THE EFFECTS OF SUBTOXIC MERCURY CONCENTRATIONS ON DOPAMINE TRANSPORT IN THE SK-N-SH CELL LINE

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Bachelor of Science

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2002

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
July, 2004

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ACKNOWLEDGEMENTS

First, I would like to express my gratitude toward my advisor, Dr. David Wallace for his guidance, encouragement, words of wisdom, and sense of humor, especially when it came to my countless random questions. I would also like to thank my advisory committee for their input and assistance on this project. My coworkers in the lab should also be recognized for their help, advice, and camaraderie; it would simply have not been the same enjoyable experience without you. Finally, I am eternally grateful to my fiancé, Eric, as well as my family and friends for their support throughout this entire process; for being the shoulder to lean on while far away from home and putting up with a flustered, overworked, under-slept graduate student; the comforts you afforded are more than you can know.

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NOMENCLATURE

CNS	Central Nervous System
Ca^{2+}	Calcium Ion
Hg^{2+}	Mercuric Ion
AD	Alzheimer's disease
DMPS	Dimercaptopropane Sulfonic Acid
DA	Dopamine
NE	Norepinephrine
DAT	Dopamine Transporter
Na^{+}	Sodium Ion
Cl^{-}	Chloride Ion
NET	Norepinephrine Transporter
PKC	Protein Kinase C
cAMP	Cyclic Adenosine Monophosphate
PLC	Phospholipase C
K_i	Inhibitory Constant
GABA	Gamma-Aminobutyric Acid
$HgCl_2$	Mercury (II) Chloride or Mercuric Chloride
ROS	Reactive Oxygen Species
SOD	Superoxide Dismutase
BBB	Blood Brain Barrier

MAPK	Mitogen-Activated Protein Kinase
PLA_2	Phospholipase A_2
$cPLA_2$	Cytosolic Phospholipase A_2
GSH	Glutathione
CSF	Cerebrospinal Fluid
Aβ	Amyloid β-Peptide
APO-E	Apolipoprotein E
FBS	Fetal Bovine Serum
LDH	Lactate Dehydrogenase
RFU	Relative Fluorescence Units
EC_{50}	Effective Concentration 50%
K_d	Dissociation Constant
ddH_2O	Double Distilled Water
B_{max}	Transporter Density

I. INTRODUCTION

I.A. Background

Mercury, a metal with no known physiological function, produces toxic effects in both the central nervous system (CNS) and peripheral nervous system (PNS). Figure 1 depicts the various effects mercury has on cellular function.

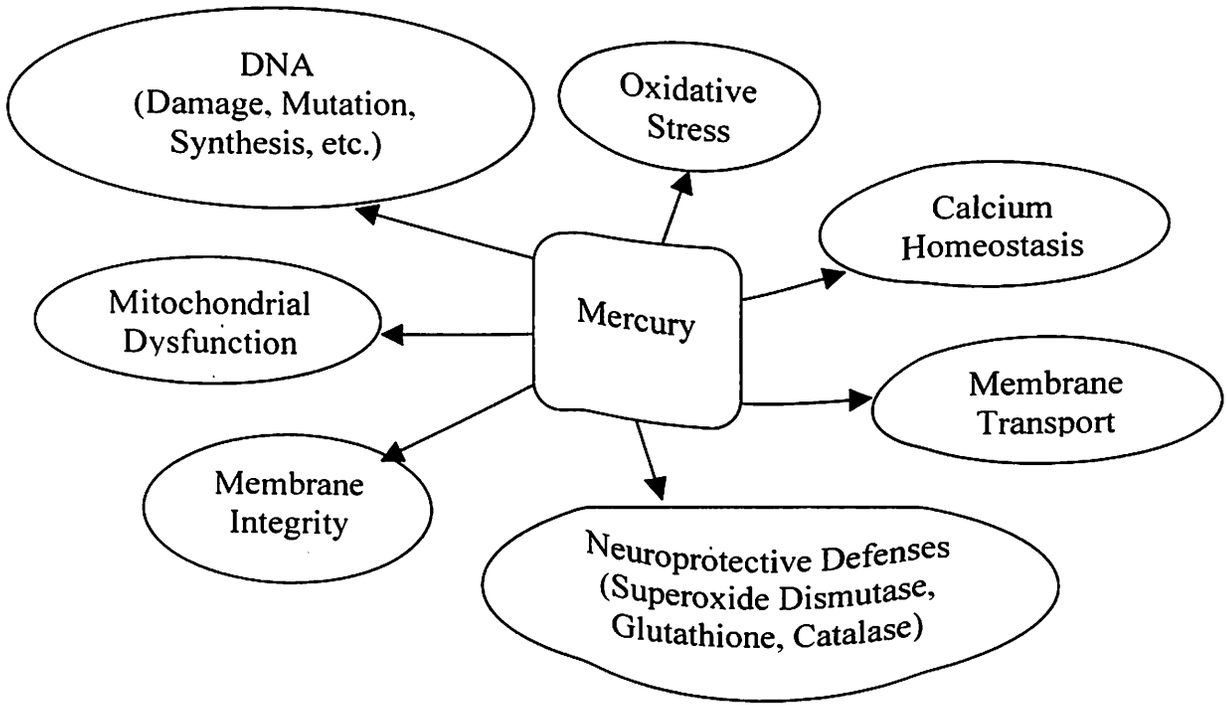

Figure 1: Diagram of the physiological components mercury is known to affect

The risk of low-level exposure to mercury is a very realistic threat since mercury exists in the environment in multiple forms, including trace metals as well as various pharmaceuticals, such as diuretics, antiseptics and skin preparations (U.S. Public Health, 1989). With its specificity for cerebellar granule cells (Fonfría et al., 2001), mercurials interact with various CNS components, altering the intracellular calcium concentrations (Ca^{2+}) and protein phosphorylation processes, both of which cause damage to the cells of the CNS. Mercury can interfere with synaptic transmission through effects on neurotransmitters, neurotransmitter receptors and the neuronal membrane. The clinical

symptoms of mercury toxicity suggest the involvement of several motor, sensory, and cortical neuropathies.

The toxicity of mercury is due, in part, to its oxidative properties. Since preceding research has shown that mercury generates reactive oxygen species and lipid peroxidation in brain tissues (Lee, et al., 2001), it can be concluded that the resulting oxidative stress may contribute to the development of neurodegenerative disorders caused by mercury intoxication (Hussain, et al., 1997). Oxidative stress induced by the divalent mercury ion (Hg^{2+}) increases the phosphorylation of tau protein in neuroblastoma cells (Olivieri et al., 2000), which is a common characteristic in patients suffering from Alzheimer's disease (AD). Perhaps this toxic metal represents an unexplored environmental factor that initiates a pathogenic cascade, resulting in CNS defects related to AD. The oxidative stress caused by low-level mercury concentrations over long periods of time may increase an individual's susceptibility to certain neurodegenerative diseases, like Alzheimer's disease.

Chelation therapy is a common treatment for metal-induced pathologies. Chelation is defined as the formation of a metal ion complex in which the metal ion is associated with a charged or uncharged electron donor referred to as a ligand (Goyer, 1991). At present, succimer is the chelation treatment used for acute mercury exposure (Dart, 2000). However it remains unclear as to whether the observed increase in mercury output upon treatment is a result of chelation action in the CNS. Dimercaptopropane sulfonic acid or 2,3-dimercapto-propane sulfonate (DMPS) has been found to remove extracellular mercury, but due to its non-lipophilic properties, this chelating agent is unable to penetrate tissues to bind and remove mercury (Goyer, 1991). While research

has demonstrated that administration of this compound to patients with chronic mercury exposure yielded an increase in urinary mercury levels (Godfrey et al., 2003). CHEMET® (succimer, 100mg capsule, NDA#019998) manufactured by Ovation Pharmaceuticals has been approved by the FDA and is available by prescription. In addition to the possible safety concerns and risk factors that prevent its approved usage, there is again no evidence that DMPS targets mercury in the CNS. While the pursuit for a treatment that focuses on the removal of mercury localized in the CNS continues, it is important to investigate both the concentration- and time-dependent effects this metal may exert on neurons representative of the areas in the CNS predisposed to mercury-induced neurotoxicity.

I.B. Significance/Objective of Study

Due to the fact that the bulk of previous research has focused on acute exposure, the purpose of this project was to examine the functional, cellular and pharmacological changes that occurred upon exposing neuronal cells to low-level mercury concentrations. The rationale for low-level toxin exposure was to define better the intermediary changes that may lead to loss of cell function because previous work has shown that in the progressive stages of neurotoxic disease, biochemical events usually precede structural changes and permanent nervous system lesions or dysfunction (Manzo, et al., 1996). Lower concentrations of a toxicant may initiate subtle changes in cellular function, which gradually gives way to cell dysfunction and/or death. It has been suggested that sequential measurements of neurochemical biomarkers may be useful to assess end points indicative of early manifestations of later-developing toxicity, recovery processes or adaptive changes during chronic exposure (Manzo, et al., 1996). The goal of this work is

to pinpoint how mercury alters normal cellular mechanisms, ultimately leading to cell death, which is seen with acute exposure.

Although clinical diagnosis and proof of chronic low-level mercury toxicity has been difficult to characterize due to the non-specific nature of the symptoms and signs (Godfrey et al., 2003), investigations of both the *in vivo* and *in vitro* effects upon mercury exposure have endeavored to identify possible biomarkers so as to prevent debilitating neurological long-term effects. The relevance of this work corresponds to the plethora of environmental mercury sources and the threat of covert exposure to a multi-faceted toxicant.

The dopaminergic system was the focus of this study since symptoms of mercury toxicity, such as sensory, cognitive and motor malfunctions, closely resemble symptoms of a dysfunctional dopamine system. Neurological diseases like Alzheimer's, Parkinson's and schizophrenia would fall into this category of dopaminergic dysfunction. Dopamine uptake was examined as an index of toxicity because the metabolism of excess dopamine in the synapse results in an increased production of reactive oxygen species. The SK-N-SH cell line was utilized to study mercury toxicity because of its similarity to dopaminergic neurons in the substantia nigra and therefore reflect what is to be expected with *in vivo* studies. Functional studies were performed to explore changes in dopamine uptake when cells were treated with mercury. Cell viability and mode of cell death was examined to understand better necrotic and apoptotic mechanisms. Pharmacological concerns were addressed by measuring both transporter affinity and density to determine how mercury may alter the efficiency of the transporter as well as its ability to upregulate or downregulate the number of transporters in the cells. Evaluating the effects of low

levels of mercury will best mimic 'natural' mercury exposure in the environment and may shed light on a possible role for mercury in the progression of neurodegenerative diseases.

II. LITERATURE REVIEW

II.A. SK-N-SH Cell Line

The SK-N-SH cell line, developed by J. L. Biedler, originated from the cells of a metastatic site in the brain of a 4-year-old female with bone marrow neuroblastoma (ATCC www.atcc.org). These cells have been used as a target cell line for induced cytotoxic studies. Researchers have found these particular cells to be noradrenergic in nature (Richards and Sadee, 1986), supporting the potential bioactivity of the neurotransmitters dopamine (DA) and norepinephrine (NE). Although it has not been officially reported that the SK-N-SH cell line specifically expresses dopamine transporters (DAT), previous studies have demonstrated that both DA and NE accumulate through a single competitive, saturable and active transport process (Richards and Sadee, 1986). Active transport is indeed both sodium- and temperature-dependent (Richards and Sadee, 1986). While the SK-N-SH cell line has been described as neuronal-like cells with the ability to produce DA and NE, these cells were found to produce larger amounts of DA than NE (Richards and Sadee, 1986). The work of Liu and colleagues (2001) revealed that the viability of SK-N-SH cells has been linked to dopamine-induced cytotoxicity, which could be due to neuronal cells' increased sensitivity to DA neurotoxicity, the mechanism of antioxidants, as well as receptor-mediated signal transduction. These cells are routinely used in studies as a model of neurotoxicity as a consequence of the cell line expressing a means for both noradrenergic and dopaminergic transport. Therefore, the SK-N-SH cell line was utilized as our model system to examine mercury toxicity due to their similarity to dopaminergic neurons in the substantia nigra and therefore reflect what is to be expected with *in vivo* studies.

II.B. Dopamine Transporter (DAT) Pharmacology

The dopamine transporter consists of twelve transmembrane spanning regions, with a large glycosylated extracellular loop between regions III and IV (Figure 2).

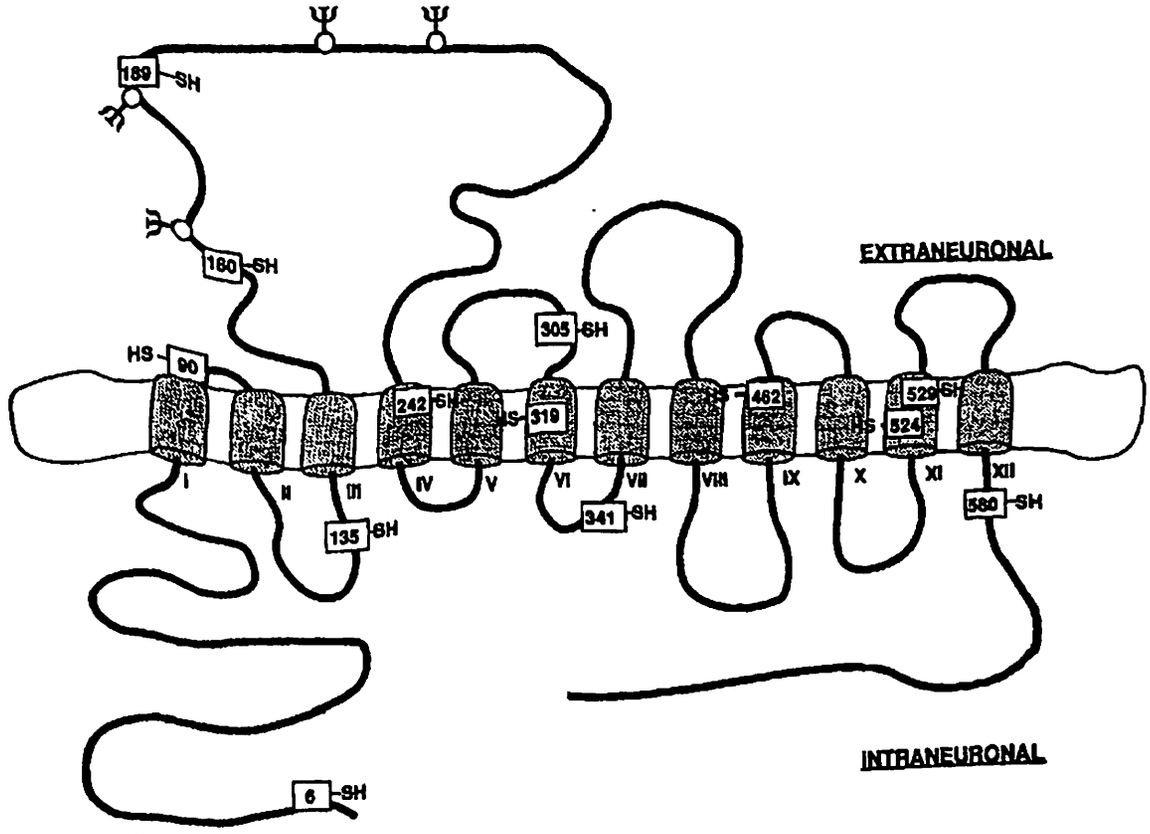

Figure 2: Schematic representation of the amino acid sequence from the rat dopamine transporter. I through XII denote transmembrane spanning regions, (-SH) symbolize suggested localization of cysteine groups, and ψ signify glycolsylation sites. (Schweri, 1994)

The mechanism of transport for the DAT, including the direction and magnitude, is dependent on membrane potential and the sodium- (Na^+) and chloride- (Cl^-) ion gradients (Falkenburger, et al., 2001). Located on the presynaptic terminal, the DAT represents a major mechanism for the inactivation of DA transmission at the synapse, thus serving a pivotal role in the regulation of DA levels in the central nervous system (Jiao, et al., 2003). Although the primary function of DAT is to move dopamine from the synaptic

space into the neuron, there is also evidence that the DA transporter can cause dopamine to be released from the pre-synaptic cell in dendritic-dendritic exchanges within the substantia nigra (Loupe, et al., 2002), leading to excess synaptic DA.

