DOPAMINE HOMEOSTASIS AND ENVIRONMENTAL RISK FACTORS IN A PARKINSON’S DISEASE MODEL

by

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The neurotransmitter dopamine (DA) is an important factor in the regulation of many biological processes, from pleasure and addiction to balance and locomotion. Therefore, understanding and defining the mechanisms and factors that are required for proper DA homeostasis is an integral component in managing and elucidating the causes of DA related diseases. Among these diseases, Parkinson’s disease (PD) is the most notable and remains one of the most researched yet puzzling motor system associated neurological disorders. PD is characterized by a preferential loss of DA neurons in the substantia nigra. Though the cause and exact mechanism of this disease remains undefined, numerous environmental factors such as metals and pesticides have been associated with the etiology of the disease process.

In the following studies, the genetic components of DA homeostasis and environmental risk factors in a Drosophila model of PD are investigated. The implication of metals as a component in the pathology of PD is examined in relationship to zinc toxicity. Catecholamines’s up (Catsup), which plays a crucial role in regulating DA homeostasis and is proposed to be a member of the mammalian KE4 ZIP transporter family, demonstrates zinc sensitivity, with the proposed underlying factors being a dysregulation of DA synthesis and DA transport. The findings of this report demonstrate that loss of dopamine transporter (DAT) function, results in a more robust sensitivity to zinc than that seen in Catsup mutants. In addition exogenous DA increases sensitivity of wild type flies to zinc, similar to that which is seen Catsup mutants.
Interestingly, LiCl ameliorates the toxic effects of zinc. The results also demonstrate a functional relationship between paraquat toxicity and DAT, which affects DA transport.

To determine the consequences of early exposure to paraquat on lifespan and mobility, the effect of a one time exposure to young adult flies was observed. The results of this experiment show that a brief exposure to paraquat illicits long term detrimental affects on survival as well as parkinsonian type phenotypes.
### LIST OF ABBREVIATIONS AND SYMBOLS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>3-IT</td>
<td>3- iodo-tyrosine</td>
</tr>
<tr>
<td>6-OHDA</td>
<td>6-hydroxydopamine</td>
</tr>
<tr>
<td>Å</td>
<td>Angstrom</td>
</tr>
<tr>
<td>AADC/DDC</td>
<td>Aromatic-L-amino-acid decarboxylase/dopa decarboxylase</td>
</tr>
<tr>
<td>Acb</td>
<td>Accubens</td>
</tr>
<tr>
<td>ADHD</td>
<td>Attention deficit hyperactivity disorder</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>ADLH2</td>
<td>Aldehyde dehydrogenase</td>
</tr>
<tr>
<td>AML1</td>
<td>Acute Myeloid Leukemia-1</td>
</tr>
<tr>
<td>AMPA</td>
<td>Alpha-amino-3-hydroxy-5-methyl-4-isoaxazolepropionic acid</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>Apaf-1</td>
<td>Apoptosis activating factor-1</td>
</tr>
<tr>
<td>APP</td>
<td>Amyloid protein precursor</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain-derived-neurotrophic factor</td>
</tr>
<tr>
<td>BH₄</td>
<td>Tetrahydrobiopterin</td>
</tr>
<tr>
<td>°C</td>
<td>Celsius</td>
</tr>
<tr>
<td>C</td>
<td>Carboxyl</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>CATSUP</td>
<td><em>Catecholamines up</em></td>
</tr>
<tr>
<td>CD 14</td>
<td>Cluster of Differentiation 14</td>
</tr>
<tr>
<td>CDF</td>
<td>Cation diffusion facilitator</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>COX 1</td>
<td>Cyclooxygenase -1</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CP</td>
<td>Choroid plexus</td>
</tr>
<tr>
<td>Cx</td>
<td>Cerebral cortex</td>
</tr>
<tr>
<td>D₂S</td>
<td>Dopamine receptor 2 short</td>
</tr>
<tr>
<td>D₂L</td>
<td>Dopamine receptor 2 long</td>
</tr>
<tr>
<td>DA</td>
<td>Dopamine</td>
</tr>
<tr>
<td>DAT</td>
<td>Dopamine transporter</td>
</tr>
<tr>
<td>dDAT</td>
<td><em>Drosophila</em> dopamine transporter</td>
</tr>
<tr>
<td>DD2R</td>
<td><em>Drosophila D₂</em>-like receptor</td>
</tr>
<tr>
<td>Df</td>
<td>Deficiency</td>
</tr>
<tr>
<td>DHPR</td>
<td>Dihydropteroine reductase</td>
</tr>
<tr>
<td>DOPAL</td>
<td>3,4-dihydroxyphenylacetaldheyde</td>
</tr>
<tr>
<td>DOPAC</td>
<td>Dihydrophenylacetic acid</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DRD</td>
<td>Dopa responsive dystonia</td>
</tr>
<tr>
<td>DTH</td>
<td><em>Drosophila</em> Tyrosine hydroxylase</td>
</tr>
<tr>
<td>DVMAT</td>
<td><em>Drosophila</em> vesicular monoamine transporter</td>
</tr>
<tr>
<td>EOP</td>
<td>Early-onset Parkinsonism</td>
</tr>
</tbody>
</table>
fmin  Fumin

g  Unit of gravity
GABA  Gamma-aminobutyric acid
GABAAR  Gamma-aminobutyric acid type A receptors
GFP  Green fluorescent protein
GFRP  GTPCH feedback regulatory protein
GSK-3  Glycogen synthase kinase 3
GTP  Guanosine triphosphate
GTPCH  Guanosine triphosphate cyclohydrolase
H2NTP  Dihydroneopterin triphosphate
H2O2  Hydrogen peroxide
HPLC  High Performance Liquid Chromatography
HSP  Heat shock protein
HVA  Homovanillic acid
kDa  Kilodalton
Ki  Inhibitor constant
Km  Michaelis-Menten constant
L  Liter
L-Dopa  L-3,4-dihydroxyphenylalaine
LZT  LIV-1 subfamily of ZIP Zn²⁺ Transporter
M  Molar
MAO  Monoamine oxidase
MAPK  mitogen activated protein kinase
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>MPP+</td>
<td>1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>MT</td>
<td>Methallothionein</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>N</td>
<td>Amino</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-asparate</td>
</tr>
<tr>
<td>NS</td>
<td>Not significant</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitrate oxide synthase</td>
</tr>
<tr>
<td>OT</td>
<td>Occipitotemporal</td>
</tr>
<tr>
<td>PAH</td>
<td>Phenylalanine hydroxylase</td>
</tr>
<tr>
<td>PC12</td>
<td>Pheochromocytoma colonal cell</td>
</tr>
<tr>
<td>PCD</td>
<td>Pterin-4a-carbinolamine dehydratase</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>PKA</td>
<td>cAMP-dependent protein kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PKU</td>
<td>Phenylketonuria</td>
</tr>
<tr>
<td>PQ</td>
<td>Paraquat</td>
</tr>
<tr>
<td>PTP</td>
<td>6-pyruvol-tetrahydropterin</td>
</tr>
<tr>
<td>PTPS</td>
<td>6-pyruvol-tetrahydropterin synthase</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>Ser</td>
<td>Serine</td>
</tr>
<tr>
<td>SN</td>
<td>Substantia nigra</td>
</tr>
<tr>
<td>SNc</td>
<td>Substantia nigra par compacta</td>
</tr>
<tr>
<td>SR</td>
<td>Sepiapterin reductase</td>
</tr>
<tr>
<td>TH</td>
<td>Tyrosine hydroxylase</td>
</tr>
<tr>
<td>TDP-43</td>
<td>TAR DNA-binding protein of 43 kDa</td>
</tr>
<tr>
<td>TPH</td>
<td>Tryptophan hydroxylase</td>
</tr>
<tr>
<td>VMAT</td>
<td>Vesicular monoamine transporter</td>
</tr>
<tr>
<td>V&lt;sub&gt;max&lt;/sub&gt;</td>
<td>Maximum enzyme velocity</td>
</tr>
<tr>
<td>VTA</td>
<td>Ventral tegmented area</td>
</tr>
<tr>
<td>µl</td>
<td>Microliter</td>
</tr>
<tr>
<td>µM</td>
<td>Micromolar</td>
</tr>
<tr>
<td>ZEN</td>
<td>Zinc enriched neurons</td>
</tr>
<tr>
<td>ZIP</td>
<td>Zrt, Irt-like protein</td>
</tr>
<tr>
<td>ZnT</td>
<td>Zinc transporter</td>
</tr>
<tr>
<td>Zn&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Zinc</td>
</tr>
<tr>
<td>ZnCl&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Zinc chloride</td>
</tr>
</tbody>
</table>
ACKNOWLEDGMENTS

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CONTENTS

ABSTRACT .......................................................................................................................... ii

LIST OF ABBREVIATIONS AND SYMBOLS .................................................................... iv

ACKNOWLEDGMENTS ....................................................................................................... ix

1. INTRODUCTION ............................................................................................................. 1

Dopamine synthesis ........................................................................................................... 2

Depamine packing, transport, and metabolism ................................................................. 2

Tyrosine hydroxylase ........................................................................................................ 8

Regulation of tyrosine hydroxylase .................................................................................. 9

Drosophila tyrosine hydroxylase .................................................................................... 12

Tetrahydrobiopterin function and dysfunction ................................................................. 13

Tetrahydrobiopterin biosynthesis .................................................................................. 15

Regulation of GTP cyclohydrolase ................................................................................. 17

Drosophila GTPCH ........................................................................................................... 20

Zinc function .................................................................................................................... 21

Zinc metabolism .............................................................................................................. 23

Zinc and the brain .............................................................................................................. 27

Zinc and dopamine .......................................................................................................... 30

Zinc and neurodegenerative diseases .............................................................................. 31
LIST OF FIGURES

1.1 Components of DA synthesis, transport and metabolism ............................................. 7

1.2 Tetrahydrobiopterin (BH₄) biosynthesis pathway ................................................................. 16

2.1 The effect of ZnCl₂ on lifespan in wild type and Catsup mutant adult flies ..................... 43

2.2 The effect of DA prefeeding on Zn²⁺-induced mortality of wild type and Catsup mutant flies ................................................................................................................................. 46

2.3 Zn²⁺ modulates the metabolism of DA ................................................................................. 48

2.4 Zn²⁺ feeding enhances mobility in wild type flies ................................................................. 50

2.5 Loss of function mutations of DAT and RNAi knockdown of DAT expression in dopaminergic neurons increase Zn²⁺ sensitivity ................................................................................................. 53

2.6 Catsup-DAT interactions in zinc toxicity ............................................................................. 56

2.7 The effect of Zn²⁺ on lifespan in foi mutants ....................................................................... 61

2.8 foi and foi/Catsup transheterozygote mutants are hypermobile ....................................... 57

2.9 LiCl suppresses the toxicity of ZnCl₂ in Catsup mutant and wild type flies .................... 59

2.10 A model of the interaction of Catsup with the genetic regulators of DA homeostasis ................................................................................................................................. 64

3.1 Over-expression of Catsup in dopaminergic cells alters parquat survival and mobility ................................................................................................................................. 75

3.2 Expression of Catsup in dopaminergic cells confers sensitivity to reserpine ................. 77

3.3 Loss of DAT function reduces sensitivity to paraquat toxicity ....................................... 80

4.1 A one time exposure to paraquat attenuates locomotor activity .................................... 92

4.2 A one time exposure to paraquat (PQ) reduces lifespan .................................................. 93
CHAPTER ONE

INTRODUCTION

Dopamine (DA), a neurohormone/neurotransmitter that is a member of the catecholamine family of molecules, is necessary for both neurological and non-neurological functions. Catecholamine is characterized by the presence of a catechol nucleus and a terminal amine group and is derived from the amino acid L-tyrosine. DA not only serves as a necessary precursor for the important vertebrate catecholamine, epinephrine and norepinephrine, but it also is the most abundant catecholamine in the nervous system, accounting for 50% of the total catecholamine levels in mammals (Carlsson, 1959). Although DA is found predominately within the central nervous system (CNS), it is also synthesized within the gastrointestinal tract and the adrenal glands (Falck et al., 1967; Axelrod, 1971; Bloom et al., 1988).

DA has been implicated in a diverse set of functions including the regulation of mood, learning and memory, attention, addiction, reward, stress management, melanin production, cuticle hardening in arthropods, development, fertility and courtship, insect tracheal morphogenesis, motor neuron activity and neuromuscular function (Kobayashi et al., 1995; Neckameyer, 1996; Neckameyer et al., 2001; Smidt et al., 2003, Wise, 2004; Schultz, 2007; Björklund & Dunnett, 2007, Hsouna et al., 2007). Given the role of DA in regulating so many important processes, it is no surprise that disruption in DA synthesis and signaling has been implicated in a variety of diseases such as Alzheimer’s disease, Parkinson’s disease, and DOPA responsive dystonia (Bernheimer et al., 1973; Segawa et al., 1976; Reinikainen et al., 1988;
Ichinose et al., 1999). In addition, DA imbalance is a key component in many psychiatric illnesses such as dementia, depression, schizophrenia and addiction (Pendelton et al., 1998; Berman et al., 1999; Iversen & Iversen, 2007). Therefore, understanding the genetic components of DA regulation and synthesis is an essential element in deciphering the cause and treatment of DA related disorders.

**Dopamine synthesis**

The process of DA synthesis is similar in both vertebrates and invertebrates. Tyrosine hydroxylase (TH) is the first and rate-limiting enzyme in the biosynthesis of DA (Nagatsu et al., 1964). DA synthesis begins with the hydroxylation of L-tyrosine to 3, 4-dihydroxyphenylalanine (L-DOPA) by TH, mediated by the binding of oxygen (O\textsubscript{2}) and the pteridine cofactor, tetrahydrobiopterin (BH\textsubscript{4}) at this enzyme’s active site (Nagatsu et al., 1964; Axelrod, 1971). In addition, ferrous iron is also required for catalysis to occur (Fitzpatrick, 1989; Haavik et al., 1992, 1993). Following the production of L-DOPA, it is converted to DA by the enzyme aromatic –L-amino acid decarboxylase or dopa decarboxylase (AADC/DDC) (Holtz et al., 1938). In *Drosophila*, the synthesis of DA is the terminal point of the catecholamine pathway, but in mammals, DA is converted to norepinephrine and epinephrine (Kumer & Vrana, 1996). Although TH is the primary enzyme responsible for the production of L-DOPA, tyrosinase, present in melanocytes, can also convert tyrosine to L-DOPA. However, it should be noted that melanocytes lack AADC/DDC and are unable to convert L-DOPA into DA (Rios et al., 1999).

**Dopamine packing, transport, and metabolism**

Once DA is synthesized, it is stored, transported and recycled in an efficient and highly regulated manner. Newly synthesized DA is packaged into vesicles by vesicular monoamine transporters (VMAT). The packaging of DA is an important aspect of neuronal health because it
stabilizes the neurotransmitter and prevents cellular damage due to its degradation. VMAT is a general transporter of all biogenic amines and is regulated by changes in the electrochemical gradient by ATPase. This process is necessary because it allows for the packaging and subsequent release of neurotransmitters (Flatmark et al., 2002; Brunk et al., 2006). In mammals, two isoforms of VMAT, VMAT1 and VMAT2, have been identified and analyzed. VMAT2 is expressed predominately in neurons, whereas VMAT1 is exclusive to neuroendocrine cells (Erickson et al., 1996). One VMAT gene has been identified in Drosophila, which produces two alternatively, spliced transcripts, VMAT A and VMAT B. These two variants are identical in structure except for changes in the C-terminal domain. Drosophila VMAT (DVMAT) has a high degree of conservation with its mammalian counterpart. The predicted motif of VMAT A, but not VMAT B, shows similarity between the carboxy terminus of VMAT A with rat VMAT2 and other mammalian VMATs. In addition, in vitro assays of VMAT A demonstrate that it is capable of transporting, serotonin, and octopamine, and is localized to dopaminergic and serotonergic neurons in the CNS (Greer et al., 2005). Following the packaging and transport of DA by VMAT, it is released into the synapse.

The DA transporter (DAT) is the main enzyme responsible for clearing DA from the synapse after completion of synaptic signaling. Located in the pre-synaptic plasma membrane, DAT maintains proper neurotransmission of DA by recycling unused DA back into the cytosol (Hitri et al., 1994). Elucidation of the primary structure of DAT has revealed that it is a member of the family of \( \text{Na}^{+}/\text{Cl}^{-} \)-dependent transporters containing 12 putative transmembrane domains (Amara & Kuhar, 1993; Giros & Caron, 1993). Analysis of homozygous DAT-deficient mice demonstrated that DA persists at least 100 times longer in the extracellular space and results in spontaneous hyper-locomotion despite major adaptive changes, such as decrease in
neurotransmitter and receptor levels (Giros et al., 1996). In addition, the DAT is a major target for psychoactive drugs such as antidepressant and abused drugs including cocaine and amphetamines (Horn, 1990; Ritz & Kuhar, 1993). In Drosophila, the cloning and characterization of dDAT indicates striking similarities to vertebrate orthologs as it relates to cocaine sensitivity and locomotion, and dDAT mRNA is found predominately within dopaminergic cells in the fly nervous system (Porzgen et al., 2001).

To maintain DA homeostasis and prevent cytological insult, DA that has been transported back into the presynaptic termini has one of two fates. It can be broken down by monoamine oxidase (MAO) into a stable nontoxic metabolite, 3, 4-dihydroxyphenylacetic acid (DOPAC) and reactive oxygen species (ROS), or it is repackaged into vesicles by VMAT (Blaschko, 1957; Kopin, 1968; Henry et al., 1998; Youdim & Bakhle, 2006). Two isoforms of MAO, MAO-A and MAO-B exists in mammals, and both have been shown to be active in the degradation of DA. In the rodent brain, mainly MAO A accomplishes DA degradation, while MAO B is responsible for this process in humans and other primates (reviewed in Bortolato et al., 2008). Moreover, examination of the expression pattern of these isoforms indicated that they were differentially expressed in mammalian tissues, suggestive of variation in functions. In humans, MAO-A is primarily localized within catecholaminergic neurons, while MAO-B is mainly expressed within serotonergic and histaminergic neurons, as well as in astrocytes (Westlund et al., 1988; Saura et al., 1994). In Drosophila, a low level of MAO activity is detected within the brain (Dewhurst et al., 1972). However, this concentration of MAO seems to be sufficient for DA metabolism because DOPAC is detectable by HPLC analysis (Chaudhuri et al., 2007).

Receptors for DA on the surface of neurons are necessary not only for receiving messages but are an integral part of maintaining homeostasis. DA receptors belong to the family
of seven transmembrane domain (7TM) G protein-coupled membrane receptors (Jackson and
Westlind-Danielsson, 1994). In mammals, five distinct DA receptors have been identified and
subdivided into two families, D1-like and D2-like, based on pharmacological properties
(Kebabian & Calne, 1979). Members of D1-like receptors are D1 and D5 receptors while the D2-like
includes D2, D3 and D4 receptors. Among the DA receptor genes, D1 and D2 have the
highest and most widespread level of expression (Jackson & Westlind-Danielsson, 1994).

