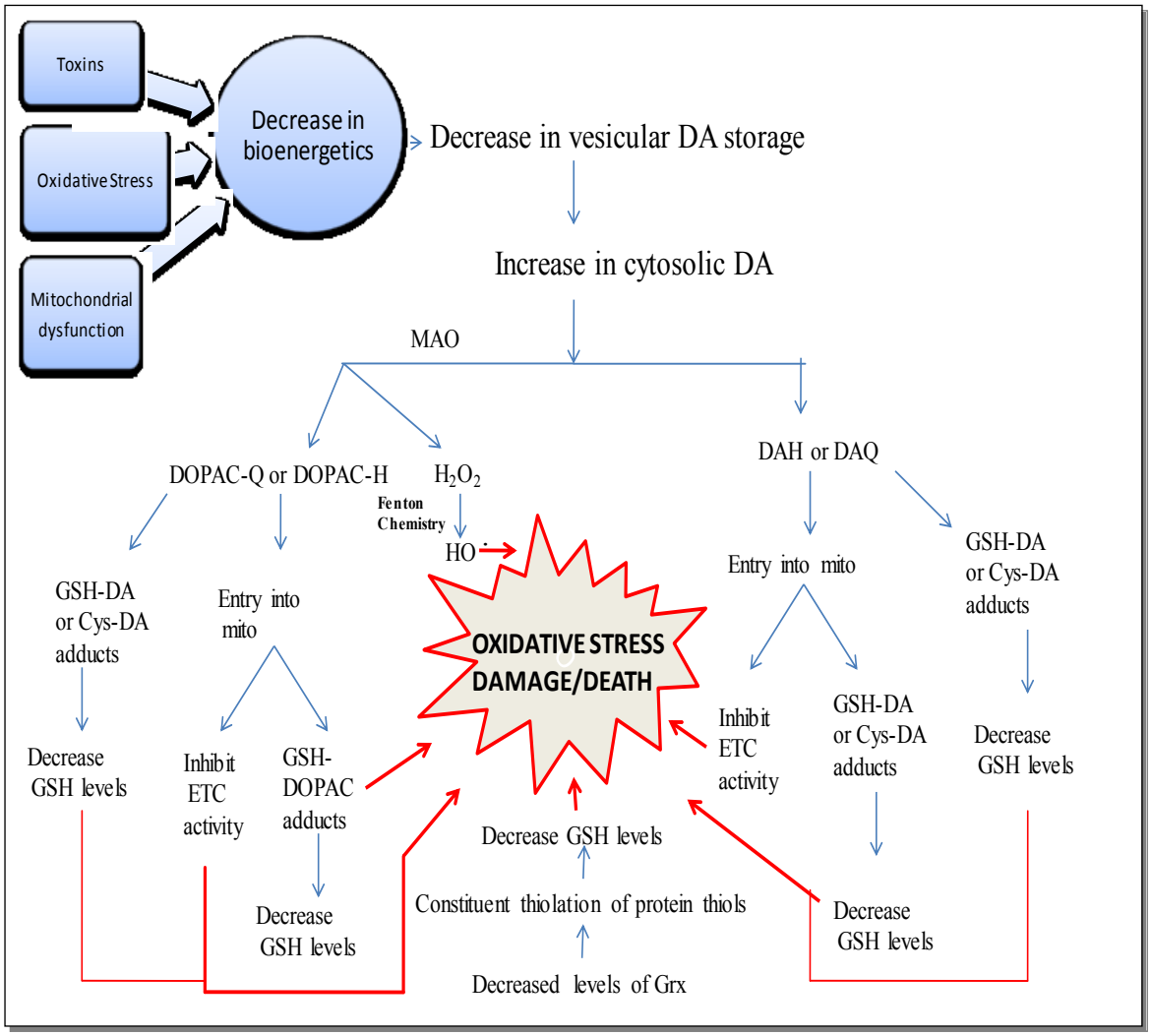

Toxins
Oxidative Stress
Mitochondrial dysfunction
Decrease in bioenergetics
Decrease in vesicular DA storage
Increase in cytosolic DA
MAO
DOPAC-Q or DOPAC-H
H_2O_2
Fenton Chemistry
HO·
DAH or DAQ
GSH-DA or Cys-DA adducts
Entry into mito
OXIDATIVE STRESS DAMAGE/DEATH
Entry into mito
GSH-DA or Cys-DA adducts
Decrease GSH levels
Inhibit ETC activity
GSH-DOPAC adducts
Inhibit ETC activity
GSH-DA or Cys-DA adducts
Decrease GSH levels
Decrease GSH levels
Decrease GSH levels
Constituent thiolation of protein thiols
Decrease GSH levels
Decreased levels of Grx

Fig 24*:* Schematic of interactions between dopamine (DA) and reduced glutathione (GSH) leading to oxidative stress damage and/or cell death. Loss of bioenergetics can be caused by various causes such as oxidative stress, mitochondrial dysfunction or exposure to toxins which could affect mitochondrial function. Deficiencies in bioenergetics could result in loss of DA sequestration in vesicles thereby increasing cytosolic DA. The DA can now be metabolized via MAO to DOPAC and hydrogen peroxide. DOPAC could autoxidize to form quinones (DOPAC-Q) and form quinone adducts with cytosolic GSH (GSH-DOPAC-Q) or free cysteines (Cys-DOPAC-Q) and decrease GSH levels. Both reduced and/or oxidized DOPAC can gain entry into mitochondria where the reduced DOAPC can be oxidized and inhibit ETC function or form adducts with the mitochondrial pool of GSH. Both reduced and oxidized DOPAC could also inhibit mitochondrial respiration. All of these events can lead to increased oxidative stress. Hydrogen peroxide can form hydroxyl radicals ($HO^{\cdot}$) through Fenton chemistry and also lead to oxidative stress. Unsequestered DA could also autoxidize to form DAQ which could also form adducts with cytosolic GSH (GSH-DA) or free cysteines (Cys-DA), both of which could decrease GSH levels adding to an oxidizing environment. Reduced and oxidized DA could also inhibit mitochondrial respiration or could gain entry into the mitochondria where it could oxidize and inhibit ETC function or form adducts (GSH-DA) with mitochondrial GSH decreasing mitochondiral GSH levels. Therefore, once inside mitochondria DA can cause damage via various pathways. In addition to increased cytosolic DA, low levels of cytosolic and mitochondrial glutaredoxin (Grx) could impede deglutathiolation of protein-mixed-disulfides (Pr-SSGs) formed during an oxidative stress episode.

This would further decrease GSH levels and maintaining the oxidative stressed environment and possibly affect the thiolated protein function.

BIBLIOGRAPHY

Abbott, L.C., Nejad, H.H., Bottje, W.G., and Hassan, A.S., (1990). Glutathione levels in specific brain regions of genetically epileptic (tg/tg) mice. *Brain Res. Bull.* **25**: 629–631.

Abeliovich, A., Schmitz, Y., Farinas, I., Choi-Lundberg, D., Ho, W.H., Castillo, P.E., Shinsky, N., Verdugo, J.M., Armanini, M., Ryan, A., Hynes, M,, Phillips, H., Sulzer, D., and Rosenthal, A., (2000). Mice lacking alpha-synuclein display functional deficits in the nigrostriatal dopamine system, *Neuron* **25**: 239–252.

Abou-Sleiman, P.M., Healy, D.G., Quinn, N.P., Lees, A.J., and Wood, N.W., (2003). The role of pathogenic DJ-1 mutations in Parkinson's disease. *Ann Neurol* **54**: 283–286.

Adams, J. D., Klaidman, L. K., and Leung, A., (1993). MPP^+ and $MPDP^+$ induced oxygen radical formation with mitochondrial enzymes. *Free Radic Biol Med* **15**:181–186.

Akterin, S., Cowburn, R.F., Miranda-Vizuete, A., Jiménez, A., Bogdanovic, N., Winblad, B., and Cedazo-Minguez, A., (2006). Involvement of glutaredoxin-1 and thioredoxin-1 in beta-amyloid toxicity and Alzheimer's disease. *Cell death and diff* **13**(9):1454-65.

Alam, M., and Schmidt, W. J., (2002) Rotenone destroys dopaminergic neurons and induces Parkinsonian symptoms in rats. *Behav Brain Res* **136** (1), 317-324.

Alam, Z.I., Daniel, S.E., Lees, A.J., Marsden, D.C., Jenner, P., and Halliwell, B., (1997). A generalized increase in protein carbonyls in the brain in Parkinson's but not Incidental Lewy Body Disease. *J Neurochem* **69**(3): 1326–1329.

Aleman, A., Kahn, R.S., and Selton, J., (2003). Sex differences in the risk of schizophrenia: evidence from meta-analysis. *Arch Gen Psychiatry* **60**:565-571.

Andersen K, Launer, L.J., Dewey, M.E., Letenneur, L., Ott, A., Copeland, J., Dartigues, J.F., Kragh-Sorensen, P., Brayne, C., Lobo, A., Martinez-Lage, J. M., Stijnen, T., and Hofman, A. (1999). Gender differences in the incidence of AD and vascular dementia: the EURODEM Studies. EURODEM incidence research group. *Neurology* **53**: 1992-1997.

Andersen, J.K., (2004). Oxidative stress in neurodegeneration: cause or consequence? *Nature Reviews Neuroscience* **5**, S18–S25.

Andersen, K., A national estimate of direct and indirect costs in Parkinson's disease using retrospective database analysis. Poster session presented at: *APHA 134th Annual Meeting and Exposition*; November 4-8 2006; Boston, MA.

Anderson, M.E., (1998). Glutathione: an overview of biosynthesis and modulation. *Chemico-Biol Interactions*, **111**:1-14.

Asahina M, Yamada T, Yoshiyama Y and Yodoi J (1998) Expression of adult T cell leukemia-derived factor in human brain and peripheral nerve tissues. Dement. *Geriatr. Cogn. Disord*. **9**: 181–185.

Bains, J. S. and Shaw, C. A., (1997). Neurodegenerative disorders in humans: the role of glutathione in oxidative stress-mediated neuronal death. *Brain Res Rev*, **25**:335–358.

Balijepalli S, Tirumalai , P.S., Swamy, K.V., Boyd, M.R., Mieyal , J.J., Ravindranath., V., (1999). Rat brain thioltransferase: regional distribution, immunological characterization, and localization by fluorescent in situ hybridization. *J Neurochem*. **72**(3):1170-1178.

Balijepalli, S., Boyd, M.R., and Ravindranath, V., (2000). Human brain thioltransferase: constitutive expression and localization by fluorescence in situ hybridization. *Mol Brain Res* **85**(1–2): 123–132.

Bandyopadhyay, S., Starke, D.W., Mieyal, J.J., and Gronostajski, R.M., (1998). Thioltransferase (glutaredoxin) reactivates the DNA-binding activity of oxidation-inactivated nuclear factor I. *J. Bio Chem* **273**: 392–397.