In an attempt to characterize the DAT, previous research has examined the number of binding sites, conformations of the transporter, and the interaction of different ligands. Earlier work has not been able to confirm whether the DAT has one or two binding sites (Dersch, et al., 1994). However, since DAT has high-affinity binding sites that are triggered at the low extracellular DA concentration such that DAT can accomplish its normal uptake function, the intracellular and extracellular balance of neurotransmitter is maintained (Liu, et al., 2001). Although both the dopamine and norepinephrine transporters (DAT and NET) can effectively transport each other's substrates, NET transports dopamine and norepinephrine with similar affinity (K_m of approximately 1μM) values, whereas the DAT displays a 10-fold higher affinity for dopamine over norepinephrine (Giros, et al., 1994). It is important to note that the DA recognition site on the DAT (where DA binds) can face the inner or outer surface of the membrane, with the direction of DA transport being dependent on which way the recognition site is facing (Eshleman, et al., 1994). Damage to the membrane, including lipid peroxidation, protein oxidation and other effects of oxidative stress, can also change the conformation of the DAT protein (Wu, et al., 1997), creating a nonfunctional transporter. Certain drugs also have a high affinity for DAT binding sites and once bound, they can modulate synaptic DA concentrations through a number of different actions. The drug can compete with other ligands, including DA, for the binding site, act

as a substrate and in turn increase the release of DA, or completely block the transporter, inhibiting the reuptake mechanism.

In addition to preventing DA reuptake, blocking the DAT prohibits the dendritic release of dopamine and self-inhibition of the neuron (Falkenburger, et al., 2001). The work of Dersch and associates (1994) suggests that the binding sites for various ligands may have overlapping domains of the DA uptake inhibitor recognition site. Due to the possibility of indistinct binding domains, it remains unclear whether different DA uptake blockers bind to one and the same domain on the DAT or to different domains (Wu, et al., 1997). This uncertainty may account for the potency of various drugs, effects on different neurotransmitters as well as other elicited responses. Potential conformational changes to the transporter can also change the activity of certain DAT inhibitors (Eshleman, et al., 1994). For example, an inhibitory drug has a higher affinity for the outward facing site. Once the drug binds, the equilibrium shifts to the outward facing site, preventing DA release through the DAT. When the outward-facing site is blocked by the bound ligand; nothing can get into the cell via the transporter and due to the effects of the bound ligand, nothing can get out of the cell by way of the transporter, resulting in complete inactivation of DAT.

The function of the DAT has been linked to apoptosis. It has been suggested that the DAT mediates a dopamine-dependent apoptotic signal transduction pathway that is independent of dopamine uptake into the cell (Liu, et al., 2001). High extracellular concentrations of DA can activate the low-affinity binding sites on the transporter, stimulating a similar signal transduction pathway, again triggering apoptotic processes (Liu, et al., 2001). In order to attenuate this phenomenon, administration of a compound

that stimulates DA uptake would reduce extracellular DA concentrations and could therefore prevent apoptotic signal transduction pathways.

II.C. Norepinephrine Transporter (NET) Pharmacology

Like the DAT, the NET also consists of twelve transmembrane spanning regions with both the amino and carboxyl terminals positioned in the cytosol. As a member of the monoamine transporter family, activity of NET is dependent on the Na^+/Cl^- gradient. However, previous research has uncovered data that suggest NET is less vulnerable to inactivation by oxidative stress than other Na^+/Cl^- dependent transporters (Fleckenstein, et al., 2000). Perhaps the difference in transporter activity when subjected to oxidative free radicals may be related to the uptake mechanism and regulation of the NET.

The primary function of NET is to terminate transmission of norepinephrine by definition (Zhu, et al., 2000). Although it is not NET's principal substrate, there is some evidence of that extracellular dopamine can be regulated by the NET (Yamamoto and Novotney, 1998). NET activity is inhibited by antidepressants, such as desipramine and reboxetine (Weinshenker, et al., 2002) and psychostimulants, like cocaine and amphetamine (Zhu, et al., 2000). By blocking the transporter, NE accumulates in the synapse. Norepinephrine signaling modulates a plethora of neurological functions including attention, mood, arousal, learning and memory (Schwartz, et al., 2003) as well as being involved in cardiovascular function, metabolism, embryonic development, susceptibility to seizure, maternal behavior and response to drugs of abuse (Weinshenker, et al., 2002). In fact, Boschmann and colleagues (2002) concluded that norepinephrine release from postganglionic adrenergic neurons has a central role in the regulation of energy metabolism and blood pressure. Thus, compounds that modulate norepinephrine

uptake can change or interfere with the connection between the transmitter and its effects throughout the body.

The NET is regulated by neuronal activity, neurotransmitters, peptide hormones and second messengers (Apparsundaram, et al., 2001), such as protein kinase C (PKC), cyclic adenosine monophosphate (cAMP), cyclic guanosine monophosphate (cGMP) and nitric oxide (NO). For example, NET protein levels have been reported to be downregulated by G protein-coupled receptors linked to protein kinase C (Apparsundaram, et al., 2001). PKC regulation begins through G protein-coupled pathways, where adenylate cyclase and phospholipase C (PLC) are activated. Increased levels of PLC lead to increased amounts of inositol phosphates and diacylglycerol (DAG). The elevation of inositol phosphates causes Ca^{++}-dependent activation of PKC (Apparsundaram, et al., 1998), which ultimately affects the number of norepinephrine transporter proteins. Recalling the systems linked to norepinephrine, it is not surprising that the regulation and expression of the NET protein has been linked to the development of psychiatric illnesses, such as depression (Zhu, et al., 2000), post traumatic stress syndrome and attention deficit disorder (Schwartz, et al., 2003). The work of Apparsundaram and associates (2001) found that impaired NET function or expression has been reported in cardiac failure, diabetic cardiomyopathy and hypertension, establishing a link between norepinephrine transport and disease.

II.D. Dopamine Uptake Inhibitors: GBR-12909 and Mazindol

In order to evaluate the relative extent of catecholamine uptake, selective and nonselective transport inhibitors are used to differentiate pharmacologically between dopamine transport through DAT and NET. If a selective drug preferentially targets one

of the transporter systems, a nonselective drug can be utilized to determine the amount of catecholamine uptake through the other transporter system. Fractional occupancy, a term used to relate the number of occupied receptor sites and the concentration of ligand (Hodgson and Smart, 2001), can be used to calculate the density of transport sites inhibited in functional assays.

1-[2[bis (4-fluorophenyl)] ethyl] 4-(3-phenyl propenyl)-piperazine (Hösli and Hösli, 1997), commonly termed GBR-12909, was used in the uptake studies due to its selectivity for the dopamine transporter. According to Elmer and collaborators (1996), GBR-12909 has a K_i of 3.7 nM for the dopamine transporter, which means this concentration of GBR-12909 inhibited 50% of dopamine uptake in comparison to control values, taking into account the affinity of GBR-12909 for the DAT. This drug selectively inhibits the uptake of dopamine with a 100-fold lower affinity for norepinephrine uptake inhibition (Hösli and Hösli, 1997). The *in vivo* and *in vitro* differences in the ability of GBR-12909 to 'successfully' block dopamine uptake by inhibiting the transporter is dependent on its pharmacokinetic properties such as permeability to the blood-brain barrier, metabolism, or protein binding (Kimura, et al., 2003). The research performed by Loupe and associates (2002) determined that GBR-12909 is a highly selective dopamine re-uptake blocker that by inhibiting presynaptic dopamine reuptake, makes dopamine more available at post-synaptic receptor sites. Kimura and colleagues (2003) confirmed that extracellular dopamine levels increased after GBR-12909 is administered when compared to control values.

Since GBR-12909 preferably inhibits uptake at the DAT more than the NET, another nonselective inhibitor was chosen for use in functional assays to account for the

dopamine being taken up by NET. The work of Richards and Sadee (1986) showed that mazindol inhibits dopamine accumulation in SK-N-SH cells. Although [^{3}H] mazindol has been reported to label both dopamine and norepinephrine uptake sites and block both DA and NE transporters (Eshleman, et al., 1994; Richards and Sadee, 1986), a greater concentration of extracellular dopamine was measured when using GBR-12909 compared to using mazindol (Loupe, et al., 2002). According to the work of Nakachi and collaborators (1995), the physiological use of dopamine uptake inhibitors like mazindol and GBR-12909 are to enhance dopaminergic neurotransmission in the central nervous system through an increase in dopamine concentration in the synaptic cleft as the result of reuptake inhibition at nerve terminals. For the functional studies employed in this project, both the selectivity and inhibitory activity of these drugs can be exploited in order to identify the transport system as well as quantify the amount of dopamine being taken back into the cells. Mazindol, reported to be a non-addictive dopamine uptake inhibitor of moderate potency, has a K_i value of 38.0 nM (Elmer, et al., 1996) for the dopamine transporter. The larger K_i value signifies that greater concentrations of mazindol are required to achieve 50% inhibition of dopamine uptake. For these studies, a concentration of 1 μM mazindol will inhibit DA uptake through the DAT or NET greater than 95% occupancy allowing for mazindol to be used to define nonspecific uptake.

II.E. Neurotoxicity: Focus on the Dopaminergic System

Within the nervous system, there are a number of molecular targets for toxicity including membrane-bound receptors, enzymes responsible for synthesis and degradation of neurotransmitters, second messengers and high-affinity uptake systems (Manzo, et al., 1996). With respect to the dopaminergic system, all five dopamine receptor subtypes

(D1-D5), classified as D1- or D2-like, as well as the dopamine transporter, the primary uptake mechanism for DA, are membrane-bound macromolecules (Siegel, et al., 1999). As a part of the biosynthetic pathway for catecholamines (Figure 3), the synthesis of dopamine and further synthesis of norepinephrine and epinephrine are dependent on specific enzymes and cofactors.

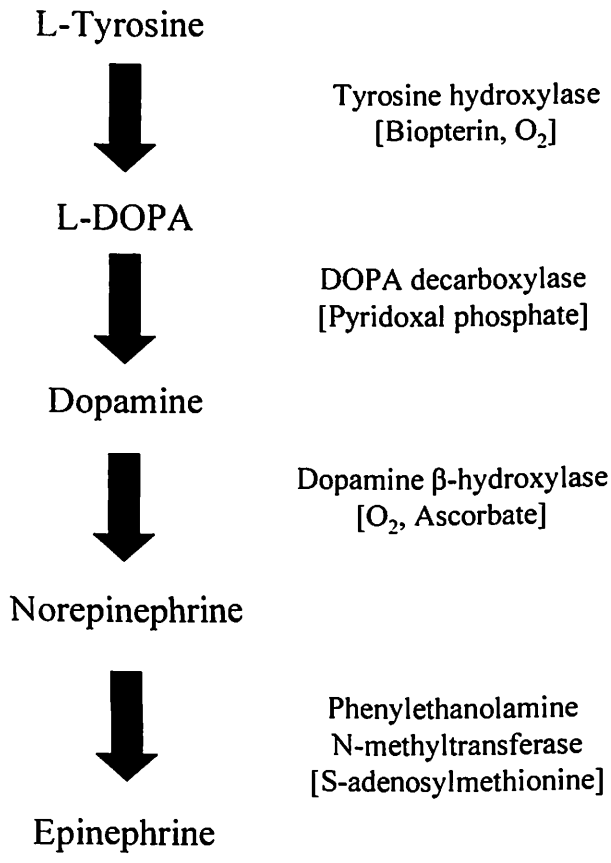

Figure 3: Simplified flowchart depicting the various steps in the pathway for catecholamine synthesis. Enzymes responsible for conversion to the next step of the pathway are shown to the right. Substrates and cofactors required for each conversion process are listed in square brackets. (Siegel, et al., 1999)

Tyrosine hydroxylase uses molecular oxygen and biopterin to convert tyrosine to 3,4-dihydroxy-$_L$-phenylalanine ($_L$-DOPA) (Siegel, et al., 1999). This product is then converted to dopamine, the final step of the pathway in dopaminergic neurons, by way of the enzyme DOPA decarboxylase and its coenzyme pyridoxal phosphate (Siegel, et al.,

1999). In non-dopaminergic neurons, dopamine β-hydroxylase, in conjunction with molecular oxygen and ascorbate, changes dopamine to norepinephrine, which can then further be synthesized to epinephrine by phenylethanolamine N-methyltransferase and S-adenosylmethionine (Siegel, et al., 1999). Monoamine oxidase (MAO), an enzyme responsible for deamination, inactivates catecholamines by converting them to their respective aldehyde (Siegel, et al., 1999). According to the research done by Manzo and colleagues (1996), neuronal second messenger systems such as intracellular calcium ion, adenylyl cyclase, guanylyl cyclase, phosphoinositides, cAMP-dependent protein kinase and protein kinase C can be affected by submicromolar concentrations of mercury. This same work contends that changes to second messenger systems may create biological "factors", which in turn regulate the long-term responses to toxic substances acting on that system (Manzo, et al., 1996). Identification and measurement of these factors could help to predict the consequences of chronic exposure to mercury.

The dopaminergic system is rooted in the basal ganglia. This portion of the brain is linked to cortical, thalamic, sensory and motor function and regulated by various neurotransmitters, including dopamine, norepinephrine, serotonin, glutamate, aspartate, acetylcholine, and gamma-aminobutyric acid (GABA) (Siegel, et al., 1999). The dopaminergic neurons of the substantia nigra innervate the striatum, making them the most important modulators of basal ganglia function (Falkenburger, et al., 2001; Siegel, et al., 1999). Currently, serum levels of dopamine β-hydroxylase and monoamine oxidase type B are being used as biomarkers to gauge dopaminergic function (Manzo, et al., 1996).

II.F. The History of Mercury: Different Forms in the Environment and Early Uses .