Analysis of the D2 receptor gene within mammals indicates that is composed of eight
exons, seven of which are coding (Gingrich & Caron, 1993). Alternative splicing of the sixth
exon generates two isoforms, DA receptor 2 short and DA receptor 2 long (D2S and D2L). Both
D1 and D2 receptors are expressed in the brain. The D1 receptor is believed to be post-synaptic
and is expressed in the choroid plexus (CP), nucleus accubens (Acb), occipitotemporal (OT),
cerebral cortex (Cx) and amygdale (Jackson & Westlind-Daniellson, 1994). In contrast, the D2
receptor is primarily pre-synaptic and is found within areas of the brain such as the CP, OT, and
Acb. In addition, the D2 receptor is also expressed in the substantia nigra par compacta (SNC)
and in the ventral tegmented area (VTA). Outside the brain, the D2 receptor is also found in the
kidney, retina, pituitary gland and vascular system (Ng et al., 1994; Jackson & Westlind-
Danielsson, 1994; Picetti et al., 1997). DA receptors regulate many chemical pathways;
however, the activation or inhibition of the cAMP pathway and modulation of calcium signaling
seem to be the most prevalent. Additionally, activation of D1-like receptors stimulates adenyl
cyclase and phosphatidylinositol-4,5 biphosphate metabolism, whereas activation of D2-like
receptors inhibits adenylyl cyclase and activates potassium channels. The inhibition of adenylyl
cyclase reduces the phosphorylation of TH, which in turn, decreases TH activity and DA
production (Jackson & Westlind-Danielsson, 1994). One of the adenosine G protein coupled
receptors, $A_{2A}$, is restricted to DA innervated regions (Ferré et al., 1997). Analysis of adenosine receptor function, in particular $A_{2A}$ and $D_2$ receptor, demonstrates that $A_{2A}$ receptor stimulation inhibits $D_2$-mediated activation in mice (Ferré et al., 1991). Moreover, it has been shown that stimulation of $A_{2A}$ receptors in neuroblastoma cells decreased the affinity of the $D_2$ receptor for DA. Therefore, modulation of DA receptor activity is an important part of maintaining DA homeostasis and function. Interestingly, analysis of ADHD patients, whose symptoms are commonly believed to be a consequence of DA dysregulation, has indicated that polymorphisms within the $D4$-R and $DAT$-1 genes are possible factors in this disorder (Cook et al., 1995; Gill et al., 1997). The importance of the DA receptor, in regulating DA homeostasis, is further underscored by the use of DA receptor agonist as potential treatment options for PD.

In *Drosophila*, DA receptors belonging to both the $D_1$-like and $D_2$-like subfamilies of DA have been cloned and characterized (Gotzes et al., 1994; Feng et al., 1996; Hearn et al., 2002). Two $D_1$-like receptor genes, *DmDop1* and *DopR99B* have been isolated. Analysis of amino acid sequence determined that DmDop1 is approximately 70% identical to human $D_1/D_5$ receptors (Gotzes et al., 1994). When expressed in mammalian cell cultures, both DmDop1 and DopR99B stimulate cAMP production in response to DA application (Gotzes et al., 1994; Feng et al., 1996). Analysis of the expression pattern of DmDop1 indicates that it is expressed in the optic lobe, whereas DopR99B is primarily expressed in the central and peripheral nervous system. Isolation and analysis of the *Drosophila* $D_2$-like receptor (DD2R) demonstrates that eight splice variants exist for this receptor (Hearn et al., 2002). Pharmacological assessment of three of these isoforms, DD2R-606, DD2R-506, DD2R-461, supports the classification of these proteins as $D_2$-like receptors. DD2R is expressed predominantly in adult fly head as well as the pupa and larva.
Figure 1.1. Components of DA synthesis, transport and metabolism (modified from Wang et al., manuscript in progress). Once DA has been produced; it is packaged by VMAT and released from the presynaptic terminal. DA that is located inside the synaptic region then interacts with DA receptors on the postsynaptic or presynaptic terminal, where it elicits a chemical response. In addition, DA within the synaptic space is reshuffled back into the presynaptic terminal by DAT, where VMAT repackages it into synaptic vesicles or MAO breaks it down into the metabolite DOPAC. All of these components work together to maintain proper homeostasis of DA.
**Tyrosine hydroxylase**

Mutations within the *Th* gene have been associated with human disorders such as L-DOPA-responsive dystonia, Parkinsonism in infancy, and progressive infantile encephalopathy with L-DOPA – nonresponsive dystonia (Lüdecke et al., 1996; Hoffmann et al., 2003; Schiller et al., 2004). Studies conducted with TH-deficient mice, generated either by complete or partial knockout of the *Th* gene or mutation within the gene, show an array of anomalies including reduced body size and hypokinesia, impaired locomotor activity, and defects in learning and memory and conditioned learning (Kobayashi et al., 2000). Additionally, conditional knockout of *Th* in dopaminergic neurons results in a wide range of phenotypes such as akinesia, cataleptic behavior, loss of drug response, blockade of dopamine receptor agonist-induced motor activation, postnatal development and death (Zhou & Palmiter, 1995; Nishii et al., 1998).

A single gene encodes TH in all organisms (Grima et al., 1987; Kobayashi & Sano, 1988). Molecular analysis of this gene indicates that it is a member of the family of tetrahydrobiopterin-dependent aromatic amino acid hydroxylases that also includes phenylalanine hydroxylase and tryptophan hydroxylase (Grenett et al., 1987). These proteins are orthologous not only in protein sequence but also in catalytic mechanism (Goodwill et al., 1997). TH is expressed in all catecholaminergic cells and has been shown to be localized to neurons of the sympathetic nervous system and the adrenal medulla as well as neurons within the ventral tegumentum, substantia nigra, hypothalamus and the olfactory bulb in vertebrates (Wong & Tank, 2007). Analysis of purified TH has demonstrated that the enzyme exists as a tetramer in its native form and as a homotetramer in most species. Each subunit consists of an N-terminal regulatory domain and a C-terminal catalytic domain (reviewed in Kumer & Vrana, 1996). Isolation and characterization of cDNA clones of rat TH has shown that the predicted molecular
weight of TH is about 55kDA, varying slightly among species (Grima et al., 1985). The catalytic core of TH is highly conserved across species but the N-terminal domain is diverse (Fitzpatrick, 1999). The divergence of the N-terminal domains is believed to be an important aspect in the regulation of TH, including phosphorylation, which occurs in this domain (Campbell et al., 1986; Haycock, 1990). In humans, four isoforms of TH (human TH variants 1-4) are produced by alternative splicing of mRNA (Grima et al., 1987; Kaneda et al., 1987). TH variants 1-4 are identical in their C terminal domain but exhibit differences within the N terminal region (Kobayashi et al., 1988). Examination of expression pattern indicates that all isoforms of human TH are expressed in every tissue including the brain (reviewed in Kumer & Vrana, 1996).

**Regulation of tyrosine hydroxylase**

The diversity in the regulation of TH synthesis and activity allows for a control mechanism of catecholamine production that is spatially and temporally suited to the requirement of the cell. Utilizing both long-term and short-term regulation, the cell is able to direct the production and activity of TH. Long-term regulation of TH includes modulation of gene expression by transcription regulation, alternative splicing of mRNA, RNA stability, and translational regulation. Conversely, TH is regulated short term by feedback inhibition, phosphorylation, and allosteric modulation of enzyme activity (Kumer & Vrana, 1996).

One of the initial observations regarding the regulation of Th gene expression was that cold stress could increase TH activity in chromaffin cells of the adrenal medulla (Thoenen et al., 1969, Mueller et al., 1970; Guidotti et al., 1975). Some of these regulatory elements in the promoter region of the Th gene include glucocorticoid, AP-1, cyclic adenosine monophosphate (cAMP) responsive element, and hypoxia inducible regulatory elements (Lewis et al., 1983; Icard-Liepkalns et al., 1992; Best et al., 1995). Other stressors in addition to cold, such as
hypoglycemia, immobilization, isolation, and excessive exercise, have been shown to lead to an increase in TH activity (Wong & Tank, 2007). The pharmalogical manipulation of TH levels can be accomplished through the use of drugs such as reserpine, cocaine, haloperidol, diazepam and nicotine (Kumer & Vrana, 1996). Reserpine, which has been shown to deplete catecholamine stores by inhibiting VMAT function, selectively increases TH levels in the adrenal gland, peripheral sympathetic cells, and the central noradrenergic cells of the locus coeruleus (Plescher, 1991; Alterio, et al., 2001). Initially, it was thought that reserpine had no affect on TH activity in the dopaminergic cells of the substantia nigra (Joh et al., 1973; Zigmond et al., 1974; Pasinetti et al., 1990). However, it has since been demonstrated that injection of reserpine in rat substantia nigra, inhibits TH activity (Sun et al., 1993). Chronic administration of cocaine, which blocks biogenic amine reuptake into nerve terminals, has been shown to increases TH mRNA levels, TH activity and TH immunoreactivity in the VTA with no change in the relative phosphorylation state of the enzyme (Beitner-Johnson & Nestler, 1991). Additionally, nerve growth factor and epidermal growth factor can affect TH expression by inducing TH activity and steady-state mRNA levels (Acheson & Thoenen, 1987; Lewis & Chikaraishi, 1987).

Phosphorylation, which increases TH activity, is a mechanism for short-term regulation of TH and is also an example of posttranslational modification of the enzyme. Therefore, it is no coincidence that the analysis of the mechanisms and components of TH phosphorylation is probably the most prevalent in the study of TH regulation. These studies have identified seven kinase systems that directly phosphorylate TH within the N- terminal regulatory domain at Ser\textsuperscript{8}, Ser\textsuperscript{19}, Ser\textsuperscript{31}, and Ser\textsuperscript{40} (Joh et al., 1978; Campbell et al., 1986; Zigmond et al., 1989; Haycock, 1990; Sutherland 1993; Toska et al., 2002; Kansy et al., 2004; Nakashima et al., 2011). Moreover, these phosphorylated residues are conserved among rats and human with the
exception of Ser\textsuperscript{8}, which has been replaced in human isoforms with threonine (Grima et al., 1987). \textit{In vitro} studies of TH phosphorylation targets have demonstrated that phosphorylation of Ser\textsuperscript{19} has no direct effect on TH activity whereas the phosphorylation of Ser\textsuperscript{31} results in a 2-fold increase in TH activity. Of the four serines phosphorylation sites for TH, Ser\textsuperscript{40} has been shown to be the most prominent in the regulation of enzyme activity. The phosphorylation of Ser\textsuperscript{40} results in a 20-fold increase in TH activity. TH is phosphorylated at Ser\textsuperscript{40} by number of kinases, such as CaM kinase II, PKC, cyclic GMP dependent protein kinase, MAPK-activated protein kinase, p38-regulated/activated kinase and mitogen- and stress- activated protein kinase (reviewed in Dunkley et al., 2004). Of these kinases, cyclic AMP-dependent protein kinase (PKA) has been shown to the most important in phosphorylating Ser\textsuperscript{40} and thus increasing TH enzyme activity (Fitzpatrick, 1999). When Ser\textsuperscript{40} is phosphorylated by PKA, it lowers the K\textsubscript{m} of TH for BH\textsubscript{4} and increases the inhibitory constant (Ki) for feedback inhibitors such as DA (Zigmond et al., 1989; Goldstein et al., 1995).

TH activity can also be influenced by its association with heparin, polyanions and phospholipids at places other than the active site of the protein (Lloyd & Kaufman, 1975; Raese et al., 1976; Katz et al., 1976; Lloyd, 1979). Allosteric effectors increase enzyme activity by decreasing the K\textsubscript{m} of the enzyme for its pterin cosubstrate, BH\textsubscript{4} (Kumer & Vrana, 1996).

The stability of TH mRNA is an important factor in the regulation and function of TH. Several studies have concluded that factors important to transcription, such as cAMP, glucocorticords, nicotine, and hypoxia are also key components in modulating stability of TH mRNA (Fossom et al., 1992; Craviso et al., 1992; Czyzyk-Krzeska et al., 1992, 1994). Czyzyk-Krzeska et al. (1992) demonstrated that hypoxia stimulated the synthesis and release of DA from O\textsubscript{2} sensitive cells in the mammalian carotid bodies but not in other catecholamine tissues such as
the adrenal gland or the sympathetic ganglia. In a follow-up study, utilizing pheochromocytoma (PC12) clonal cell line as an experimental model, Czyzk-Krzeska and colleagues (1994) further demonstrated that hypoxia enhances the rate of transcription of the \( Th \) gene and results in a 3-fold increase in stability of TH mRNA.

To maintain TH homeostasis and prevent cellular damage, two forms of catecholamine feedback inhibition can negatively regulate TH activity. The first method is based on the concentrations of catecholamine product and is readily reversible. During this process, catecholamine such as DA competitively inhibits TH activity by binding to TH at the same site as BH4 (Goldstein, 1995). The second mechanism of inhibition involves the association of DA with ferric iron (Fe\(^{3+}\)) at the active site of TH. This method, which is nearly irreversible, not only decreases enzyme activity but also stabilizes TH (Okuno & Fujisawa, 1991). The formation of this catecholamine-metal complex results in oxidation of Fe\(^{3+}\) and consequently the inactivation of the enzyme (Okuno & Fujisawa, 1985; Okuno & Fujisawa, 1991).

**Drosophila tyrosine hydroxylase**

As in mammals, TH is the rate-limiting enzyme in the production of DA in *Drosophila*, and is encoded by the *pale (ple)* locus (Neckameyer & White, 1993). *ple*, which maps to the same chromosomal region as *Drosophila* TH (DTH) was first recovered as a recessive embryonic lethal (Nüsslein-Volhard et al., 1985). Later, the isolation and characterization of DTH by Neckameyer and Quinn (1989) indicated that DTH is the homologue of rat TH and shares 50% sequence identity with its mammalian counterpart. Additionally, many of the regulatory components of mammalian TH are conserved within the fly (Vie et al., 1999). The discovery of the *Drosophila* gene encoding TH has led to many studies that seek to evaluate the molecular kinetics of TH regulation and function in insects. The *pale* locus encodes two
isoforms, DTH 1 and DTH 2, which are generated by alternative splicing (Neckameyer & White, 1992; Birman, 1994). Although both isoforms are expressed in all stages of development, the minor isoform, DTH 1, is expressed only in the CNS where as the major isoform, DTH 2, is expressed predominately in the hypoderm, where it participates in the synthesis of cross-linked cuticles (Birman, 1994). There are also structural differences between both isoforms that may affect the regulatory properties of DTH. DTH 2 adds an acidic domain of 71 amino acids in the regulatory part of the enzyme, causing it to have a higher specific activity than unphosphorylated DTH 1, which must be phosphorylated to achieve maximum activity (Birman, 1994). Analysis of purified DTH recombinant proteins revealed that the mammalian equivalent of Ser\textsuperscript{40}, which is important for phosphorylation in mammals, is conserved in Drosophila at Ser\textsuperscript{32} and is phosphorylated by PKA (Vie et al., 1999). Interestingly, site-directed mutagenesis of Ser\textsuperscript{32} abolished the phosphorylation of both isoforms. Additionally, the results of this study suggest that there are several important regulatory differences between DTH 1 and DTH 2 that is most likely related to differences in structure. DTH 2 in comparison to DTH 1 has a lower affinity for BH\textsubscript{4}, a broader pH profile, increased specific activity, and the inability to be stimulated by PKA phosphorylation (Vie et al., 1999).

**Tetrahydrobiopterin function and dysfunction**

The pteridine, BH\textsubscript{4}, is a required cofactor for the catalytic activities of phenylalanine hydroxylase, tyrosine hydroxylase, tryptophan hydroxylase, all isoforms of nitric oxide synthase, and glycerol ether monoxygenase (Kaufman, 1963; Nagatsu et al., 1964; Lovenberg et al., 1967; Kwon et al., 1989; Kaufman et al., 1990). These enzymes are essential for the maintenance of diverse body functions such as blood pressure regulation, immune function, neurotransmission and the conversion of phenylalanine to tyrosine (Thony et al., 2000. Tight regulation of BH\textsubscript{4}
Physiological studies have demonstrated that BH₄ is involved in neuronal cell proliferation and survival (Koshimura et al., 1999). As it relates to neuronal health, several mechanisms have been proposed for the neuroprotective capabilities of BH₄. BH₄ is subject to oxidation by a number of reactive species such as the hydroxyl radical and reactive nitrogen species (Heales et al., 1988; Heales et al., 2004). Therefore, its ability to react with oxidants suggests antioxidant properties, a function that is supported by the evidence of Gramsbergen et al., (2002) who noted that under conditions of glutathione depletion and incubation of nigrostriatal cultures with the BH₄ precursor, sepiapterin, enhanced production of BH₄ substituted for a loss of the autoxidant. In another study, Nakumara and colleagues (2000) demonstrated that BH₄ functions as an antioxidant and prevented cell death in glutathione-depleted neurons. Moreover, it has also been suggested that BH₄ functions as a scavenger of reactive oxygen species and regulates cell survival during paraquat exposure (Kojima et al., 1995). However, the neuroprotective function of BH₄ is challenged by the reports of some studies, that demonstrate that exogenous BH₄ can elevate oxidative stress and adversely affect neuron survival in some disease models (Choi et al., 2004; Kim et al., 2004; Choi et al., 2006).

Several of the BH₄-requiring enzymes noted above catalyze the rate-limiting reactions in the synthesis of neurotransmitters, and mis-regulation or defective synthesis of this cofactor has been implicated in many neurological disorders and diseases. Loss of BH₄, due to mutations in the gch-1 gene, is one cause of hyperphenylalaninemia, an atypical form of phenylketonuria (PKU), and dopa responsive dystonia (Ichinose et al., 1995; Nagatsu et al., 1997; Hirano et al., 1998). Limited availability of this cofactor has also been implicated in features of several
disorders such as Alzheimer’s disease, Parkinson’s disease, depression, and autism (Hamon & Blair, 1986; Thöny et al., 2000; Richardson et al., 2007; Nagatsu et al., 2007). Moreover, loss of BH$_4$ results in a reduction in TH levels, which consequently leads to neurological anomalies such as physical and mental retardation, seizures, and hypotonia. Based on these findings, it can be hypothesized that maintaining BH$_4$ homeostasis is a crucial component in the regulation of cell function and health (Longo, 2009).

_Tetrahydrobiopterin biosynthesis_

BH$_4$ is synthesized via two pathways: the _de novo_ synthesis and the ‘salvage’ pathways. The _de novo_ synthesis pathway requires Mg$^{2+}$-, Zn$^{2+}$-, and NADPH-dependent reactions, and begins with the conversion of guanosine triphosphate (GTP) to the first intermediate, dihydromeopterin triphosphate (H$_2$NTP) by GTP cyclohydrolase 1 (GTPCH1) (Thöny et al., 2000). This process is the first and rate-limiting step in the _de novo_ biosynthesis of BH$_4$ (Nichol et al., 1985; Weisberg & O’Donnell 1986). Following this conversion, 6-pyruvoyl-tetrahydropterin synthase (PTPS) modifies H$_2$NTP to form the second intermediate in the BH$_4$ biosynthesis pathway, 6-pyruvol tetrahydropterin (PTP). The final step in this pathway is the reduction of PTP, by sepiapterin reductase, to BH$_4$ (Matsubara & Akino, 1964; Matsubara et al., 1966).

The ‘salvage’ pathway begins with the conversion of sepiapterin into BH$_2$ by the enzyme sepiapterin reductase. BH$_2$ is then is converted into BH$_4$ by the NADPH dependent enzyme, dihydrofolate reductase (Nichol et al., 1985). However, it appears that the salvage pathway function cannot compensate entirely for BH$_4$ deficiencies resulting from defects in the _de novo_ biosynthesis pathway (Thöny et al., 2000).
Figure 1.2. Tetrahydrobiopterin (BH₄) biosynthesis pathway. The *de novo* synthesis of BH₄ begins with the rate-limiting step within this pathway, the conversion of guanosine triphosphate to dihydroleptopterin triphosphate. This is followed by the modification of the two intermediates by the enzymes 6-pyruvol-tetrahydropterin synthase (PTPS) and sepiaterin reductase (SR), respectively, into the end product, BH₄. The salvage pathway begins with the conversion of pterin 4 a-carbinolamine by the enzymes pterin-4a-carbinolamine dehydratase (PCD) and dihydropteridinede reductase (DHRP) into BH₄.
Regulation of GTP cyclohydrolase

Mammalian GTPCH is encoded by a single copy gene, gch1, which is comprised of six exons spanning 30kb (Ichinose et al., 1995). As previously discussed, GTPCH is the rate-limiting enzyme in the production of BH4 which is a necessary cofactor for the catalytic activity of TH, the rate limiting enzyme in dopamine synthesis. Structural analysis of *E. coli* GTP cyclohydrolase 1 reveals that the holoenzyme is a homodecamer consisting of two tightly associated pentamers. The protein ranges in mass from 300-500kDA, depending on the species, but all forms of GTPCH I seem to be comprised of homodecamers (Nar et al., 1995). Each subunit consists of a catalytic domain and an N-terminal helical domain that extends outward from the enzyme’s core. Sequence analysis reveals that the C-terminal domain of the protein is evolutionarily conserved with 60% residue identity between human and *E. coli* forms of the enzyme (Witter et al., 1996). However, the N-terminal regulatory domain is variable among vertebrates and invertebrates. In mammals, six alternatively spliced transcripts produce GTPCH polypeptides, although only the longest polypeptide has been demonstrated to assemble into a catalytically active enzyme (Gütlich et al., 1994). Because GTPCH is the rate-limiting enzyme in BH4 synthesis, mutations in the gch1 are a major cause of disorders associated with BH4 deficiencies. More than 100 mutations have been identified in patients with GTPCH1 deficiencies, most of which are heterozygous and result in the dominant genetic disorder DOPA-responsive dystonia (Thöny & Blau, 2006). Other GTPCH mutations are recessive, and homozygous individuals are afflicted with severe BH4 deficiencies that are characteristic of the disorder. Analysis of these patients reveals that, in the absence of BH4 supplementation, they suffer from convulsions, severe developmental retardation, muscular hypotonia, and frequent hyperthermia, as a consequence of defects in the biosynthesis of catecholamine and serotonin.
(Niederwieser et al., 1984). GTPCH1 deficiency has also been implicated in vascular diseases such as diabetes, hypertension, and atherosclerosis (Stroes et al., 1997; Meininger et al., 2000; Cosentino et al., 2001). Moreover, these disorders are also associated with the abnormal function of the enzyme nitric oxide synthase, which, like the aromatic amino acid hydroxylases, also requires the BH₄ cofactor for catalytic function.