Bandopadhyay R., Kingsbury, A.E, Cookson, M.R, Reid A.R., Evans, I.M., Hope, A.D., Pittman, A.M., Lashley, T., Canet-Aviles, R., Miller, D.W., McLendon, C., Strand, C., Leonard, A.J., Abou-Sleiman, P.M., Healy, D.G., Ariga, H., Wood, N.W., de Silva, R., Revesz, T., Hardy, J.A., and Lees, A.J., (2004). The expression of DJ-1 (PARK7) in normal human CNS and idiopathic Parkinson's disease. *Brain* **127**(Pt 2):420-430.

Barnes, L.L.,Wilson, R.S., Bienias, J.L., Schneider, J.A., Evans, D.A., and Bennett, D.A., (2005). Sex differences in the clinical manifestations of Alzheimer disease pathology. *Arch Gen Psychiatry* **62**: 685-691.

Bassan M., Zamostiano R., Davidson A., Pinhasov A., Giladi E., Perl O., Bassan, H., Blat, C., Gibney, G., Glazner, G., Brenneman, D.E., and Gozes, I., (1999) Complete sequence of a novel protein containing a femtomolar-activity-dependent neuroprotective peptide. *J. Neurochem* **72**: 1283–1293.

Beal, M.F., (2001). Experimental models of Parkinson's disease. *Nat Rev Neurosci* **2**:325–334.

Beal, M. F. (2003). Mitochondria, oxidative damage, and inflammation in Parkinson's disease. *Ann NY Acad Sci* **991**:120-131.

Beal, M.F., (2004). Aging, energy, and oxidative stress in neurodegenerative diseases. *Ann. Neuro* **38**(3): 357 – 366.

Beal, M.F., (2005). Mitochondria take center stage in aging and neurodegeneration. *Ann Neurol* **58**: 495–505.

Becker, J.B., Arnold A.P., Berkley K.J., Blaustein J.D., Eckel L.A., Hampson E., Herman J.P., Marts S., Sadee W., Steiner M., Taylor, J., and Young, E., (2005). Strategies and methods for research on sex differences in brain and behavior. *Endocrinology* **146**:1650–1673.

Beer, S.M., Taylor, E.R., Brown, S.E., Dahm, C.C., Costa, N.J., runswick, M.J., and Murphy, M.P., (2004). Glutaredoxin 2 catalyses the reversible oxidation and glutathionylation of mitochondrial membrane thiol proteins: implications for mitochondrial redox regulation and antioxidant defense. *J.Biol Chem* **279**: 47939-47951.

Beilina A, Van Der Brug M, Ahmad R, Kesavapany S, Miller DW, Petsko GA, and Cookson, M. R., (2005) Mutations in PTEN-induced putative kinase 1 associated with recessive parkinsonism have differential effects on protein stability. *Proc Natl Acad Sci* **102**:5703–5708.

Ben-Shachar, D., Zuk, R., and Glinka, Y., (1995) Dopamine neurotoxicity: inhibition of mitochondrial respiration, *J Neurochem*. **64** , 718–723.

Ben-Shachar, D., Zuk, R., Gazawi H., and Ljubuncic, P., (2004) Dopamine toxicity involves mitochondrial complex I inhibition: implications to dopamine-related neuropsychiatric disorders. *Biochem Pharm* **67** (10):1965-1974.

Benecke, R., Strumper, P., and Weiss, H. (1993). Electron transfer complexes I and IV of platelets are abnormal in Parkinson's disease but normal in Parkinson-plus syndromes *Brain* **116**:1451-146.

Benzi, G., (Ed.), (1990) Peroxidation, Energy Transduction and Mitochondria During Aging, John Libbey Eurotext, Paris.

Berg, D., Schweitzer, K., Leitner, P., Zimprich, A., Lichtner, P., Belcredi, P., Brussel, T., Schulte, C., Maass, S., Nagele, T., (2005) Type and frequency of mutations in the LRRK2 gene in familial and sporadic Parkinson's disease. *Brain*. **128**:3000–3011.

Berg, JM.; Tymoczko, J.L.; Stryer, L., (2007), Biochemistry (6 ed.), New York: WH Freeman and Co.

Berman, B.M., and Hastings, T.G., (1999). Dopamine Oxidation Alters Mitochondrial Respiration and Induces Permeability Transition in Brain Mitochondria. Implications for Parkinson's disease. *J Neurochem*, **73** (3): 1127–1137.

Bernard, O., and Balasubramanian K. A., (1995). Effect of oxidized glutathione on intestinal mitochondria and brush border membrane. *Int. J. Biochem. Cell Biol* **27**:589–595.

Bernheimer, H., Birkmayer, W., Hornykiewicz, O., Jellinger, K., and Seitelberger, F. (1973). Brain dopamine and the syndromes of Parkinson and Huntington. Clinical, morphological and neurochemical correlations. *J Neurol Sci* **20**; 415–455.

Betarbet, R., Sherer, T.B., MacKenzie, G., Garcia-Osuna, M., Panov, A.V., and Greenamyre, J.T. (2000). Chronic systemic pesticide exposure reproduces features of Parkinson's disease. *Nat Neurosci* **3**:1301–1306.

Betarbet, R., Sherer, T.B., and Greenamyre, J.T., (2002). Animal models of Parkinson's disease. *Bioessays* **24**:308–318.

Bindoff, L.A., Birch-Machin, M., Cartlidge, N.E., Parker, W.D. Jr., Turnbull, D.M., (1991).Respiratory chain abnormalities in skeletal muscle from patients with Parkinson's disease. *J Neurol Sci* **104**:203-208.

Bindoli A, Rigobello MP, Deeble D.J., (1992). Biochemical and toxicological properties of the oxidation products of catecholamines. *Free Radic Biol Med* **13**:391–405**.**

Birch-Machin, M.A., Briggs, H.L., Saborido, A.A., Bindoff, L.A., and Turnbull, D.M., (1994) An evaluation of measurement of the activities of Complexes I-IV in the respiratory chain of human skeletal muscle mitochondria. *Biochem. Med. Metab. Biol.* **51** (1):35–42.

Blum, D., Torch, S., Lambeng, N., Nissou M., Benabid A.L., Sadoul R., Verna, J.M. (2001) Molecular pathways involved in the neurotoxicity of 6-OHDA, dopamine and MPTP: contribution to the apoptotic theory in Parkinsons disease. *Prog Neurobiol* **65**:135–172.

Bolaños J.P., Heales S.J.R., Land J.M., Clark J.B.,(1995). Effect of peroxynitrite on the mitochondrial respiratory chain: differential susceptibility of neurons and astrocytes in primary culture. *J. Neurochem* **64**:1965–1972.

Bonifati,V., Rizzu, P., Van Baren, M.J., Schaap, O., Breedveld, G.J., Krieger, E., Dekker, M.C., Squitieri, F., Ibanez, P., Joosse, M., van Dongen, J,W., Vanacore, N., van Swieten, J.C., Brice, A., Meco, G., van Duijn, C.M., Oostra, B.A., Heutink, P., (2003). Mutations in the DJ-1 gene associated with autosomal recessive early-onset parkinsonism, *Science* **299**:256–259.

Bonifati, V., (2007) Genetics of parkinsonism. *Parkinsonism Relat. Disord*., **13** (SUPPL. 3)., S233-S241.

Borges, C.R., Geddes, T., Watson, J.T., Kuhn, D.M., (2002). Dopamine biosynthesis is regulated by S-glutathionylation: potential mechanism of tyrosine hydroxylase inhibition during oxidative stress. *J Biol Chem* **277**:48295-48302.

Brenner-Lavie H, Klein E, Zuk R, Gazawi H, Ljubuncic P, and Shachar, D. (2008). Dopamine modulates mitochondrial function in viable SH-SY5Y cells possibly via its interaction with complex I: relevance to dopamine pathology in schizophrenia. *Biochim Biophys Acta.***1777**:173–185.

Cassarino D.S., Fall C.P., Smith T.S., and Bennett J.P.J r., (1998) Pramipexole reduces reactive oxygen species production in vivo and in vitro and inhibits the mitochondrial permeability transition produced by the parkinsonian neurotoxin methylpyridinium ion. *J. Neu*rochem. **71**:295–301.

Castello, P.R., Drechsel, D.A., and Patel, M., (2007). Mitochondria are a major source of paraquat-induced reactive oxygen species production in the brain. *J Biol Chem* **282**:14186–14193.

Caudle, W.M., Richardson, J.R., Wang, M.Z., Taylor, T.N., Guillot, T.S., McCormack, A.L., Colebrooke, R.E., Di Monte, D.A., Emson, P.C., and Miller, G.W., (2007) Reduced vesicular storage of dopamine causes progressive nigrostriatal neurodegeneration. *J Neurosci* **27**:8138–8148.

Chai, Y.C., Hendrich, S., and Thomas, J.A., (1994). Protein S-thiolation in hepatocytes stimulated by t-butyl hydroperoxide, menadione, and neutrophils. *Arch. Biochem Biophys* **310**:264 272.

Chen, L., Ding, Y., Cagniard, B., Van Laar, A.D., Mortimer, A., Chi, W., Hastings, T.G., Kang U.J., Zhuang., X., (2008). Unregulated cytosolic dopamine causes neurodegeneration associated with oxidative stress in mice. *J Neurosci* **28**:425–433.

Chen, T.S., Richie, J.P Jr., and Lang, C.A., (1989). The effect of aging on glutathione and cysteine levels in different regions of the mouse brain. *Proc Soc Exp Bio Med* **190**:399–402.

Chiba, K., Trevor, A.J., and Castagnoli Jr. N., (1985). Active uptake of MPP+, a metabolite of MPTP, by brain synaptosomes. *Biochem and Biophysl Res Comm*, **128** (3):1228-1232.

Chinta SJ, and Andersen J.K., (2006). Reversible inhibition of mitochondrial complex I activity following chronic dopaminergic glutathione depletion in vitro: implications for Parkinson's disease. *Free Radic Biol Med* **41**(9):1442-1448.

Chrestensen, C.A., D.W. Starke and J.J. Mieyal, (2000). Acute cadmium exposure inactivates thioltransferase (glutaredoxin), inhibits intracellular reduction of protein–glutathionyl-mixed disulfides and initiates apoptosis. *J Biol Chem* 275 **34**, 26556–26565.

Chung, K.K, Dawson, V.L, and Dawson, T.M., (2001). The role of the ubiquitin-proteasomal pathway in Parkinson's disease and other neurodegenerative disorders. *Trends Neurosci* **24** (11 Suppl): S7–14.

Chung, K.K.K., B. Thomas, X. Li, O. Pletnikova, J.C. Troncoso, L. Marsh, V. L. Dawson and Dawson, T.M., (2004). S-Nitrosylation of Parkin regulates ubiquitination and compromises Parkin's protective function. *Science* **304**:1328-1331.

Clark, J.B., and Nicklas, W.J., (1970) Metabolism of rat brain mitochondria: Preparation and characterization. *J Biol Chem* **255**, 4724-4731.

Cohen, G. (1988) Oxygen radicals and Parkinson's disease. In: *Oxygen radicals and tissue injury* (Halliwell, B., ed,), 130-135, FASEB, Bethseda, MD.

Cohen, G. and Werner, P., (1994). Free radicals oxidative stress and neurodegeneration, In D.B. Calne (Ed.), Neurodegenerative Diseases, W.B. Saunders, Philadelphia, PA, 139-161.