Mercury exhibits toxicity in the central and peripheral nervous systems, as well as in other organ systems. The general population is constantly subjected to exposure to various forms of mercury, including organic mercury, inorganic mercurial salts, and elemental mercury in the form of mercury vapor. Methylmercury, a form of organic mercury, tends to bioaccumulate in the food chain (Tchounwou, et al., 2003), especially in fish. Organic mercury can be absorbed through the lungs or gastrointestinal (GI) tract, as well as through the skin in some cases (Gochfeld, 2003). Inorganic forms of mercury are extremely potent enzyme inactivators (Godfrey, et al., 2003), disrupting numerous enzymatic reactions. The different forms of inorganic mercury vary in solubility and adsorptivity (Gochfeld, 2003), and therefore in potency and intrinsic activity. Common inorganic salts include: mercuric chloride ($HgCl_2$) which is used in certain fungicides, mercurous chloride (Hg_2Cl_2, also known as calomel) which has been used in medicine, mercuric fulminate ($Hg(OCN)_2$) which is used as a detonator for explosives and mercuric sulfide (HgS, also called vermillion) which is used as a high-grade paint pigment (Tchounwou, et al., 2003). Elemental mercury, also termed 'quicksilver', is readily volatilized (Gochfeld, 2003), producing mercury vapor. Mercury vapor, a common contaminant of industrial applications, has been found to be very toxic to the lungs and nervous system (Asano, et al., 2000). Researchers have also revealed that the natural deterioration of mercury-containing dental amalgams can lead to mercury vapor production (Godfrey, et al., 2003). The risk of low-level exposure to mercury is a serious threat due to environmental contamination as a result of mining, smelting, and industrial

discharge including ingestion via inhalation and the food chain (Stohs and Bagchi, 1995), as well as commercial, medicinal, and agricultural usage.

While society may not have been aware of mercury's toxic effects at the time, use of this metal has been dated back as early as the 15th or 16th century (Asano, et al., 2000). Mercury is a naturally occurring trace metal found in the earth's crust, mainly in sulfide ores, such as cinnabar (Kazantis, 2002). Besides the mining of such ores, additional mercury can be released into the environment by way of erosion and volcanic eruption (Tchounwou, et al., 2003). Mercury has also been used to extract gold from its ores (Kazantis, 2002), creating yet another potential exposure route. Industrial use for mercury has included lamps, measurement, medical instruments as well as a mildew-proofing agent for paints (Kazantis, 2002).

At one time, industrial mercury discharge into waterways was commonplace because people believed that mercury sank to the bottom and bound to the sediment, producing no harmful effects (Tchounwou, et al., 2003); unfortunately they were very wrong. In the 1950's, the first major mercury outbreak of mercury poisoning (in the form of methylmercury) occurred in Minimata, Japan where local industrial discharge created mercury-polluted waters and people were contaminated by eating poisoned fish (Castoldi, et al., 2001). The chemical company responsible for this deadly pollution was not forced to curtail its mercury releases until 1968 (Gochfeld, 2003), allowing for the methylmercury to make its way up through food chain to affect the health of innocent citizens. During the early 20th century, the fungicidal properties of mercurial compounds led to commercial agricultural applications (Tchounwou, et al., 2003). Mercury's use in both pesticides and fungicides afforded this toxic metal another entrance to both the food

chain and water supplies. Another significant mercury-poisoning incident took place in Iraq in the 1970's when people had eaten bread made from grain that had been treated with an organomercuric fungicide (Castoldi, et al., 2001).

In the realm of medicine, a type of inorganic mercury known as calomel (sweet mercury) was once commonly used to treat many ailments, including yellow fever, typhus and syphilis (Weinstein and Bernstein, 2003). In fact, it wasn't until the 1940's that people realized its toxicity when calomel-based teething powders caused a scourge of mercury poisoning called pink disease among infants and children (Weinstein and Bernstein, 2003). At one point in time, different forms of mercury were used in cosmetic-based pharmaceutical products; ammoniated mercury in skin lightening creams and mercuric iodide in skin-lightening soaps (Kazantis, 2002). More recently, the American Academy of Family Physicians, the Advisory Committee on Immunization Practices and the U.S. Public Health Service have advised that thimerosal, a derivative of ethylmercury, should no longer be used as a vaccine preservative or biological agent for medical therapy (Tchounwou, et al., 2003). In response to the combination of a desire for health preservation and a growing knowledge of its toxic effects, scientists set out to investigate the different targets and mechanisms involved in mercury toxicity.

II.G. Toxic Action: Pathophysiological Effects Resulting from Mercury Exposure

The toxicity of a metal is contingent upon several factors, including but not limited to: solubility, the ability to be absorbed and distributed into tissues, transport, chemical reactivity and complexes formed when the metal binds to certain biological molecules (Stohs and Bagchi, 1995). According to the work of Tchounwou and colleagues (2003), mercury toxicity specifically depends on exposure route, frequency,

dose level, nutritional status, individual susceptibility and genetic disposition. Though they may have different mechanisms of action, inorganic and organic forms of mercury exhibit a wide range of toxic properties including nephrotoxicity, neurotoxicity and gastrointestinal toxicity with ulceration as well as hemorrhage (Frisk, et al., 2000).

Studies have shown that inorganic mercury causes toxicity in the kidney; the proximal convoluted tubule being the main target for damage in response to acute exposure (Rodilla, et al., 1998). The inorganic form of mercury quickly induces nephrotoxicity as the mercuric salts accumulate rapidly in the kidneys, producing acute tubular necrosis within hours of administration (Rodilla, et al., 1998). According to the work of Aleo and collaborators (2002), a possible mechanism in which mercury causes nephrotoxicity is via the biochemical damage of mitochondrial function and calcium metabolism as well as immune mechanism.

Exposure to mercury is known to elicit neurotoxic consequences, although the pathogenesis of mercury toxicity depends on several factors, including the chemical form of mercury, and the developmental period at which intoxication occurs (Monnet-Tschudi, et al., 1996). The research of Tchounwou and associates (2003) found that mercury damages DNA, impairs mitosis, and disrupts neuronal migration, which illustrates how mercury can be especially damaging to a developing child. Previous work has examined the symptoms and general pathology of mercury toxicity with long- and short-term exposures. Mattingly and colleagues (2001) found that human exposure to moderate levels of mercury can cause neurotoxic manifestations, including sensory, cognitive and motor abnormalities. Long-term effects of mercury neurotoxicity include central hearing loss, vestibular dysfunction, poor concentration, mental deterioration, speech difficulty,

chronic headaches, impaired vision, autism, chronic fatigue, weakness of extremities, insomnia, ataxia, tremor, delirium and rigidity (Asano, et al., 2000; Godfrey, et al., 2003; Gopal, 2003). In addition to defects in the CNS, mercury exposure has also been associated with erythrism, arrhythmias and cardiomyopathy (Tchounwou, et al., 2003). Though lower concentrations of mercury may have delayed cytotoxic effects, the presence of mercury may still interfere with signal transduction and stimulate protein tyrosine phosphorylation (McCabe, et al., 1999) as well as reduce DNA synthesis. Even short-term, low-concentration exposure to mercury indicate that mercuric chloride increases post-transcriptional elevation of protein and intracellular Ca^{2+} concentrations, as well as oxidative phosphorylation in the mitochondria (Rao, et al., 2001).

According to the work of Godfrey and associates (2003), mercury is very destructive at the mitochondrial level where catalase can demethylate organic mercury species into highly reactive inorganic mercury, which can then further damage to cellular function. The research done by Uversky, Li and Fink (2001) ascertained that metal-induced oxidant stress can damage critical biological molecules and initiate a cascade of events including mitochondrial dysfunction, excitotoxicity, and a rise in cytosolic free calcium, leading to cell death. Mercury has been shown to disrupt cellular metabolism and activities through interaction with membrane proteins (Worth, et al., 2001), resulting in membrane polarization and production of both nitric oxide (NO) and other reactive oxygen species (ROS).

Generation of ROS propagates further damage to aspects of cellular function such as alteration to DNA, breakage of DNA strands, disruption of protein function, reduction of glutathione, inhibition of glutathione synthesis, alteration in calcium homeostasis and

lipid peroxidation (Rao, et al., 2001; Stohs and Bagchi, 1995). In addition to increased ROS production, exposure to mercury decreased the activity of superoxide dismutase (SOD), Cu/Zn-SOD and Mn-SOD (Hussain, et al., 1997), all of which are enzymes that serve as crucial antioxidant defenses. Mercury's ability to interact with the membrane can also lead to malfunctions with transporter proteins, inhibiting activity at the Na^+-K^+-Cl^- co-transporter (Jacoby, et al., 1999). The variety of effects seen in mercury-induced neurotoxicity moved scientists' focus to mercury's mechanism of action in the central nervous system.

II.H. Localized Mechanism of Action: Mercury's Effects in the CNS

Early studies in the CNS began to shed some light on the activity of mercury in the brain. Brookes (1988) found that mercury compounds inhibit amino acid transport. Mercury has also been shown to alter protein function due to interaction with sulfhydryl groups (Jacoby, et al., 1999). Others determined that exposure to mercury predisposes affected cells to further damage from reactive oxygen species (Aleo, et al., 2002) brought on by oxidative stress.

Mercury's mechanism of action begins with gaining access to the CNS, and exerting its toxic effects at certain receptors in specific brain regions, causing dysfunction in the sensitive balance of neurological systems. Mercury enters the CNS through a variety of different mechanisms. The brain's main defense mechanism against toxicants is the blood brain barrier (BBB). According to Zheng and colleagues (2003), a series of active or receptor-mediated transport systems inherent to the BBB vasculature serve to control the transport of metals into the brain. However, the permeability across the BBB is dependent upon the toxicant's lipophilicity and size (Zheng, et al., 2003), in this case

mercury. Organic mercury is more lipophilic, allowing it to easily diffuse across membranes. Mercury can also gain entry to the CNS via "piggy-back" transport due to its affinity for sulfhydryl groups; by forming complexes with molecules which already have well-established physiological transport systems, such as glutathione, methionine or cysteine (Hultberg, et al., 2001, Zheng, et al., 2003). Though most inorganic mercury salts are less toxic due to their decreased rate of transport across the BBB, certain inorganic mercury salts, like $HgCl_2$ can act as a direct barrier toxicant (Zheng, et al., 2003) or a choroid plexus toxicant (Zheng, 2001).

Active defense systems located in the brain, like superoxide dismutase, glutathione peroxidase and catalase, work to prevent free radical-initiated oxidative stress (Zheng, et al., 2003). However, the effectiveness of the barrier systems may also be compromised either in pathological situations or following toxic insults by compounds that target the blood brain interface (Zheng, et al., 2003), such as heavy metals. The breakdown of said barriers, possibly caused by metal toxicity, can lead to the "leakage" of neural macromolecules and immune cells (Manzo, et al., 1996), allowing them to become more vulnerable to further damage by metal toxicants such as mercury.

Earlier studies have suggested that mercury is taken up by microvesicles in neurosecretory terminals and conveyed to neuronal bodies by retrograde axonal transport (Villegas, et al., 1999). The research of Hare and associates (1990) determined that introduction of Hg^{2+} into the system increases the permeability of the plasma membrane to small molecules, enhancing the cellular injury sustained by such toxic action. Other work has shown that mercury deposits directly in the choroid plexus; causing damage to the structure itself as well as mediating entry for metals and other neuroactive toxicants

into brain tissues (Zheng, 2001). Due to the fact that mercury disrupts the normal function of ion channels (Asano, et al., 2000), this may serve as a means for mercury to gain access to brain tissue. Once the toxin has made its way into the cells, mercury can bind to a variety of enzyme systems producing non-specific cell injury or cell death (Frisk, et al., 2000).

Relatively low concentrations of mercury (1 – 1000 μM) can inhibit activity at muscarinic, nicotinic, NMDA, $GABA_A$ and dopamine receptors (Fonfría, et al., 2001). Mercury disturbs the activity of enzymes by binding to electron-rich sulfhydryls, carboxyls, and imidizoles (Kumar, et al., 2002) but has the highest affinity for sulfhydryl groups, which can be found in glutathione, metallothionine and cysteine. To a lesser degree, mercury has also been found to bind to hydroxyl, carboxyl and phosphoryl groups (Tchounwou, et al., 2003), all of which are imperative for protein function. Previous research has also uncovered mercury's ability to alter neuronal differentiation, which can affect the production of neurotransmitters and ultimately activate the apoptotic cascade (Rossi, et al.1997). The work of Faro, and others (2001) has indicated $HgCl_2$ exerts a well-known inhibitory effect on membrane transport that is generally attributed to its high-affinity interaction with protein sulfhydryl groups. Mercury's reactivity at sulfhydryl and other electron-donating groups can cause protein unfolding (Brookes, 1988), which can result in a breakage of the lipid bilayer membrane or disruption of membrane transporter function. The role of metallothionine in metabolism, transport, and homeostasis of metals determines whether the pathway leads to toxicity or detoxification (Rodilla, et al., 1998). Dithiols, another form of sulfhydryl group, enhance the toxic effects of mercury due to its inability to form stable complexes, which releases

mercury ions, allowing for direct mercury uptake into cells (Hultberg, et al., 2002). The work of Tchounwou and colleagues (2003) confirmed that divalent inorganic mercury (Hg^{2+}) binds to multiple cell surface receptors via free sulfhydryl groups and results in nonspecific receptor clustering, dysregulated signal transduction, and disorders of cellular function.

Due to its electrophilic nature, mercury can complex with DNA by binding to negatively charged sites; altering base pairs and forming cross-linkages between strands, resulting in mutagenesis (Rao, et al., 2001), which can lead to the production of dysfunctional proteins. Mercury can also interfere with protein production and DNA synthesis by binding to the sulfhydryl groups contained in the amino acid sequences that make up proteins associated with replication (Rao, et al., 2001), ultimately distorting the genetic information so that the affected cell cannot maintain normal function. Furthermore, prior work has shown that mercury can bind to the electron-rich bases of mitochondrial DNA (Bhattacharya, et al., 1997), causing mutation, fragmentation and eventual degradation of DNA, rendering afflicted mitochondria useless.

II.I. Targeted Brain Regions

There are several factors that determine what regions of the brain are affected by the toxic actions of mercury. Areas of the brain affected may be defined by transport efficiency (Brookes, 1988); the more efficient the transport systems, the more likely mercury will gain entry to initiate its toxic effects. Prior work has also suggested that mercury has achieved access to striatal tissue (Faro, et al., 2001), due to the increased levels of dopamine detected when exposed to mercury ($HgCl_2$).

One specific cell type in the brain that seems to be targeted by mercury toxicity is microglia. According to the work of Monnet-Tschudi and associates (1996), even at sub-cytotoxic concentrations distinct glia-specific reactions could be observed with both organic and inorganic mercury compounds. Microglial cells have proven to be extremely sensitive to the toxic effects of mercury, exhibiting damage before any sign of neuronal degeneration in multiple brain regions (Monnet-Tschudi, et al., 1996). When microglia are activated by neuronal insult resulting in inflammation, the release of superoxide anion ($^{\cdot}O_2^{-}$), hydrogen peroxide (H_2O_2), and nitric oxide (NO) is increased, further contributing to oxidative neurotoxicity (Wang, et al., 2003). The presence of these free radicals allows for the formation of peroxynitrite ($ONOO^{-}$), a highly reactive free radical which damages lipids, membranes, proteins, DNA and sulfhydryls, as well as inactivates critical enzymes (Wang, et al., 2003). Activated microglia can also release cytokines or proteases, which may trigger secondary cellular responses on astrocytes or neurons (Monnet-Tschudi, et al., 1996), possibly initiating apoptotic mechanisms.