GTPCH protein levels and enzymatic activity are regulated by transcriptional and post-translational mechanisms that range from the induction of transcription by cytokines to the regulation of enzyme activity by phosphorylation. At the transcriptional level, it has been demonstrated that pro-inflammatory cytokines, such as interferon-γ, interleukin-1β and tumor necrosis factor-α can increase intracellular levels of GTPCH in a variety of mammalian cell lines and tissue cultures (Frank et al., 1998; Katusic et al., 1998; Franscini et al., 2003). Other factors such as lipopolysaccharides, neurotrophic factor, cAMP, the transcription factor Nurr-1 and glucocorticoids have been shown to have positive effects on GTPCH levels (Serova et al., 1997). Interestingly, glucocorticoid interaction with the glucocorticoid receptor has also been demonstrated to have a negative effect on GTPCH expression (Mitchell et al., 2004). The basis for this discrepancy is unclear. Additionally, it has been reported that alternative splicing in human peripheral blood mononuclear cells can have both positive and negative effects on GTPCH expression with the negative effect acting as a dominant-negative mutation (Hwu et al., 2003). Two alternatively spliced mRNAs, type I and type II, have been reported for GTPCH (Togari et al., 1992). Both mRNAs are identical for the 5’ region but differ in their 3’ make up between exons 5 and 6. Type 1 represents the full length mRNA with a long noncoding 3’ region encoded by exon 6, whereas type II is a truncated mRNA due to a splice between intron 5 and exon 6. Examination of type I and type II effect on GTPCH protein levels in peripheral
blood mononuclear cells indicated that type II has a dominant –negative effect by disrupting the catalytic activity of wild-type GTPCH.

The labeling of mammalian cell cultures with $^{32}$P first revealed that GTPCH activity could be increased by phosphorylation (Hesslinger et al., 1998). A similar study conducted by Lapize and colleagues (1998), implicated protein kinase C in this modification. These investigators were able to decrease GTPCH activity by treating cells with the PKC-specific inhibitor, RO-31-8220. Recently, it was proposed that Ser$^81$ is an important phosphorylation site in human GTPCH, and that this site is phosphorylated by casein kinase II (Widder et al., 2007).

Tumor necrosis factor-α and Janus kinase-2 were also reported to have positive effects on GTPCH activity (Vann et al., 2002; Shimizu et al., 2008). Few of these studies, however, included stringent structure function analysis to clearly identify activating residues in GTPCH.

While phosphorylation is an important component in the up-regulation of GTPCH activity, end product feedback inhibition of BH$_4$ is probably the most efficient down-regulation mechanism. In mammals, GTP cyclohydrolase 1 feedback regulatory protein (GFRP) functions in both positive and negative regulation of the enzyme, maintaining intracellular levels of BH$_4$ at or below that needed by BH$_4$ requiring enzymes (Harada et al., 1993). Harada and colleagues (1993) noted that the inhibition of GTPCH1 occurred by the formation of a complex between BH$_4$, a small protein termed “p35” in that study, and GTPCH. In the presence of BH$_4$, p35, which was subsequently named GFRP, inhibited GTPCH activity in a dose-dependent manner with no inhibition occurring in the absence of BH$_4$. Subsequent gel filtration purification and crystallographic studies led to a model for this association, in which two pentamers of GFRP associate with the decameric GTPCH1, lowering the Vmax for the GTP substrate in a noncompetitive manner when BH$_4$ is also bound to GFRP (Yoneyama & Hatakeyama, 1998;
Maita et al., 2001; Harada et al., 1993). As a positive regulator, the association of GFRP with GTPCH1 and phenylalanine increases GTPCH activity (Harada et al., 1993). The inhibition of GTPCH activity by the association of GTPCH, BH₄, and GFRP can be reversed by phenylalanine, and it is postulated that phenylalanine displaces BH₄ during this process (Yoneyama & Hatakeyama, 1998; Xie et al., 1998; Maita et al., 2004). The association of phenylalanine with GFRP reduces the positive cooperativity of GTPCH1 and as a result, increases the enzyme’s activity in saturating amounts of GTP. However, it should be noted that in the absence of GFRP, phenylalanine has no effect on GTPCH1 activity (Harada et al., 1993; Maita et al., 2001).

**Drosophila GTPCH**

*Drosophila* GTPCH is evolutionarily conserved with all other forms of the enzyme and shares 80% sequence identity in its catalytic domain with its mammalian counterparts (McLean et al., 1993). As in mammals, *Drosophila* GTPCH is the rate-limiting enzyme in the production of BH₄ (Weisburg & O’Donnell, 1986; Hatakeyama et al., 1989). There are four isoforms of GTPCH in *Drosophila*, all encoded by the *Punch (Pu)* locus (Mackay & O’Donnell, 1983; McLean et al., 1993). These forms, unlike alternative forms of the mammalian protein, have identical C-termini, and distinct N-terminal domains (McLean et al., 1993), and at least three of the four forms are catalytically active (Funderburk et al., 2006). The fourth form has not yet been assayed *in vitro* to confirm its activity.

The *Drosophila* transcripts, as well as the isoforms encoded by them, are spatially and temporally regulated in complex patterns (Mackay et al., 1993; McLean et al., 1993; Chen et al., 1994). Analysis of the expression patterns of these transcripts revealed that Transcript A is expressed primarily in the newly eclosed heads of adult flies, Transcript B is abundantly
expressed in the larvae and adult head, and Transcript C has been found in all stages of development (McLean et al., 1993). Previous research in our lab demonstrated that the N-terminal domains are significantly longer than in any other organism, and they are functionally similar to GFRP in mammals in that these domains serve to down-regulate catalytic activity when in an unphosphorylated state, while serving as targets for activating phosphorylation events (Funderburk et al., 2006). Additional roles of GTPCH in *Drosophila* include the regulation of pigments in the developing eye, regulating brain and tracheal development, and mediating embryonic nuclear division (O’Donnell, 1989; Chen et al., 1994; Hsouna et al., 2007).

**Zinc function**

The small size of zinc (Zn$^{2+}$), 65Å, as well as the physical and chemical properties of this element, makes it highly adaptable for meeting the needs of enzymes and other proteins that carry out diverse biological functions (Caujungco & Gordan, 1997). Advances in molecular techniques in detecting and determining Zn$^{2+}$ levels *in vivo* as well as more efficient methods in the isolation, purification, and characterization of macromolecules, have led to a wealth of knowledge establishing this trace element as an integral part of many biological processes and an essential nutrient for all organisms on earth (Caujungco & Gordan, 1997; Gaither & Eide, 2001). The importance of Zn$^{2+}$ function is further underscored by the requirement of this element for over 300 enzymes in microorganisms, plants, and animals (Vallee & Falchuk, 1993). Thus, the requirement of this element for many biological processes, means that Zn$^{2+}$ is usually found associated with proteins within the cell (Williams, 1989).

Zn$^{2+}$ is an integral component in the metabolism of proteins, lipids, carbohydrates, and nucleic acids (Valle & Falchuk, 1993). Additionally, Zn$^{2+}$ has been demonstrated to have an inhibitory and catalytic effect on several enzymes (Caujungco & Gordan, 1997). In this
capacity, Zn$^{2+}$ is required for numerous ligases, oxioreductases, transferases, hydroxylases, and isomerases (Valle & Falchuk, 1993). Zn$^{2+}$ is also an essential structural component of many proteins including the ubiquitous Zn$^{2+}$ finger DNA binding proteins (Rhodes & Klug, 1993). In addition, calcium-binding proteins, with helix-loop-helix motif, contain high affinity Zn$^{2+}$ binding sites and have been implicated in an array of biological processes from cell membrane rearrangement to brain-related diseases (Baudier & Kuznicki, 1983; Filipek et al., 1990; Sheng et al., 1998; Mbele et al., 2002). Studies of brain disorders have also shown that Zn$^{2+}$ is an integral factor in the mis-regulation of protein folding (Mantyh et al., 1993; Bush et al., 1994; Clements et al., 1996; Chattopadhyay & Valentine, 2009). As it relates to gene expression, Zn$^{2+}$ modulates DNA and RNA activity by regulating activator proteins, which in turn, control transcription and replication of DNA.

Although there is an abundance of Zn$^{2+}$ within the cell, the amount of free Zn$^{2+}$ is minimal due to the association of Zn$^{2+}$ with proteins and the efficient transport and storage of Zn$^{2+}$ within vesicles (Caujungco & Gordan, 1997). To maintain Zn$^{2+}$ homeostasis and prevent oxidative damage, free Zn$^{2+}$ may be sequestered by metallothioneins, taken up by organelles, or transported in or out of the cell by Zn$^{2+}$ transporter proteins (Cole et al., 1999). This tight regulation of Zn$^{2+}$ homeostasis is a contributing factor to the idea that Zn$^{2+}$ is a relatively non-toxic molecule (Plum et al., 2010). Moreover, no disorder has been linked to an abundance of Zn$^{2+}$ (Vallee & Falchuk 1993; Plum et al., 2010). In contrast, studies in a variety of organisms have concluded that Zn$^{2+}$ deficient cells fail to divide and differentiate as a consequence of cell cycle arrest during development (Vallee & Falchuk, 1993).
**Zinc metabolism**

The Zn\(^{2+}\) requirement of various enzymes and the need to tightly regulate the homeostatic condition of the cell is accomplished through the manipulation of ion influx and efflux by Zn\(^{2+}\) transporters, and the chelation of Zn\(^{2+}\) by apothionein or other Zn\(^{2+}\) sequestering proteins and amino acids (Caujungco & Gordan, 1997). Methallothioneins, a group of low molecular weight proteins, aid in maintaining Zn\(^{2+}\) homeostasis by binding to and therefore detoxifying reactive metals and free radicals (Caujungco & Gordan, 1997; Palmiter, 2004). However, metallothionein apparently is not used for long-term storage of Zn\(^{2+}\) as it has been shown to have a short biological half-life in mammalian cell cultures (Krezoski et al., 1988). Zn\(^{2+}\) transport is tissue and condition dependent and is regulated according to the needs of particular cell types. Under conditions of Zn\(^{2+}\) toxicity, when extracellular Zn\(^{2+}\) levels are high, Zn\(^{2+}\) may enter into neurons via N-methyl-D-aspartate (NMDA) receptors, \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainite receptors, voltage-dependent calcium channels or transporter-mediated exchange with intracellular sodium (Sensi et al., 1997).

Whether transporting Zn\(^{2+}\) into intracellular organelles, to serve as cofactors for various enzymes, or operating as a detoxifying agent by facilitating the sequestration of Zn\(^{2+}\) within organelles or the efflux of this ion across the plasma membrane, Zn\(^{2+}\) transporters (ZnTs) play an essential role in modulating Zn\(^{2+}\) metabolism (Gaither & Eide, 2001). Much of what was known initially regarding ZnT function is due to the results of studies conducted in yeast. These studies have revealed the presence of at least two transport systems within this organism. One system has a high affinity for Zn\(^{2+}\) and is active in Zn\(^{2+}\)-limited cells (Zhao & Eide, 1996a), while, the other system has a low affinity for Zn\(^{2+}\) and is active in Zn\(^{2+}\) depleted cells (1996b). Therefore, it is suggested that both systems are specific for Zn\(^{2+}\) and probably do not contribute to the
accumulation of other metals (Zhao & Eide, 1996a). ZrT1 and ZrT2 are genes that encode the transporter proteins of the high and low affinity systems, respectively (Zhao & Eide, 1996a, b). Both genes are transcriptionally regulated by the transcriptional activator ZAP1 and encode members of the Zrt, Irt-like protein (ZIP) family of metal ion transporters (Zhao & Eide, 1997). In addition, yeast Zn\(^{2+}\) levels are regulated by the endocytosis of ZrT1, a plasma membrane protein, which prevents the import of excess Zn\(^{2+}\) within the cell (Gitan et al., 1998).

ZnT function is conserved from prokaryotes to eukaryotes. In bacteria, two ZnT families that function in Zn\(^{2+}\) uptake and export have been identified. The ABC family of ZnT proteins functions in Zn\(^{2+}\) uptake. One such transporter, the ZnuABC is a major source of Zn\(^{2+}\) accumulation in the cells of *E. coli* (Patzer & Hantke, 1998). Unlike the ABC family of proteins, the family of P-type ATPases has been shown to demonstrate Zn\(^{2+}\) export function in bacteria. For example, *E. coli* ZntA plays an important role in Zn\(^{2+}\) detoxification by pumping the metal ion out of the cell when intracellular levels are too high (Rensing et al., 1997). In eukaryotes, ZIP family plays a prominent role in uptake and the ‘cation diffusion facilitator’ (CDF) family promotes Zn\(^{2+}\) efflux. The ZIP family transports Zn\(^{2+}\) from outside the cell into the cytoplasm and has been found to mobilize stored Zn\(^{2+}\) by transporting the metal from intracellular compartment into the cytoplasm (Gaither & Eide, 2001). Alternatively, the CDF family pumps Zn\(^{2+}\) from the cytoplasm out of the cell or into the lumen of an organelle (Gaither & Eide, 2001).

The ZIP family of proteins was first discovered in *Saccharomyces cerevisiae* and *Arabidopsis thaliana* and has been shown to transport Zn\(^{2+}\) and iron, respectively (Zhao & Eide 1996a; Eide et al., 1996). More than 100 members of this family have been identified in bacteria, fungi, protozoa, insects, plants, and mammals (Gaither & Eide, 2001; Taylor & Nicholson, 2003; Lichten & Cousins, 2009). ZIP family members are characterized by a predicted topology in
which the amino and carboxy termini are found on the extracellular surface of the plasma membrane. In addition, most ZIP proteins have approximately eight transmembrane domains and the presence of a cytoplasmic histidine rich loop (Gaither & Eide, 2001). Based on the number of members of the ZIP family of proteins and the higher degree of sequence conservation among groups, the ZIP family of proteins is divided into four subfamilies: subfamilies I and II, gufA and LIV-1 subfamily of ZIP Zn\(^{2+}\) transporters (LZT) (Gaither & Eide, 2001; Lichten & Cousins, 2009). Subfamily I consists largely of fungal and plant members, whereas subfamily II is a smaller group of nematode and mammalian proteins (Guerinot, 2000). The GufA group consists of prokaryotes and eukaryotic members and are proteins of unknown function (McGowan et al., 1993). The LZT subfamily is restricted to eukaryotes; its founding member is a protein of unknown function that has been linked to metastatic breast cancer gene, LIV-1 (Manning et al., 1995). Like other members of the ZIP family of proteins, the LZT subfamily also has histidine-rich repeats present in the cytosolic loop between transmembranes III and IV. In addition, this subfamily also has histidine residues on the extracellular loop between transmembranes II and III and in the extracellular N terminus (Rogers et al., 2000). Though the function of these additional histidine residues is unknown, the presence of these repeats on both sides of the membrane is unprecedented and are believed to be involved in the transport of metal ions (Rogers et al., 2000). In addition, conserved amino acid regions include a novel metalloprotease motif that is not found in other three subfamilies of ZIP transporters. Taylor et al. (2004) has further divided this subfamily into a KE4 subgroup. The KE4 subgroup, which was first identified in the mouse, has been demonstrated to localize to the endoplasmic reticulum and not the plasma membrane (Suzuki & Endo, 2002). Analysis of the cellular location of HKE4 protein, the human homologue of the mouse KE4 gene, demonstrates that it
also localizes to intracellular membranes, including the endoplasmic reticulum (Taylor et al., 2004). In addition, HKE4 has been shown to increase the intracellular Zn\textsuperscript{2+}. Among this group is CATSUP from *Drosophila*, which is a negative regulator of tyrosine hydroxylase. Although the exact zinc related function of CATSUP has not been determined, analysis of the KE4 subfamily has implicated this protein family in ion transport and the regulation of tyrosine hydroxylase (Stathakis et al., 1999; Lasswell et al., 2000). The main physiological function of ZIP proteins is yet to be determined. However, mutations within the hZIP4 gene in humans have been linked to the Zn\textsuperscript{2+} deficiency disease acrodermatitis enteropathica, which is caused by a defective uptake of Zn\textsuperscript{2+} in the small intestine (Kury et al.; 2002; Wang et al., 2004).

As previously stated, the CDF family of proteins is involved mostly in the transport of Zn\textsuperscript{2+} from the cytoplasm out of the cell or into the compartments of organelles. Like the ZIP family of ZnT, groups of protein within this family have been divided into subfamilies, I, II, and III, based on sequence similarities. Subfamily I consists mostly of prokaryotic members, while subfamilies II and III contain an approximately equal representation of prokaryotic and eukaryotic members. The CDF family of proteins is characterized by six transmembrane domains with a great degree of conservation among proteins within transmembrane domains I, II, and V. In addition, most members of the CDF family share a similar predicted topology where the amino and carboxy termini are cytoplasmic. Topological analysis of the CzcD protein in bacteria has confirmed some elements of this topology (Anton et al., 1999). Several members of the CDF family of ZnT have been identified in humans and mouse. To date, 10 ZnTs, ZnT 1-10, of the CDF family of proteins have been identified in humans. ZnT1 is a Zn\textsuperscript{2+} efflux protein found within the plasma membrane of neuron and glial cells and in this capacity is believed to play a role in cellular detoxification of Zn\textsuperscript{2+} by exporting excess metal ion out of the cell (Gaither
& Eide, 2001; Sekler et al., 2007). Analysis of mouse brains, demonstrates that ZnT1 is localized to regions rich in synaptic Zn\(^{2+}\) and its expression is transcriptionally regulated in correlation with the appearance of synaptic Zn\(^{2+}\) (Sekler et al., 2002; Nitzan et al., 2002). Moreover, over-expression of ZnT-1 has been shown to protect against Zn\(^{2+}\) toxicity in neurons and glial cells (Palmiter, 2004; Nolte et al., 2004). Znt-2 is believed to be involved in intracellular Zn\(^{2+}\) sequestration and is located in the membranes of an acidic compartment that accumulates Zn\(^{2+}\) when cells are grown in concentration of 100 or 200 µM Zn\(^{2+}\) (Gaither & Eide, 2001). Like ZnT-2, ZnT-3 also sequesters Zn\(^{3+}\) and is required for transport of Zn\(^{3+}\) into synaptic vesicles where it is presumed to play a neuromodulatory role (Palmiter et al., 1996; Cole et al., 1999). Similarly, ZnT4-ZnT10 are also involved in Zn\(^{2+}\) export (Luizzi & Cousins, 2004).

**Zinc and the brain**

The importance of Zn\(^{2+}\) for proper brain function is demonstrated by the abundance of this element in brain tissue with respect to other organs. The average total brain Zn\(^{2+}\) concentration is approximately 150 µmol/L (Takeda, 2000). However, much of this Zn\(^{2+}\) is associated with other proteins and therefore estimation of free Zn\(^{2+}\) is sub-nanomolar and is estimated to be approximately 500 nM in brain extracellular fluids (Weiss et al., 2000).