Cohen, G., (1999). Oxidative stress and Parkinson's disease. In: *Reactive oxygen species in biological systems*. Gilbert, D.L., and Colton, C.A., (eds.), Kluwer Academic/Plenum, New York. 593-608.

Cohen, G., and Kesler, N., (1999). Monoamine oxidase and mitochondrial respiration. *J Neurochem* **73**(6):2310–2315.

Conway K. A., Lee, S. J., Rochet, J. C., Ding, T. T., Williamson, R. E. and Lansbury, P. T., Jr. (2000) Acceleration of oligomerization, not fibrillization, is a shared property of both alpha-synuclein mutations linked to early-onset Parkinson's disease: implications for pathogenesis and therapy. *Proc. Natl. Acad. Sci. USA* **97:**571-576.

Conway, K.A., Rochet, J.C., Bieganski, R.M., and Lansbury, P.T., Jr., (2001). Kinetic stabilization of the alpha-synuclein protofibril by a dopamine-alpha-synuclein adduct. *Science* **294**:1346–1349.

Cookson MR. (2005). The biochemistry of Parkinson's disease. *Annu Rev Biochem.* **74**:29-52.

Cooper, A.J.L., (1997). Glutathione in the brain: Disorders of glutathione metabolism. In: Rosenberg, R.N., Prusiner, S.B,, Dimauro, S,, Barchim R.L., Kunk, L.M., (eds) The molecular and genetic basis of neurological disease. Boston: Butterworth-Heinemann. pp 1195-1230.

Currie, L.J., Harrison, M. B. , Trugman, J. M. , Bennett, J. P. , and Wooten, G. F., (2004). Postmenopausal estrogen use affects risk for Parkinson Disease. *Arch Neurol* **61**(6): 886 - 888.

Cyr, M., Beaulieu, J.M., Laakso, A., Sotnikova, T.D., Yao, W.D., Bohn,L.M., Gainetdinov, R.R., Caron, M.G., 2003. Sustained elevation of extracellular dopamine causes motor dysfunction and selective degeneration of striatal GABAergic neurons. Proc *Natl Acad Sci* **100**:11035– 11040.

Daily D, Vlamis-Gardikas A, Offen D, Mittelman L, Melamed E, Holmgren A and Barzilai A., (2001). Glutaredoxin protects cerebellar granule neurons from dopamine-induced apoptosis by activating NF-kappa B via Ref-1. *J Biol Chem* 276 1335–1344.

Dalle-Donne, I., Rossi, R., Giustarini, D., Colombo, R., and Milzani, A., (2007). S-glutathionylation in protein redox regulation, *Free Radic Biol Med* **43**:883–898.

Dauer, W. and Przedborski, S. (2003). Parkinson's disease: mechanisms and models, *Neuron* **39**(8):889-909.

Dauer, W., Kholodilov, N., Vila, M., Trillat, AC, Goodchild, R, Larsen, K.E., Staal, R., Tieu, K., Schmitz, Y., Yuan, C..A, Rocha, M., Jackson-Lewis, V., Hersch, S., Sulzer, D., Przedborski, S., Burke, R., and Hen, R., (2002). Resistance of alpha-synuclein null mice to the parkinsonian neurotoxin MPTP. *Proc Natl Acad Sci* **99**:14524–14529.

Dawson, T.M., and Dawson, V.L., (2003). Rare genetic mutations shed light on the pathogenesis of Parkinson disease. *J Clin Invest* **111**: 145–151.

Desagher S., Glowinski J., and Premont J., (1996). Astrocytes protect neurons from hydrogen peroxide toxicity. *J Neurosci* **16**:2553–2562.

Dexter, D.T., Wells, F.R., Lees, A.J., Agid, F., Agid, Y., Jenner, P.,and Marsden, C.D., (1989). Increased nigral iron content and alterations in other metal ions occurring in brain in Parkinson's disease. *J Neurochem* **52**(6):1830-1836.

Dexter, D.T., Sian, J, Rose, S., Hindmarsh, J.G., Mann, V.M., Cooper, J.M., Wells, F.R., Daniel,S.E., Less, A.J., and Schapira, A.H., (1994). Indices of oxidative stress and mitochondrial function in individual with incidental Lewy body disease. *Ann Neurol* **35:**38-44.

Diwakar, L., Kenchappa, R.S., Annepu, J., and Ravindranath, V., (2007). Downregulation of glutaredoxin but not glutathione loss leads to mitochondrial dysfunction in female mice CNS: implications in excitotoxicity. *Neurochem Int* **51**(1):37-46.

Do, K.Q., Trabesinger, A.H., Kirsten-Krüger, M., Lauer, C.J., Dydak, U., Hell, D., Holsboer, F., Boesiger, P, and Cuenod, M., (2000). Schizophrenia: glutathione deficit in cerebrospinal fluid and prefrontal cortex in vitro. *Eur J Neurosci* **12**: 3721-3728.

Dringen, R., and Hirrlinger, J., 2003. Glutathione Pathways in the Brain. *Biol. Chem*., **384**:505–516.

Ehrhart J, and Zeevalk G.D., (2001). Hydrogen peroxide removal and glutathione mixed disulphide formation during metabolic inhibition in mesencephalic cultures. J *Neurochem* **77**:1496-1507.

Ehrhart, J., Gluck, M., Mieyal, J., and Zeevalk, G. D. (2002) Functional glutaredoxin(thioltransferase) activity in rat brain and liver mitochondria. *Parkinsonism Related Disord* **8**:395-400.

Ehrhart J., and Zeevalk, G.D., (2003). Cooperative interaction between ascorbate and glutathione during mitochondrial impairment in the mesencephalic cultures. *J. Neurochem* **86**: 1487-1497.

Eisenhofer, G., Kopin, I.J., and Goldstein, D.S., (2004) Catecholamine metabolism: a contemporary view with implications for physiology and medicine. *Pharmacol Rev* **56**:331–349.

Fahn S. and Cohen G. (1992) The oxidant stress hypothesis in Parkinson's disease: Evidence supporting it. *Ann Neurol* **32**:804–812.

Ferrante, R.J., Schulz, J.B., Kowall, N.W., and Beal, M.F. (1997). Mutation Systemic administration of rotenone produces selective damage in of the E6-AP ubiquitin ligase reduces nuclear inclusion frequency the striatum and globus pallidus, but not in the substantia nigra. *Brain Res* **753**(1):157-162.

Forno, L.S. (1982) Pathology of Parkinson's disease. In *Movement Disorders*, (C.D. Marsden and S. Fahn), 25-40, Butterworth Scientific, London.

Forno LS, DeLanney LE, Irwin I, Di Monte D, Langston, .JW., (1992). Astrocytes and Parkinson's disease. *Prog Brain Res* **94**:429-36

Forno, L.S. (1996). Neuropathology of Parkinson's disease. *J. Neuropathol. Exp. Neurol.* **55**:259–272.

Fornstedt, B., Pileblad, E., and Carlsson A., (1990). In vivo autoxidation of dopamine n guinea pig striatum increases with age. *J. Neurochem* **55**:655-659.

Freeman, M., Smith, M., Nazian, S., and Neill, J., (1974). Ovarian and hypothalamic control of the daily surges of prolactin secretion during pseudopregnancy in the rat. *Endocrinology* **94**:875-882.

Freeman, M.E., (1994). The neuroendocrine control of the ovarian cycle of the rat. In: E. Knobil and J.D. Neill, Editors, *The Physiology of Reproduction*, Raven, New York pp. 613–658.

Ghezzi, P., (2005). Regulation of protein function by glutathionylation. *Free Radic Res.* **39**: 573–580.

Giasson, J., Duda, E., Quinn, S.M., Zhang, B., Trojanowski, J.Q., and Lee, V.M. (2002). Neuronal alpha-synucleinopathy with severe movement disorder in mice expressing A53T human alpha-synuclein, *Neuron* **34**: 521–533.

Giedde, A. Modeling metabolite and tracer kinetics. In: Feinendegen, L.E., Shreeve, W.W., Eckelmann, W.C., Bahk, Y.W., Wagner, H.N. Molecular Nuclear Medicine, The Challenge of Genomics and Proteomics to Clinical Practice. Berlin, Heidelberg: Springer, Germany, 2003, pp 252-300.

Gilbert, M.F., (1995). Thiol/disulfide exchange equilibria and disulfide bond stability. *Methods Enzymol* **251**:123-133.

Giovanni A., Sieber B. A., Heikkila R. E. and Sonsalla P. K. (1991). Correlation between the neostriatal content of the 1-methyl-4-phenylpyridinium species and dopaminergic neurotoxicity following 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine administration to several strains of mice. *J Pharmacol Exp Ther* **257**:691-697.

Gladyshev, V.N., L. Aimin, S.V. Novoselov, K. Krysan, Q. Sun, V.M. Kryukov, G.V. Kryukov and. Lou, M.F., (2001). Identification and characterization of a new mammalian glutaredoxin (thioltransferase), Grx2. *J Biol Chem* **276** (32): 30374–30380.

Gluck, M.R., Ehrhart, J., Jayatilleke, E., and Zeevalk, G.D., (2002). Inhibition of brain mitochondrial respiration by dopamine: involvement of H2O2 and hydroxyl radicals but not glutathione-protein-mixed disulfides. *J Neurochem* **82** (1), 66–74.

Gluck, M.R., and Zeevalk, G.D., (2004). Inhibition of brain mitochondrial respiration by dopamine and its metabolites: implications for Parkinson's disease and catecholamine-associated diseases. *J Neurochem* **91**(4):788–795.

Goldberg, M.S., Fleming, S.M., Palacino, J.J., Cepeda, C., Lam, H.A., Bhatnagar, A., Meloni, E.G., Wu, N., Ackerson, L.C., Klapstein, G.J., Gajendiran, M., Roth, B.L., Chesselet, M.F., Maidment, N.T., Levine, M.S., and Shen, J., (2003) Parkin-deficient mice exhibit nigrostriatal deficits but not loss of dopaminergic neurons. *J Biol Chem* **278**: 43628–43635.

Goodman, Y., and Mattson, M.P., (1994). Secreted forms of β-amyloid precursor protein protects hippocampal neurons against amyloid β-peptide-induced oxidative injury. *Exp Neurol* **128**: 1-12.

Gorell, J.M., Johnson, C.C., Rybicki, B.A., Peterson, E.L., Kortsha, G.X., Brown, G.G., and Richardson, R.J., (1997). Occupational exposures to metals as risk factors for Parkinson's disease. *Neurol* **48**(3): 650-658.

Graham D.G., (1978). Oxidative pathways for catecholamines in the genesis of neuromelanin and cytotoxic quinones. *Mol Pharmacol* **14**:633-643.

Grasbon-Frodl, E.M., Andersson, A., and Brundin, P., (1996). Lazaroid treatment prevents death of cultured rat embryonic mesencephalic neurons following glutathione depletion. *J Neurochem* **67**:1653–1660.

Gravina, S.A., and Meiyal J.J., (1993). Thioltransferase is a specific glutathionyl mixed disulfide oxidoreductase. *Biochem J* **32:**3368-3376.