II.J. On A Cellular Level: Systems Affected by Mercury Exposure

The various toxic effects of mercury play a role in a number of neurological systems, including the glutamate/glutamine cycle, dopaminergic, synaptic transmission, GABAergic, glutathione, calcium homeostasis, immune response, and motor activity. Sub-micromolar concentrations of $HgCl_2$ have been shown to inhibit selectively glutamate uptake (Faro, et al., 2001), increasing the concentration of glutamate in the synaptic space. Since uptake is the primary inactivation mechanism for synaptically released glutamate in the brain, inactivation of glutamate uptake could nevertheless accelerate processes of excitotoxic neurodegeneration associated with disease or aging

(Brookes, 1992). Excessive stimulation of the glutamate/glutamine cycle results in a state of excitotoxicity and ends up overwhelming neuroprotective systems, increasing DA release, which in turn increases arachidonic acid production and leads to transferritin dysfunction (Weber, 1999). Activation of ionotropic and metabotropic glutamate receptors can trigger the Ca^{2+}-independent efflux of dopamine (Falkenburger, et al., 2001). Even though it is typically an excitatory transmitter, research has suggested that glutamate can inhibit dopaminergic pathways in the basal ganglia (Jiao, et al., 2003), which would still lead to neurotoxicity because inhibition of the pathways increases the amount of excess dopamine in the synaptic space. Although the inhibition of glutamate transport can be reversed, this process becomes less reversible when exposed to mercury for longer periods of time (Brookes, 1988). Similarly, inhibition of glutamate transport is also less reversible with higher concentrations of mercury (Brookes, 1988) due to the toxicant's oxidative properties and interaction with membrane proteins.

Mercury also affects the dopaminergic system. Previous work has shown that mercury blocks the degradation pathway of catecholamines (Weinstein and Bernstein, 2003), including dopamine. Neuronal dopamine uptake is both sodium- and temperature-dependent, indicating an active mechanism for dopamine transport (Hösli and Hösli, 1997). The dopamine transporter, located on the presynaptic terminal, terminates dopaminergic neurotransmission by accumulation of released dopamine into presynaptic neurons (Dutta, et al., 2001). The functional importance of the dopamine transporter lies in its ability to clear excess synaptically released dopamine (Wu, et al., 1997). In fact, DAT sites represent a major mechanism for the inactivation of DA transmission at the synapse, thus serving a pivotal role in the regulation of DA levels in the central nervous

system (Jiao, et al., 2003). Dopamine levels can be regulated by drugs that bind to DAT, modulating synaptic DA concentrations through different mechanisms (Eshleman, et al., 1994), including blocking the transporter, acting as a substrate and increasing DA release or competing for the binding site. Excessive dopamine in the synapse is metabolized by one of two oxidation mechanisms. The one mechanism consists of monoamine oxidase-A or –B (MAO-A, MAO-B) oxidation of dopamine to produce 3,4 dihydroxyphenyl acetate (DHPA), ammonia and hydrogen peroxide (Hodgson and Smart, 2001).

$$\mathbf{DA + O_2 + H_2O \rightarrow DHPA + NH_3 + H_2O_2}$$

Hydrogen peroxide can be further converted to hydroxyl radical, a toxic reactive oxygen species, through the Fenton reaction. The second mechanism for oxidative breakdown of dopamine is auto-oxidation, where dopamine reacts with divalent oxygen to produce a semiquinone, a hydrogen ion and a superoxide radical, which can react with another molecule of dopamine to form a second semiquinone and more hydrogen peroxide (Hodgson and Smart, 2001).

$$\mathbf{DA + O_2 \rightarrow SQ^{\bullet} + {}^{\bullet}O_2^{-} + H^{+}}$$

$$\mathbf{DA + {}^{\bullet}O_2^{-} + 2H^{+} \rightarrow SQ^{\bullet} + H_2O_2}$$

The production of hydrogen peroxide and other reactive oxygen species can be especially damaging to membrane integrity, DNA synthesis and overall cellular function. Although removing dopamine via reuptake by the DAT can prevent initiation of the dopamine metabolism mechanisms, this process can also be enhanced by the stimulation of dopamine release or production of reactive oxygen species (Weber, 1999). Excess dopamine can bring about dopamine-induced apoptosis in neuronal cells; however, the extent of cell death depends on reuptake of dopamine, intracellular thiol modulation, and

mitochondrial function (Zhang, et al., 1998). Alteration and/or disruption of dopamine transport leads to dopamine accumulation in the synaptic space, resulting in the production of destructive reactive oxygen species via dopamine metabolism.

Mercury-induced toxicity is known to affect synaptic transmission. Mercury interferes with signal transduction pathways due to the fact that this toxicant changes tyrosine phosphorylation of proteins and pathways regulated by receptor-associated tyrosine kinases, like nerve growth receptors (Mattingly, et al., 2001). The research of Wang and colleagues (2003) concluded oxidants such as mercury, are involved in the activation of multiple signaling pathways contributing to cytotoxicity, including the phosphorylation cascades leading to the activation of mitogen-activated protein kinases (MAPK). In addition to evidence of MAPK activation in brains of Alzheimer's patients, this enzyme has also been linked to mediation of apoptosis in neuroblastoma cells (Wang, et al., 2003). The work of Shanker, Hampson and Aschner (2004) tried to correlate methylmercury exposure with phospholipase A_2 (PLA_2) activity, an enzyme that plays a key role in the signal transduction pathways of many receptors. Due to its highly lipophilic nature, methylmercury was found to quickly activate cytosolic PLA_2 ($cPLA_2$) levels (Shanker, et al., 2004). Activation of cPLA2 not only catalyzes the breakdown of membrane lipids, but it also triggers the release of arachidonic acid (AA), whose pro-inflammatory metabolites can activate voltage dependent and ligand-gated channels as well as contribute to ROS generation and impaired mitochondrial energy synthesis (Shanker, et al., 2004). The excessive stimulation of this enzyme, $cPLA_2$, has been linked to the etiology of metal encephalopathies, stroke, seizures, cerebral ischemia and Alzheimer's disease (Shanker, et al., 2004), which potentially links the effects of

mercury-induced neurotoxicity to certain pathologies. Mercury triggers neurotoxicity by increasing and then suppressing the spontaneous release of neurotransmitters in the periphery (Yuan and Atchison, 1994). The effects of neurotoxic mercurials depend on the site and potency of action, which is directly linked to the lipophilicity of the mercury compound, as well as the possible reversibility of that binding action (Yuan and Atchison, 1994). Hg^{2+} has been shown to increase neurotransmitter release by changing intracellular calcium concentrations (Hare, et al., 1990).

Mercury has also been shown to influence GABAergic function. Alterations in GABAergic function have been associated with various neurological and psychiatric disorders, such as Huntington's disease, epilepsy, tardive dyskinesia, alcoholism, schizophrenia, sleep disorders, Parkinson's disease and mental retardation (Siegel, et al., 1999). The two categories of GABA receptors, $GABA_A$ and $GABA_B$, are coupled to chloride and potassium ion channels, respectively (Siegel, et al., 1999). The research of Gopal (2003) established the $GABA_A$ receptor as another neuronal target for mercury, interrupting GABA signaling pathways and therefore hindering GABA function. Activation of the $GABA_A$ receptor mediates Cl^- influx, depolarizing the cell membrane, which then activates Ca^{2+} influx (Siegel, et al., 1999) and can trigger apoptotic mechanisms.

As a primary protective mechanism against oxidative stress, one can easily predict that mercury-induced toxicity affects glutathione (GSH), an essential antioxidant neuroprotector mainly localized in glial cells (Lee, et al., 2001). Previous research has found that incubation with inorganic mercury at higher concentrations leads to lipid peroxidation and a reduction in glutathione (Stacey and Kappus, 1982), causing a loss in

cell viability. Glutathione depletion can be resultant of synthesis inhibition, conjugation of GSH to electrophilic xenobiotics or the effects of oxidative stress (Hultberg, et al., 2001). Although exposure to mercury has shown an initial increase in extracellular glutathione (Hultberg, et al., 2001), the toxic process ultimately ends in glutathione depletion. The initial surge of glutathione may represent a compensatory mechanism to protect against further damage from oxidative stress or a reserve mechanism to replenish the glutathione that has complexed mercury ions. In addition to cellular GSH, mitochondrial GSH levels are also diminished under oxidative conditions. As defensive systems like glutathione deteriorate due to the generation of ROS, normal mitochondrial activity becomes especially susceptible to disruptions in the energy production pathways. The research of Stohs and Bagchi (1995) noted that the addition of mercury to a system enhances hydrogen peroxide formation under conditions of impaired respiratory chain electron transport. The resulting depletion of glutathione leads to an increase in reactive oxygen species, like superoxide ion, hydrogen peroxide and hydroxyl radical (Stohs and Bagchi, 1995). Resultant damage induced by ROS includes: lipid peroxidation, DNA fragmentation as well as altered calcium and sulfhydryl homeostasis.

As previously mentioned, mercury-induced oxidative stress leads to impairment of calcium homeostasis. Oxidative stress due to free radical oxygen species increases intracellular calcium by bringing calcium into the cell and releasing inner stores of calcium (Stohs and Bagchi, 1995), which are commonly found in the mitochondria. An increase in Ca^{2+} concentration can also trigger apoptotic mechanisms (Amoroso, et al., 2002). Prior research has revealed that Hg^{2+} inhibits ATP-dependent calcium uptake into intracellular stores (Hare, et al., 1990), such as the mitochondria. The toxic actions of

mercury interrupts calcium's role in activating proteases, endonucleases and phospholipases (Stohs and Bagchi, 1995). The lack of calcium activity allows for the generation of more free radicals, initiating an intracellular signal transduction cascade, which can result in long-term potentiation (Weber, 1999) and excitotoxicity.

Due to the interaction between regulatory mechanisms in the CNS and immune systems, it should not be surprising to learn that mercury's neurotoxic effects can elicit peripheral immune responses. Down-regulation of DNA synthesis occurs with mercury-induced toxicity because mercury hinders this process by binding to the sulfhydryl-containing amino acids of proteins/enzymes associated with DNA replication; less DNA production leads to less cell proliferation, weakening the system. Mercury has been shown to influence growth control and regulation of apoptotic mechanisms in lymphocytes (Mattingly, et al., 2001), cells whose main function is cell-mediated immunity. This same research also observed that Hg^{2+} alters tyrosine phosphorylation, interfering with Ras-mediated signal transduction (Mattingly, et al., 2001). Ras, a small G protein that controls growth factor and MAP kinase pathways, mediates cell growth (Siegel, et al., 1999); inactivation or dysfunction of this protein can impede essential physiological functions. Due to its high affinity, mercury binds to sulfhydryl groups on the cell membrane, creating disulfide bridges, which promotes cellular dysfunction (McCabe, et al., 1999). This is a proposed mechanism to explain how mercury interrupts the normal activation of tyrosine kinase, an enzyme involved in tyrosine phosphorylation. Tyrosine phosphorylation plays a fundamental role in the CNS, including the development and function of a neuron, survival and differentiation, the extension of axons to their targets and synapse formation and function (Siegel, et al., 1999). This

phosphorylation process is also important to synaptic transmission; neurotransmitter receptors, voltage-gated ion channels, enzymes and proteins in neurotransmitter release are all dependent on tyrosine phosphorylation (Siegel, et al., 1999). The oxidative properties and consequential neurological effects of mercury influence essential transduction pathways linked to the immune system, therefore allowing physiological systems to become more susceptible to other harmful substances.

As the clinical symptoms suggest, mercury toxicity affects activity in motor neurons. Even at low concentrations, Hg^{2+} potently depolarizes skeletal muscle and reduces the effective resistance of the fibers (Yuan and Atchison, 1994). This depolarization could be brought on by the disruption of Ca^{2+} homeostasis that occurs when mercury blocks Ca^{2+} uptake into nerve terminals (Yuan and Atchison, 1994), resulting in neuronal dysfunction and possible neuronal death. The work of Pamphlett and Pang (1998) demonstrated that the presence of inorganic mercury within motor neurons therefore appears to behave as a slow-acting neurotoxin that shrinks motor neurons. This same effect could shrink the axons of sensory neurons (Pamphlett and Pang, 1998), which might explain some of the symptoms seen with mercury neurotoxicity, such as hearing loss and impaired vision.

II.K. Factors of Toxicity and Possible Chelation Therapy

The neurological effects of mercury-induced toxicity are dose-, concentration- and time-dependent. Although low concentrations of mercury were more toxic to undifferentiated brain cells (Monnet-Tschudi, et al., 1996), higher (near cytotoxic) concentrations of organic mercury had more neurospecific effects (Monnet-Tschudi, et al., 1996). Accumulation of mercury in targeted brain regions is concentration-

dependent. The work of Monnet-Tschudi, and associates (1996) offers that mercury compounds accumulate in a concentration dependent manner. Mercury-induced nephrotoxicity is both concentration-and time-dependent (Rodilla, et al., 1998). The toxic effects of dopamine in the striatum are also dependent on the duration of mercury exposure (Faro, et al., 2001). Perhaps this could be due to mercury's direct interaction with dopaminergic neurons, affecting membranes, vesicular release, reuptake system and transport of dopamine or due to mercury's effects on central synaptic transmission in respect to its potency and reversibility of action (Faro, et al., 2001).

In order to remove mercury from the central and peripheral nervous systems, the search for chelators remains an on-going venture. For example, vitamin E and EDTA, two commonly used chelators, had no effect on ultimate cell death induced by mercury, though they did prolong cell death by protecting against oxidative stress (Stohs and Bagchi, 1995). The later work of Gassó and colleagues (2001) support this conclusion, also finding that vitamin E did not in fact reduce $HgCl_2$ cytotoxicity. Perhaps mercury elicits its toxic effects by displacing other divalent ions, which may contribute to more oxidative stress, depletion of protective resources and the cell finally succumbs to the damage. Due to its strong antioxidant and nucleophilic nature (Rao, et al., 2001), vitamin C works against mercury-induced genotoxicity by directly binding to mercury ions or removing and/or reducing free radicals generated by mercury toxicity. Though the chelation of excess free Ca^{2+} brought on by chemically induced oxidative stress seems like a logical treatment, Ca^{2+}-chelation does not halt free-radical-induced cell death (Amoroso, et al., 2002). Melatonin has also been suggested as a possible treatment for mercury-induced toxicity. And although melatonin may not directly chelate mercury, it

has been shown to decrease the effects of mercury-induced oxidative stress (Olivieri, et al., 2000), perhaps due to an upregulation in glutathione. Currently DMPS is considered an effective therapeutic agent because the mercury levels in urine increased about 10-fold (Godfrey, et al., 2003). However, there has been no indication that the mercury present in the urine is resultant of successful chelation from the brain, which suggests administration of this drug is not a comprehensive treatment for mercury-induced neurotoxicity.