The main supply of Zn\(^{2+}\) to the brain is through the blood-brain barrier system (Pullen et al., 1990; Franklin et al., 1992). Found within the plasma are large components of exchangeable Zn\(^{2+}\) associated with albumin and amino acids such as histidine and cysteine (Hallman et al., 1971; Harris & Keen, 1989). L-histidine, both in the plasma and in cerebrospinal fluid (CSF), plays a significant role in transferring Zn\(^{2+}\) to target sites (Takedo et al., 2002). Inside the brain, Zn\(^{2+}\) is transferred freely throughout the cerebrospinal and the brain extracellular fluid compartments.
Once Zn$^{2+}$ has been taken up by a neuron, it is transported anterogradely and retrogradely via the axonal transporting system. These Zn$^{2+}$-containing neurons sequester Zn$^{2+}$ in presynaptic vesicles and release it in a calcium-dependent manner (Takeda, 2001). Additionally, Zn$^{2+}$ sequestered in synaptic vesicles is released with glutamate and may modulate neurotransmission (Takeda, 2001). Once inside the synaptic cleft, Zn$^{2+}$ may be taken up by postsynaptic and presynaptic neurons (Takeda et al., 1997). The uptake of Zn$^{2+}$ into postsynaptic neurons as a result of excessive excitation of Zn$^{2+}$-containing glutamate neurons, can lead to degeneration of these cells (Sloviter, 1985; Choi et al., 1988; Tonder et al., 1990; Choi & Koh, 1998).

Examination of Zn$^{2+}$ levels within various sections and tissues of the brain have demonstrated that excitatory glutamatergic neurons, located in the forebrain regions, are Zn$^{2+}$-enriched neurons (ZEN) or cells that contain free Zn$^{2+}$ ions in the vesicles of their presynaptic boutons (Slomianka, 1992; Frederickson and Moncrieff, 1994). These regions include the amygdala, the hippocampus, and the neocortex. ZEN have also been found in the spinal cord and the cerebellum. However, most of the ZEN in the spinal cord are inhibitory γ-aminobutyric acid (GABA)ergic (Danscher et al., 1994).

The storage and release of Zn$^{2+}$ within glutaminergic and GABA neurons has led to the idea that Zn$^{2+}$ may function as a neuromodulator. In this capacity, Zn$^{2+}$ that is released from synaptic vesicle is believed to interact with certain neurotransmitter receptors. Several studies have indicated that the release of Zn$^{2+}$ from synaptic vesicles results in the inhibition of NMDA receptors (Paoletti et al., 1997; Vogt et al., 2000; Kay, 2003). Glutamate is the major excitatory transmitter employed in the nervous system. The release of Zn$^{2+}$ with glutamate reduces the ability of glutamate to activate NMDA receptors, by direct interaction with NMDA receptors at the post-synaptic level (Smart et al., 1994). Moreover, the Zn$^{2+}$ effect on glutamate neurons is
significant because glutamate is the most abundant neurotransmitter in the brain and Zn$^{2+}$ is found predominately within the synaptic vesicles of glutamate neurons. In addition, Zn$^{2+}$ has been shown to inhibit GABA receptors (Ruiz et al., 2004). The results of these studies indicate that Zn$^{2+}$ has an inhibitory effect on the major excitatory and inhibitory neurotransmitters of the CNS.

Based on the abundance of Zn$^{2+}$ in brain tissues and increasing evidence of its role in diverse cellular functions in neurons, it is now generally accepted that Zn$^{2+}$ transport is an essential component in modulating brain function. As previously mentioned, ZnTs are responsible for transporting Zn$^{2+}$ into synaptic vesicles. In mammals, ZnT-3 has been implicated as the main transporter that functions in this capacity. Cole et al. (1999) has demonstrated that knockout of ZnT3 in mice results in a complete loss of Zn$^{2+}$ within synaptic vesicles and a 20% reduction of Zn$^{2+}$ levels in the hippocampus and cortex (Cole et al., 1999). Loss of ZnT3 in these animals did not affect memory or sensorimotor function, but these animals were more prone to seizures and seizure-related neurodegeneration than controls (Cole et al., 2000, 2001). However, the lack of phenotype in these animals remained puzzling to researchers. Recently, it was determined that ZnT3 knockout mice exhibited age-dependent learning deficits that were manifested at 6 months but were not present at 3 months. Additionally, manipulation of vesicular Zn$^{2+}$ by Zn$^{2+}$ deprivation diets and the use of the Zn$^{2+}$ chelator, cliniquol, resulted in reduced hippocampal neurogenesis in mice and rats (Suh et al., 2009).

Neuronal mitochondria can also uptake Zn$^{2+}$ as a way of maintaining Zn$^{2+}$ homeostasis in the cell (Sensi & Jeng, 2004). This finding is significant because mitochondrial dysfunction is thought to be a key component in many neurological disorders such as Parkinson’s and Alzheimer’s disease (Cappaso et al., 2005; Jones, 2010; Santos et al., 2010). Moreover, excess
mitochondrial Zn$^{2+}$ can lead to dysfunction within this organelle due to prolonged ROS generation (Sensi & Jeng, 2004; Frazzini et al., 2006; Sensi et al., 2008).

**Zinc and dopamine**

The DAT located at the presynaptic termini is responsible for modulating DA neurotransmission by rapidly clearing DA from the synapse and hence, terminating DA signaling and controlling the duration of synaptic inputs into the brain (Jones et al., 1998; Pifl et al., 1995; Norregard et al., 1998). Structure-function analysis of endogenous Zn$^{2+}$ binding sites within the DAT has revealed that Zn$^{2+}$ inhibits DAT function and attenuates DA content (Norregard et al., 1998; Loland et al., 1999; Meinild et al., 2004). Interestingly, Zn$^{2+}$ modulates DAT function not by preventing DA binding but by inhibiting the translocation of this neurotransmitter. Moreover, it has been demonstrated that Zn$^{2+}$ inhibition of DAT function is not due to a conformational change of the protein but rather a change in the membrane potential by the depolarization of DAT (Meinild et al., 2004). Others studies have indicated that DAT also mediates DA efflux (Levi & Raiteri, 1993; Pifl et al., 1995). Therefore, Zn$^{2+}$ may function as a neuromodulator of DA homeostasis by not only regulating the clearance of DA from the synapse, but also the exocytotic release of DA.

Zn$^{2+}$ binding sites have also been identified within DA receptors (Liu et al., 2006) Studies of the molecular interaction of the DAT and Zn$^{2+}$ have determined that Zn$^{2+}$ allosterically modulates D$_2$ receptor function by preventing DA from binding to the receptor (Schetz & Sibley, 1997; Schetz et al., 1999; Liu et al., 2006). In addition, Zn$^{2+}$ also affects post-synaptic dopamine interactions by inhibiting D1 receptor function (Schetz & Sibley, 1997). Moreover, inhibition of DA receptor function is specific to Zn$^{2+}$, given the fact that other metals such as manganese had no effect on receptor function (Schetz & Sibley, 1997).
Sequence analysis of proteins involved in the metabolism of DA has also identified Zn\(^{2+}\) binding sites on GTPCH1. Analysis of the crystal structure of GTPCH in mammals and bacteria revealed the presence of Zn\(^{2+}\) ions at the active site of both the inhibitory and stimulatory complexes of this enzyme (Maita et al., 2002; Maita et al., 2004). Moreover, Zn\(^{2+}\) is required for the catalytic activity of purified GTPCH in bacteria and mammals (Auerbach et al., 2000). This is significant because the synthesis of BH\(_4\), a necessary cofactor in the synthesis of DA, is regulated by GTPCH1. In addition, concentrations greater than 1mM Zn\(^{2+}\) are capable of inhibiting purified GTPCHs from bacteria and mammals (Kohashi et al., 1980; Blau et al., 1985; Kwon et al., 1989). Thus, these studies suggest that Zn\(^{2+}\) can regulate DA neurotransmission and function by directly interacting with components of DA homeostasis. However, further studies need to be conducted to determine if Zn\(^{2+}\) is a neuromodulator of DA.

**Zinc and neurodegenerative diseases**

Increasingly, metals, including Zn\(^{2+}\), are being implicated in the etiology of many diseases. Although alteration of Zn\(^{2+}\) homeostasis may be a component of brain dysfunction and neurological diseases, the mechanism of alteration is poorly understood. Moreover, the requirement of Zn\(^{2+}\) for so many biological processes and the abundance of this metal within the brain make it difficult to decipher whether alteration in Zn\(^{2+}\) homeostasis is the end result of neurological disease or a contributing factor in this process. Therefore, researchers have been on a quest to examine the biological significance of Zn\(^{2+}\) metabolism in many neurological disorders.

As previously discussed, Zn\(^{2+}\) is tightly regulated within the brain by the blood brain barrier, ZnTs, metallothionein, and other Zn\(^{2+}\)-binding proteins. The inability of a diseased cell to maintain proper Zn\(^{2+}\) homeostasis subsequently leads to a toxic accumulation of Zn\(^{2+}\) and is
believed to be a causative component in the neurodegeneration of brain cells. Koh and colleagues (1996) have demonstrated that excessive increase of intracellular Zn\textsuperscript{2+} in the brain due to transient forebrain ischemia in rats’ results in neurodegeneration. In addition, some studies have indicated that Zn\textsuperscript{2+} enters into post-synaptic neurons in toxic excess during seizures and traumatic brain injury (Frederickson et al., 1989; Suh et al., 2000). Moreover, the influx of toxic amounts of Zn\textsuperscript{2+} from pre-synaptic vesicles into post-synaptic neurons seems to be a factor in the neurodegenerative process (Koh et al., 1996; Suh et al., 2000). All together, this body of evidence not only demonstrates that excess Zn\textsuperscript{2+} is toxic to cells, but supports the concept that Zn\textsuperscript{2+} may be a constituent in the onset and development of neurological disorders such as Amyotrophic lateral sclerosis (ALS), Alzheimer’s disease (AD), and Parkinson’s disease (PD).

ALS is a progressive neurodegenerative disease that results in the deterioration of anterior horn cells in the spinal cord and brain motor cortex cells, which in turn, leads to progressive muscle degeneration, paralysis, and death (Boillée et al., 2006; Pasinelli & Brown, 2006; Banks et al., 2008). Though this disease is mostly sporadic, approximately 10% of all cases are inherited or familial (Pasinelli & Brown, 2006). Among the few genes that have been identified, mutation of the gene encoding the ubiquitously expressed enzyme superoxide dismutase (SOD-1) accounts for 20% of familial ALS and 1% of sporadic ALS (Rosen et al., 1993; Pasinelli & Brown, 2006). The causes of ALS remain undefined; however, the recent discovery of TAR DNA binding protein 43 (TDP-43) in the inclusion bodies of ALS degenerating neurons have led to new insights into the pathophysiology of this disease (Boillée et al., 2006; Arai et al., 2006; Caragounis et al., 2010). As a result, ubiquitinated neuronal aggregates with TDP-43 inclusions are now understood to be a pathological hallmark of ALS.
The ubiquitously expressed TDP-43 has RNA recognition and glycine rich motifs and may normally function to regulate transcription (Ayala et al., 2005). Although, TDP-43 is a nuclear protein, aggregates often occur in the cytoplasm and in the cerebrospinal fluid (Geser et al., 2008). The biochemical mechanisms underlying the modification of TDP-43 in affected neurons, is the result of C-terminal fragmentation, phosphorylation, and ubiquitination (Nonaka et al., 2009). Analysis of molecular determinants of TDP-43 inclusion in yeast has demonstrated that alteration in TDP-43 localization is a consequence of increased TDP-43 expression (Johnson et al., 2008). The mis-localization of TDP-43 led to a dramatic inhibition in the growth of yeast cells and provided initial direct evidence that TDP43 cytoplasmic aggregation may be a factor in the pathogenesis of TDP-43 related neuropathies. Additionally, TDP-43 cytoplasmic expression and aggregation resulted in oxidative damage and induced the generation of superoxide dismutase, indicating that TDP-43 aggregation may be a component in the induction of cellular toxicity. It was recently determined that Zn\(^{2+}\) modulates TDP-43 metabolism by decreasing nuclear expression of endogenous TDP-43 and resulted in the formation of TDP-43 positive inclusion in SY5Y neuronal like cells (Caragounis et al., 2010). Moreover, analysis of the role of altered metal homeostasis in this disease process revealed that aggregation of TDP-43 was Zn\(^{2+}\) specific, since neither copper nor iron affected TDP-43 metabolism (Caragounis et al., 2010). Therefore, Zn\(^{2+}\) is now thought to be an integral component in the neurodegenerative process in ALS; however, further studies need to be conducted to determine if Zn\(^{2+}\) is indeed a contributing factor in this disease.

The neurological disorder AD is one of the main causes of dementia in older adults and is characterized by a loss of cholinergic neurons as well as the progressive deterioration of cognitive function and memory (Drachman & Levitt, 1974; Davis & Maloney, 1976). The main
pathological hallmark of this disease is the accumulation of amyloid-β peptide (AB) within senile plaques in the brain as well as the deposition of neurofibrillary tangles (NFT) and neurophil threads (Cras et al., 1991; Perry et al., 1991). AB peptide is generated from the amyloid precursor protein (APP) by the proteolytic activity of β- and γ-secretase (Checler, 1995). Though most cases of AD are sporadic, about 5-10% of AD patients suffer from familial AD that is marked by an autosomal dominant inheritance of a mutation within the APP gene (Chartier-Harlin et al., 1991; Murrell et al., 1991). Specific and saturable binding sites for zinc and copper have been identified within the cysteine-rich region of the APP-695 conserved ectodomain indicating that these metals may be an intergral component in the proper regulation of this protein (Bush et al., 1993; Hesse et al., 1994). In addition, the AB peptide, which is central to progression of AD, has been shown to directly produce hydrogen peroxide (H₂O₂) due to metal ion reduction (Huang et al., 1999). Analysis of the role of metal ion homeostasis in the pathogenesis of AD indicated that the induction of H₂O₂ displaced Zn²⁺ from metalloproteins, and metallothioneins and may be a contributing factor in the up-regulation of Zn²⁺ in AD brains (Fliss & Menard, 1992; Danscher et al., 1997; Cornett et al., 1998). These findings are supported by evidence indicating that metal chelators are able to combat the progressive decline of AD. Cherny et al. (2001) have shown that the ingestion of the metal chelator clioquinol (CQ) in transgenic mice expressing human Aβ peptide reduced Aβ plaque deposits and improved the overall health and weight of the animals. Moreover, the use of metal chelators as a therapeutic agent in reducing the progression of AD has shown promising results in a pilot phase II clinical trial of CQ among moderate to severely affected AD patients. These patients exhibited a reduction in cognitive decline most likely due to a decrease in Aβ42 plasma levels (Ritchie et al., 2003).
While the exact cause of PD is unknown, many studies have indicated that Zn\(^{2+}\)-induced neurotoxicity may be a significant player in the pathogenesis of this disease (Gorrel et al., 1999; Kim et al., 2000). Post-mortem analysis of PD patients demonstrates an accumulation of free zinc within the nigrostriatal dopaminergic system (Dexter et al., 1989). However, given the requirement of Zn\(^{2+}\) for so many processes within the brain, it is currently unclear whether Zn\(^{2+}\) is a contributing factor in PD. In fact, some studies have suggested that Zn\(^{2+}\) may actually be neuroprotective. Ahn et al. (1998) noted that the depletion of endogenous Zn\(^{2+}\) in mouse cortical neuronal cultures resulted in the induction of cell death by modulating NMDA receptor function. This finding is supported by the results of Peters et al. (1987) and others (Choi et al., 1998; Ma & Zhao, 2001) who noted that blockage of NMDA function, attenuates NMDA receptor-mediated neurotoxicity. Although the exact mechanism is still undefined, the blockage of NMDA receptors by Zn\(^{2+}\) is believed to prevent the binding of NMDA receptor antagonists. Consequently, activated NMDA receptors are unable to mediate glutamate-induced excitotoxicity (Choi, 1992). As it relates to PD, dysfunction in trafficking and subunit composition of NMDA receptors is believed to be involved in the pathogenesis of dyskinesia or abnormal involuntary muscle movement (Bagetta et al., 2010). Moreover, NMDA receptor antagonists have been demonstrated to inhibit the induction of nitric oxide (NO) synthesis by acting on alpha synuclein (Radenovic & Selakovic, 2005; Adamczyk et al., 2009). Alpha-synuclein (α-synuclein), a small presynaptic protein, has been found in the Lewy bodies of PD patients and is believed to be an important component in the pathogenesis of PD. Therefore, by blocking NMDA receptor function through the use of antagonist, alpha synuclein levels are reduced and NO synthesis is inhibited. Studies have indicated that increased synthesis of NO results in the activation of a neuroinflammatory response in which macromolecular oxidation as
well as mitochondrial and DNA damage occurs (Chalimoniuk et al., 2006; Aquilano et al., 2008).

In contrast to the reported neuroprotective abilities of Zn\(^{2+}\), other studies have indicated that Zn\(^{2+}\) may be an integral component of the neurodegenerative process of PD. These studies have demonstrated that infusion of Zn\(^{2+}\) (10-1000 mM) induced neurotoxicity in cultured cortical neurons and in the hippocampus and nigrostriatal dopaminergic system of mice (Yokoyama & Koh, 1986; Choi et al., 1988; Canzoniero et al., 1997; Lin, 2001). More recently, Lin et al. (2003) have established that zinc-induced oxidative stress may result in apoptosis and reduced DA function in the nigrostriatal dopaminergic system of rats. Interestingly, the administration of vitamin D3 abolished this Zn\(^{2+}\)-stimulated oxidative stress and prevented apoptosis.

There are two proposed mechanism for vitamin D3 neuroprotection. First, vitamin D3 reportedly up-regulates factors such as glial cell line-derived neurotrophic factor (GDNF). Analysis of GDNF function indicates that it enhances DA uptake and promotes the survival of DA neurons in disassociated rat embryo cultures (Lin et al., 1993). In addition, the administration of vitamin D3 attenuated 6-OHDA induced neurodegeneration by reducing DA and its metabolites in rats (Wang et al., 2001). Secondly, vitamin D3 prevented zinc-induced lipid peroxidation. This is significant because the products of lipid peroxidation have been demonstrated to inhibit DA catabolism by reducing mitochondrial aldehyde dehydrogenase [ADLH2] (Jinsmaa et al., 2009). ADLH2 is necessary for the oxidation of 3,4-dihydroxyphenylacetaldehyde (DOPAL) to DOPAC. Thus, the inhibition of ALDH2 function by lipid peroxidation prevents the proper metabolism of DA and results in an increase in DOPAL levels. Interestingly, DOPAL has been shown to be more toxic than DA and its metabolites and is therefore relevant to PD pathogenesis (Burke et al., 2003).
Alpha synuclein is the major filamentous component of Lewy bodies and is therefore considered an integral factor in the pathology of PD (Heintz & Zoghbi, 1997; Clayton & George, 1998). Transgenic mice over-expressing α-synuclein developed inclusions and a loss of dopaminergic terminals. Interestingly, Zn\(^{2+}\) has been shown to induce the aggregation of α-synuclein in vitro by causing a conformational change in the protein (Kim et al., 2000; Lowe et al., 2004). To examine the role of Zn\(^{2+}\) in the substantia nigra (SN) and determine the effect of Zn\(^{2+}\) on the intrinsic electrical properties of this region of the brain, Chung et al. (2000) employed a whole cell recording method. This study concluded that Zn\(^{2+}\) hyperpolarized SN neurons and modulated their voltage-gated ion content, which contributed to an increase of neuronal excitability to incoming stimuli. This excitatory effect may compensate for the loss of dopaminergic neurons as PD develops, as well as the ability to relay chemical messages. Collectively, these findings suggest that Zn\(^{2+}\) may be an essential factor in the pathophysiology of PD.
CHAPTER 2
ZINC AND DOPAMINE: A SYNERGISTIC RELATIONSHIP?

Introduction

Dopamine (DA) regulates various processes from development to locomotion. Therefore, the dysregulation of DA homeostasis is implicated in the pathogenesis of many neurological diseases. Probably the most prominent of these DA related disorders is Parkinson’s disease (PD). PD is characterized by a preferential loss of DA neurons within the substantia nigra leading to a reduction of DA content, locomotor deficits and ultimately, death (Farrer, 2006). The rich biological evidence that exists in this area of research has implicated many genetic and environmental factors in the etiology of this disease. Epidemiological studies have associated environmental factors such as pesticide use, rural living, and heavy metals as potential factors in the neurodegenerative process of PD (Tanner & Goldman, 1996; Firestone et al., 2005; Jankovic, 2005; Anderson, 2004; Priyadarshi et al., 2001). Moreover, these studies suggest that exposure to environmental risk factors may subsequently lead to neurodegeneration due to biochemical abnormalities such as oxidative stress and mitochondrial dysfunction (Schapira, 2006).