Greenamyre, J.T., Sherer, T.B., Betarbet, R., and Panov, A.V. (2001).Complex I and Parkinson's disease. *IUBMB Life* **52**:135–141.

Greenamyre, J.T., and Hastings, T.G., (2004). Parkinson's–Divergent causes, convergent mechanisms. *Science* **304**:1120-1122.

Griffith, O.W., and Meister, A., (1985). Origin and turnover of mitochondrial glutathione. *Proc of the Nat Acad of Sci of the United States of America*, **82** (14): 4668-4672.

Gutman, M., Mersmann, H., Luthy, J., and Singer, T.P.,(1970). Action of sulfhydryl inhibitors on different forms of the respiratory chain-linked reduced nicotinamide-adenine dinucleotide dehydrogenase. *Biochem* **9:** 2678–2687.

Haas, R.H., Nasirian, F., Nakano, K., Ward, D., Pay, M., Hill, R., and Shults, C.W. (1995). Low platelet mitochondrial complex I and complex II/III activity in early untreated Parkinson's disease. *Ann Neurol* **37**:714-722.

Halliwell B, and Gutteridge J.M.C., (1989) Free radicals in biology and medicine. Clarendon Press, Oxford, pp 96–98.

Halliwell, B., (1992). Reactive oxygen species and central nervous system. *J Neurochem* **59**:1609-1623.

Hardy, J.A., and Higgins, G.A., (1992). Alzheimer's disease: the amyloid cascade hypothesis. *Science* **256**: 184-185.

Harrower, T.P., Michell, A.W., and Barker, R.A., (2005). Lewy bodies in Parkinson's disease: protectors or perpetrators? *Exp Neurol* **195**: 1–6.

Hasegawa, E., Takeshige, K., Oishi, T., Murai, Y., Minakami, S., (1990). 1-Methyl-4-phenylpyridinium (MPP+) induces NADH-dependent superoxide formation and enhances NADH-dependent lipid peroxidation in bovine heart submitochondrial particles. *Biochem Biophys Res Commun* **170**:1049–1055

Hastings, T.G., and Zigmond, M.J., (1994). Identification of catechol-protein conjugates in neostriatal slices incubated with [3H] dopamine: impact of ascorbic acid and glutathione. *J Neurochem* **63**:1126 –1132.

Hasting T.G., (1995). Enzymatic oxidation of dopamine: the role of prostaglandin H synthase. *J Neurochem*. **64**: 919-924.

Hastings, T.G., Lewis, D.A., and Zigmond, M..J., (1996). Role of oxidation in the neurotoxic effects of intrastriatal dopamine injections. *Proc Natl Acad Sci* **93**: 1956-1961.
Hazelton, G.A., and Lang, C.A., (1980). Glutathione contents of tissues in the aging mouse. *Biochem J* **188**:25–30.

Healy, D.G., Abou-Sleiman, P.M., Gibson, J.M., Ross, O.A., Jain, S., Gandhi, S., Gosal, D., Muqit, M.M., Wood, N.W., and Lynch, T., (2004). PINK1 (PARK6) associated Parkinson disease in Ireland. *Neurology* **63**: 1486–1488

Hebert, L.E., Scherr, P.A., McCann, J.J., Beckett, L.A, Evans, D.A., 2001. Is the risk of developing Alzheimer's disease greater for women than for men? Am J Epidemiol. 153, 132-136.

Heikkila R. E., Manzino L., Cabbat F. S. and Duvoisin R. C., (1984). Protection against the dopaminergic neurotoxicity of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine by monoamine oxidase inhibitors. *Nature* **311**:467-469.

Herkenham, M., Little, M.D., Bankiewicz, K., Yang, S.C., Markey, S.P., and Johannessen, J.N. (1991). Selective retention of MPP+ within the monoaminergic systems of the primate brain following MPTP administration: an in vivo autoradiographic study. *Neurosci* 40, 133–158.

Herrero, M.T., Hirsch, E.C., Kastner, A., Ruberg, M., Luquin, M.R., Laguna, J., Javoy-Agid, F., Obeso, J.A., and Agid, Y., (1993). Does neuromelanin contribute to the vulnerability of catecholaminergic neurons in monkeys intoxicated with MPTP. *Neurosci* **56**: 499–511.

Hirota K, Matsui M, Murata M, Takashima Y, Cheng FS, Itoh T, Fukuda K and Yodoi J., (2000). Nucleoredoxin, glutaredoxin, and thioredoxin differentially regulate NF-kappaB, AP-1, and CREB activation in HEK293 cells. *Biochem and Biophys Res Comm* **274** 177–182.

Hirsh, E.C., Brandel, J.P. and Galle, P., 1991. Iron and aluminum increase in the substantia nigra of patients with Parkinson's disease: an X-ray microanalysis. *J Neurochem* **56**: 446–451

Hoglinger, G.U., Feger, J., Annick, P., Michel, P.P., Karine, P., Champy, P., Ruberg, M., Wolfgang, W.O., and Hirsch, E. (2003).Chronic systemic complex I inhibition induces a hypokinetic. *J Neurochem* **84**:1–12.

Holmgren, A. and Åslund, F., (1995). Glutaredoxin. *Methods Enzymol* **252**:283–292.

Holmgren, A., (1989) Thioredoxin and glutaredoxin systems. *J Bio Chem* **264**: 13963 13966.

Hornykiewicz O. and Kish S. J.,(1986). Biochemical pathoshysiology of Parkinson's disease. *Adv Neurol* **45**: 19-34.

Hornykiewicz, O., and Kish, S.J. (1987). Biochemical pathophysiol of Parkinson's disease. In Parkinson's Disease, M. Yahr and K.J. Bergmann, eds. (New York: Raven Press), pp. 19–34.

Hösli E., and Hösli L., (1997). Autoradiographic studies on the uptake of 3H-dopamine by neurons and astrocytes in explant and primary cultures of rat CNS: effects of uptake inhibitors. *Int J Dev Neurosci* ;**15**(1):45-53.

Hubscher, C.H., Brooks, D.L. and Johnson, J.R., (2005). A quantitative method for assessing stages of the rat estrous cycle. *Biotech Histochem* **80** (2):79–87.

Hurd, T.R., Requejo, R., Fillpovska, A., Brown, S., Prime, T.A., Robinson, A.J., Fearnley, I.M., and Murphy, M.P., (2008). Complex I within oxidatively stressed bovine heart mitochondria is glutathionylated on cys-531 and cys-704 of the 75-kDa subunit. Potential role of cys residues in decreasing oxidative damage. *J Biol Chem* **283** (36): 24801-24815.

Inestrosa, N.C., Marzolo, M.P., and Bonnefont, A.B., (1998). Cellular and molecular basis for estrogen's neuroprotection; Potential relevance for Alzheimer's disease. *Mol Neurobiol* **17**: 73-86.

Ischiropoulos, H., and Beckman, J.S., (2003) Oxidative stress and nitration in neurodegeneration: cause, effect or association? *J Clin Invest* **111**:163–169.

Jana, S., Kumar, A., Maria, M., Bagha, B.,Banerjeea, K., Dasa, A., Royb., A. and. Chakrabartia, S., (2007). Dopamine but not 3,4-dihydroxy phenylacetic acid (DOPAC) inhibits brain respiratory chain activity by autoxidation and mitochondria catalyzed oxidation to quinone products: Implications in Parkinson's disease. *Brain Res* **1139**:195-200.

Javitch, J.A., D'Amato, R.J., Strittmatter, S.M., and Snyder, S.H. (1985). Parkinsonism-inducing neurotoxin, N-methyl-4-phenyl- 1,2,3,6-tetrahydropyridine: uptake of the metabolite N-methyl-4- phenylpyridinium by dopamine neurons explain selective toxicity. *Proc Natl Acad Sci* **82**: 2173–2177.

Jenner, P., Dexter, D.T., Sian, J., Schapira, A.H.V., and Marsden, C.D., (1992). Oxidative stress as a cause of nigral cell death in Parkinson's disease and incidental Lewy body disease. *Ann. Neurol*. **32** (Suppl) S82-S-87.

Jenner P. (1993) Altered mitochondrial function, iron metabolism and glutathione levels in Parkinson's disease. *Acta. Neuro. Scand.* Suppl. **146**, 6-13.
Jenner, P., (2003) Oxidative stress in Parkinson's disease. *Ann Neurol* **53** (Suppl 3): S26–S36

Jeon, B.S., Jackson-Lewis, V., and Burke, R.E., (1995). 6-Hydroxydopamine lesion of the rat substantia nigra: time course and morphology of cell death. *Neurodegeneration* **4**:131–137.

Jha, N., Jurma, O., Lalli, G., Liu, Y., Pettusi, E.H., Greenamyre, J.T., Liu, R.M., Forman, H.J. and Andersen, J.K., (2000). Glutathione depletion in PC12 results in selective inhibition of mitochondrial complex I activity: implications for Parkinson's disease. *J. Biol Chem* **275**: 26096–27001.

Jiang, Y., Pei, L., Wang, M., and Liu, F., (2008). Extracellular dopamine induces the oxidative toxicity of SH-SY5Y cells. *Synapse* **62** (11)**:**797-803.

Jung, C.H., and Thomas, J.A., (1996). S-Glutahiolated hepatocyte proteins and insulin disulfides as substrates for reduction by glutaredoxin, thioredoxin, protein disulfide isomerase and glutathione. *Arch Biochem Biophys* **335**(1):61-72.

Karunakaran, S., Saeed, U., Ramakrishnan, S., Koumar, R.C., and Ravindranath, V., (2007). Constitutive expression and functional characterization of mitochondrial glutaredoxin (Grx2) in mouse and human brain. *Brain Res* **14**:(1185):8-17.

Kedar, N.P. (2003) Can we prevent Parkinson's and Alzheimer's disease? *J Postgrad Med* **49**: 236-245.

Kenchappa, RS, and Ravindranath, V., (2003). Glutaredoxin is essential for maintenance of brain mitochondrial complex I: studies with MPTP. *FASEB J* **17**(6): 717-719.

Kenchappa, R.S., Diwakar., L, Annepu., J., and Ravindranath., V.,(2004). Estrogen and neuroprotection: higher constitutive expression of glutaredoxin in female mice offers protection against MPTP-mediated neurodegeneration. *FASEB J* **18** (10):1102-4.

Khan, F.H., Saha, M., and Chakrabarti, S., (2001). Dopamine induced protein damage in mitochondrial-synaptosomal fraction of rat brain. *Brain Res* **895**(1 and 2): 245-249.

Khan, F.H., Sen, T., Maiti, A.K., Jana, S., Chatterjee, U., and Chakrabarti, S., (2005). Inhibition of rat brain mitochondria electron transport chain activity by dopamine oxidation products during extended in vitro incubation: Implications for Parkinson's disease. *Biochimica et Biophysica Acta* **1741**: 65-74.

Kikuchi A., Takeda A., Onodera H., Kimpara T., Hisanaga K., Sato N., Nunomura A., Castellani R.J., Perry G., Smith M.A., and Itoyama Y., (1999). Systemic Increase of Oxidative Nucleic Acid Damage in Parkinson's Disease and Multiple System Atrophy. *Neurobio of Dis* **9** (2); 244-248.