II.L. Links to Alzheimer's Disease

The work of Zheng, et al. (2003) showed that a toxin's influence on the blood brain barrier can be linked to neurological disorders, abnormal brain development, chemically-induced swelling of the brain or the onset of neurodegenerative disease. Previous research has suggested links between mercury-induced neurotoxicity and neurodegenerative disease, mainly Alzheimer's disease (AD). In their research, Hock and collaborators (1998) found a correlation between high blood mercury levels and increased cerebrospinal fluid (CSF) levels of amyloid β-peptide (Aβ), a characteristic closely associated with AD. Although mutations in the amyloid precursor protein, the protein responsible for Aβ production, have been linked to genetic factors, these mutations may also be linked to environmental factors (Hock, et al., 1998), such as exposure to heavy metals like mercury. An increase in Aβ levels has been implicated in oxidative stress and free radical production (Olivieri, et al., 2000). It has been theorized that mercury alters the action of amyloid precursor protein because the toxicant inhibits PKC activity, which is responsible for the abnormal cleavage product, Aβ (Hock, et al., 1998) and therefore causes the formation of Aβ plaques.

Oxidative stress induced by Hg^{++} has also been shown to increase the phosphorylation of tau protein in neuroblastoma cells (Olivieri et al., 2000), a constituent of neurofibrillary tangles and classic feature of Alzheimer's. Other studies have also found increased amounts of mercury in brains of AD patients (Markesbery, 1997). Mercury-induced toxicity may contribute to the oxidized protein and DNA found in conjunction with Alzheimer's disease; so thus, with elevated Fe, Al and Hg levels, and the potential of acting synergistically, the microenvironment of the brain in AD is fertile for enhancing free radical generation and lipid peroxidation (Markesbery, 1997). Even though it has not been isolated as a clinical diagnostic tool for Alzheimer's, the blood mercury levels in early onset AD patients was 3-fold higher than those in control groups (Hock, et al., 1998). However, current research has not been able to offer any evidence of a direct causal effect of mercury in the pathogenesis of AD (Hock, et al., 1998).

Later research investigated use of Apolipoprotein E (Apo-E) genotyping as a biomarker to determine those with an increased risk of developing AD (Godfrey, et al., 2003). Apo-E is a crucial element in the mechanisms that dictate amyloid β secretion, plaque formation and oxidative stress (Weber, 1999). In addition to the ε_4 allele being identified as a risk factor for AD with a 70% successful prediction rate, Alzheimer's patients with mercury-associated symptoms had higher amounts of the ε_4 allele (Godfrey, et al., 2003), suggesting a link between mercury toxicity and the genetic factors that determine onset of the neurodegenerative disease. This work suggests that individuals with a combination of homozygous ε_4 allele genotype and mercury exposure would have an increased risk of early-onset Alzheimer's disease (Godfrey, et al., 2003). Other Apo-E alleles, ε_2 and ε_3, contain sulfhydryl groups, which allow for them to bind and chelate

mercury, removing the toxicant from the CNS (Godfrey, et al., 2003) and therefore preventing the pathway from proceeding to β-amyloid production.

II.M. Summary and Hypothesis

In summary, mercury-induced toxicity has been linked to several cellular functions, including membrane transport, DNA synthesis, mitochondrial efficiency, calcium homeostasis and neuroprotective defense systems. Through experimentation with lower concentrations of mercury, this research aims to determine at what levels toxicity occurs by performing functional, cellular and binding studies. Based on a literature value which defines acute neurotoxicity as having blood mercury levels exceeding 200 μg/L (Hock, et al., 1998), the loss of cellular function indicated by changes in dopamine uptake, lactate dehydrogenase and caspase enzymatic activity as well as affinity and density of the transporter is expected to occur at mercury treatments greater than 1 μM (Hg MW=200.59). However, since this toxicity threshold specifically describes blood mercury levels, which can be measured in the CSF, the concentration of toxicant in the blood may not truly reflect the concentration of toxicant in the synapse. Therefore, this project explores the potential functional, cellular and pharmacological changes while focused on potential synaptic concentrations of mercury.

The **HYPOTHESIS** of this project is: Concentrations of mercuric chloride, at or below levels reported as cytotoxic will have effects on the dopaminergic system in SK-N-SH cells. These changes could have a profound psychological/motor impact *in vivo* prior to cytotoxic effects normally observed with high concentrations of mercury.

III. RESEARCH DESIGN AND METHODS

III.A. General Methodologies

III.A.1. Cell Culture

SK-N-SH human neuroblastoma cells were obtained from American Type Culture Collection (ATCC, Manassas, VA) and cultured in RPMI 1640 without L-glutamine (Cellgro, MediaTech Inc., Herndon, VA) supplemented with 10% heat inactivated fetal bovine serum (FBS; Hyclone, Logan, UT) and 1% Penicillin/Streptomycin solution—10,000 I.U./mL and 10,000 µg/mL (Cellgro, MediaTech Inc., Herndon, VA) in vented 25 cm^2 cell culture flasks (Corning Inc., Corning, NY). Cultures were maintained at 37°C in a 5% CO_2 humidified atmosphere. The culture medium was changed twice a week, as recommended by ATCC, and confluent monolayers were subcultured once a week at a 1:3 ratio using 0.25% Trypsin, 1.0 mM ethylenediaminetetraacetic acid (EDTA) in HBSS without calcium and magnesium salts (Atlanta Biologicals, Lawrenceville, GA) at room temperature for 15 minutes in a sterile environment. The cells from passages 4 to 14 were used to perform the characterization, functional and binding studies.

For subsequent portions of the project, 1 x 10^6 cells were seeded into 24-well plastic cell culture plates (Costar® 3524, Corning, Inc., Corning, NY) and maintained in the complete growth medium. After plating, the cells were allowed at least 24 h to adhere to the surface of the well before further experiments were performed.

III.A.2. [^{3}H] Dopamine Uptake

After the specified incubation time, the growth medium/assay buffer/nonspecific drug/$HgCl_2$ treatment was removed from all wells. Then 100 µL of assay buffer (consisting of 25 mM HEPES, 120 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM

$MgSO_4$, 300 mM Ascorbic Acid, 1 μM Pargyline and 2 mg/mL D-(+) Glucose) was . added to the control and treatment (drug or metal) wells. The [7, 8 ^{3}H] Dopamine (DA), 0.02 M acetic acid: ethanol (1:1) solution (Amersham Biosciences, UK) required for this portion of the assay was prepared by making 1.2X concentration solution diluted with the assay buffer, yielding a final in-well concentration of 20 nM. Next, 400 μL of the 20 nM [^{3}H] DA solution was added to all wells and the plate was incubated for twenty minutes on a plate shaker (Clinical Rotator, Cat. No. 2500, Eberbach Corp., Ann Arbor, MI) at room temperature. Dopamine uptake was terminated by removing the [^{3}H] DA solution and washing the cells with 2 mL of ice-cold 0.9% NaCl. After the NaCl was removed, the cells were detached from the plate upon addition of 150 μL Trypsin-EDTA, transferred to scintillation vials (Fisher Scientific, Pittsburgh, PA), and 5 mL scintillation cocktail was added to each vial (ScintiVerse®, Fisher Scientific, Pittsburgh, PA), which was then capped and vortexed. As well as the vials prepared from each individual well, a scintillation vial containing 150 μL of 20 nM [^{3}H] DA and 5 mL scintillation cocktail was included a means of calculating the amount of dopamine available for actual uptake. Uptake was determined by liquid scintillation spectrophotometry using a Beckman Coulter LS 1801 (Beckman Coulter Inc., Fullerton, CA).

III.A.3. Cytotoxicity and Necrosis: Measuring Lactate Dehydrogenase (LDH)

These experiments were carried out using a colorimetric LDH enzyme detection kit: CytoTox 96 ® Non-Radioactive Cytotoxicity Assay from Promega Corporation (Madison, WI). This kit quantitatively measures lactate dehydrogenase through the series of enzymatic reactions seen below:

$$\text{LDH}$$
$$NAD^+ + \text{Lactate} \rightarrow \text{Pyruvate} + NADH$$

$$\text{Diaphorase}$$
$$NADH + \text{INT} \rightarrow NAD^+ + \text{Formazan}$$

In the first step of this process, NAD^+ is reduced to NADH as the LDH released from affected cells catalyzes the conversion of lactate to pyruvate. In the second step, an electron-accepting catalyst, such as diaphorase, transfers the proton from NADH to the tetrazolium salt (INT), which is reduced to formazan, a red-colored product.

The general protocol begins by transferring 50 µL of each sample into a clear 96-well flat-bottom cell culture plate (Costar ® 3599, Corning Inc., Corning, NY) in duplicate. Blanks were included as a means of subtracting out any background absorbance attributed to assay buffer or media, again using 50 µL aliquots of assay buffer (refer to section III.A.3) or media. The assay buffer was then thawed, and while protected from the light, acclimated to room temperature. A 12-mL portion of the LDH assay buffer was used to reconstitute a bottle of substrate mix. Then 50 µL of substrate mix solution was then added to each well of the 96-well plate. After the reaction mixture was incubated for 30 minutes at room temperature, again protected from light, 50µL of stop solution (1 M acetic acid) is added to each well. Within an hour of when the reaction was stopped, the absorbance was measured at 490 nm with the Synergy™ HT Multi-Detection Microplate Reader (Bio-Tek Instruments, Inc., Winooski, VT.), which uses KC4™ PC Software to report absorbance data.

III.A.4. Initiation of Apoptotic Mechanisms: Detection of Caspase-3 and –7

These experiments were done using a caspase enzyme apoptosis kit: Apo-ONE™ Homogeneous Caspase-3/7 Assay, from Promega Corporation (Madison, WI). Toxin-

induced apoptosis activates different caspases, which are responsible for triggering a cascade of cleavage events that result in the methodical destruction of cells. The caspase 3/7 substrate, consisting of rhodamine110, bis-(N-CBZ-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid amine) (Z-DEVD-R110) allows for the fluorescent detection of caspase 3/7 activity. The amount of caspase 3/7 activity can be measured because as the caspase 3/7 enzymes present in the sample initiate the cleavage and removal of DEVD peptides (Asp-Glu-Val-Asp), an intensely fluorescent product (the rhodamine 110 leaving group) is created, which has an excitation wavelength of 499 nm and a maximum emission wavelength of 521 nm. Therefore, the total amount of fluorescence measured is proportional to the amount of caspase 3/7 contained in the cells from each sample.

First, 100 μL from each individual sample was put into a black 96-well clear-bottom cell culture plate (Costar ® 3603, Corning Inc., Corning NY) in duplicate. Blanks were included (also performed in duplicate) with each assay to account for any background fluorescence contributed by the various solutions used during toxin incubation, such as the growth medium or uptake buffer, again using 100 μL aliquots. While protected from the light, the Apo-ONE™ Homogeneous Caspase-3/7 Buffer and Caspase Substrate Z-DEVD-R110 were thawed to room temperature. The Caspase Substrate was diluted 1:100 with the Caspase-3/7 buffer to the desired volume and mixed by inversion. Then 100 μL of the caspase reagent was then added to each well of the plate and incubated on a plate shaker for the desired amount of time, anywhere from 30 minutes to 18 hours depending on the expected amount of caspase activity at room temperature, again protected from light. Within 18 hours of the post-incubation time, the fluorescence was measured at 499 nm (excitation wavelength) and 521 nm (emission

wavelength) with the Synergy™ HT Multi-Detection Microplate Reader (Bio-Tek Instruments, Inc., Winooski, VT.), which uses KC4™ PC Software to report absorbance data.

III.A.5. Binding Studies: Affinity and Density of the Dopamine Transporter

After treatment with mercury, the cells were trypsinized, centrifuged to form a pellet and frozen to later perform saturation analysis.

Cell pellets were resuspended in assay buffer to yield a final protein concentration of 0.003 – 0.03 mg/mL, using assay buffer. Assay buffer consists of 50 mM Tris-HCl, 120 mM NaCl and 0.01% bovine serum albumin adjusted to pH of 7.7. After resuspension, 400 µL of the cell preparation is added to 12 x 75mm polypropylene binding tubes followed by 50 µL of either buffer (control) or unlabeled drug. The binding reaction is initiated by the addition of 50 µL of increasing concentrations of [^{3}H] GBR-12935 (6 concentrations, 1 nM – 30 nM) both in the absence and presence of 5 µM GBR-12909 and allowed to proceed at room temperature for 60 minutes. Binding is terminated by filtration under reduced pressure using a Brandel Tissue Harvester (Brandel Instruments, Gaithersburg, MD) onto GF/B fiberglass filters which had been presoaked in 0.3% polyethyleneimine to reduce nonspecific binding. Filters are then washed for 15 seconds (approximately 15 mL) with ice-cold 50 mM Tris-HCl (pH 7.4). Specific [^{3}H] GBR-12935 binding is then determined by the subtraction of binding in the presence of 5 µM GBR-12909 from binding in the absence of any drugs (total binding). These data will allow for the determination of the density and affinity of the dopamine transporter. [^{3}H] GBR-12935 binding to the dopamine transporter is then determined by liquid scintillation spectrophotometry.

III.B. Data Analysis and Statistics

The data from the previously described experiments was recorded as % Control [^{3}H] Dopamine Uptake, Absorbance at 490 nm, Relative Fluorescence Units (RFU), Affinity of [^{3}H] GBR-12935 for DAT (nM) and Density (pmol/ mg protein) of DAT. Appropriate statistical analyses were performed using GraphPad Prism® Version 4.0 (San Diego, CA). Parametric analysis using one- and two-way analysis of variance (ANOVA) was accepted since the data met both the concept of normal distribution and robustness. Post-hoc analysis was performed using the Bonferroni correction for multiple comparisons or Dunnett's Multiple Comparison Test that compares treatment values to control values. A value of $P < 0.05$ was used to indicate significant differences.

III.C. Experimental Design

III.C.1. Cell Culture

The SK-N-SH cultures were prepared as described above and all procedures were performed while utilizing 'sterile' techniques.

Maintenance of cells included changing the complete growth medium twice a week and splitting the cultures once a week. To change the media, the old media was taken out using a sterile pipette; careful not to disturb the monolayer of cells. 5 mL of warmed complete media (heated in 37°C water bath for 20-30 minutes) was added to the flasks without creating bubbles.

Upon achieving confluence, within 7 days, the cells were split and subcultured in a 1:3 ratio. Using a pipette, the medium was removed and 1.5 mL of trypsin (thawed in 37°C water bath) was added to each 25 cm^2 flask. Typically, cells will loosen from the culture surface in 5-15 minutes; the flasks can also be returned to the incubator if the cells

begin to clump or do not appear to be detaching from the plastic; gentle agitation can be used as a last resort. Once the cells are removed from the surface, 3.5 mL of complete media was added to each flask to stop the action of the trypsin. Pipetting the solution up and down breaks up any clumps and disperses the cells in the suspension. Depending on the confluence of the monolayers, 0.5-1.0 mL of the cell suspension was transferred to a new sterile 25 cm^2 culture flask and 4.5-4.0 mL of complete media was added to each new flask, again avoiding bubbles, maintaining a 5 mL final volume. After the splitting procedures were completed, all culture flasks are recapped and returned to the incubator.