Analyses of PD brains have revealed a buildup of free Zn$^{2+}$ within the substantia nigra, for which there is no clear biological requirement (Dexter et al., 1989, 1991). Based on the evidence that high concentrations of free Zn$^{2+}$ can be toxic (Revuelta et al., 2005), the role of Zn$^{2+}$ toxicity in combination with the genetic components of DA homeostasis was investigated
as possible mechanism in pathogenesis of PD. In this study, it was hypothesized that feeding flies excess Zn\(^{2+}\) in combination with mutations in genes that are involved in DA synthesis and transport would increase susceptibility to this transition metal. Furthermore, these mutants provide a strong tool for modeling the \textit{in vivo} conditions of extreme DA dysregulation and may provide new insights into the disease process of PD. One of the mutants used in this study, \textit{Catsup}, is predicted to contain seven transmembrane domains and is a member of the ZIP family of Zn\(^{2+}\) transporters that include the mammalian KE4 protein (Begum et al., 2002; Carbone et al., 2006). Additionally, Catsup is a negative regulator of TH, and is therefore an important modulator of DA synthesis (Stathakis et al., 1999). Thus, loss of Catsup protein results in an elevation in DA pools, but these flies are surprisingly, paraquat resistant (Chaudhuri et al., 2007).

The results of this study demonstrate that \textit{Catsup} mutant strains are Zn\(^{2+}\) sensitive. Interestingly, the \textit{Drosophila} DA transporter mutant \textit{fumin (fmn)} is also Zn\(^{2+}\) sensitive. Moreover, Zn\(^{2+}\) exposure at low concentrations for 12 hrs, increases mobility in wild type strains but reduces DA content. Finally, we show that exogenous DA in combination with Zn\(^{2+}\) exposure increases \textit{Drosophila}’s susceptibility to Zn\(^{2+}\) toxicity. These findings suggest that Zn\(^{2+}\) is a possible trigger in the etiology and pathophysiology of PD.

\textbf{Materials and Methods}

\textit{Drosophila strains and culture maintenance}: All strains were maintained on standard cornmeal-agar medium at 25\(^\circ\) C. Strains used as controls in this experiment included Canton S and a white-eyed mutant, \textit{Df(1)w, y}. Both are wild type for the DA-regulating genes under examination. The \textit{Catsup} mutant alleles used in this study, \textit{Catsup}\(^{26}/CyO} and \textit{Catsup}\(^{12}/CyO} flies, have been previously described (Stathakis et al. 1999). These mutant heterozygotes were crossed into either \textit{Df(1)w, y} or Canton S backgrounds. The \textit{fumin} mutant flies (\textit{fmn}) were a
generous gift from J. Hirsh, University of Virginia. These flies have a genetic lesion abolishing dopamine transporter function (Kume et al., 2005) and were crossed into Df(1) w, y backgrounds unless otherwise noted. Foi mutants were obtained from Bloomington stock center and have been previously described (Matthews et al., 2006). The UAS-DAT RNAi strains were obtained from the Vienna Drosophila RNAi Center (Vienna, Austria). The TH-GAL-4 strain (Friggi-Grelin et al., 2003) was a generous gift from J. Hirsh (University of Virginia).

**Zinc feeding experiments.** Adult male flies (n=10 per vial) were collected and aged 24 to 72 hrs post-eclosion before being placed in vials of 1% agar containing 5% sucrose, or 5% sucrose and 1 mM, 5 mM, or 10 mM ZnCl₂ (Sigma, St. Louis, MO). Their survival was monitored at 12 hrs intervals until zinc-fed flies had died. Approximately, five vials and 50 flies were used per treatment.

**Locomotion assay.** Adult male flies, 48 hrs post-eclosion, were collected, and each fly was transferred, individually, to a food vial for 1 hr. The vial then was gently tapped to bring the fly down to the bottom, and mobility was measured by counting the number of seconds the fly was in motion during a 45 sec time period following the tapping. All assays were conducted during the afternoon. Fifteen to twenty flies were assayed once for each data set.

**DA analysis.** Heads from adult male flies, 48 hrs post-eclosion, were collected by freezing the flies in liquid nitrogen, vortexing and sieving to separate the heads from bodies. Approximately 75-100 heads were homogenized in 75-100 µl of cold 0.1 M perchloric acid by using a motorized tissue grinder. The extracts were chilled on ice for 15 min to allow proteins to precipitate. Samples were pelleted by centrifugation at 9300 x g for 10 min at 4°C. The supernatants were removed and immediately stored at –80°C before HPLC analysis. Catecholamine levels were determined using a CoulArray HPLC system (model 5600A; ESA, Chelmsford, MA) and a
Synergi 4 µm Hydro column (4.6 X 150 mm; Phenomenex, Torrance, CA) as described by McClung and Hirsh (1999) with modifications. Extracts were filtered through 0.2 µm filters. Ten µl of each filtrate were injected. The mobile phase contained 75 mM sodium phosphate, pH 3.0, 1.4 mM octanesulfonic acid, 25 µM ethylene diamine tetraacetic acid, 100 µL/L triethylamine, and 7% acetonitrile. Separations were performed with isocratic flow at 1ml/min. Amines were detected with an ESA electrochemical analytical cell (model 5011; channel 1 at-50mV, channel 2 at 300mV). Pool sizes were determined relative to freshly prepared standards (Sigma). Analysis was performed using ESA CoulArray software.

Statistical analysis. Analyses of all data were conducted using GraphPad Prism (San Diego, CA), using a two-tailed Student’s t test assuming equal variances, or using a one-way ANOVA followed by the Dunnett’s post test. Details of analyses are described in the figure legends.

Results

Catsup mutants are Zn\textsuperscript{2+} sensitive

Since Catsup is a putative Zn\textsuperscript{2+} transporter and functions as a regulator of DA synthesis and transport, the hypothesis that this protein is an integral component in Zn\textsuperscript{2+} and DA interactions that are suggested to play a role in the etiology of PD was tested. Specifically, the combination of elevated DA synthesis and transport with the proposed Zn\textsuperscript{2+} transport dysfunction expected in Catsup mutants could interact to alter neuronal function and to enhance the susceptibility of Catsup mutants to Zn\textsuperscript{2+}. This prediction was analyzed by comparing the effects of Zn\textsuperscript{2+} ingestion by Catsup\textsuperscript{12} and Catsup\textsuperscript{26} mutants with the response of wild type adult flies. Wild type flies that were fed Zn\textsuperscript{2+} exhibited neurological symptoms such as tremor and paralysis, 24-48 hrs after the exhibition of these symptoms in Catsup mutants. Moreover, survival rates of Catsup mutants compared to those of wild type flies fed 1 or 10 mM ZnCl\textsubscript{2} were
significantly reduced, indicating that some component of Catsup function is an essential factor in modulating the toxic effects of Zn\(^{2+}\) (see Figure 2.1).
Figure 2.1. The effect of ZnCl₂ on lifespan in wild type and Catsup mutant adult flies. Mutations in Catsup cause sensitivity to zinc. Canton S and Df(1)w, y male flies served as Catsup⁺ controls for Catsup^{26/+/} (A) and Df(1)w, y, Catsup^{12/+} flies (B), respectively. Adults, 1-3 days post eclosion, were fed for 5 days with 1 mM ZnCl₂ dissolved in 1% agar and 5% sucrose. The number of dead flies was counted twice each day for 5 days, a period of time in which approximately 50% of the flies had died and the data were expressed as average percent mortality. Each value is the average of 5-10 replications, and each replication was made up of 10 flies. Statistical analysis was performed using Student’s t-test (*, p < 0.05, **, p<0.01). Error bars indicate ± SEM.
**Dopamine increases Zn\(^{2+}\) sensitivity in Catsup mutant and wild type flies**

*Catsup* mutants have elevated DA pools due to the increased activity of GTPCH and TH. Since synergistic interactions between DA and Zn\(^{2+}\) have been shown to lead to increased death in PC12 cell lines (Lo et al., 2004), the hypothesis that the elevation of DA pools in *Catsup* mutants contribute to their hypersensitivity to Zn\(^{2+}\) was tested. If the sensitivity of *Catsup* mutants is associated with DA-Zn\(^{2+}\) interactions, then elevating DA pools in wild type flies through ingestion of DA should increase the toxic effects of Zn\(^{2+}\), while ingestion of DA by *Catsup* mutants might be expected to have little or no additional consequence.

Wild type and *Catsup* mutants were pre-fed DA for 24 hrs and then fed Zn\(^{2+}\) for 3 days, a period of time resulting in approximately 50% mortality in flies fed Zn\(^{2+}\) only. In this experiment, flies were fed a higher concentration of Zn\(^{2+}\) than employed in the previous experiment (10 mM vs 1 mM) to obtain a more rapid response in the induction of toxicity under conditions of DA pre-feeding. Prior trials at lower concentrations of Zn\(^{2+}\) did not show a synergistic effect between Zn\(^{2+}\) and DA, probably because DA pools had most likely returned to basal levels by the time Zn\(^{2+}\) toxicity ensued. Though the concentrations of Zn\(^{2+}\) and assay time points were different for this experiment, similar results in survival on Zn\(^{2+}\) alone was manifested (Figs 2.1 and 2.2A). Feeding of 1 mM DA alone did not affect the survival of either wild type control or *Catsup* mutant flies during the time course of these experiments (data not shown). However, the implementation of increased DA in wild type flies dramatically increased Zn\(^{2+}\) toxicity in comparison to flies that had been fed only Zn\(^{2+}\) (see Fig 2.2 A). In contrast, the Zn\(^{2+}\) and DA combination resulted in a non-significant increase in sensitivity in *Catsup* mutants (Fig 2.2B), demonstrating that Zn\(^{2+}\) had a more robust effect on wild type flies than *Catsup* mutants under conditions of DA supplementation. This difference in Zn\(^{2+}\) sensitivity between wild type
and *Catsup* mutants presumably is due to the fact that DA pools in *Catsup* mutants are already elevated several folds, creating conditions in which toxic DA-Zn$^{2+}$ interactions are so severe that additional DA cannot further accelerate the damage to neurons. It is also plausible, that *Catsup* mutant flies have compensated for increased DA synthesis by modulating packaging and transport. Previous research in our lab has shown that Catsup mutants are resistant to reserpine, which attenuates VMAT function (Wang et al, submission in progress). Therefore, as it relates to the sensitivity that is seen in Catsup mutants, it is believed that some factor other than elevated DA is integral in this process. Taken together, these findings demonstrate that DA in combination with Zn$^{2+}$ increased the toxicity of Zn$^{2+}$ mostly in wild type flies and further support models that predict an integral role for this metal in the neurodegenerative process of DA-associated diseases such as PD. However, the evidence from this experiment also suggests that the sensitivity of Catsup mutant to zinc toxicity is due to some other factor other than elevated DA synthesis.
Figure 2.2 The effect of DA prefeeding on Zn²⁺-induced mortality of wild type and Catsup mutant flies.

DA increases Zn²⁺ sensitivity in wild type (A) and does not affect Catsup mutant (B) flies. Adult flies, 1-3 days post eclosion, were fed 1 mM DA for 24 hrs, removed and then fed continuously with 10 mM ZnCl₂ dissolved in 1% agar and 5% sucrose. The number of dead flies was determined twice each day for 3 days, the time period in which approximately 50% of the flies had died, and the data were expressed as average percent mortality. Each value is the average of 5-10 replications, and each replication was made up of 10 flies. Statistical analysis was performed using Student’s t-test (***, p<0.001). Error bars indicate ± SEM.
**Zinc increases adult mobility but reduces DA pools**

It is believed that the metabolism of DA is an essential component in the neurodegenerative process of PD. Moreover, it has been demonstrated that free radicals can be generated by the autoxidation of DA (Dunnett & Björklund, 1999). Since we noted that exogenous DA enhances zinc related mortality, here we explored possible Zn- DA interactions by assaying for Zn- mediated effects on DA- dependent mobility and on the oxidative metabolism of DA. For this experiment, flies were fed 1 mM ZnCl₂ for 6 hrs, after which, they were placed on regular fly medium for the duration of the experiment. Mobility assays were performed post-Zn⁺⁺ exposure, and DA pools in the heads of these flies were then measured to determine whether Zn⁺⁺ was affecting DA metabolism.

As seen in Figure 2.3, ingestion of excess Zn⁺⁺ for a period of only 6 hrs is associated with reduced DA content in head extracts and increased DOPAC levels. We have previously shown that oxidative stress increases DOPAC levels and results in DA neuron degeneration (Chaudhuri et al., 2007). Therefore, the increased DOPAC levels as a consequence of exposure to excess Zn⁺⁺ is suggestive of elevated oxidative stress and is in agreement with the findings of previous studies of zinc-exposed cells (Hsiao et al., 2004). In addition, the reduced DA pools are consistent with the conversion of cytoplasmic DA to DOPAC and to other oxidative metabolites in the neuron.
Figure 2.3. Zn$^{2+}$ modulates the metabolism of DA. Df(1)w,y flies were aged 48 hrs post eclosion and fed 5% sucrose or 1 mM Zn$^{2+}$ in 5% sucrose for 6hrs. (A) DA pools were significantly reduced in Zn$^{2+}$ fed flies. (B) DOPAC/DA ratios were significantly elevated in the presence of Zn$^{2+}$. DA and DOPAC levels were determined by HPLC analysis. Statistical analysis was performed using Student’s t-test (*, p<0.05). Error bars represent ± SEM.
Previous studies in our laboratory and others (Pendleton et al., 2002; Chaudhuri et al., 2007) indicate that diminished DA pools, as detected above, are associated with decreased mobility. It was, therefore, surprising that flies exposed to either 0.1 or 1 mM Zn$^{2+}$ for 6 hrs demonstrate increased mobility 24 hrs after Zn$^{2+}$ exposure (see Figure 2.4.A). Even more surprising, is that mobility remained elevated in this group for three weeks post initial Zn$^{2+}$ exposure (see Figure 2.4.B). Therefore, these findings suggest that Zn$^{2+}$ may be directly or indirectly affecting the availability of synaptically active DA, and that the alterations in DA metabolism or transport induced by Zn$^{2+}$ are permanent synaptic changes.
Figure 2.4. Zn$^{2+}$ feeding enhances mobility in wild type flies. (A) Df(1)w, y male flies 2-3 days post eclosion were fed 5% sucrose, 0.1 mM ZnCl$_2$ and 1 mM ZnCl$_2$ for 6 hrs then removed and placed on regular fly medium. These flies were then assayed for mobility 24 hrs post feeding by measuring each individual fly’s time in motion for 45 seconds. Zn$^{2+}$ fed flies were hypermobile in comparison with sucrose fed flies. (B) These flies were analyzed again for mobility three weeks later. Once again Zn$^{2+}$ fed flies had greater mobility than sucrose fed flies. Each value is the average of the mobility of 15-20 flies. Statistical analysis was performed using one way Anova (**, p<0.01, ***, p<0.001). Error bars indicate ± SEM.
Loss of DAT results in increased sensitivity to Zn$^{2+}$

Even though Zn$^{2+}$ decreases DA pool in wild type flies, it also increases mobility and is most likely due to alteration in DAT function. Interestingly, high affinity Zn$^{2+}$ binding sites have been identified in the human dopamine transporter (hDAT), and Zn$^{2+}$ was found to act as a potent, non-competitive blocker of DA uptake in hDAT-expressing COS cells (Norregaard et al., 1998). Furthermore, it has been demonstrated that the inability to properly clear DA from the synaptic region of the brain results in hypermobility in mice and flies lacking the DAT (Giros et al., 1996). These studies, along with the fact that Catsup mutant are hypermobile and Zn$^{2+}$ sensitive, suggest that DAT function may be compromised in Catsup mutants and is further enhanced by Zn$^{2+}$ exposure. Therefore, we hypothesized that loss of DAT function will result in increased Zn$^{2+}$ sensitivity. To test this hypothesis, wild type and DAT mutant (fmn) flies, as well as flies in which DAT RNAi was expressed in dopaminergic cells, were fed 1 mM ZnCl$_2$ and average survival duration was measured. As seen in Figures 2.5 and 2.6, the reduction of DAT function, in DAT mutants and DAT knock-down flies, increased Zn$^{2+}$ sensitivity relative to the effect of Zn$^{2+}$ on control flies. Thus, we conclude that a reduced level of DAT is an important factor in not only modulating the physiological effects of DA but also in regulating survival under conditions of exposure to toxic levels of Zn$^{2+}$.

Simultaneous reduction in Catsup and DAT function enhances Zn$^{2+}$ sensitivity

To further explore the functional relationship between Catsup and DAT, trans-heterozygous double mutants flies were created. These flies were fed 1 or 10 mM ZnCl$_2$ for 4 days, the point at which approximately 50 % of the Zn$^{2+}$ fed flies had died. As shown in Figure 2.6, double mutant flies that were fed 1mM ZnCl$_2$ were significantly more sensitive to Zn$^{2+}$ than flies’ mutant for Catsup alone. In addition, the results of Figure 2.6 A demonstrate that the
transheterozygous fnn/Catsup\textsuperscript{26} mutants display an intermediate zinc-sensitivity relative to either single heterozygous mutant. Thus, the findings of this study indicate that both of these proteins are significant players in mediating the toxic effects of Zn\textsuperscript{2+} and that loss of Catsup partially rescues DAT mutant sensitivity. The fact that we saw no significant differences in survival between DAT and transheterozygous mutants that were fed 10 mM Zn\textsuperscript{2+} is most likely a consequence of the increased toxicity of Zn\textsuperscript{2+} at high concentrations and demonstrates why it was necessary to repeat this experiment at 1 mM ZnCl\textsubscript{2}. However, it is intriguing to note that the sensitivity of the heterozygous mutants was similarly affected at both concentrations. The increased sensitivity of the fnn/Catsup\textsuperscript{26} mutants in comparison to Catsup heterozygotes suggest that there is a functional relationship between these proteins in regulating DA metabolism and that this relationship is a significant factor in modulating the response to Zn\textsuperscript{2+} toxicity.
Figure 2.5. Loss of function mutations of DAT and RNAi knockdown of DAT expression in dopaminergic neurons increase Zn$^{2+}$ sensitivity.