Kitada, T., Asakawa, S., Hattori, N., Matsumine, H., Yamamura, Y., Minoshima, S., Yokochi, M., Mizuno, Y. and Shimizu, N. (1998). Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. *Nature* **392**:605–608.

Kitimura, Y., T., Taniguchi., and Shimohama. S., (1999). Apoptotic cell death in neurons and glial cells: implications for Alzheimer's Disease. *Japan J Pharmacol* **79:**1-5.

Koller W, Vetere-Overfield B, Gray C, Alexander, C., Chin, T., Dolezal, J., Hassanein, R., and Tanner, C., (1990). Environmental risk factors in Parkinson's disease. *Neurology* **40**:1218-1221.

Korr, D., Toschi, L., Donner, P., Pohlenz, H.D., Kreft, B., Weiss, B. (2006). LRRK1 protein kinase activity is stimulated upon binding of GTP to its Roc domain. *Cell Signalling* **18**:910–920.

Kosower, N.S. and Kosower, E.M., The glutathione-glutathione disulfide system. In: W.A. Pryor, (Ed), Free Radicals in Biology Vol. II, Academic. Press, New York (1976), pp 55–84.

Koutsilieri E., Scheller C., Grunblatt E., Nara K., Li J. and Riederer P. (2002) Free radicals in Parkinson's disease. *J Neurol* **249**(Suppl 2):1–5.

Krahenbuhl, S., Chang, M., Brass, E.P., and Hoppel, C.L., (1991) Decreased activities of ubiquinol:ferricytochrome c oxidoreductase (Complex III) and ferrocytochrome c: oxygen oxidoreductase (Complex IV) in liver mitochondria from rats with hydroxycabalamin[c-lactam]-induced methylmalonic aciduria. *J Biol Chem* **266**(31):20998-21003.

Krahenbuhl, S., Talos, C., Wiesmann, U., and Hoppel, C.L., (1994) Development and evaluation of a spectrophotmetric assay for complex III in isolated mitochondria, tissues and fibroblasts from rats and humans. *Clinica Chimica Acta* **230***,* 177-187.

Langeveld C.H., Jongenelen A.M., Schepens E., Stoof J.C., Bast A., and Drukarch B., (1995). Cultured rat striatal and cortical astrocytes protect mesencephalic dopaminergic neurons against hydrogen peroxide toxicity independent of their effect on neuronal development. *Neurosci Lett* **192**: 13–16.

Langston, J.W.,Ballard, P., and Irwin, I. (1983).Chronic parkinsonism in humans due to a product of meperidine-analog synthesis. *Science* **219**: 979–980.

LaVoie, M.J., and Hastings, T.G., (1999a). Dopamine quinone formation and protein modification associated with the stratal neurotoxicity of methamphetamine: evidence against a role for extracelleular dopamine. *J Neurosci* **19**(4):1484-1491.

LaVoie, M.J., and Hastings, T.G., (1999b). Peroxynitrite- and nitrite-induced oxidation of dopamine : Implications for nitric oxide in dopaminergic cell loss. *J. Neurochem*. **73** (6):2546 – 2554.

LaVoie, M.J., Ostaszewski, B.L., Weihofen, A., Schlossmacher, M.G., and Selkoe, D.J., (2005). Dopamine covalently modifies and functionally inactivates parkin. *Nat Med* **11**:1214–1221

Leroy, E., Boyer, R., Auburger, G., Leube, B., Ulm, G., Mezey, E., Harta, G., Brownstein, M.J., Jonnalagada, S., Chernova, T. Dehejia, A., Lavedan, C., Gasser, T., Steinbach, P.J., Wilkinson, K.D., and Polymeropoulos, M.H., (1998). The ubiquitin pathway in Parkinson's disease. *Nature* **395**:451–452.

Li, Y., Liu, W., Oo, T.F., Wang, L., Tang, Y., Jackson-Lewis, V., Zhou, C., Geghman, K., Bogdanov, M., Przedborski, S., Beal, M.F., Burke, R.E., and Li, C., (2009) Mutant LRRK2(R1441G) BAC transgenic mice recapitulate cardinal features of Parkinson's disease. *Nat Neurosci* **12**:826–828.

Lind, C., Gerdes, R., Hamnell, Y., I., Schuppe-Koistinen, I., von Lowenhielm, H.B., Holmgren A., and Cotgreave, I.A., (2002). Identification of S-glutathionylated cellular proteins during oxidative stress and constitutive metabolism by affinity purification and proteomic analysis, *Arch Biochem Biophys* **406**:229–240.

Liou, H.H., Tsai,M.C., Chen, C.J., Jeng, J.S., Chang, Y.C.,Chen, S.Y., and Chen, R.C. (1997). Environmental risk factors and Parkinson's disease: a case-control study in Taiwan. *Neurology* **48**: 1583–1588.

Lippoldt., A , Padilla C A , Gerst H , Andbjer B , Richter E , Holmgren A , Fuxe K (1995). Localization of thioredoxin in the rat brain and functional implications. *J Neurosci* **15**:6747–6756

Livak, K.J., and Schmittgen, T.D., (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta}$ CT method. *Methods* **25**: 402–408.

Llopis, J., McCaffery, J. M., Miyawaki, A., Farquhar, M. G. and Tsien, R. Y., (1998) Measurement of cytosolic, mitochondrial, and golgi pH in single living cells with green fluorescent proteins. *Proc Natl Acad. Sci* 6803–6808.

Lotharius, J. and O'Malley, K.,(2000)The Parkinsonism-inducing drug 1-methyl-4-phenylpyridinium triggers intracellular dopamine oxidation: a novel mechanism of toxicity. *J Biol Chem* **75**: 38581-38588.

Lotharius, J. and Brundin, P., (2002a) Pathogenesis of Parkinson's disease: dopamine, vesicles and alpha-synuclein *Nat Rev Neurosci*. **3:**932– 942.

Lotharius, J. and Brundin, P., (2002b) Impaired dopamine storage resulting from alpha-synuclein mutations may contribute to the pathogenesis of Parkinson's disease *Hum Mol Genet* **11**:2395– 2407.

Lovell, M. A., Xie, C., and Markesbery, W.R., (1998). Decreased glutathione transferase activity in brain ventricular fluid in Alzheimer's disease. *Neurol* **51**:1562-1566.

Lundberg, M., C. Johansson, J. Chandra, M. Enoksson, G. Jacobsson, J. Ljung, M. Johansson and Holmgren, A., (2001). Cloning and expression of a novel human Glutaredoxin (Grx2) with mitochondrial and nuclear isoforms. *J Biol Chem* **276**: (28)26269–26275.

Lundstrom-Ljung, J.A., Vlamis-Gardikas, F., Aslund, F., and Holmgren, A., (1999). Reactivity of glutaredoxins 2,3 and 3 from Escherichia coli and protein disulfide isomerase towards glutathionyl-mixed disulfides in ribonuclease A. *FEBS Lett* **443**:85-88.

Luthman, J., Fredriksson, A., Sundstrom, E., Jonsson, G., and Archer, T., (1989) Selective lesion of central dopamine or noradrenaline neuron systems in the neonatal rat: motor behavior and monoamine alterations at adult stage. *Behav Brain Res* **33**:267–277.

MacKeigan, J.P., Murphy, L.O. and Blenis, J. (2005). Sensitized RNAi screen of human kinases and phosphatases identifies new regulators of apoptosis and chemoresistance. *Nat Cell Biol* **7**:591–600.

Mahadik, S.P., and Mukherjee, S. (1996) Free radical pathology and antioxidant defense in schizophrenia: a review. *Schizophr Res* **19**:1–17

Manning-Bog, A.B., McCormack, A.L., Li, J., Uversky, V.N., A.L., and Di Monte, D.A. (2002). The herbicide paraquat causes up-regulation and aggregation of alpha-synuclein in mice: *J Biol Chem* **277**:1641–1644.

Markey,S.P., Johannessen, J.N., Chiueh, C.C., Burns, R.S., and Herkenham, M.A. (1984). Intraneuronal generation of a pyridinium metabolite may cause drug-induced parkinsonism. *Nature* **311**: 464–467.

Maroteaux, L., Campanelli, J.T., and Scheller, R.H., (1988). Synuclein: a neuron-specific protein localized to the nucleus and presynaptic nerve terminal. *J Neurosci* **8**: 2804–2815.

Martinez-Vicente, M., Talloczy, Z., Kaushik, S., Massey, A.C., Mazzulli, J., Mosharov, E.V., Hodara, R., Fredenburg, R., Wu, D.C., Follenzi, A., Dauer, W., Przedborski, S., Ischiropoulos, H., Lansbury, P.T., Sulzer, D., and Cuervo, A.M., (2008). Dopamine-modified alpha-synuclein blocks chaperone-mediated autophagy. *J Clin Invest* **118**:777–788.

Mata, I.F., Lockhart, P.J. and Farrer, M.J. (2004) Parkin genetics: one model for Parkinson's disease. *Hum Mol Genet* **13**:R127–R133.

Mayer, R.A., Kindt, M.V., and Heikkila, R.E. (1986). Prevention of the nigrostriatal toxicity of 1-methyl-4-phenyl-1, 2,3,6-tetrahydropyridine by inhibitors of 3,4-dihydroxyphenylethylamine transport. *Neurochem* **47**:1073–1079.

McCormack, A.L., Thiruchelvam, M., Manning-Bog, A.B., Thiffault, Pendleton, C., Langston, J.W., Cory-Slechta, D.A., and Di Monte, D.A. (2002). Environmental risk factors and Parkinson's disease: generation of nigral dopaminergic neurons caused by the herbicide paraquat. *Neurobiol Dis* **10**:119–127.

McNaught, K.S., and Jenner, P., (2001). Proteasomal function is impaired in substantia nigra in Parkinson's disease. *Neurosci Lett* **297**:191–194.

McNaught, K.S., Perl, D.P., Brownell, A.L., and Olanow, C.W. (2004) Systemic exposure to proteasome inhibitors causes a progressive model of Parkinson's disease. *Ann Neurol* **56**:149–162.

Meister, A, and Anderson, M.E., (1983).Glutathione. *Annu Rev Biochem* **52**:711–760.

Meister, A., (1991). Glutathione deficiency produced by inhibition of its synthesis, and its reversal; Applications in research and therapy. *Pharmacol Ther* **51**:155–194.

Meister, A., (1994). Glutathione-ascorbic acid antioxidant system in animals. *J Biol Chem* **269**:9397-9400.

Meister, A., (1995). Mitochondrial changes associated with glutathione deficiency. *Biochim Biophys Acta* 1271, 35–42.

Menegon, A., Board, P.G., Blackburn, A., Mellick, G.D., and LeCouteur, D.G., (1998). Parkinson's disease, pesticides and glutathione transferase polymorphisms. *Lancet* **352**:1344-1346.

Meredith, G.E., Totterdell, S., Potashkin, J.A., and Surmeier, D.J., (2008). Modeling PD pathogenesis in mice: advantages of a chronic MPTP protocol. *Parkinsonism Relat Disord* **14**(Suppl 2):S112–S115.