III.C.2. Characterization of the Dopamine Transporter Using GBR-12909 and Mazindol

In order to determine a drug's affinity for the dopamine transporter, competition binding assays are performed to measure the competition between a radioligand and an unlabeled drug for a specific receptor site (Hodgson and Smart, 2001). The cells were treated with a range of drug concentrations and incubated with [^{3}H] DA to measure changes in dopamine uptake. The uptake inhibitory drugs examined include: Mazindol, Desipramine, GBR-12909 and Nomifensine.

The cells were plated as described above. The drugs (Mazindol and GBR-12909 from RBI, Inc., Natick MA) were prepared by making 5X concentrations diluted with the assay buffer, accounting for the 1:5 dilutions into each well (100µL drug in a total volume of 500µL/well). The growth medium was removed, 100 µL assay buffer was added to control wells (defining total uptake) and 100µL of drug concentrations ranging from 0.1-1000 nM were added to the treatment wells. After administration of drug, the dopamine uptake procedures explained earlier were carried out to quantify the amount of

dopamine uptake blocked by the various inhibitors, which correlates to the drug's affinity for the dopamine transporter.

The uptake data was reported as Mean % Control Total [^{3}H] Dopamine Uptake ± SEM of four experiments performed in duplicate and evaluated using one-site competition analysis. The inhibitory constant (K_i), defined as the concentration of drug that will inhibit 50% of the observed response taking into account the affinity of the drug for its site of action with respect to the affinity of the substrate for its receptor, was calculated for the different tested using the equation:

$$K_i = \frac{EC_{50}}{1 + [\text{ligand}]/K_d}$$

The effective concentration 50% (EC_{50}) value reflects the ability of the drug to bind to its receptor (affinity) and the ability of the drug to cause a response once it is bound (efficacy); essentially the concentration of drug that competes for half the specific binding. For these calculations, the EC_{50} value for the individual drugs was supplied by the analysis from one-site competition, the K_d of [^{3}H] Dopamine for DAT was found to be 70 nM and 100 nM was used for the concentration of ligand to best approximate the concentration of [^{3}H] Dopamine having the affinity K_d.

The term fractional occupancy is used to describe receptor occupancy as a function of ligand concentration; for these assays, the amount of dopamine transported (uptake) as a function of drug concentration. The formula for fractional occupancy is shown below:

$$\text{Fractional Occupancy} = \frac{[\text{Ligand/Drug}]}{[\text{Ligand/Drug}] + K_d}$$

K_d, the dissociation constant, defines the affinity of the ligand for the binding site of interest; in this case, the affinity of the uptake inhibitor for the dopamine transporter. Based on the data collected for the different inhibitory drugs, appropriate DA uptake inhibitors were chosen to use later in the uptake assays after mercury exposure.

As prior research indicated, GBR-12909 was shown to have a higher affinity (approximately 150-fold) for the DAT compared to NET. Mazindol has been reported to have a slightly higher affinity for NET (7-fold) compared to DAT. Thus, suggesting that GBR-12909 is a selective uptake inhibitor for the dopamine transporter (DAT) over the norepinephrine transporter (NET). Use of appropriate concentrations of both drugs could facilitate the determination of DAT and NET density. By incubating with low concentrations of GBR-12909 (50 nM/DAT) and high concentrations of mazindol (1 μM/DAT+NET), one can mathematically calculate the density of DAT and NET using a single radioligand.

III.C.3. $HgCl_2$ Treatments

SK-N-SH cells were plated as described above and exposed to varying concentrations of Mercury (II) Chloride ($HgCl_2$; Sigma Aldrich, St. Louis, MO) over different periods of time. Incubation with $HgCl_2$ was carried out in assay buffer, complete media and modified assay buffer (without ascorbic acid).

For the experiments performed in the buffer, the inorganic salt was accurately dissolved in Milli-Q water to make a 1 mM stock solution; then aliquots of the original stock solution were diluted with the assay buffer up to the final $HgCl_2$ concentration used for the various treatments. Throughout the course of experimentation, two stock solutions were made for the mercury treatments done in the buffer because over time, the

mercury in the first solution had begun to visibly precipitate out of solution. The first stock solution (1mM) was prepared by dissolving 3.30 mg of $HgCl_2$ into 12.15 mL of Milli-Q water, while the second stock solution (1mM) was prepared by dissolving 3.50 mg of $HgCl_2$ into 12.89 mL of Milli-Q water. The mercury treatments were prepared, being mindful of the 1:5 dilution factor (100µL of treatment in 500µL total). The growth medium was removed and 400 µL of assay buffer was added to each well. Then 100 µL of the different $HgCl_2$ concentrations, ranging from 1 nM to 1 mM, were added to each of the treatment wells and incubated at room temperature for the specified exposure time, beginning with 1h and extending to 6h.

For the experiments performed in the growth medium, the inorganic salt was accurately dissolved in Milli-Q water to make a 51 mM stock solution. This stock solution was prepared by dissolving 13.85 mg of $HgCl_2$ into 100.54 mL of Milli-Q water and then autoclaved to sterilize the solution. Then aliquots of the sterile stock solution were diluted with sterile water up to the final $HgCl_2$ concentrations, varying from 10µM to 1 mM to be used for the different treatments. Preparation of these mercury treatments required a different dilution factor of 1:51 because 10µL of treatment was added to the 500µL of media already in the wells, creating a total volume of 510µL. The rationale for using a lower volume was to minimize the dilution of media constituents. 10 µL of the various sterile $HgCl_2$ concentrations were added directly to the complete growth medium in each of the treatment wells and maintained in a 37°C, 5% CO_2 humidified atmosphere for the specified exposure time, ranging from 1h to 48h.

Later experiments investigated the potential effects of specific components in the original assay buffer, namely ascorbic acid. These treatments were performed the same

as the other 'in buffer' studies, except that the aforementioned 300 mM ascorbic acid was not added to the assay buffer used in this part of the experiment. This 'modified' buffer was utilized to make the mercury treatments and was put in the wells during the exposure period. This limited study focused on moderate concentrations, ranging from 1 μM to 100 μM and an exposure time of 6h.

III.C.4. Measuring [^{3}H] Dopamine Uptake After Toxin (Hg^{2+}) Exposure

This procedure was performed in accordance to the aforementioned protocol with a few modifications. Once the exposure period expired and the solution (media/buffer/mercury) was removed from all wells, 100 μL of 250 nM GBR-12909 and 100 μL of 5 μM mazindol (diluted with assay buffer (refer to section III.A.3)) were added to designated wells (with resultant 'in well' concentrations of 50 nM GBR-12909 and 1 μM mazindol) while 100 μL of assay buffer (refer to section III.A.3) was put into control and mercury-treated wells. Nonspecific binding was defined as [^{3}H] DA uptake in the presence of 50 nM GBR-12909 and uptake in the presence of 1 μM mazindol. All other steps in the process were carried out as previously described.

Data were reported as Mean % Control [^{3}H] Dopamine Uptake ± SEM of four experiments done in duplicate. Data were analyzed by either two-way ANOVA (time by concentration) or one-way ANOVA (concentration) to determine effects on [^{3}H] dopamine uptake.

III.C.5. Cytotoxicity and Cell Viability: Measuring Lactate Dehydrogenase (LDH)

The concentration(s) and exposure time period(s) were chosen in conjunction with the changes noted in the dopamine uptake data. Based on the uptake studies, the cells were exposed to 5 μM and 50 μM $HgCl_2$ for 24 hours in both media and assay buffer

(refer to section III.A.3). Because of the 1:10 dilution (a 50 μL mercury treatment in 500 μL total volume), 50 μM and 500 μM solutions were prepared; dissolving 7.1 mg of $HgCl_2$ in 52.30 mL of Milli-Q water for the 500 μM solution and dissolving 1.2 mg $HgCl_2$ in 88.40 mL of Milli-Q water for the 50 μM solution. To maintain a sterile environment during the 24h incubation period, the two mercury treatments were autoclaved, as well as a portion of assay buffer.

To begin this procedure, SK-N-SH cells were plated out in a 24-well plate as previously described. After allowing time for the cells to adhere to the culture surface, the growth medium was removed and 450 μL of incubation solution (complete media or assay buffer) was added to the respective wells. Then, 50 μL of the 5 μM and 50 μM $HgCl_2$ treatments was added to the proper wells and the plate was returned to the incubator (37°C) for 24h.

Once the 24h treatment period had finished, the procedures for measuring LDH commenced. The aforementioned protocol was followed with minimal modifications. In order to account for background absorbance contributed by both incubation solutions, duplicate 50 μL aliquots of complete media and assay buffer served as blanks for the experiment.

Data were reported as Mean Absorbance at 490 nm ± SEM of four experimental samples run in duplicate. Data were analyzed by one-way ANOVA (absorbance at 490 nm) to determine any differences between the various groups: control, 5 μM treatment and 50 μM treatment.

III.C.6. Initiation of Apoptotic Mechanisms: Caspase-3 and –7 Detection

Immediately following procedures for the LDH experiment, preparations for measuring caspase activity began. From the same 24-well plate, all remaining solution was removed and 150 μL of trypsin was added to each well. After the cells had loosened from the culture surface, 50 μL aliquots of trypsinized cells from each of the 24 sample wells were transferred in duplicate to a black 96-well plate. In order to maintain the cells during incubation period for the caspase assay, 50 μL of complete media was added to each of the wells on the 96-well plate, resulting in a total volume of 100 μL per well, diluting the cells by a 1:1 ratio (50 μL of cells in 100 μL total). The 96-well black plate was allowed to sit for approximately 2h so that the trypsinized cells from each sample could re-adhere to the culture surface.

In order to account for any background fluorescence contributed by the combination of incubation solutions, blanks consisting of 50 μL trypsin and 50 μL complete media were also performed in duplicate. As indicated in the general protocol, the 100X caspase reagent solution required for the assay was prepared by mixing 100 μL of Z-DEVD-R110 caspase substrate with 9.9 mL of caspase buffer. The aforementioned procedures concerning the Caspase-3/7 Assay were followed, incubating the covered 96-well black plate for 6h at room temperature, mixing the contents for the first 30 minutes on a plate shaker.

By using KC4^{TM} PC Software to measure fluorescence, the parameters for the excitation and emission wavelengths were slightly modified. Excitation wavelength was set at 485 ± 20 nm and emission wavelength was set at 528 ± 20 nm; both of these ranges contain the original wavelengths recommended by the general protocol. The data were

reported as Mean Relative Fluorescent Units (RFU) ± SEM of four experimental samples run in duplicate. Data were analyzed by one-way ANOVA (RFU) to establish if there are changes in relative fluorescence between groups: control, 5 μM treatment and 50 μM treatment.

III.C.7. Binding Studies: Affinity and Density of the Dopamine Transporter

For these experiments cells were subcultured in 25 cm^2 flasks and flasks were randomly subdivided into 3 groups; control/vehicle, 5 μM $HgCl_2$, and 50 μM $HgCl_2$. Cells were allowed to adhere for at least 24 hours and then treated by the addition of the appropriate solution. Control groups received 500 μL of sterile double distilled water (ddH_2O), whereas the mercury groups received either (final concentration) 500 μL of 5 or 50 μM $HgCl_2$. The flasks were then returned to the incubator (37°C) and allowed to sit for 24h. The reaction was terminated by the removal of growth media with ddH_2O or mercuric chloride and addition of 1.5 mL of 0.25% trypsin/0.03% EDTA. Following trypsinization, cells were centrifuged to form a pellet and the pellet frozen until assayed.

The saturation analysis was performed as described above. The data were analyzed by fitting a rectangular hyperbola to determine K_d (affinity) and B_{max} (density) values.

IV. RESULTS

IV.A. Characterization of the Dopamine Transporter Using GBR-12909 and Mazindol

Analysis of the data revealed the different affinities each drug had for the dopamine transporter. Figure 4 shows competition analysis curves that were used to evaluate the inhibitory constants (K_i values) for mazindol and desipramine.

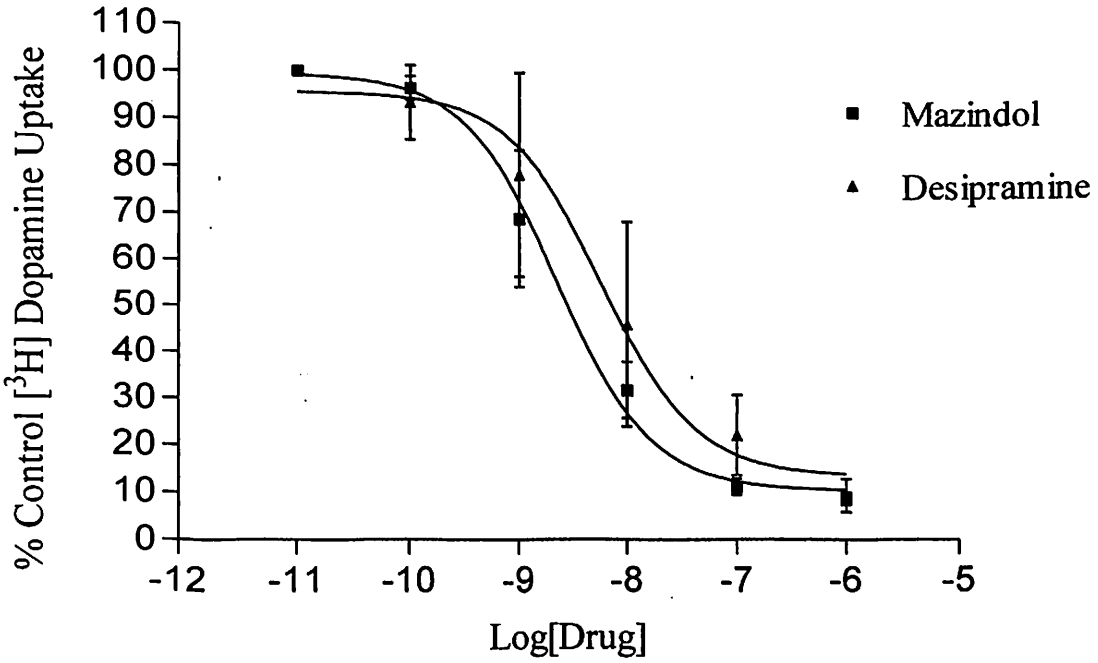

Figure 4. Effects of Mazindol and Desipramine on [^{3}H] Dopamine Uptake in SK-N-SH cells. Uptake in the presence of 0.1nM - 10μM of inhibitory drug is expressed as Mean % Control [^{3}H] Dopamine Uptake ± SEM of 4 experiments, each run in duplicate. By using One-Site Competition Nonlinear Regression Analysis, the Inhibitory Constant (K_i) values for the two drugs of interest were calculated.

K_i values were used to determine which drugs were more selective for DAT. According to Hodgson and Smart (2001), K_i is the equilibrium dissociation constant of the unlabeled competitor mazindol, desipramine, GBR-12909 and cocaine, while K_d is the dissociation constant of the radioligand the [^{3}H] Dopamine. K_i values of different toxicants or drugs

can be compared to determine the rank order of potency of toxicants in competing for the radiolabeled receptor site, where a low K_i indicates a high affinity and a higher K_i indicates a lower affinity. The data in Table 1 summarizes the inhibitory constants obtained from the competition analysis yielding a potency series for the four drugs examined.