(A) Flies heterozygous for loss of the DAT allele, fmnub, were significantly more sensitive to 1 mM Zn than the Df(1)w,Y control flies. (B) UAS-DAT RNAi expression driven in dopaminergic neurons by TH-Gal4, similarly resulted in elevated Zn$^{2+}$ sensitivity when compared to that of flies containing either the UAS-DAT-RNAi or the TH-GAL4 transgenes alone. Adult flies, 1-3 days post eclosion, were fed for 4 days with 1mM (A) or 5mM (B) ZnCl$_2$ dissolved in 1% agar and 5% sucrose. The number of dead flies was counted twice each day and the average percent mortality, the point at which approximately 50% of the Zn$^{2+}$ fed flies had died, was determined at day 4. Each value is the average of 5-10 replications, and each replication was made up of 10 flies. Statistical analyses were performed using Student’s t-test and one way ANOVA (**, p<0.001, ***, p<0.0001). Error bars indicate ± SEM.
**Figure 2.6. Catsup-DAT interactions in zinc toxicity.**

*DAT* and *Catsup* double mutation increases Zn$^{2+}$ sensitivity, relative to *Catsup*^{26}/+ alone. *DAT (fmnub), fmnub/Df(1)w, y; Catsup*^{26}, Df(1)w, y; *Catsup*^{26}/+ and Df(1)w, y flies 1-3 days post-eclosion were fed continuously with 1 mM (A) or 10 mM ZnCl$_2$ dissolved in 1% agar and 5% sucrose. The number of dead flies was counted twice each day for 5 days and the data were expressed as average percent mortality. Each value is the average of 5-10 replications, and each replication was made up of 10 flies. Statistical analysis was performed using Student’s t-test and one way ANOVA (**, p<0.01). Error bars indicate ± SEM.
Fears of intimacy (foi) mutants are Zn\textsuperscript{2+} sensitive and hypermobile

Given the proposed role of Catsup as a Zn\textsuperscript{2+} transporter, we sought to determine whether the loss of another Zn\textsuperscript{2+} transporter function similarly affected Zn\textsuperscript{2+} sensitivity. To address this issue, we tested another mutant member of the LIV-1 subfamily of Zn\textsuperscript{2+} transporters, fear of intimacy (foi), for zinc sensitivity. The results of this experiment (see Figure 2.7) demonstrate that heterozygous foi mutants are significantly more sensitive to Zn\textsuperscript{2+} exposure than wild type flies. In addition, loss of foi function is associated with hypermobility in comparison to wild type flies (see Figure 2.8). These findings are similar to the phenotypes of Catsup mutant. Although both proteins are proposed members of the ZIP family of transporters, we did not anticipate that foi mutants would demonstrate a phenotype similar to Catsup mutants given Catsup’s distinct function as a regulator of DA synthesis. Interestingly, reduction of both Catsup and foi expression further enhances mobility in comparison with flies that are mutant for Catsup only (see Figure 2.8). However, we did not note a significant difference between foi mutants and the transheterozygous mutants. Thus, the component that is modulating mobility is not enhanced by the loss of Catsup in a foi mutant background. Taken together, these findings suggest that disruption of Zn\textsuperscript{2+} transporter function makes cells more susceptible to Zn\textsuperscript{2+} toxicity probably as a result of an inability to properly regulate Zn\textsuperscript{2+} homeostasis. Thus we conclude that mutations in modulators of DA biosynthesis, metabolism and transport, as well mutations in Zn\textsuperscript{2+} transport, enhances susceptibility to Zn\textsuperscript{2+} toxicity and may be a useful tool in assessing the role of Zn\textsuperscript{2+} exposure in DA associated neurodegenerative diseases.
Figure 2.7. The effect of Zn$^{2+}$ on lifespan in foi mutants. Mutation of foi increases Zn$^{2+}$ sensitivity. A strain containing the mutant foi allele, foi$^{20.71}$, and Df(1)w, y flies 1-3 days post-eclosion were fed continuously with 1 mM ZnCl$_2$ dissolved in 1% agar and 5% sucrose. The number of dead flies was counted twice each day for 4 days, the time period at which approximately 50% of the Zn$^{2+}$ fed flies had died and the data analyzed as average percent mortality. Each value is the average of 5-10 replications, and each replication was made up of 10 flies. Statistical analysis was performed using Student’s t-test (***p<0.001). Error bars indicate ± SEM.
Figure 2.8. *foi and foi/Catsup transheterozygous mutants are hypermobile.* Mobility was determined in 2-3 day old *Catsup*/*+, foi/+, and *Catsup/foi* transheterozygous mutants relative to wild type flies. Mobility assays were conducted by measuring time in motion of each fly for a period of 45 seconds. Each value is the average of 15-20 replications. Statistical analysis of the difference between *Catsup*/*26+/+, foi/+, and *Catsup/foi* was performed using one way Anova (**, p<0.01, ***, p<0.0001). Error bars indicate ± SEM. NS: not significant
LiCl rescues the sensitivity of Catsup mutants to Zn$^{2+}$

LiCl is used predominately as a mood stabilizer to treat mood disorders such as bipolar disease. However, lithium ($\text{Li}^+$) also been shown to be effective in preventing neurodegeneration (Bhalla et al., 2010). Here, I sought to determine if LiCl is an effective agent in preventing or reducing Zn$^{2+}$ toxicity in this *Drosophila* model. To address the possible neuroprotective role of LiCl, several different combinations of treatments were performed with wild type and *Catsup* flies. The final concentrations used were based on preliminary dosage trials to identify non-toxic concentrations. In the experiments presented here, 20 mM LiCl, a concentration that had almost negligible effects on the viability of either wild type or *Catsup* mutant flies, was employed. As expected, ZnCl$_2$ alone at a concentration of 10 mM was very toxic to both wild type and *Catsup* mutant flies, with the latter being more sensitive to Zn$^{2+}$ exposure, displaying an average mortality of nearly 70% by day 3, relative to approximately 20% mortality for the wild type strain. Combining Li$^+$ with Zn$^{2+}$ resulted in a striking reduction in Zn$^{2+}$ toxicity, producing a 5-6-fold reduction in mortality for both the wild type and *Catsup* mutant strains (see Figure 2.8). This finding is particularly intriguing, suggesting that further exploration of the effects of lithium on Zn$^{2+}$-DA interactions will be a fruitful avenue for investigating the mechanisms of zinc toxicity as well as the mechanisms of Li$^+$ rescue of neurodegeneration observed in other models.
Figure 2.9. LiCl suppresses the toxicity of ZnCl$_2$ in Catsup mutant and wild type flies. 
Catsup mutant and Df(1)w, y (WT) flies, 1-3 days post eclosion, were fed continuously with 10 mM ZnCl$_2$, 20 mM LiCl or 10 mM ZnCl$_2$ and 20 mM LiCl together, dissolved in regular fly media. The number of dead flies was counted twice each day for 3 days, the time point at which approximately 50% of the flies had died, and the data were expressed as average percent mortality. Each value is the average of 5-10 replications, and each replication was made up of 10 flies. (A) Li$^+$ in combination with Zn$^{2+}$ rescues Zn toxicity in WT. (B) Ingestion of Li$^+$ with Zn$^{2+}$ rescues Catsup sensitivity to Zn$^{2+}$. Statistical analysis were performed using one way ANOVA ***p<0.001). Error bars indicate ± SEM.
Discussion

This report demonstrates several novel findings related to genes that are components of DA synthesis and transport and their susceptibility to Zn$^{2+}$ toxicity. For the first time, we show that *Catsup*, *DAT* and *foi* mutants are zinc sensitive. In addition, we also demonstrate that exogenous DA increases susceptibility to Zn$^{2+}$ toxicity and that the mood stabilizer, LiCl, enhances survival in Zn$^{2+}$ fed flies. We also noted that Zn$^{2+}$ reduced DA pools and caused long-term modulation of mobility resulting in hypermobile flies.

Previously, we demonstrated that *Catsup* mutants are resistant to oxidative stressors such as paraquat (Chaudhuri et al., 2007). Here, we demonstrate that this resistance does not apply to Zn$^{2+}$ toxicity. In addition, exposing *Catsup* flies to other metals resulted in no significant differences in sensitivity and thus confirmed the idea that *Catsup* mutant sensitivity is zinc-specific (K. Lackey and J. O’Donnell, unpublished results; Taylor et al., 2004). This finding is rather intriguing given the fact that the predicted topology of *Catsup* indicates that it is a transmembrane protein with several histidine repeats and shares structural homology with known members of a zinc transporter family of ZIP proteins. Interestingly, we noted that excess Zn$^{2+}$ not only reduces life span, but also results in parkinsonian-like phenotypes such as tremors and paralysis. Based on these findings, we sought to gain a better understanding of the functional relationship between zinc toxicity and the regulation of DA homeostasis.

*Catsup* functions as a negative regulator of TH and GTPCH and mutations within this gene result in elevated DA pools (Stathakis et al., 1999; Wang et al., submitted). To assess the potential role of DA in enhancing the response of *Catsup* mutants to Zn$^{2+}$ exposure, we pre-fed wild type flies DA and then exposed them to Zn$^{2+}$. In the present study, we noted that DA and Zn$^{2+}$ enhanced susceptibility to Zn$^{2+}$ toxicity *in vivo* with no significant effects on survival with
flies fed DA alone. Previously, Zn\(^{2+}\) and DA have been shown to synergistically enhance the apoptosis of PC12 cells and the depletion of striatal DA (Lo et al., 2004). The evidence from our study suggests that the concerted action of Zn\(^{2+}\) and DA may be responsible for PD pathogenesis. However, the mechanism of this reaction is still unclear. Thus, we hypothesized that the combination of increased Zn\(^{2+}\) and DA enhances oxidative stress by modulating DA transport and synthesis. This concept is supported by evidence that Zn\(^{2+}\) can modulate DA homeostasis by binding to and inhibiting DA re-uptake by DAT and ligand binding to D\(_2\) DA receptors (Richfield, 1993; Schetz & Sibley, 1997). Moreover, various substrates of DAT, including DA and MPTP (1-methyl-4-phenyl-1, 2, 5, 6-tetrahydropyridine), have been shown to be neurotoxic (Miller et al., 1999). Here, we demonstrate that loss of DAT enhances sensitivity to Zn\(^{2+}\). The findings of our study also revealed that a one time exposure to exogenous Zn\(^{2+}\) results in an increase in mobility and reduction of DA pools, and intriguingly, that these effects are long term. Additionally, these findings suggest that this single exposure to Zn\(^{2+}\) elicits permanent changes in DA regulation. However, we were surprised that these flies were hypermobile since loss of DA pools should result in decreased mobility. Though we cannot definitively conclude from these findings the mechanism by which Zn\(^{2+}\) modulation of DA content results in hypermobility, we hypothesized that this is due to the inhibition of the DAT by Zn\(^{2+}\). Moreover, this hypothesis is supported by the findings of several studies that have concluded that a loss of DAT function creates excess extracellular synaptic DA and hypermobility in mice (Jones et al., 1998; Zhuang et al., 2000). Therefore, the observed increase in DOPAC levels indicates an enhancement in DA turnover and possibly signals the induction of oxidative stress.

The idea that oxidative stress is contributing to neurodegeneration is supported by the reduction in DA pools following six hours of Zn\(^{2+}\) exposure. Additionally, this hypothesis is
supported by evidence in DAT knockout mice which revealed that, under normal conditions, the tissue content of DA metabolites was unaltered; however, inhibition of DAT by cocaine, which also blocks DA reuptake, increased DOPAC levels and oxidative stress (Jones et al., 1998). Though the ability of Zn\(^{2+}\) to modify mobility while reducing DA content was fascinating, we did not expect this behavioral phenotype to continue three weeks following the initial exposure to Zn\(^{2+}\). Therefore, if Zn\(^{2+}\) is truly acting on DAT to create this behavioral phenotype, this result suggest that Zn\(^{2+}\) exposure has long term effects on DA transport and homeostasis, perhaps by mechanisms similar to those involved in long-term alterations following exposure to cocaine (Meyer et al., 1993; Jones et al., 1998; Bhide, 2009; Kubrusly & Bhide, 2010).

So, is there a connection between DAT and Catsup that contributes to the similarity in phenotypes? We have previously shown that Catsup mutant flies are extremely hypermobile, which appears to be the result of a combination of increased synthesis, packaging and release of synaptically active DA (Wang et al., submitted). Based on the findings that both Catsup and DAT mutants are Zn\(^{2+}\) sensitive, the effect of Zn\(^{2+}\) on the survival of Catsup and DAT double mutants were examined. We hypothesized that the loss of both these genes would augment susceptibility to Zn\(^{2+}\) toxicity. Transheterozygous loss-of-function mutants for DAT and Catsup genes demonstrate enhanced sensitivity to Zn\(^{2+}\) at both high and low concentrations in comparison to heterozygous Catsup mutant alone. It also could be argued that the presence of the Catsup mutation rescued DAT sensitivity to Zn\(^{2+}\) at lower concentrations. However, based on our findings that this effect is abolished at higher concentration (see Figure 2.6 B), along with the fact that Catsup mutants are hypermobile, suggesting a deficit in DAT function, we conclude that the double mutation most likely enhances susceptibility to Zn\(^{2+}\) toxicity due to a further attenuation of DAT function. Consequently, Catsup and DAT regulation of DA homeostasis is
an integral factor in the toxicology of Zn$^{2+}$ exposure. Although many of findings of this study are novel and interesting, there are limits to what can be extrapolated from this information. For example, it is unknown if the double mutation changes extracellular DA content, which would indicate differences in the inhibition of DAT. Interestingly, mutants that are heterozygous for DAT were more sensitive to 1mM Zn$^{2+}$ exposure than double mutants and Catsup mutants. Thus, the amount of DAT that is functionally available may be an important factor in Zn$^{2+}$ induced toxicity. Taken together, the results of this study lead to a model whereby Catsup regulates DAT function by maintaining Zn$^{2+}$ homeostasis such that a reduction in the function of either gene causes DA dysregulation and increases susceptibility to neurotoxicity under conditions of excess Zn$^{2+}$ exposure.
Figure 2.10. *A model of the interaction of Catsup with the genetic regulators of DA homeostasis.*

Loss of Catsup results in an elevation in TH activity and thus an increase in DA synthesis. It has been demonstrated that Catsup physically interacts with TH and thus acts a negative regulator of this enzyme (Wang et al., submitted). As it relates to VMAT and DAT, there is no evidence that Catsup physically associates with these proteins, however, we proposed that loss of Catsup enhances VMAT function and down regulates DAT to compensate for the enhanced metabolism of DA. Specifically, as it relates to DAT, our data suggest that any loss in Catsup function also compromises DAT function resulting in an inability to properly recycle DA and a dysregulation of DA homeostasis.
In addition to inhibiting DAT, Zn\(^{2+}\) also has been shown to modulate DA receptor antagonist interactions through allosteric mechanisms (Schetz et al., 1999). These regulatory properties of Zn\(^{2+}\) are also important to endogenous Zn\(^{2+}\) exposure, because synaptic concentrations of Zn\(^{2+}\) within the synapse can reach up to 300uM (Assaf & Chung, 1984). Therefore maintaining Zn\(^{2+}\) homeostasis is an important factor in neuronal health and may be an important component in the pathogenesis of PD.

Although *Catsup* mutants are Zn\(^{2+}\) sensitive, we cannot definitively conclude that this phenotype is due to Catsup’s proposed role as a Zn\(^{2+}\) transporter, as the protein has not been biochemically analyzed for transport function, nor has Zn\(^{2+}\) distribution in *Catsup* mutants been examined. However, it has been shown that Catsup localizes with TH in the synaptic regions of dopaminergic neurons and that *Catsup* mutants demonstrate increased synaptic activity in comparison to wild type. Additionally, mammalian DAT is a pre-synaptic plasma membrane protein that is susceptible to inhibition by Zn\(^{2+}\). Therefore, Catsup may function by modulating the access of the DAT to Zn\(^{2+}\) and if so, then Catsup’s proposed role as a zinc transporter could be a factor in Zn\(^{2+}\) sensitivity. This concept is supported by evidence that under toxic zinc conditions, when extracellular Zn\(^{2+}\) is high, Zn\(^{2+}\) has many routes to enter neurons including transporter-mediated exchange with intracellular sodium (Sensi et al., 1997). To examine the role of Zn\(^{2+}\) transport in modulating the toxic response to Zn\(^{2+}\) exposure, flies that are mutant for the *fear of intimacy* (*foi*) gene, which has been characterized as a Zn\(^{2+}\) transporter protein in *Drosophila* were analyzed for Zn\(^{2+}\) sensitivity. Interestingly, *foi*, like *Catsup*, is a member of the ZIP family of Zn\(^{2+}\) transporters and has been identified as an essential component of tissue architecture and in the regulation of development in *Drosophila*, particularly in the modulation of the migration of germ cells, glia and tracheal cells (Matthews et al., 2005; Van Doren et al.,
In addition, *foi* belongs to the LIV-1A group of the LIV-1 subfamily of ZIP transporters, unlike Catsup, which belongs to the LIV-1B group (Matthews et al., 2005). The ZIP family of zinc transporters has been identified as the main regulator of Zn$^{2+}$ influx into the cytoplasm. To determine the importance of Zn$^{2+}$ transport in Zn$^{2+}$ toxicity, we fed *foi* mutants Zn$^{2+}$ and measured survival. Like *Catsup* mutants, *foi* mutants are also Zn$^{2+}$ sensitive. Thus, the ability to regulate Zn$^{2+}$ influx is an important factor in maintaining Zn$^{2+}$ homeostasis and may play a significant role in modulating oxidative stress. Interestingly, the disruption of the ZnT-3 gene, which is responsible for zinc uptake into synaptic vesicles in mammals, revealed no morphological differences in brain development, or any differences in Zn$^{2+}$ content in other organs such as the pancreatic B-islet cells, germ cells in the testis or the cuboidal cells of the choroid plexus (Cole et al., 1999). However, examination of degenerating neurons after seizure induction in ZnT3 null mice indicated zinc accumulation in both mutant and wild type strains (Lee et al., 2000). Moreover, it in a later study, Lee et al. (2003) suggested that the release of Zn$^{2+}$ from metallothionein maybe a contributing factor in neurodegenerative process following acute brain injury.

All together, the findings of this study have demonstrated that excess DA in combination with Zn$^{2+}$, loss of Zn$^{2+}$ transporter function, loss of DAT, and loss of DAT and Catsup enhances susceptibility to Zn$^{2+}$ toxicity. Are all these processes working together under conditions of oxidative stress or are they separate factors that are involved independently in the toxic process of excess Zn$^{2+}$ exposure? As it relates to *Catsup* mutants, we suggest that these are factors that work together in increasing susceptibility to Zn$^{2+}$ with DAT being the main regulator of all of these processes. The proposed Zn$^{2+}$ transporter dysfunction of *Catsup* mutants may result in increased extracellular Zn$^{2+}$ and ultimately affect DA neurotransmission. This idea is
corroborated by the increased mobility seen in *Catsup* mutant flies and *DAT* mutant mice. However, it should be noted that *Catsup* mutants are heterozygous and have one functional copy of the *Catsup* gene. The fact that excess Zn$^{2+}$ can affect other components of DA regulation suggests that exposure to this metal exacerbates the oxidative process. It is unlikely, however, that Zn$^{2+}$ directly causes oxidative damage like metals such as copper, because Zn$^{2+}$ does not undergo redox changes which can catalyze the generation of increased reactive oxygen species (Fenton, 1894).

Finally, we examined the effect of lithium, which has been shown to have neuroprotective property in both mammals and *Drosophila* (Berger et al., 2005; French and Heberlein 2009, Toledo and Inestrosa, 2010), on Zn$^{2+}$-induced toxicity. Feeding flies LiCl in combination with Zn$^{2+}$ significantly reduced Zn$^{2+}$ induced toxicity. For this experiment several different concentrations of LiCl were used before determining that 20mM was the most effective at reducing the toxic effects of Zn$^{2+}$. Lithium appears to exert its neuroprotective properties by preventing the activation of glycogen synthase –3 (GSK-3) and by inducing several pathways including the Wnt/Wg signaling pathway in *Drosophila* and mammals (Phiel and Klein, 2001). Interestingly, lithium has been shown to prevent cocaine from binding to DAT, unlike Zn$^{2+}$, which stimulates the binding of this DAT inhibitor (Wu et al., 1997). These data suggest that lithium also regulates DAT function. The ability of both of these cations to modulate DAT function corroborates our findings, which suggest that the modulation of DAT is an integral component of the cytotoxic process induced by Zn$^{2+}$. Our findings along with the wealth of information on the neuroprotective quality of lithium suggest that this cation may be an important therapeutic agent in alleviating PD induced neurodegeneration.
The mechanism by which lithium attenuates Zn$^{2+}$ toxicity remains undefined. However, based on an examination of the literature, we hypothesize that there are two possible mechanisms by which lithium may exert its effect. It is possible that lithium competes with Zn$^{2+}$ for binding to DAT and thus prevent Zn$^{2+}$ induced DA dysregulation. The other mechanism by which lithium could exert it effect is by inhibiting GSK3β under conditions of Zn$^{2+}$ induced toxicity due to DA dysregulation. Interestingly, GSK3β activation, has been shown to be a key component in the modulation of apoptosis (Pap & Cooper, 1998; Bijur et al, 2000; Cross et al., 2001).

While the results from this study provide fascinating insights into the role of Zn$^{2+}$ toxicity as a possible contributing factor in PD, there are many questions that are yet to be answered as it relates to the mechanism of this action. 1) Does Zn$^{2+}$ exposure result in the neurodegeneration of DA neurons? Here, we noted an increase in the metabolism of DA in Zn$^{2+}$ fed flies and a reduction in DA content; however, the effect of Zn$^{2+}$ on neuronal health is yet to be determined. In addition, if there are differences in DA neuron count between the respective groups, is this difference in DA neuron insult specific to dopaminergic neurons? Using DA specific markers and confocal microscopy we will determine neurodegeneration of DA neuron. To determine if Zn$^{2+}$ affects other neuronal subsets, non-DA specific markers will be employed. In addition, the proposed role of Catsup as a Zn$^{2+}$ transporter may mean that it is an important component in regulating Zn$^{2+}$ homoeostasis in the brain. The dysregulation of Zn$^{2+}$ homeostasis may be apparent if there are differences in Zn$^{2+}$ levels or localization in Catsup mutants versus wild type flies. This could be accomplished by staining the brains of both strains with the free zinc marker, Newport green.