Mieyal, J.J., Srinivasan, U., Starke, D.W., Gravina, S.A., and Mieyal, P.A., (1995). Glutathionyl specificity of the thioltransferases: mechanistic and physiological implications. In: L. Packer and E. Cadenas, Editors, *Biothiols in Health and Disease*, Marcel Dekker, Inc., pp305–372.

Milosevic, J., Schwarz, S.C., Ogunlade, V., Meyer, A.K., Storch, A., and Schwarz, J., (2009). Emerging role of LRRK2 in human neural progenitor cell cycle progression, survival and differentiation. *Mol Neurodegener* **15**;4:25.

Mitsumoto A, and Nakagawa Y., (2001). DJ-1 is an indicator for endogenous reactive oxygen species elicited by endotoxin. *Free Radic Res* **35**: 885–93.

Mizuno, Y., Ohta, S., Tanaka, M., Takamiya, S., Suzuki, K., Sato, T., Oya, H, Ozawa, T., and Kagawa, Y., (1989). Deficiencies in complex I subunits of the respiratory chain in Parkinson's disease. *Biochem Biophys Res Commun* **29:**163(3):1450–1455.

Mizuno, Y., Hattori, N., Mori, H., Suzuki, T., and Tanaka, K., (2001). Parkin and Parkinson's disease, *Curr Opin Neurol* **14**:477–482.

Montine, T.J., Picklo, M.J., Amarnath, V., Whetsell, W.O. Jr., and Graham, D.G., (1997). Neurotoxicity of endogenous cysteinylcatechols. *Exp Neurol* **148**:26-33.

Moratalla, R., Quinn, B., DeLanney, L.E., Irwin, I., Langston, J.W., and Graybiel, A.M. (1992). Differential vulnerability of primate caudate putamen and striosome-matrix dopamine systems to the neurotoxic effects of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. *Proc Natl Acad Sci* **89**:3859–3863.

Morikawa, N., Nakagawa-Hattori, Y., and Mizuno, Y., (1996) Effect of dopamine, dimethoxyphenylethylamine, papaverine, and related compounds on mitochondrial respiration and complex I activity. *J Neurochem* **66** (3):1174–1181.

Muthane,U.,Ramsay, K.A., Jiang,H., Jackson-Lewis, V.,Donaldson, D., Fernando, S., Ferreira,M., and Przedborski, S.(1994). Differences in nigral neuron number and sensitivity to 1-methyl-4-phenyl- 1,2,3,6-tetrahydropyridine in C57/bl and CD-1mice. *Exp Neurol* **126**:195–204.

Nagakubo D., Taira T, Kitaura H., Ikeda M., Tamai K., Iguchi-Ariga S.M., and Ariga H., (1997). DJ-1, a novel oncogene which transforms mouse NIH3T3 cells in cooperation with ras. *Biochem Biophys Res Commun* **231**: 509–13.

Nicklas, W.J., Vyas, I., and Heikkila, R.E. (1985). Inhibition of NADH- linked oxidation in brain mitochondria by MPP_, a metabolite of the neurotoxin MPTP. *Life Sci* **36**: 2503–2508.

Olanow, CW. and Tatton, WG., (1999). Etiology and Pathogenesis of Parkinson's disease. *Ann Rev of Neurosci* **22**:123-144.

Paganini-Hill, A., and Henderson, V.W., (1994) Estrogen deficiency and risk of Alzheimer's disease in women. *Am J Epidemiol* **140**:256-261.

Paganini-Hill, A., Henderson, V.W., (1996) Estrogen replacement therapy and risk of Alzheimer disease. *Arch Intern Med* **156**:2213-2217.

Paisan-Ruiz, S., Jain, E.W., Evans, W.P., Gilks, J., Simon, M. ,van der Brug, A., Lopez de Munain, A., Aparicio, S., Perez-Tur, J., Wood, N.W., and Singleton, A.B., (2004). Cloning of the gene containing mutations that cause PARK8-linked Parkinson's disease, *Neuron* **44**:595–600.

Parker, W.D., Jr., Boyson, S.J., and Parks, J.K., (1989). Abnormalities of the electron transport chain in idiopathic Parkinson's disease. *Ann Neurol* **26**:719–72

Parkinson, J., (1817) An essay on the shaking palsy. *J Neuropsychiatry Clin. Neurosci* **14**: 223–236.

Penn, A.M.W., Roberts, T., Hodder, J., Allen, P.S., Zhu, G., and Martin, W.R.W., (1995) Generalized mitochondrial dysfunction in Parkinson's disease detected by magnetic resonance spectroscopy of muscle. *Neurology* **45** (11):2097-2099.

Perry, T.L, and Yong, V.W., (1986). Idiopathic Parkinson's disease, progressive supranuclear palsy and glutathione metabolism in the substantia nigra of patients. *Neurosci. Lett.* **67**:269−274.

Perry, T.L., Godin, D.V. and Hansen, S., (1982). Parkinson's disease: a disorder due to nigral glutathione deficiency? *Neurosci Lett* **33**:305–310.

Perumal, A.S., Gopal, V.B., Tordzro, W.K., Cooper, T.B., and Cadet, J.L., (1992). Vitamin E attenuates the toxic effects of 6-hydroxydopamine on free radical scavenging systems in rat brain. *Brain Res Bull* **29**:699–701.

Pileblad, E., Eriksson, E.S., and Hansson, E. (1991). The presence of glutathione in primary neuronal and astroglial cultures from rat cerebral cortex and brain stem. *J Neural Trans* **86** (1): 43-49.

Polymeropoulos, M.H., Lavedan, C., Leroy, E., Ide, S.E, Dehejia, A., Dutra, A., Pike, B., Root, H., Rubenstein, J., Boyer, R., Stenroos, E.S., Chandrasekharappa, S., Athanassiadou, A., Papapetropoulos, T., Johnson, W.G., Lazzarini, A.M., Duvoisin, R.C., Di Iorio, G., Golbe, L.I., and Nussbaum, R.L., (1997). Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. *Science*. **276**: 2045–70.

Pràtico, D., and Delanty, N., (2000). Oxidative injury in diseases of the central nervous system: focus on Alzheimer's disease. *Am J Med* **109**: 577-585.

Price, P.A., Parkes, J.D., and Marsden, C.D., (1978). Sodium valproate in the treatment of levodopa-induced dyskinesia. *J Neurol Neurosurg Psychiatry* **41**(8):702–706.

Prinz, W.A., Åslund, F., Holmgren, A., and Beckwith, J. (1997) The role of the thioredoxin and glutaredoxin pathways in reducing protein disulfide bonds in the Escherichia coli cytoplasm. *J Biol Chem* **272**: 15661 15667.

Ragan, C.I., Wilson, M.T., Darley-Usmar, V.M, and Lowe, P.N., (1988) Subfractionation of mitochondria and isolation of the proteins of oxidative phophorylation. In: *Mitochondria: a practical approach* (Darley-Usmar VM, Rickwood D, Wilson MT, Deds.). Oxford, England: IRL Press, pp79-113

Ramachandiran, S., Hansen, J.M., Jones, D.P., Richardson, J.R., and Miller, G.W., (2007). Divergent mechanisms of paraquat, MPP+, and rotenone toxicity: oxidation of thioredoxin and caspase-3 activation. *Toxicol Sci* **95**:163–171.

Ramsay R. R., Kowal A. T., Johnson M. D., Salach J. I., and SingerT. P. (1987) The inhibition site of MPP+, the neurotoxic bioactivationproduct of I -methyl+phenyI- I ,2,3,6-tertrahydropyridine is near the Q-binding site of NADH dehydrogenase. *Arch Biochem Eiophys* **259**: 645-649.

Ramsay, R.R., and Singer, T.P. (1986). Energy-dependent uptake of N-methyl-4-phenylpyridinium, the neurotoxic metabolite of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, by mitochondria. *J Biol Chem* **261**:7585–7587.

Ramsay, R.R.., Krueger, M.J., Youngster, S.K., Gluck, M.R., Cesida, J.E., and Singer, T.P., (1991). Interaction of 1-methyl-4-phenylpyridinium ion (MPP+) and its analogs with the rotenone/piericidin binding site of NADH dehydrogenase. *J Neurochem* **56**(4):1184-1190.

Ravindranath, V. and Reed., D.J., (1990). Glutathione depletion and formation of glutathione–protein mixed disulfide following exposure of brain mitochondria to oxidative stress. *Biochem Biophys Res Commun* **169** (3):1075–1079.

Reinhard, J.F. Jr., Diliberto, E.J., Jr., Viveros, O.H and Daniels, A.J., (1987). Subcellular compartmentalization of 1-methyl-4-phenylpyridinium with catecholamines in adrenal medullary chromaffin vesicles may explain the lack of toxicity to adrenal chromaffin cells. *Proc Natl Acad Sci* **84**:8160–8164.

Rice, M.E., and Russo-Menna, I., (1998). Differential compartmentalization of brain ascorbate and glutathione between neurons and glia. *Neurosci* **82:**1213-1223**.**

Richardson, J.R., Quan, Y., Sherer, T.B., Greenamyre, J.T., and Miller, G.W., (2005). Paraquat neurotoxicity is distinct from that of MPTP and Rotenone. *Toxicol Sci* **88**:193–201.

Riederer P., Sofic E., Rausch W.D., Schmidt B., Reynolds G. P., Jellinger K., and Youdim M. B. H., (1989). Transition metals, ferritin, glutathione and ascorbic acid in parkinsonian brains. *J Neurochem* **52**:515-520.

Ritz, D., Patel, H., Doan, B., Zheng, M., Åslund, F., Storz, G., and Beckwith, J., (2000). Thioredoxin 2 is involved in the oxidative stress response in Escherichia coli. *J. Biol Chem* **275**:2505-2512.

Rosengren E., Linder-Eliasson E., and Carlsson A., (1985). Detection of 5-S-cysteinyl-DA in human brain. *J Neural Transm* **63**: 247–253.

Sachs, C., and Jonsson, G., (1975). Mechanisms of action of 6-hydroxydopamine. *Biochem Pharmacol* **24**(1):1-8.

Sáez, G. T.; Viña, J. R.; and Viña, J., (1989). The role of free radicals in the cytotoxicity of cysteine. In: de Miquel, J., ed. CRC handbook of free radicals and antioxidants in biomedicine. Boca Raton, FL: CRC Press Inc.

Saggu, H., Cooksey, J., Dexter, D.,Wells, F.R., Lees, A., Jenner, P., and Marsden, C. D., (1989). A selective increase in particulate superoxide dismutase activity in Parkinsonian substantia nigra. *J Neurochem* **53(3)**:692–697.

Saigoh, K., Wang, Y.L., Suh, J.G., Yamanishi, T., Sakai, Y., Kiyosawa, H., Harada, T., Ichihara, N., Wakana, S., Kikuchi, T. and Wada, K., (1999). Intragenic deletion in the gene encoding ubiquitin carboxy-terminal hydrolase in gad mice. *Nat Genet* **23**: 47–51.

Schafer, F.Q., and Buettner, G.R., (2001). Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. *Free Radic Biol Med* **30**: 1191–1212.

Schapira, A.H., Cooper, J.M., Dexter, D., Clark, J.B., Jenner, P., and Marsden, C.D., (1990) Mitochondrial complex I deficiency in Parkinson's disease, *J. Neurochem.* **54**:823–827.