Table 1. Dissociation Constants for DAT ligands calculated using One-Site Competition Nonlinear Regression Analysis

Ligand	**N**	**$K_i \pm$ SEM (nM)**
Mazindol	4	$1.52 \pm .52$
Desipramine	4	6.91 ± 6.2
GBR-12909	3	21.6 ± 5.8
Cocaine	3	56.6 ± 20.5

As the data indicate, GBR-12909 was shown to have a high affinity for the dopamine transporter, suggesting that GBR-12909 is a potent uptake inhibitor for the dopamine transporter (DAT). Interestingly, all curves were best fit to a single, non-interacting, site. One would have expected that if there were two sites (DAT and NET) on the SK-N-SH cells, two sites would be observed based on the drugs' inherent selectivity. Or, if there were only one site, for example DAT, that GBR-12909 would exhibit a high affinity and desipramine a low affinity. Additional work is needed to further elucidate the exact type of transport system that is present on SK-N-SH cells. One hypothesis is that in undifferentiated cells, a "generalized" transport system is expressed with high affinity for both DAT and NET uptake inhibitors and that following differentiation; more distinct DAT and NET transport sites would be observed.

Based on Table 2, which summarizes the approximate affinities for various drugs based on literature values, the fractional occupancy of GBR-12909 and mazindol was calculated (Table 3) with respect to the different transporters.

Table 2. Approximate reported affinities of different drugs for the dopamine (DAT), norepinephrine (NET) and serotonin (SERT) transporters

Drug	DAT Affinity	NET Affinity	SERT Affinity
GBR 12909	1 nM	150 nM	>1 μM
GBR 12935	6 nM	220 nM	>5 μM
Indatraline	6 nM	2 nM	0.5 nM
Mazindol	15 nM	1-2 nM	100 nM
Nomifensine	98 nM	10 nM	840 nM
Nisoxetine	>1 μM	5 nM	>1 μM
Desipramine	>5 μM	4 nM	60 nM
Fluoxetine	>5 μM	>5 μM	3 nM

Table 3. Fractional Occupancy of the two DAT ligands utilized to define nonspecific binding in measuring [^{3}H] Dopamine Uptake for SK-N-SH cells.

Transporter	GBR-12909 (% Receptor Occupied)	Mazindol (% Receptor Occupied)
DAT	98.04	98.5
NET	25.0	99.9
SERT	4.76	90.9

GBR-12909 occupied a greater percentage of receptors on dopamine transporters when compared to norepinephrine and serotonin. The percentages of occupied transporter receptors for mazindol were approximately the same whether they were dopamine, norepinephrine or serotonin transporters. These data support that GBR-12909 is more selective for the dopamine transporter, while the nonselective nature of mazindol is confirmed by the comparable percentages of occupied receptors for the different transporter proteins.

IV.B. Measuring [^{3}H] Dopamine Uptake After Mercury Exposure

For the experiments where the incubation period was carried out in assay buffer at room temperature prior to performing the dopamine uptake procedures, lower concentrations (1nM-1μM) of mercury elicited little to no change in dopamine uptake when compared to control values, as seen in Table 4. A similar trend was seen at similar concentrations over more moderate periods of time (6h).

Table 4. Dopamine Uptake Values in Assay buffer (refer to section III.A.3). Data expressed as Mean % Control [^{3}H] Dopamine Uptake ± SEM of 4 experiments done in duplicate. ND = Not Determined. Control values defined as 16.0 ± 1.2 fmol/min of dopamine being taken up for these sets of experiments.

[$HgCl_2$]	*1 Hour*	*3 Hour*	*6 Hour*
1 nM	79.4 ± 6.1	90.6 ± 7.6	ND
10 nM	83.0 ± 3.8	93.8 ± 6.7	ND
100 nM	83.7 ± 3.9	103.9 ± 7.5	87.6 ± 10.8
1 μM	98.1 ± 12.4	85.2 ± 7.6	82.6 ± 8.2
10 μM	106.3 ± 7.5	90.3 ± 5.8	72.5 ± 7.8
100 μM	112.8 ± 8.8	93.5 ± 3.6	54.4 ± 17.9

Although there was no significant effect of concentration ($F_{5,54}$=0.20; p = 0.9619) on dopamine uptake, the data does indicate a significant concentration X time interaction ($F_{10,54}$=2.49; p = 0.0154) and a significant ($F_{2,54}$=6.35; p = 0.0033) effect of time. There was also a significant ($F_{5,36}$=2.79; p = 0.0315) concentration X time interaction with comparing 1h and 3h data sets (Figure 5).

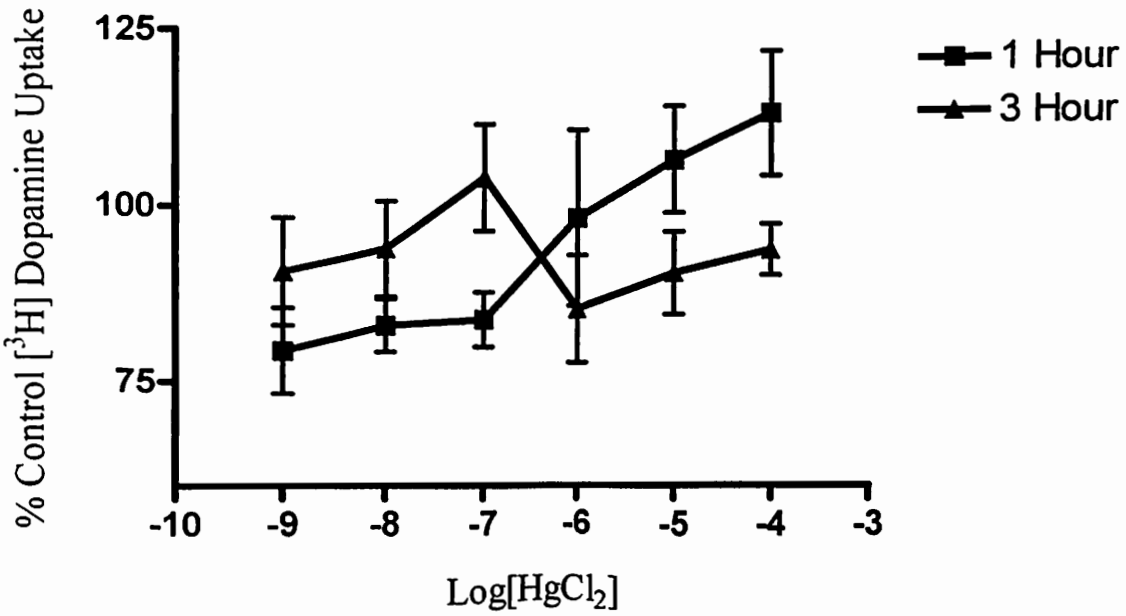

Figure 5. Effects of mercuric chloride on [^{3}H] Dopamine Uptake in SK-N-SH cells for 1- and 3-hour exposure periods. Uptake in the presence of 1nM - 100 μM $HgCl_2$ was expressed as Mean % Control [^{3}H] Dopamine Uptake ± SEM of four experiments, each run in duplicate. These mercury treatments were performed in assay buffer. The data indicate a significant interaction between both concentration and time in mercury-exposed cells at shorter time points and smaller concentrations when compared to control values. Control values defined as 18.6 ± 1.3 fmol/minute of dopamine being taken up for these sets of experiments.

At moderate concentrations of mercury (100nM-100μM), significant time-dependent effects ($F_{1,36} = 7.91$; $p = 0.0079$) were observed between 1- and 6-hour exposure periods performed in the assay buffer (refer to section III.A.3). The data in Figure 6 demonstrate that as the incubation time for exposing the SK-N-SH cells to the same concentrations of mercury increases, there is more of a defined decrease in dopamine uptake.

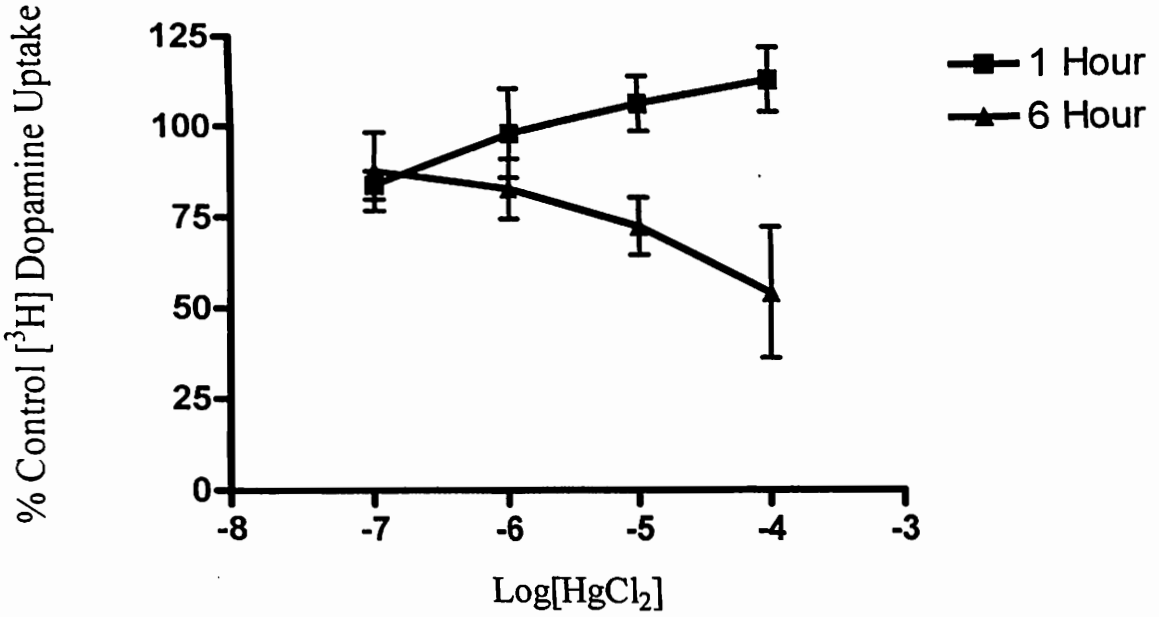

Figure 6. Effect of mercuric chloride on [^{3}H] Dopamine Uptake in neuroblastoma cells for 1h and 6h exposure periods. Uptake in the presence of 100nM - 100μM $HgCl_2$ was expressed as Mean % Control [^{3}H] Dopamine Uptake ± SEM of four experiments, each run in duplicate. Mercury treatments were performed in the DAT assay buffer. A significant effect of time was observed ($p<0.01$) as the exposure time increases, dopamine uptake decreases. Control values defined as 15.1 ± 1.6 fmol/minute of dopamine being taken up for these sets of experiments.

A significant effect of concentration ($F_{3,32} = 43.93$; $p < 0.0001$) in dopamine uptake was seen when the neuroblastoma cells were treated with mercury for longer periods of time (24h) in the complete RPMI-1640 at 37°C compared to DA uptake buffer (Figure 7). During the actual experimentation, after treatment with higher concentrations of $HgCl_2$ (> 10 μM) it was observed that the cells were no longer attached to the culture surface; appearing much like the cells had been trypsinized. The data typifies a biphasic response pattern; where incubation with moderate concentrations of mercury reflects control values and as the concentration of toxin increases, there is a distinct upsurge in

dopamine uptake on the order of 3 times control values followed by a severe reduction in dopamine uptake.

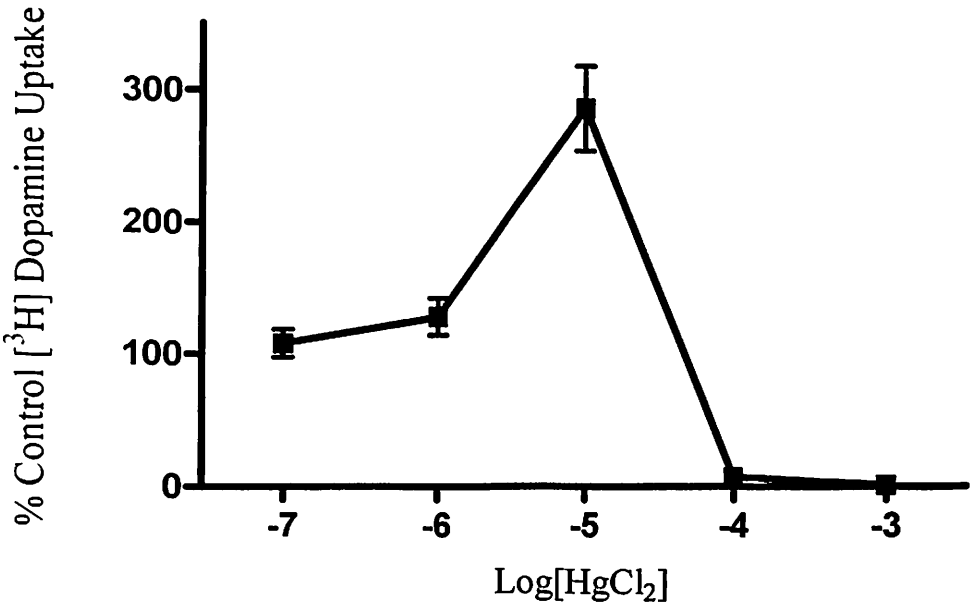

Figure 7. Effects of mercuric chloride on [^{3}H] Dopamine Uptake in SK-N-SH cells after 24-hour exposure treatments. Uptake in the presence of 100 nM - 1 mM $HgCl_2$ was expressed as Mean % Control [^{3}H] Dopamine Uptake ± SEM of four experiments, each run in duplicate in complete RPMI-1640. A significant effect of concentration ($p < 0.0001$) was observed. Control uptake was 10.3 ± 1.8 fmol/minute of dopamine being taken up for these sets of experiments.

A similar phenomenon was noted in the uptake studies performed using greater concentrations of mercury over a range of time points, again treating the SK-N-SH cells with mercury in the growth medium at 37°C (See Figure 8). In addition to the experiments run for 24-hours, cells were also visibly floating in the growth media after 1- and 6-hour exposure periods to concentrations greater than 10 μM $HgCl_2$. The data reveal that dopamine uptake increased by 2.5- to 3-fold at all monitored time points (1, 6, 24 h) when treated with lesser concentrations (10μM) of mercury. Incubation with higher concentrations of mercury (100μM-500μM) resulted in extreme inhibition of

dopamine uptake, again at all monitored time points (1, 6, 24 h). Statistical analyses of the data reveal a significant effect of concentration ($F_{3,36} = 298.2$; $p < 0.0001$) in dopamine uptake. Bonferroni posttests were employed to compare the uptake measured with each individual concentration to the other concentrations. As the data illustrate, these analyses revealed a significant difference ($p < 0.001$) between dopamine uptake measured after 10 μM $HgCl_2$ treatment and the dopamine uptake measured after 100, 250, and 500 μM $HgCl_2$ treatments at all time points (1, 6, 24h). No significant effect of time ($F_{2,36} = 1.04$; $p = 0.3641$) in dopamine uptake between 100, 250 and 500 μM $HgCl_2$ treatments. It is also important to point out that the amount of dopamine uptake did not appear to vary over the range of time points at any one individual concentration.