The findings of this study also demonstrated that a one time exposure to Zn$^{2+}$ for six hours resulted in reduced DA content and increased mobility. 2) Does Zn$^{2+}$-induced reduction of
DA pools continue at week three? Having observed that mobility remains elevated three weeks following initial Zn\textsuperscript{2+} exposure, it would be interested to note if DA pools remained reduced at the three week time period. Also, if there is a reduction in DA content, is it progressive reduction or is DA levels unchanged overtime? Finally, we demonstrate that lithium can be protective against Zn\textsuperscript{2+} toxicity. The works of Berger et al. supports this finding, (2005) by demonstrating that lithium protected against aggregate prone proteins by inhibiting GSK3\textbeta through the Wnt/Wg signaling pathway in Drosophila. The Drosophila homologue of GSK-3 is Zeste-white\textsuperscript{3}\textsuperscript{Shaggy} and has been shown to be an essential component of the wingless signaling pathway. 3) Is Zn\textsuperscript{2+} toxicity due to induction of apoptosis and does this occur by the modulation of GSK-3 activity? Given the proposed mechanism by which lithium is thought to be neuroprotective and based on our evidence that lithium protects against Zn\textsuperscript{2+} induced toxicity, it would be rather informative to determine the mechanism by which Zn\textsuperscript{2+} may trigger neurodegeneration. Both a mutant strain and an over-expression line of Shaggy is available and would be a wonderful tool in dissecting the mechanism by which lithium protects against Zn\textsuperscript{2+}, as well as, provide information regarding the signaling pathway that is involved in this process.
CHAPTER THREE

ALTERATION IN DA HOMEOSTASIS BY A REDUCTION IN DAT FUNCTION ATTENUATES THE TOXIC EFFECTS OF THE HERBICIDE PARAQUAT

Introduction

Dopamine (DA) plays an essential role in proper brain function and is required for learning and memory, reward, cognitive processing, regulating movement, and the sleep wake cycle (Graybiel et al., 1994; Schultz et al., 2000; Nieoullon, 2002; Wise, 2004; Dzirasak, et al., 2006). The dopamine transporter (DAT) is an important component in maintaining DA homeostasis by modulating DA synthesis and transport (Amara & Kuhar, 1993). This significant role is underscored by the implication of DAT function in regulating psychological and neurological processes. Loss of DAT function has been associated with cognitive disorders such as attention deficit hyperactivity disorder (ADHD) and schizophrenia, as well as neurodegenerative diseases such as Alzheimer and Parkinson’s (Cohen & Servan-Schreiber, 1992; Cerruti et al., 1993; Swanson et al., 1997; Uhl, 2003; Pritchard et al., 2008). As it relates to Parkinson’s disease, DAT is believed to be responsible for regulating the access of neurotoxins such as MPTP (1-methyl-1, 2, 3, 6-tetrahydropyridine), and paraquat to dopaminergic neurons (Shimizu et al., 2001, 2003; Yang & Tiffany-Castiglioni, 2005; Ritz et al., 2009; Kumar et al., 2010). Therefore, deciphering the mechanisms by which environmental toxins gain access to dopaminergic neurons, and particularly, their interactions with the DAT, are critical to understanding the roles of environmental interactions with gene products implicated in idiopathic PD, approximately 90% of all PD cases.
The herbicide paraquat has been shown to be a DA neuron-specific neurotoxin and is thought to be an environmental trigger in the neurodegeneration of DA neurons in some PD patients (McCormack et al., 2002; Li et al., 2005). Structural similarities between DAT and the toxic MPTP derivative, MPP⁺, has led some investigator to suggest that paraquat gains access to DA neurons through the DAT (Shimuzu et al., 2001, 2003). However, whether this hypothesis is correct and if so, the mechanism by which this is accomplished, remains undefined and contested. The notion that paraquat exerts its affect on DA neuron through transport by DAT is refuted by the findings of Richardson et al. (2005), which suggest that paraquat is neither a substrate nor an inhibitor of DAT.

Analysis of DAT function within *Drosophila* indicates that this gene is essential to the regulation of the circadian process and may be critical to DA homeostasis (Kume et al., 2005). As in mammals, the *Drosophila DAT* mutants, *fumin* (*fmn*), exhibit hyperactivity and decreased periods of rest and arousal threshold. The conservation of fly DAT function is supported by recent data that demonstrated that the neurochemical function of fly DAT in regulating DA synthesis and transport is similar to that of its mammalian counterparts. In this study, electrochemical analysis of exogenously applied DA indicated a significant elevation in synaptic DA in *fmn* mutants, due to a failure to properly recycle DA, compared to wild type controls (Makos et al., 2009). In addition, clearance of exogenously applied DA was significantly reduced in wild type flies following cocaine treatment, but no differences were noted in *fmn* flies with identical treatments (Makos et al., 2010). Both of these studies provide neurochemical evidence that dopamine uptake is decreased in *fmn* flies, and makes this mutant a useful tool to investigate the role of the DAT in DA associated diseases. Previously, we have shown that *Drosophila* is a good model organism to study the susceptibility of DA neurons to paraquat.
based on genetic variation in DA associated genes. In addition, the results of this study demonstrated that loss of Catsup, which results in elevation of DA synthesis coupled to enhanced VMAT activity, which increases the efficiency of DA packaging in vesicles, reduces sensitivity to paraquat. Due to phenotypic similarities between DAT and Catsup mutants in locomotor response and in regulating sensitivity to paraquat toxicity, we hypothesized that loss of Catsup results in a reduction of DAT function. The results of this experiment show that over-expression of Catsup in TH neurons does not alleviate resistance. Furthermore, the findings of this report demonstrate that a reduction in DAT function attenuates sensitivity to paraquat and provides evidence of DAT-Catsup functional interactions.

**Materials and Methods**

*Drosophila strains and culture maintenance.* Drosophila strains were cultured on standard corn-yeast-sugar based media at room temperature (23-25°C). The Catsup mutants used have been previously described (Stathakis et al., 1999). The w; TH-Gal4 and fmn (DAT) [Kume et al., 2005] stocks were a generous gift from J. Hirsh, University of Virginia. The w, UAS-DAT-RNAi was obtained from the Vienna Drosophila RNAi Center (Vienna, Austria). Dr. Kevin Bowling, a former graduate student in our lab, generated the UAS-Catsup strain. The strains used as controls were: Df (1) w, y, w; UAS-DAT RNAi, and w; TH-Gal4.

*Behavior.* Locomotion was assayed by time-in-motion as described previously (Carbone et al., 2006) with modifications. Adult males (15-20 individuals of each genotype), 3-7 day old post-eclosion, were collected and each fly was transferred to a food vial for 1 hour. Each test vial was given a gentle mechanical disturbance before beginning the locomotor assay. The mobility of the fly was measured by counting the number of seconds the fly was in motion during the 45 sec period immediately following the disturbance. All assays were conducted during the afternoon.
Negative geotaxis assays were conducted for some experiments by placing a single fly in an empty plastic vial. After a ten-minute rest period, the fly was tapped to the bottom of the vial, and the time to cross 5 cm (within a 60 second time frame) was recorded. The assays were repeated for 15-20 flies and the scores for each replication were averaged.

**Paraquat and reserpine exposure.** Two slightly different methods were used for paraquat and reserpine exposure. For paraquat, separated adult male flies, 24 to 48 hrs old after eclosion, were placed in vials containing 10 mM paraquat mixed with 5% sucrose and 1% agar. For reserpine exposure, adult male flies, 24-48 hrs after eclosion and aged for 72 hrs, were placed on filter paper saturated with 5% sucrose or 5% sucrose, 60 mM reserpine. Feeding was continued for a period of 8-10 hrs.

**Statistical analysis.** Analyses of all data were conducted using GraphPad Prism (San Diego, CA) using two-tailed Student’s T-test assuming equal variances or one way ANOVA followed by Bonferroni’s post-test as required. Details of analyses are described in figure legends.

**Results**

*Expression of a wild type Catsup transgene in dopaminergic neurons does not ameliorate resistance to paraquat*

Having observed previously that loss of Catsup decreased sensitivity to paraquat (Chaudhuri et al., 2007), the effects of expressing a *Catsup* transgene in the dopaminergic neurons of *Catsup* + adults in combination with feeding the toxic herbicide, paraquat was evaluated. If loss-of-function *Catsup* mutants resulted in elevated DA pools, increased synaptic release of DA as determined by hypermobility, and resistance to paraquat, then over-expression of wild type Catsup should result in opposing phenotypes. Thus, Catsup O.E. flies should demonstrate decreased DA synthesis, less efficient synaptic release and increased sensitivity to paraquat. As expected, the positive control (*Catsup*36) demonstrated decreased sensitivity to
paraquat relative to wild type flies (see Figure 3.1A). However, expression of a Catsup\(^+\) transgene in dopaminergic neurons did not result in increased sensitivity to paraquat, but demonstrated resistance to the herbicide similar to that of Catsup mutants (Fig 3.1 A). Based on this unexpected result and the knowledge that Catsup is a negative regulator of DA synthesis, the mobility of the Catsup over-expression (O.E.) strain was assayed to determine if the hypermobility phenotype of Catsup mutants would also be observed when Catsup is expressed in dopaminergic neurons.

**Catsup over-expression flies are hypermobile**

Since Catsup mutants are hypermobile, the hypothesis was formulated that over-expression of Catsup in dopaminergic neurons would result in decreased mobility. This hypothesis seemed plausible based on the fact that a loss of Catsup results in elevation of DA synthesis due to increase TH and GTPCH activity. To ascertain differences in mobility, flies were subjected to a time in motion assay in which the amount of time each individual fly was in motion in a 45 sec interval for both Catsup transgenic lines and wild type controls was determined (Fig 3.2 B). Unexpectedly, O.E. of the Catsup protein significantly potentiated mobility in comparison to controls. In addition, the hypothesis of lower rates of mobility was not supported by the findings of this report. Therefore, the conclusion that O.E. of Catsup modulates some component of DA synthesis resulting in phenotypes similar to that of Catsup mutants seems plausible.
Figure 3.1. Over-expression of Catsup in dopaminergic cells alters paraquat survival and mobility.

(A) Over-expression of *Catsup* in dopaminergic neurons increases resistance to paraquat (PQ). *Catsup* over-expression (O.E.) flies, *Catsup*^{26}/+ and wild type (WT) controls [Df(1)w,y transgenic strains UAS-Catsup (without the Gal4 driver) and TH-Gal4 (without the UAS promoter element)] were fed 10 mM PQ to determine differences in survival. *Catsup* O.E and *Catsup* mutants demonstrate decreased sensitivity to PQ in comparison to WT controls. Ten male flies were scored per replication, and the mean values represent the average of 5 replications. (B) Over-expression of *Catsup* in dopaminergic neurons increases mobility. Time-in-motion assays were conducted for a period of 45 sec for 20 adult male flies from the *Catsup* O.E. strain and WT control to determine differences in mobility. The assays were performed for each individual fly and the mean values represent the average of 20 independent replication per strain. The * indicates the significance of differences between *Catsup* O.E. flies and control strains. Statistical analysis was performed using one way Anova (**, p < 0.01, *** p < 0.001). Error bars indicate ± SEM.
**Over-expression of Catsup modulates VMAT function**

Previously, we have demonstrated that loss of *Catsup* results in increased VMAT activity as determined by the hypermobility of *Catsup* mutant flies after reserpine treatment (Wang et al., manuscript in progress). Reserpine is an inhibitor of VMAT function, which prevents the packaging of DA within synaptic vesicles. The attenuation of VMAT activity by reserpine feeding results in decreased mobility in *Drosophila* (Chang et al., 2006). Based on the known behavioral phenotype of *Catsup* mutants, we hypothesized that increased *Catsup* expression would down-regulate VMAT function, resulting in flies that were sensitive to reserpine. The *Catsup O.E.* flies and wild type controls were fed 60 mM reserpine for 8 hrs and a negative geotaxis-climbing assay was conducted. As expected, reserpine caused reduced mobility in both wild type flies and *Catsup* O.E. flies, relative to the sucrose-fed controls (see Figure.3.2). Since the *Catsup O.E.* strain and *Catsup* mutant adults both had increased resistance to paraquat, we anticipated that resistance to the effects of reserpine would be comparable for these strains. Surprisingly, this expectation was not met. Whereas the climbing rate of control strain was decreased approximately 2.75 fold with reserpine ingestion, the *Catsup* over-expression flies were slowed by nearly 5-fold, and are approximately 20% slower in the time required for them to cross 5cm than are the control flies. This observation suggests that *Catsup* over-expression reduces VMAT activity and increases susceptibility to the pharmacological manipulation of VMAT function.
Figure 3.2. Expression of Catsup in dopaminergic cells confers sensitivity to reserpine. To determine effects on VMAT function, 15 to 20 male Catsup O.E. flies and WT strains were fed 60 mm reserpine and 5 % sucrose solutions for 8 hrs. Flies were removed and mobility calculated by measuring the time to cross 5 cm. Both wild type and Catsup O.E. flies were significantly more sensitive to reserpine than their sucrose fed counterparts. In addition, reserpine-fed Catsup overexpression flies were significantly slower than wild type flies on reserpine. No significant difference was noted between Catsup O.E. and wild type sucrose fed flies. Each value is the average of 15-20 replications and replication was made up of one fly per strain. Statistical analysis was performed using one way Anova (*, p<0.05, ***, p<0.001). The * indicates the significance of differences between Catsup over-expression and WT controls and the differences between reserpine fed and sucrose fed flies. Error bars indicate ± SEM.
Loss of DAT function reduces sensitivity to paraquat

Though the insecticide paraquat has been implicated in the pathophysiology of Parkinson’s disease (PD), the mechanism by which this compound triggers neurodegeneration is unclear. Some studies have suggested that paraquat may gain access to DA neurons through the DAT. This hypothesis is based on the structural similarities between paraquat and a neurotoxic metabolite of MPTP, MPP+, which gains access to DA neurons through the DAT. Utilizing the availability of a Drosophila DAT mutant strain fumin (fmn), we sought to explore the possible relevance of DAT to PQ toxicity and to examine the proposed functional interactions between Catsup and DAT. Here, we hypothesized that reduction of DAT function would decrease susceptibility to paraquat toxicity based on the concept that diminishing DAT would decrease access of PQ to DA neurons.

To determine susceptibility to paraquat, survival in heterozygous DAT (fumin) mutants and DAT RNAi flies fed 10 mM paraquat was measured. Reduction of DAT function reduced susceptibility to paraquat relative to wild type controls (Fig 3.3A). In addition, DAT mutants survived almost twice as long as wild type flies. A similar resistance to paraquat was observed when DAT product was knocked down by expression of DAT RNAi in dopaminergic neurons (see Figure 3.3B). Although we cannot conclude that access of paraquat to the dopaminergic neurons is reduced in DAT mutants, in the absence of transport studies, it appears that the amount of DAT that is functionally available is an important factor in the toxic response to paraquat exposure. These results suggest either that DAT function is an important component in regulating paraquat access to the neuron or that neuronal homeostatic responses to reduction in DAT levels trigger protective mechanisms to detoxify paraquat or otherwise modulate oxidative stress responses. Moreover, these findings also reveal another instance in which genetic variation
within DA homeostasis interacts with environmental factors and recapitulates PD related phenotypes.
Figure 3.3. Loss of DAT function reduces sensitivity to paraquat toxicity. (A, B) Loss of DAT function enhances resistance to paraquat. The results show that attenuation of DAT function enhances survival against paraquat toxicity in comparison to wild type and transgenic controls. Ten flies were scored per replication and the mean values represent the average of 5 independent replication. The * indicates the significance of differences DAT deficient and control flies. Statistical analysis was performed using Student’s t-test and one way Anova (*, p < 0.05, ***, p < 0.001). Error bars indicate ± SEM.
Discussion

Given the proposed role of Catsup as a negative regulator of TH and GTPCH, a UAS-
Catsup transgenic line was generated by a former graduate student, Dr. Kevin Bowling, with the
intent of better defining the role of Catsup in regulating DA homeostasis by driving expression
of the transgene specifically in dopaminergic cells. The expression of the transgene clearly
affects DA-dependent characteristics; however, it should be noted here, that we are currently
uncertain of the degree to which these flies are expressing Catsup. Western blot analysis of this
Catsup transgenic line indicated that the Catsup band appeared to have a more intense signal
than the 50kD band of Catsup expressed by the wild type control, suggesting an increase in the
concentration of this protein. Unfortunately, this analysis was conducted without proper controls
and will need to be repeated with proper loading controls so that the relative signals from
transgenic and wild type control flies can be quantified. Thus, we can only speculate about the
relationship of Catsup over-expression and the phenotypes observed within this study. However,
our knowledge of Catsup function and the evidence presented with this study are very suggestive
that this protein is being over-expressed and modulating components of DA homeostasis.

The phenotypes observed in this study, related to dopamine synthesis and function
resulting from expression of the Catsup transgene, such as increased resistance to paraquat and
increased mobility relative to wild type controls, were unexpected. Both phenotypes were
observed previously in heterozygous loss of function Catsup mutants; we expected the opposite
result from over-expressing the wild type Catsup protein, decreased sensitivity to paraquat and
decreased mobility. There are two possible explanations for the observed phenotypes of the
Catsup over-expression flies. 1) If this is a true over-expression line, then a compensatory
mechanism to deal with the increased expression of Catsup protein may alter the expected
outcome. Such a mechanism is very plausible given the importance of Catsup expression for development, survival, and the regulation of DA homeostasis (Stathakis et al., 1999). 2) If Catsup expression is not up regulated, there may be an auto-regulatory response to *Catsup* transgene over-expression that down-regulates the expression of the endogenous *Catsup* gene. This mechanism may be a necessary adaptive response in an effort to maintain proper DA homeostasis. If this is so, then Catsup expression levels within the over-expression lines may be comparable to wild type or even mutant, in the case of an excessive response. Based on the observed phenotypes of increased mobility in time-in-motion assays and resistance to paraquat toxicity within these flies, the down-regulation would most likely mimic mutant expression levels.

Previously, we have shown that loss of Catsup results in increased VMAT activity, which in turn decreases the effect of reserpine on locomotor activity (Wang et al., manuscript in progress). In this study, it was hypothesized that Catsup functioned as a negative regulator of VMAT (either via direct or indirect mechanisms) and that the increase in VMAT activity diminished the susceptibility of *Catsup* mutants to paraquat and reserpine exposure by efficiently packaging DA and preventing the production of toxic metabolites. Based on this proposed mechanistic interaction between Catsup and VMAT in the regulation of DA homeostasis, we hypothesized that over-expression of Catsup should result in decreased VMAT activity as measured by increased sensitivity to reserpine. The findings of this experiment are consistent with this model; we noted that exposing the presumed over-expression line to reserpine treatment increased sensitivity to this drug. While *TH>Catsup* transgenic flies exhibited elevated mobility in time-in motion assays and increased paraquat sensitivity relative to control flies, they nevertheless were far more strongly affected by reserpine exposure. However, time in motion
and negative geotaxis are two different assays used to determine locomotor activity. Time in motion is geared towards determining differences in any form of mobility while negative geotaxis measures climbing ability. For this experiment, negative geotaxis was a more conclusive assay to assess the effect of reserpine on locomotor activity. What is clear from either assays, is that any modulation in Catsup activity results in a change in locomotor phenotype due to dysregulation in DA homeostasis. Consequently, the time required for the transgenic flies to cross 5 cm increased approximately 4.33 fold, from 1.5 to 6.5 sec while the control flies increased from 2.0 sec to 5.5 sec or a 2.75% increase. These data suggest that the initial elevated activity of the transgenic Catsup line results from a compensatory response in DA synthesis with a concentration dependent synaptic release, while the role of Catsup in modulating VMAT function is not subject to the same degree of compensation. We observed previously that feeding adult Drosophila L-DOPA or DA similarly results in somewhat elevated activity, but also strongly elevated DOPAC pools, indicating that much of the supplied DA remains in the cytoplasm where it is subject to oxidation.