Schapira, A.H., Mann, V.M., Cooper, J.M., and Dexter, D., (1991) Anatomic and disease specificity of NADH CoQ1 reductase (Complex I) deficiency in Parkinson's disease. *J. Neurochem* **55** (6): 2142–2145.

Schapira, A.H.V., (1994) Mitochondrial dysfunction in neurodegenerative disorders and aging. In *Mitochondrial disorders in neurology.* (Schapira, A.H.V., Di Mauro, S., Eds) Butterworth-Heinemann, Oxford.

Schapira, A.H.V., (2004) Evidence for mitochondrial dysfunction in Parkinson's disease - a critical appraisal. *Movement Disorder* **9** (2), 125-138.

Schuppe-Koistinen, I., Gerdes, R., Moldéus, P., and Cotgreave, I.A., (1994). Studies on the reversibility of protein S-thiolation in human endothelial cells. *Arch Biochem Biophys* **315**:226 234.

Seniuk, N.A., Tatton, W.G., and Greenwood, C.E., (1990). Dose-dependent destruction of the coeruleus-cortical and nigral-striatal of the dopaminergic nigrostriatal pathway induced by intrastriprojections by MPTP. *Brain Res* **527**:7–20.

Shen, J. (2004) Protein kinases linked to the pathogenesis of Parkinson's disease. *Neuron*, **44**: 575–577.

Shen, X., and Dryhurst, G., (1996). Further Insights into the Influence of L-Cysteine on the Oxidation Chemistry of Dopamine: Reaction Pathways of Potential Relevance to Parkinson's Disease. *Chem Res Toxicol* **9** (4):751 -763.

Shendelman, S., Jonason, A., Martinat, C., Leete, T. and Abeliovich, A., (2004). DJ-1 is a redox-dependent molecular chaperone that inhibits alpha-synuclein aggregate formation. *PLoS Biol 2 e362*.

Sherer T.B, R. Betarbet, A.K., Stout, S., Lund, M., Baptista, A.V., Panov, M.R., Cookson, and Greenamyre, J.T., (2003a). An *In Vitro* Model of Parkinson's disease: Linking mitochondrial impairment to altered *alpha*-synuclein metabolism and oxidative damage. *J Neurosci* **22**(16):7006-7015.

Sherer, T. B., Betarbet, R., Testa, C. M., Seo, B. B., Richardson, J. R., Kim, J. H., Miller, G. W., Yagi, T., Matsuno-Yagi, A., and Greenamyre, J. T., (2003b). Mechanism of toxicity in rotenone models of Parkinson's disease. *J Neurosci* **23**:10756–10764.

Sherman, M.Y., and Goldberg, A.L., (2001). Cellular defenses against unfolded proteins: a cell biologist thinks about neurodegenerative diseases, *Neuron* **29**:15–32.

Shoffner, J.M., Watts, R.L., Junces, J.L., Torrom, A., Wallace, D.C., (1991). Mitochondrial oxidative phosphorylation defects in Parkinson's disease. *Ann Neurol* **30**:332-9.

Shimizu K, Ohtaki K, Matsubara K, Aoyama K, Uezono T, Saito O, Suno M, Ogawa K, Hayase N, Kimura K, and. Shiono, H., (2001). Carrier-mediated processes in blood–brain barrier penetration and neural uptake of paraquat. *Brain Res* **906**:135–142.

Shimura, H., Hattori, N., Kubo, S., Mizuno, Y., Asakawa, S., Minocell, S., Shimizu, N., Iwai, K., Chiba, T., Tanaka, K., and Suzuki, T., (2000). Familial Parkinson disease gene product, parkin, is a ubiquitin-protein ligase. *Nat Genet* **25**:302–305.

Shivakumar, B.R., and Ravindranath, V., (1993): Oxidative stress and thiol modification induced by chronic administration of haloperidol. *J Pharmacol Exp Ther* **3**:1137-1141.

Shivakumar, B.R., Kolluri, S.V.R. and Ravindranath, V., (1995). Glutathione and protein thiol homeostasis in brain during reperfusin after cerebral ischemia. *J Pharmacol Exp Ther* **274**:1167-1173.

Sian, J., Dexter, D.T., Lees, A.J., Daniel, S., Agid, Y., Javoy-Agid, F., Jenner, P., and Marsden, C.D., (1994a). Alterations in glutathione levels in Parkinson's disease and other neurodegenerative disorders affecting the basal ganglia. *Ann Neurol* **36**:348-355.

Sian, J., Dexter, D.T., Lees, A.J., Daniel, S., Jenner, P. and Marsden, C.D., (1994b) Glutathione-related enzymes in brain in Parkinson's disease, *Ann Neurol* **36**: 356–361.

Sies, H., (1991). Oxidative Stress: Oxidant and Antioxidant. , Academic Press, London.
Silvestri, L., Caputo, V., Bellacchio, E., Atorino, L., Dallapiccola, B., Maria-Valente E, and Casari, G., (2005). Mitochondrial import and enzymatic activity of PINK1 mutants associated to recessive parkinsonism. *Hum Mol Genet* **14**:3477–3492.

Singer, T. P., Ramsay, R. R., McKeown, K., Trevor, A., and Castagnoli, N. E., Jr. (1988). Mechanism of the neurotoxicity of 1-methyl-4-phenylpyridinium (MPP)+, the toxic bioactivation product of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). *Toxicology* **49**:17-23

Sirinathsinghji, D.J., Kupsch, A., Mayer, E., Zivin, M., Pufal, D., and Oertel, W.H. (1992). Cellular localization of tyrosine hydroxylase mRNA and cholecystokinin mRNA-containing cells in the ventral mesencephalon o the common marmosets. *Brain Res Mol* **12***:*267–274.

Slivka A, Mytilineou C, and Cohen G., (1987). Histochemical evaluation of glutathione in brain. *Brain Res* **409**: 275–284.

Smith, C.D., Carney, J.M., Stake-Reed, P.E., Oliver, C.N., Stadtman, E.R., Floyd, R.A., and Markesbury, W.R., (1991). Excess brain protein oxidation and enzyme disfunction in normal aging and Alzheimer's disease. *Proc Natl Acad Sci* 10540–10543.

Smith, W.W., Pei, Z., Jiang, H., Moore, D.J., Liang, Y., West, A.B., Dawson, V.L., Dawson, T.M., and Ross, C.A., (2005) Leucine-rich repeat kinase 2 (LRRK2) interacts with parkin, and mutant LRRK2 induces neuronal degeneration. *Proc Natl Acad Sci* **102**:18676–18681.

Sofic E, Lange KW, Jellinger K, and Riederer P., (1992). Reduced and oxidized glutathione in the substantia nigra of patients with Parkinson's disease. *Neurosci Lett* **142**:128–130.

Speciale, S.G., Liang, C.L., Sonsalla, P.K., Edwards, R.H., and German, D.C., (1998). The neurotoxin 1-methyl-4-phenylpyridinium is sequestered within neurons that contain the vesicular monoamine transporter. *Neuroscience* **84**:1177–1185.

Spencer, J.P.E., Jenner, P., Daniel, S.E., Lees, A.J., Marsden, C.D., and Halliwell, B., (1998). Conjugates of catecholamines with cysteine and GSH in Parkinson's disease: Possible mechanisms of formation involving reactive oxygen species. *J Neurochem* **71**:2112-2122.

Staal, R.G., Mosharov, E.V., and Sulzer, D., (2004). Dopamine neurons release transmitter via flickering fusion pore. *Nat Neurosci* **7**:341–346.

Stokes, A.H., Hastings, T.G., and Vrana, K.E., (1999). Cytotoxic and genotoxic potential of dopamine. *J Neurosci Res* **55**:659–665.

Subbaiah, M.T.R., Kessel, B., Agrawal, M., Rajan, R., Abplanalp, W. and Rymaszewski, R. (1993). Antioxidant potential of specific estrogens and lipid peroxidation. *J Clin Endocrinol Meth* **77**: 95-105.

Sugioka, K., Shimosegawa, Y., and Nakano, M., (1987). Estrogens as natural antioxidants of membrane phospholipids perioxidation. *FEBS Lett* **210** (11): 37-39.

Sugumaran, M., Dali, H., and Semensi, H., (1992). Mechanistic studies on tyrosine-catalyze oxidative decarboxylation of 3, 4-dihydroxymendelic acid. *J Biochem* **281**:353-357.

Takagi, Y., Nishuyama, A., Nozake, K., Tanaka, T., Hashimoto, T., and Yodoi, J., (1999). Localization of Glutaredoxin (Thioltransferase) in the rat brain and possible functional implications during focal ischemia. *Biochem and Biophys Res Comm* **258** (2): 390-394.

Takahashi, K., Taira, T., Niki, T., Seino, C., Iguchi-Ariga, S.M., and Ariga, H., (2001). DJ-1 positively regulates the androgen receptor by impairing the binding of PIASx alpha to the receptor. *J Biol Chem* **276**:37556-63.

Takashima, Y., Hirota, K., Nakamura, H., Nakamura, T., Akiyama, K., Cheng, F.S., Maeda, M., and Yodoi, J., (1999). Differential expression of glutaredoxin and thioredoxin during monocytic differentiation. *Immon Lett* **68**: 397–401.

Taylor, J.P., Mata, I.F., and Farrer, M.J., (2006) LRRK2: a common pathway for parkinsonism, pathogenesis and prevention? *Trends Mol Med* **12**:76–82.

Thiruchelvam, M., Brockel, B.J., Richfield, E.K., Baggs, R.B., and Cory-Slechta, D.A. (2000). Potentiated and preferential effects of combined paraquat and maneb on nigrostriatal dopamine systems: risk factors for Parkinson's disease? *Brain Res.* **873**:225–234.

Thomas, J.A., Poland, B., and Honzatko, R., (1995). Protein sulfhydryls and their role in the antioxidant function of protein S-thiolation. *Arch Biochem Biophys* **319**:1- 9.

Tse, D.C.S., McCreery, R.L., and Adams, R.N., (1976). Potential oxidative pathways of brain catecholamines. *J Med Chem* **19**:37-40.

Unoki, M. and Nakamura, Y., (2001). Growth-suppressive effects of BPOZ and EGR2, two genes involved in the PTEN signaling pathway. *Oncogene* **20**:4457–4465.

Valente, E.M., Abou-Sleiman, P.M., Caputo, V., Muqit, M.M., Harvey, K., Gispert, S., Ali Z., Del Turco, D., Bentivoglio, A.R., Healy, D.G., Albanese, A., Nussbaum, R., González-Maldonado, R., Deller, T., Salvi, S., Cortelli, P., Gilks, W.P., Latchman, D.S., Harvey, R.J., Dallapiccola, B., Auburger, G., and Wood, N.W., (2004). Hereditary early-onset Parkinson's disease caused by mutations in PINK1. *Science* **304**:1158–60.

Van Laar, V.S., Dukes, A.A., Cascio, M., and Hastings, T.G., (2008a). Proteomic analysis of rat brain mitochondria following exposure to dopamine quinone: Implications for Parkinson's disease. *Neurobiol Dis* **29**:477-489.

Van Laar, V.S., Dukes, A.A., Cascio, M. and Hastings, T.G., (2008b) Proteomic identification of dopamine-conjugated proteins from isolated rat brain mitochondria and SH-SY5Y cells. *Neurobiol Dis* **34** (3) 487-500

Vila, M., and Przedborski, S., (2004) Genetic clues to the pathogenesis of Parkinson's disease. *Nat Med* **10** Suppl:S58-62.

Volles, M.J., Lee, S.J., Rochet, J.C., Shtilerman, M.D., Ding, T.T., Kessler, J.C., and Lansbury, P.T., Jr. (2001). Vesicle permeabilization by protofibrillar alpha-synuclein: implications for the pathogenesis and treatment of Parkinson's disease, *Biochemistry* **40**:7812–7819.

Wallace, D.C., Lott, M.T., and Brown, M.D., (1997). Mitochondrial defects in neurodegenerative diseases and aging. In: Beal, M.F, Howel, N., Bodis-wollner, I. (Eds), Mitochondria and free radicals in neurodegenerative diseases. Wiley-Liss Inc., New York, pp277-289.

Watts, L.T., Rathinam, M.L., Schenker, S., and Henderson, G.I., (2005). Astrocytes protect neurons from ethanol-induced oxidative stress and apoptotic death. *J Neurosci Res* **80**: 655-666.

Wei Q., Yeung, M., Jurma, O.P., and Anderson, J.K., (1996). Genetic elevation of monoamine oxidase levels in dopaminergic PC12 cells results in increased free radical damage and sensitivity to MPTP. *J Neurosci Res* **46**:666-673.

Weihofen, A., Ostaszewski, B., Minami, Y., and Selkoe, D.J., (2007) Pink1 Parkinson mutations, the Cdc37 / Hsp90 chaperones and Parkin all influence the maturation or subcellular distribution of Pink1. *Human Mol Genet* **17**:602-16.

Wood, T.K., Sullivan, A.M., and McDermott, K.W., (2003). Viability of dopaminergic neurons is influenced by serum and astroglial cells in vitro. *J Neurocyto* **32**:97–103.

Wooten, G.F., Currie, L.J., Boubjerg, V.E., Lee, J.K., and Patrie, J., (2008). Are men at greater risk for Parkinson's disease than women? *J Neurosurg Psych* **75**: 637-639.

Wu, D.C., Teismann, P., Tieu, K., Vila, M., Jackson-Lewis, V., Ischiropoulos, H., and Przedborski, S. (2003). NADPH oxidase mediates oxidative stress in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine model of Parkinson's disease. *Proc Natl Acad Sci* USA **100**; 6145–6150.

Wullner, U., Loschmann, A., Schulz, J.B., Schmid, A., Dringen, R., Eblen, B., Turski, L., and Klockgether, T., (1996). Glutathione depletion potentiates MPTP and MPP+ toxicity in nigral dopaminergic neurons. *Neuroreport* **7**:921-926.

Xu, Y., Stokes, A.H., Roskoski, R. Jr., and Vrana K.E., (1998) Dopamine, in the presence of tyrosinase, covalently modifies and inactivates tyrosine hydroxylase. *J Neurosci* Res **54**:691-697.

Xu, J., Zhong, N., Wang, H., Elias, J.E., Kim, C.Y, Woldman, I., Pifl, C., Gygi, S.P., Geula, C., and Yankner, B.A., (2005) The Parkinson's disease-associated DJ-1 protein is a transcriptional co-activator that protects against neuronal apoptosis. *Hum Mol Genet* **14**:1231–1241.

Yang, L., Matthews, R.T., Schultz, J.B., Klockgether, T., Liao, A.W., Martinou, J.C., Penney, J.B., Hyman, B.H., and Beal, M.F. (1998). 1-methyl-4-phenyl-1,2,3,6-tetrahydropyride neurotoxicity is attenauted in mice overexpressing Bcl-2*, J Neurosci* **18**:8145–8152.

Yang, Y., Nishimura, I., Imai, Y., Takahashi, R. and Lu, B. (2003). Parkin suppresses dopaminergic neuron-selective neurotoxicity induced by Pael-R in Drosophila. *Neuron* **37**:911–924.

Yao J.K., Reddy, R.D., van Kammen, D.P., (2001). Oxidative damage and schizophrenia: an overview of the evidence and its therapeutic implications. *CNS Drugs* **15**(4):287–310.

Yao, J.K., Leonard, S., Reddy, R.D., (2006). Altered glutathione redox state in schizophrenia. *Dis Markers* **22**: 88-93.

Yoritaka, A., Hattori, N., Uchida, K., Tanaka, M., Stadtman, E.R., and Mizuno, Y., (1996). Immunohistochemical detection of 4-hydroxynonenal protein adducts in Parkinson disease. *Proc Natl Acad Sci U S A*. **93**(7): 2696–2701.

Yoshikawa, M., Hirai, S., (1967). Lipid peroxide formation in the brain of aging rats. *J Gerontol* **22**:162-165.

Zarranz, J.J., Alegre, J., Gómez-Esteban, J.C., Lezcano, E., Ros, R., Ampuero, I., Vidal, L., Hoenicka, J., Rodriguez, O., Atarés, B., Llorens, V., Gomez Tortosa, E del Ser T, Muñoz, D.G., and de Yebenes, J.G. (2004). The new mutation, E46K, of alpha-synuclein causes Parkinson and Lewy body dementia. *Ann Neurol.* 2004 **55**(2):164-73.

Zeevalk, G.D., Bernard, L.P. and Nicklas, W.J., (1998). Role of oxidative stress and the glutathione system in loss of dopamine neurons due to impairment of energy metabolism. *J. Neurochem*. **70**:1421–1430.

Zeevalk, G.D., Bernard, L.P., and Ehrhart, J., (2003). Glutathione and ascorbate: Their role in protein glutathione mixed disulfide formation during oxidative stress and potential relevance to Parkinson's disease. *Ann NY Acad Sci* **991**:342–345.

Zeevalk, G.D., and Bernard, L.P., (2005). Energy status, ubiquitin proteasomal function, and oxidative stress during chronic and acute complex I inhibition with rotenone in mesencephalic cultures. *Antioxid Redox Signal* **7**:662-672.

Zeevalk, G.D., Bernard, L.P., Song, C., Gluck, M., Ehrhart, J., (2005). Mitochondrial inhibition and oxidative stress: reciprocating players in neurodegeneration. *Antioxid Redox Signal* **7**(9-10):1117-39.

Zhang F and Dryhurst G., (1993) Oxidation of dopamine: Possible insights into the age-dependent loss of dopaminergic nigrostriatal neurons. *Bioorg Chem* **21**:392-410.

Zhang, Y., Gao, J., Chung, K.K., Huang, H., Dawson, V.L., and Dawson, T.M. (2000). Parkin functions as an E2-dependent ubiquitin-protein ligase and promotes the degradation of the synaptic vesicle-associated protein, CDCrel-1. *Proc Natl Acad Sci* **97**:13354–13359.

Zhang, L., Shimoji, M., Thomas, B., Moore, D.J., Yu, S.W., Marupudi, N.I., Torp, R., Torgner, I.A., Ottersen, O.P., Dawson, T.M., and Dawson, V.L. (2005) Mitochondrial localization of the Parkinson's disease related protein DJ-1: implications for pathogenesis. *Hum. Mol Genet* **14**:2063–2073.

Ziegler, D.M., (1985). Role of reversible oxidation-reduction of enzyme thiols-disulfides in metabolic regulation. *Annu. Rev. Biochem* **54**:305 329.

ALPA H GAUTAM

ahgautam@gmail.com

1645 Emerald St.

Unit 1-J

San Diego, CA 92109

(917) 674-3396

EDUCATION

UMDNJ/Rutgers University New Brunswick, NJ

Ph.D., Neuroscience 2003-2010

Hunter College, City University of NY NY, New York

BA, Biology 1996-2003

RESEARCH EXPERIENCE

UMDNJ, University of Dentistry and Medicine of New Jersey Piscataway, NJ

Graduate Student with Dr. Gail D Zeevalk 2005 – 2010

Thesis Research: GSH-Dopamine Interactions: Relevance to Cell Vulnerability in Parkinson's disease

- Developed and modified enzymatic assays to study effects of reduced and oxidized dopamine and DOPAC on lysed and intact mitochondrial function.
- Analyzed dopamine uptake into intact mitochondria.
- Characterized developmental, regional, gender and cell specific changes in glutaredoxin function in rat brain.

Bronx Veterans Affairs Bronx, NY

Undergraduate Research with Dr. Martin Gluck 2001 – 2003

Project: To detect oxidative markers in rat brain slices after exposure to dopamine

- Used immunohistochemistry to detect formation of 3-nitrotyrosylated proteins.

SKILLS AND TECHNIQUES

- Isolation of intact mitochondria from rat brain and other organs
- Establishing mitochondrial RCR rates via oxygen consumption to establish mitochondria membrane integrity
- Perform, modify and troubleshoot enzyme assays using spectrophotometry
- Cryostat sectioning and mapping of rat brain
- Immunocytochemistry
- rtPCR
- Statistical Analysis

TEACHING EXPERIENCE

Rutgers University, The State University of New Jersey New Brunswick, NJ

Teaching Assistant, Introductory Biology 2003-2005,2007-2008

- Instruct two Biology Laboratory classes
- Develop lesson plans and testing material
- Instruct new teaching assistants

POSTERS AND CONFERENCES

A. H. Gautam, G. Zeevalk. Downstream Impairment of Mitochondrial Function by Dopamine and its metabolites: protection by Glutathione but not other antioxidants. Neuroscience Meeting Planner. Atlanta, GA: Society for Neuroscience, 2006. Online.

A. H. Gautam, G. Zeevalk. Mitochondrial inhibition by dopamine quinone and relevance to Parkinson's disease. Neuroscience Meeting Planner. San Diego,CA: Society for Neuroscience, 2007. Online.

*M. Polavarapu**, A.H. Gautam**, G. Zeevalk . The Effects of Glutathione and Dopamine on Glutarexoxin-1 activity. Aresty Research Center for Undergraduates Symposium, 2007.

A. H. Gautam, J.R.Richardson, and G. Zeevalk. Regional, age and gender characterization of glutaredoxin in rat brain: relevance to Parkinson's disease.Neuroscinece Meeting Planner. Washington, D.C.: Society for Neuroscience, 2007. Online.

REFERENCES

1- **Gail D. Zeevalk, Ph. D.**
Associate Professor Neurology
UMDNJ-Robert Wood Johnson Medical School

Department of Neurology
Piscataway, NJ 08854

Email: gzeevalk@gmail.com

2- **Jason Richardson, M.S., Ph. D.**
Assistant Professor
Robert Wood Johnson Medical School

Toxicology Division
Environmental and Occupational Health Sciences Institute

Email: jricha3@eohsi.rutgers.edu

Phone: 732 445 0174

3- **Diana Martin, Ph. D.**
Director of General Biology
Rutgers, The State University of New Jersey

Life Sicence Department

Email: DiaMarGBio@aol.com

Phone: 732 445-0778