Loss of VMAT activity, which also elevates cytoplasmic DA and oxidative load, has been shown to enhance DA neurodegeneration (Hanson et al., 2004). Given the enhanced sensitivity of VMAT to reserpine, indicating a decrease in VMAT activity, when Catsup is over-expressed, we would expect that these flies would exhibit heightened sensitivity to paraquat. Surprisingly, these flies demonstrate resistance to paraquat toxicity. Indeed, it is plausible that VMAT activity in conjunction with DAT function may play a role in modulating the phenotypic response seen here as consequence of paraquat exposure. It has been shown that interruption of DAT function reduces sensitivity to paraquat, presumably by diminishing the accessibility of paraquat to DA neurons (Shimizu et al., 2003). Behavioral analysis of loss of function alleles of
DVMAT-A indicated a reduction in sensitivity to cocaine, which binds to DAT, reduced DA content and increased locomotion in adult flies (Simon et al., 2009). This study not only demonstrates the ability of flies to adapt to loss of VMAT function, but provides evidence that one component of such adaptation is a reduction of DAT function. Recently it has been determined that VMAT (2), which is responsible for loading of dopamine into synaptic vesicles, associates with components of DA synthesis, TH and AADC, at synaptic vesicles (Cartier et al., 2010). Moreover, the authors of this study provided evidence that the formation of this complex has a functional role in regulating DA synthesis and transport. This finding is in line with previous results in our lab that demonstrates that the coupling of TH and GTPCH has a direct effect on the synthesis of DA in Drosophila (Bowling et al., 2008). In addition, Catsup directly associates with both GTPCH and TH and this association is an integral component in the regulation of DA synthesis. Therefore, we propose that over-expression of Catsup down-regulates VMAT and DAT function, resulting in enhanced sensitivity to reserpine, hyperlocomotion, and resistance to paraquat. It is also likely that DA synthesis is attenuated by the over-expression of this protein. Interestingly, lost of DAT function, which prevents clearance of DA within the synapse, in mammals has been linked to increased mobility notwithstanding the modulation of DA production and release (Jones et al., 1998). Interruption of Drosophila DAT function has been credited with modulation of sleep pattern (Kume et al., 2005). Moreover, the idea that the DAT function is compromised within Catsup mutants is further supported by evidence demonstrating that polymorphism within Catsup contributes to variation in sleep pattern (Harbison et al., 2009).

To determine if Catsup is really being over-expressed, Western blot analysis will be performed for these flies with the proper controls. In addition, FLAG and Venus epitope tagged
lines for the over-expression of Catsup will be used to evaluate mobility and paraquat sensitivity. Another benefit of these fusion protein tag lines will be the ability to quantify differences in the relative levels of endogenous Catsup protein in the transgenic lines compared to wild type, allowing an assessment of possible autoregulatory responses to transgene over-expression. To ascertain whether phenotypes demonstrated by Catsup O.E. are DA specific the pan-neuronal driver, Elav-Gal4 will be used to over-express Catsup. Finally, based on the evidence that VMAT activity is down regulated in Catsup O.E. flies, Western blot analysis will be performed to determine levels of VMAT expression within these lines.

Is there a real link between DAT function and paraquat toxicity? Arguments for, as well as against, the requirement of the DAT in regulating paraquat exposure to DA neurons (Shimizu et al., 2003; Richardson et al., 2005) have been proposed. Based on the suggestions that DAT function is compromised in both Catsup mutants and Catsup O.E. lines, we hypothesized that loss of DAT function reduces sensitivity to paraquat toxicity. This was confirmed by the results of this experiment, which indicated that DAT mutants as well DAT-RNAi flies are less susceptible to morbidity as a consequence of paraquat exposure. Moreover, post-mortem neuroanatomical analysis of control and PD patient brain revealed that areas with high DAT expression are most sensitive to damage in PD (Miller et al., 1997). To determine the effect of paraquat exposure on DA neuron survival, immunohistochemistry analysis will be performed. This is information is vital because loss of DA neurons within the substantia nigra is the main pathological hallmark of PD. In addition, we have previously shown that paraquat selectively destroys subsets of DA neurons by inducing oxidative stress and apoptosis (Chaudhuri et al., 2007). Thus, it is possible that Catsup O.E. may reduce the susceptibility of DA neurons to paraquat insult. Given the proposed role of oxidative stress in contributing to the neurotoxic
process of paraquat exposure, differences in DOPAC levels as well as catalase activity will be determined. Both of these assays are important indicator of oxidative stress. In addition, Catsup has been proposed to modulate both DAT and VMAT function. Therefore, exposing DAT mutants to reserpine and analyzing locomotor activity will evaluate the effect of loss of DAT activity on VMAT function. Finally, western blot analysis with VMAT antibody will be conducted to determine if VMAT levels are modulated in DAT mutants.
CHAPTER FOUR

A BRIEF EXPOSURE TO PARAQUAT RESULTS IN REDUCED LIFE SPAN AND PARKINSONIAN-RELATED PHENOTYPES IN YOUNG ADULT DROSOPHILA

This work is a continuation of a project begun by Arati Inamdar who contributed Fig. 4.1 D and Fig. 4.2 B. I conducted all remaining experiments with the help of my undergraduate assistant Eric Cook.

Introduction

Parkinson’s disease (PD) is the second most common progressive neurological disorder, which results in a preferential loss of dopamine (DA) neurons in the substantia nigra (Olanow & Tatton, 1999). Although cell culture and animal models have provided important information regarding some of the genetic and environmental factors that may result in Parkinsonism, the exact mechanisms and cause of this disease remain elusive. Moreover, the misregulation of DA homeostasis is thought to play an important role in the etiology of PD (Hastings et al., 1996; Guillot & Miller, 2009). Loss of DA neurons results in DA deficiency and has been attributed to early symptoms of the disease such as tremor and rigidity (Fahn, 2003). Additionally, DA dysregulation, which results in the release of DA into the cytoplasm, contributes to increase in oxidative cascades and may ultimately lead to the demise of DA neurons (Stokes et al., 1999). Although several genes have been linked to rare cases of familial Parkinsonism, such as mutations in genes encoding alpha synuclein, DJ-1, and ubiquitin C-terminal hydroxylase L1, more than 90% of the cases of PD are the result of sporadic PD (Miller et al., 2005). As it relates to sporadic PD, a combination of environmental factors and genes involved in DA regulation are believed to components in the neurodegenerative process. The factors that may ultimately lead
to a diagnosis of PD vary among individuals; however, there are some factors that are universal in the pathophysiology of PD. First, PD selectively targets DA neurons in the midbrain. Second, mitochondrial dysfunction leads to the activation of apoptosis and is often implicated in the neurodegenerative process (Parker et al., 1989; Bywood & Johnson, 2003; Tretter et al., 2004). Third, the loss of DA neurons results in motor impairment (Fahn, 2003).

Parkinson’s disease primarily affects individuals between the ages of 50 and 60. However, there have been documented cases of young adults and even children being affected by this dreadful disease (Lang & Lozano, 1998). Cases of PD in individuals under the age of 50 are classified as early-onset Parkinsonism (EOP). Patients who exhibit symptoms under the age of 21 are classified as belonging to the EOP subset Juvenile Parkinsonism, while patients over the age of 21 are classified as belonging to the EOP subset Young-Onset PD.

Neurotoxins, such as MPTP, and pesticides such as rotenone and paraquat (PQ), have been implicated as environmental contributors of PD (Liou et al., 1997; Betarbet et al., 2000; Uversky, 2004). Previously, we have shown that the herbicide PQ induced neurodegeneration of DA neurons and resulted in PD related phenotypes. Based on these results and epidemiological studies that suggest that PQ is an environmental trigger of PD, the goal of this study was to determine if a one-time exposure to PQ at early stages of development is sufficient to elicit behavioral phenotypes that are associated with PD or to increase risk later in life. Currently, it is undetermined if a one time exposure to PQ is sufficient to mimic or accelerate the PD neurodegenerative process. This is an important issue because there is a lag time between onset of neuronal loss and diagnosis, and considerable time may have elapsed between initial exposure to an environmental agent and neuron loss. Moreover, the probable time between the initial triggering event and onset of observable symptoms can be a period of several decades, making it
difficult to effectively treat or delay the progressive neurodegenerative process. Therefore, by
the time the disease process has been diagnosed, PD patients would have lost approximately 80%
of their DA neuron. In addition, this study may provide useful insights into the pathogenesis of
PD as it pertains to the correlation between PQ exposure and Juvenile Parkinsonism.

The results of this study demonstrate that a brief exposure to low doses of PQ among
newly eclosed young adult flies reduces lifespan and mobility. Both of these phenotypes have
been previously identified as key components in the pathogenesis of PD and in a Drosophila
model of PD exposure to PQ (Jellinger, 2000; Chaudhauri et al. 2007).

Material and Methods

Drosophila strains and culture maintenance. All Drosophila stocks were maintained at 25° C
on standard cornmeal-yeast-sugar media, and all the experiments were performed at the same
temperature. A transgenic reporter strain, TH-GAL4; UAS-eGFP was employed in all
experiments. The transgenic strain UAS- 2X eGFP (Chromosome II) was obtained from the
Bloomingon, IN Drosophila stock center and a TH-GAL-4 strain (Friggi-Grelin et al., 2003) was
a generous gift from Jay Hirsh (University of Virginia).

Feeding experiments. Newly eclosed flies (0-6 hours) were fed 5% sucrose and 0.1 мМ, 1 мМ,
and 10 мМ PQ. The feeding solution were mixed with 1% agar. For all experiments, flies were
fed for 12 hours.

Locomotion assays. A time in motion assay was conducted as described previously (Carbone et
al., 2006) with a few modifications. The flies were immediately removed from their respective
feeding vials and allowed to acclimate in vials with regular fly media for 24 hours. The assay
was then performed by gently tapping the vial and measuring the length of time the fly remained
in motion for a period of 45 seconds. Mobility for each treatment set was calculated as the
average of 20 independent replications. These assays were performed subsequently for the same
treatment sets at two and three weeks with the original set of flies.

**Statistical analysis.** All data were analyzed by one-way ANOVA with Dunnett’s post-test or by
one-tailed Student’s t-test using GraphPad Prism (San Diego, CA). Details of the analyses are
described in the figure legends.

**Results**

*Early PQ exposure to young adult flies modulates mobility both short-term and long-term*

Motor impairment is a pathological hallmark of PD due to loss of DA neurons (Fahn, 2003). To ascertain whether a one time exposure to low and high does of PQ could affect
mobility, a time in motion assay was performed immediately following PQ and sucrose
exposures. As seen in Fig 4.1 A-C, transient exposure of young adult flies to 10 mM PQ causes a
significant reduction in mobility 24 hrs after the 12 hr ingestion period. Ingestion of 0.1 mM or 1
mM PQ did not affect mobility 24 hrs post PQ exposure. Similarly, Inamdar (manuscript in
progress) noted a concentration dependent reduction in locomotion in a negative geotaxis assay,
due to one time exposure to PQ for 12 hrs (Fig 4.1 D). Though our assays were different, these
results concur in demonstrating that a high concentration of PQ for a 12 hr period, affects the
locomotor activity of PQ treated flies.

To determine if locomotor activity is progressively reduced over time as a consequence
of early exposure to PQ, time in motion assays were conducted two weeks following the initial
PQ exposure. Here, we noted no significant difference in mobility between control flies and
those exposed to 0.1 mM PQ, but saw a significant and progressive reduction in mobility among
flies that were fed 1 mM and 10 mM PQ. Interestingly, we also noted that 1 mM PQ fed flies
appeared to be more sensitive than flies that had been exposed to 10 mM PQ. Based on this
result, we also did a three-week follow up. Here we noted that flies that were initially fed 10 mM PQ continued to show a significant reduction in mobility compared to non-fed controls but we saw no significant differences between sucrose fed flies and flies that were fed 0.1 mM and 1 mM PQ. It should be noted, however, that by 5 weeks post-eclosion, control flies were beginning to slow dramatically as they experienced normal age-dependent loss of DA neurons. Thus, we do not observe evidence of accelerated onset of symptoms after exposure to the lower doses of PQ at these much later times. Overall, these results demonstrate that transient exposure to PQ reduces locomotor activity with flies fed 10 mM PQ and results in a robust and progressive decline in mobility.

**Early paraquat exposure reduces lifespan in adult flies**

We have previously demonstrated that the environmental toxin PQ is capable of initiating Parkinsonian type phenotypes in flies such as loss of DA neurons and reduced lifespan (Chaudhuri et al., 2007). To determine the effect of a one time exposure on long-term survival, we fed flies 0.1 mM, 1 mM or 10 mM PQ for 12 hrs, then placed them on regular fly media and measured lifespan. The same procedures were followed for controls, which were fed 5% sucrose for 12 hrs. All PQ-fed flies showed a reduction in lifespan by approximately twenty days in comparison to control. However, flies that were fed 10 mM PQ appeared to have the most detrimental effect on longevity with these flies surviving approximately 30 days less than wild-type controls. We should note, however, that the survival of 10 mM PQ fed flies were not significantly different than 0.1 mM and 1 mM PQ fed flies. As seen in figure 4.2 B, the results of this experiment are supported by the findings of Inamdar (manuscript in progress) who also demonstrated that one time exposure to PQ has a significant effect on lifespan.
Figure 4.1. A one time exposure to paraquat attenuates locomotor activity. Twenty newly eclosed (between 0hrs and 6hrs post eclosion) TH-Gal4-UAS GFP flies were fed 5% sucrose, 0.1 mM, 1 mM and 10 mM PQ for 12 hrs. After feeding for 12 hrs, flies were then removed and allowed to acclimate on regular fly media for 24 hrs. Following the acclimation period, time in motion assays were performed for each feeding set. The assays were performed for each individual fly and the mean value represents the average of 20 independent replications per strain. Flies were placed back on regular media and transferred twice per week for the duration of the experiment. Time in motion assays were also determined at two weeks and three weeks post PQ exposure. (A) At 24 hrs post-PQ exposure, 10 mM PQ flies exhibited reduced mobility compared to sucrose fed controls. (B) 1 mM and 10 mM PQ fed flies demonstrated reduced mobility two weeks following initial exposure. (C) 10 mM PQ reduces mobility compared to sucrose control three weeks following PQ exposure. (D) Young adult male flies demonstrate reduced mobility after 12 hrs of exposure to PQ (1 mM and 10 mM). Mobility was determined by the negative geotaxis assay. The * indicates the significance of differences between control and PQ-fed flies. Statistical analysis was performed using one way Anova (*, p< 0.05, **, p <. 0.01, ***, P < 0.001). Error bars represent standard error of the mean.
Figure 4.2. A one time exposure to paraquat (PQ) reduces lifespan. (A) Newly eclosed (0-6 hrs) TH-Gal4-UAS-GFP flies were fed 5% sucrose, 0.1 mM PQ, 1 mM PQ, and 10 mM PQ for 12hrs, removed and then placed on regular fly media. Lifespan was determined by counting the number of dead flies for each data set over the duration of the experiment at 2-3 day intervals. Each data point represents five replications of 10-15 flies each. (B) Young adult flies, aged to 6 hrs post-eclosion, were exposed to 10 mM and 1 mM PQ, or 5% sucrose for 12 hrs and then transferred to the normal food. The flies exposed to PQ in early adulthood showed decreased survival duration. Each data point represents at least 5 replications of 10-15 flies each. The * indicates the significance of differences between control and PQ-fed flies. Statistical analysis was performed using one way Anova, (*p<0.05, **, p < 0.01, ***p < 0.001). Error bars represent standard error of the mean.
Discussion

Epidemiological studies associate PQ exposure to increased incidents of PD cases in agricultural communities (Lai et al., 2002; Landrigan et al., 2005; Brown et al., 2006). However, there are discrepancies as to the actual effect of PQ on PD. In addition, many of these studies are based on the premise of repeated exposure to the herbicide and the implicit effect of the impact of short-term exposure to PQ has not been fully explored. For example, what length of exposure is sufficient to result in parkinsonian phentoypes? Moreover, is age of exposure a determining factor in the impact of PQ on PD related phenotypes? In this report we demonstrate that a one time exposure to PQ, early in life, is sufficient to elicit the parkinsonian-related phenotype of loss of mobility, as well as a subsequent shortening of life span. While it has been previously demonstrated that PQ exposure resulted in PD related symptoms in Drosophila (Chaudhuri et al., 2007), the previous study was conducted using higher concentrations of PQ and acute dosing. In addition, it was undetermined whether a one time exposure could mimic the disease process. Taken together, the findings of this study indicated that PQ exposure at the young adult stage of development has detrimental long-term effects.

Analysis of locomotor activity following the initial PQ feeding (see Figure 4.1 A) showed a decreased in mobility for 10 mM PQ but no effect for 0.1 mM and 1 mM PQ. Therefore, we assumed there was a concentration dependent effect occurring for this time period of exposure. This finding is slightly contradicted by the findings of Inamdar (manuscript in progress), who noted a difference in mobility at 1 mM and 10 mM PQ. In addition, this difference in motor impairment is probably due in part to the different assays used to detect mobility in flies exposed to PQ for these two experiments. Inamdar measured mobility by determining the time it took a fly to cross 5cm, and we measured mobility by determining the time in motion of the fly over a
45 sec period. These two assays, while corresponding in general, have differences in the type of neural responses being assessed, and therefore, outcomes can diverge somewhat with respect to time of onset in locomotion deficits and in sensitivity to some treatments. In addition, Inamdar performed the assay immediately following PQ exposure, unlike our experiment in which we waited 24 hrs before measuring mobility. The decision to allow for an acclimation period of 24 hrs prior to performing the mobility assays was based on the findings of previous experiments in which we noted great fluctuation in numbers among individual groups following PQ treatment. Upon allowing for a 24 hr acclimation period prior to measuring mobility, the results were more consistent for individual groups. In addition, the recovery time should not negatively impact the results of this experiment because PQ toxicity is believed to be a progressive process. However, it is possible that the difference in assay and the time period of measurement employed by Inamdar, could have been more sensitive in detecting mobility deficits at 1 mM PQ.

The findings of this report also demonstrated that a one time exposure to the herbicide PQ, at the highest concentrations, resulted in a progressive decline in locomotor activity, as seen in the increased decline in activity for flies fed 10 mM PQ at two and three weeks following the initial PQ exposure. Interestingly, we also saw a decline in mobility for 1 mM PQ fed flies at two weeks post exposure, but noted no difference in mobility at three weeks post exposure. Additionally, we saw no difference in mobility for 0.1 mM PQ fed flies compared to 5% sucrose control at any time point. Therefore, it is possible that these flies have developed resistance to oxidative stress as the flies responded to the short term, low dose treatment, or they are simply unaffected by this low dose. Oxidative stress measurements such as catalase or lipid peroxidation assays can be used to distinguish these alternatives.
Here we sought to determine if a one time exposure to PQ for a relatively short period (12 hrs) could also generate a reduction in lifespan. Interestingly, a one time exposure to PQ at 0.1 mM, 1 mM, and 10 mM significantly reduced in lifespan compared to sucrose fed control. As in mobility deficit, 10 mM PQ had the most pronounced effect. These finding are also supported by the results of Inamdar (manuscript in progress). All together, these results suggest that a one time exposure to PQ could be a factor in Juvenile Parkinsonism.

Although the findings of this report are intriguing, there are several questions that are yet to determined. For example, is there a direct correlation with mobility deficit and DA? To address this question, we will repeat the parameters of the experiment and follow-up with HPLC analysis in which DA and DOPAC levels will be determined. Determining DOPAC levels will be a good gauge of oxidative damage because DA is a highly reactive amine that generates neurotoxic quinones and reactive oxygen species. To determine if DA neurons are undergoing apoptosis, as a result of PQ exposure, we will do immunohistochemistry and confocal analysis. If there are indications that DA, DOPAC and DA neurons are affected by PQ exposure, a will follow-up catalase assays to ascertain the role of oxidative response will be performed.

We also report here that PQ exposure at all concentrations significantly reduced lifespan in adults. It would be interesting to determine if a two-week follow-up hit of PQ at 0.1 mM and 1 mM will further reduce lifespan. We anticipate that re-exposure of these flies after a two weeks post initial exposure will accelerate the decline in lifespan compared to flies that were subjected to only one hit of PQ exposure.
REFERENCES