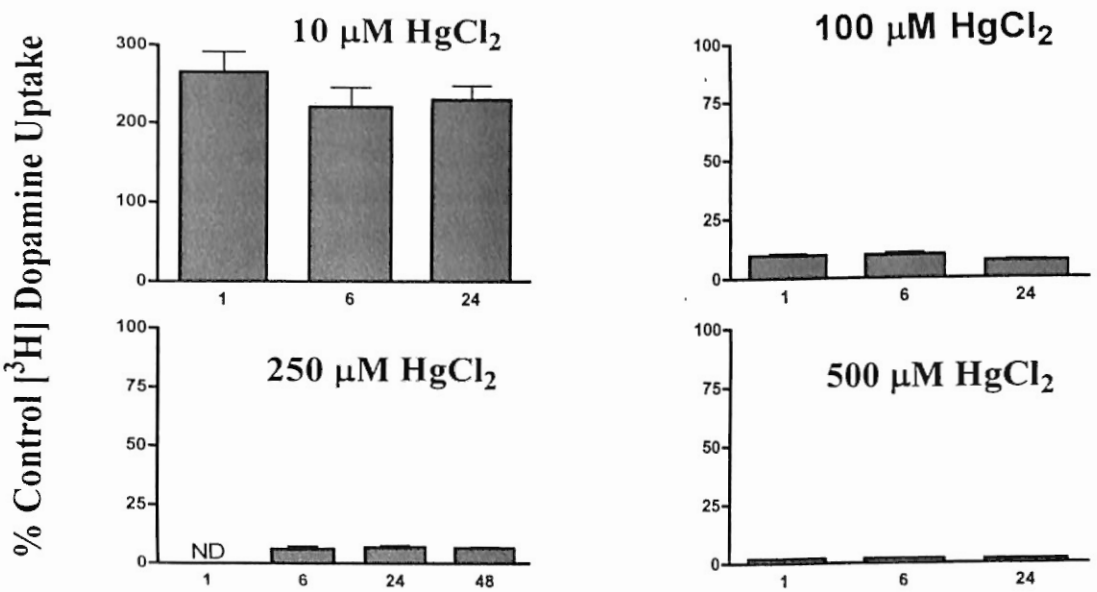

Figure 8. Effect of various exposure times and multiple concentrations of mercuric chloride on [^{3}H] Dopamine Uptake in SK-N-SH cells. Uptake in the presence of 10-500 μM $HgCl_2$ is expressed as Mean % Control [^{3}H] Dopamine Uptake ± SEM of four experiments, each run in duplicate. Control values defined as 8.0 ± 1.3 fmol/minute of dopamine being taken up for these sets of experiments. All treatments were performed in the growth medium. The data reveal a significant effect of concentration between the dopamine uptake measured after exposure to 10 μM $HgCl_2$ and all other concentrations ($P < 0.001$, two-way ANOVA, Bonferroni Multiple Comparison Test).

As reported earlier, there was minimal difference noted in the dopamine uptake compared to control values during the experiments run in assay buffer (refer to section III.A.3) when the neuroblastoma cells were exposed to mercury for 6 h. However, when the same experiments were carried out in assay buffer (refer to section III.A.3) containing no ascorbic acid (Vitamin C), there was a substantial decrease in dopamine uptake upon exposure to 10 μM $HgCl_2$, which was not indicated by the previous data. Alterations in assay buffer (with or without ascorbate) resulted in significant effects of buffer composition ($F_{1,30} = 14.60$; $p = 0.0006$), concentration ($F_{2,30} = 11.04$; $p = 0.0003$) and a significant interaction ($F_{2,30} = 4.369$; $p = 0.0216$) between the two parameters (Figure 9).

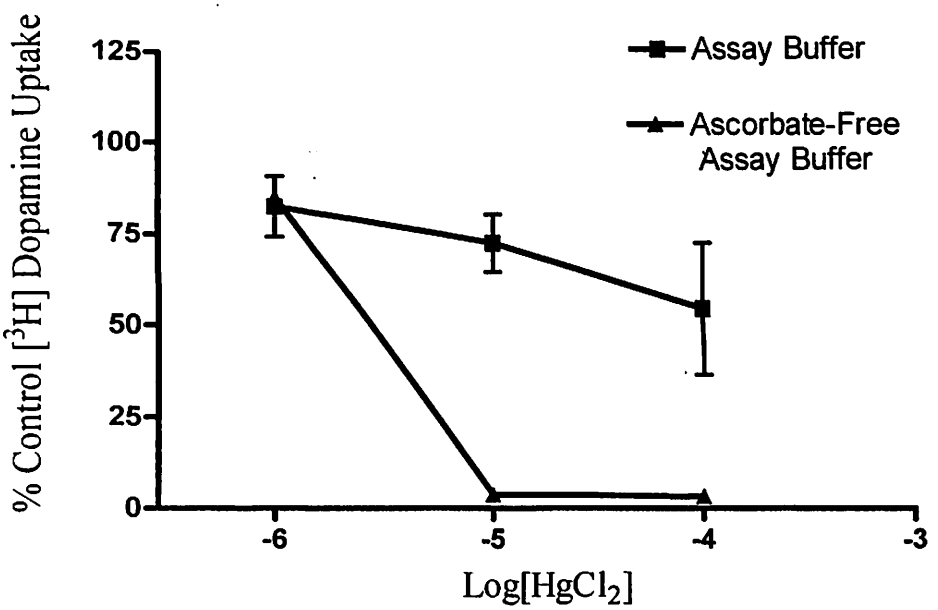

Figure 9. Effects of mercuric chloride on [^{3}H] Dopamine Uptake for 6 hour treatment; comparing dopamine uptake when the neuroblastoma cells were treated in assay buffer or ascorbate- free assay buffer. Uptake in the presence of 0.1-200 μM $HgCl_2$ is expressed as Mean % Control [^{3}H] Dopamine Uptake ± SEM of four experiments, each run in duplicate. Control values defined as 12.4 ± 1.3 fmol/minute of dopamine being taken up for these sets of experiments. The data shows a significant effect of buffer composition during the 6 h treatment period ($P < 0.001$, two-way ANOVA).

The differences in the control values of dopamine taken up (fmol/min) are a result of several contributing factors, such as what passage the cells were in when the assays were performed. how many cells were seeded into the wells, and how much time had passed between initial plating and actual experimentation. To standardize these variables and normalize the raw information gathered, data were expressed as Mean %Control [^{3}H] Dopamine Uptake.

IV.C. Cytotoxicity and Cell Viability

The amount of absorbance measured using the CytoTox 96® Assay relates to the quantity of LDH released from the cells in that the colored product is resultant of catalysis in the presence of LDH. Absorbance of each sample is obtained by subtracting out the average value for the appropriate blank.

Analysis of the absorbance data using one-way ANOVA revealed a significant ($F_{2,11} = 14.18$; $p = 0.0017$) difference between groups when treated with these intermediary concentrations of mercury. Since there is a difference seen in the results of ANOVA, post-hoc analysis was employed to identify changes in overall main effects. To determine which group means differ and to what extent, Dunnett's Multiple Comparison Test, a conservative statistical test which compares treatment groups to control values, was used. According to the analysis, a significant difference in cytotoxicity was noted between the 50 μM $HgCl_2$ treatments and control values ($p < 0.01$), while no significant difference was detected between the 5 μM $HgCl_2$ treatments and control values ($P > 0.05$). As seen in Figure 10, there is a distinct increase in absorbance when the cells are exposed to mercury, especially the larger concentration; this signifies an increase in LDH release upon treatment with mercury.

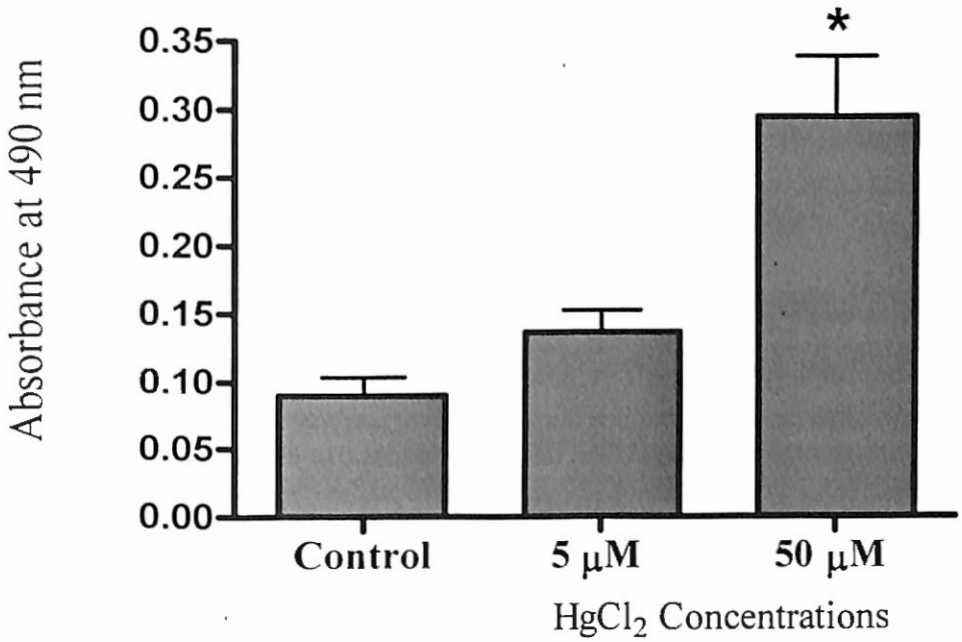

Figure 10. Effects of mercuric chloride on LDH release in SK-N-SH cells for 24h exposure period carried out in complete media. LDH release in the presence of 5 and 50 μM $HgCl_2$ is designated as Mean Absorbance at 490 nm ± SEM of four samples run in duplicate. There was a significant difference between the 50 μM $HgCl_2$ and control values ($P < 0.01$, Dunnett's Multiple Comparison Test), yet no difference was observed between 5 μM $HgCl_2$ and control values.

IV.D. Initiation of Apoptotic Mechanisms: Caspase-3 and -7 Detection

The amount of fluorescence measured using the Apo-ONE™ Homogeneous Caspase 3/7 Assay is relative to the quantity of caspase 3/7 enzymatic activity contained within the control and treated cells. The Relative Fluorescence Units (RFU) of each sample is determined by subtracting out the average value for the trypsin/media blank.

Statistical analysis of the fluorescence data using one-way ANOVA exhibited an overall significant ($F_{2,11} = 4.794$; $p = 0.0383$) difference between groups when treated with 5 μM mercury concentrations. Similar to the procedures used to evaluate the LDH data, Dunnett's Multiple Comparison Test was the post-hoc analysis employed to discern differences between treatment groups and control as well as the magnitude of said

differences. The data showed the resultant significant difference to be between the fluorescence measured after the 5μM $HgCl_2$ treatments and control values ($P < 0.05$) and not between the fluorescence measured after the 50 μM $HgCl_2$ treatments and control values ($P > 0.05$). The data depicts a decrease in RFUs when the cells are exposed to 5 μM $HgCl_2$ treatments, which represents a decrease in caspase 3/7 enzymatic activity (See Figure 11). However, the amount of relative fluorescence then increases back to control values when the cells are treated with 50 μM $HgCl_2$, indicating there is no change in caspase 3/7 enzymatic activity for the larger concentration.

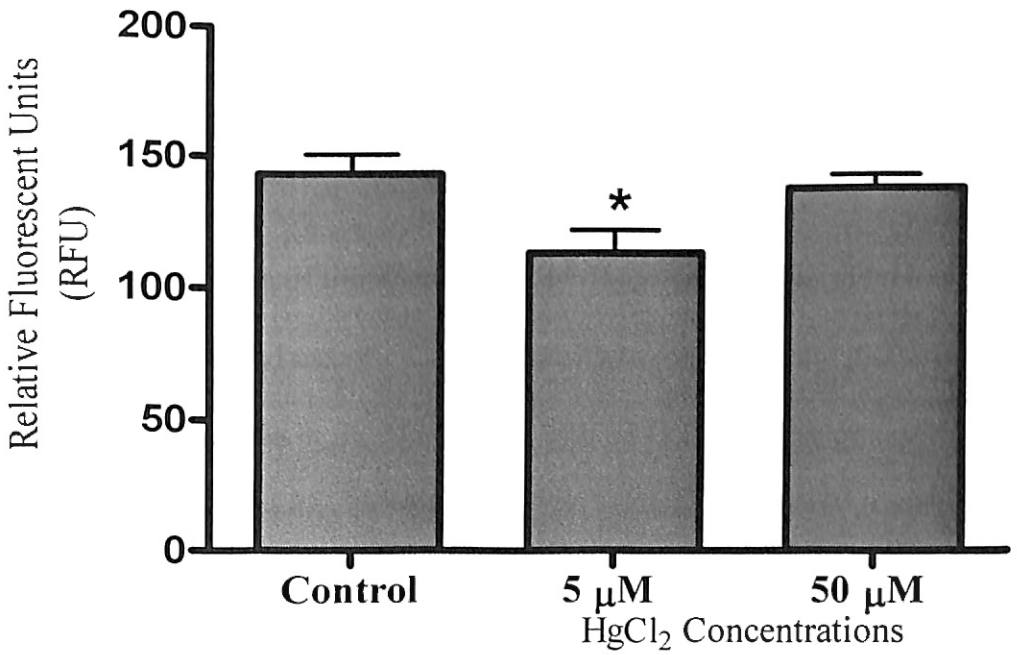

Figure 11. Effects of mercuric chloride on Caspase 3/7 enzymatic activity in SK-N-SH cells during 24h incubation period performed in growth medium. Caspase 3/7 enzymatic activity is expressed as Mean RFU ± SEM of four samples run in duplicate. The data indicate a significant difference between the absorbance measured in the 5 μM $HgCl_2$ treatments and control values ($P < 0.05$).

IV.E. Binding Studies: Affinity and Density of the Dopamine Transporter

All curves were fit to a single site with a range of K_d values of 6.5 to 27.4 nM (Table 5). Affinity for DAT is represented by K_d values, where a higher K_d corresponds to a lower affinity and a lower K_d indicates a higher affinity.

Table 5: Summary of K_d Values for [^{3}H] GBR-12935 Binding to DAT After Mercuric Chloride Treatment (24h)

	Control	***5 μM $HgCl_2$***	***50 μM $HgCl_2$***
K_d (nM)	6.574	27.43	9.257

Interestingly, control K_d values resembled previous report for [^{3}H] GBR12935 binding to DAT (6.5 nM). There was a four-fold shift in affinity of [^{3}H] GBR12935 in the 5 μM $HgCl_2$ group (27.4 nM), which returned close to control values in the 50 μM $HgCl_2$ group (9.3 nM).

Examination of transporter density (B_{max}) values reflected the changes that were observed in the LDH assays. Control density of 49.7 pmol/mg protein was similar to observed B_{max} values in the 5μM $HgCl_2$ group (41.0 pmol/mg protein). Treatment with the highest concentration of $HgCl_2$ (50 μM) resulted in a 69.1% reduction in [^{3}H] GBR12935 binding (49.7 pmol/mg protein to 15.4 pmol/mg protein) (Figure 12).

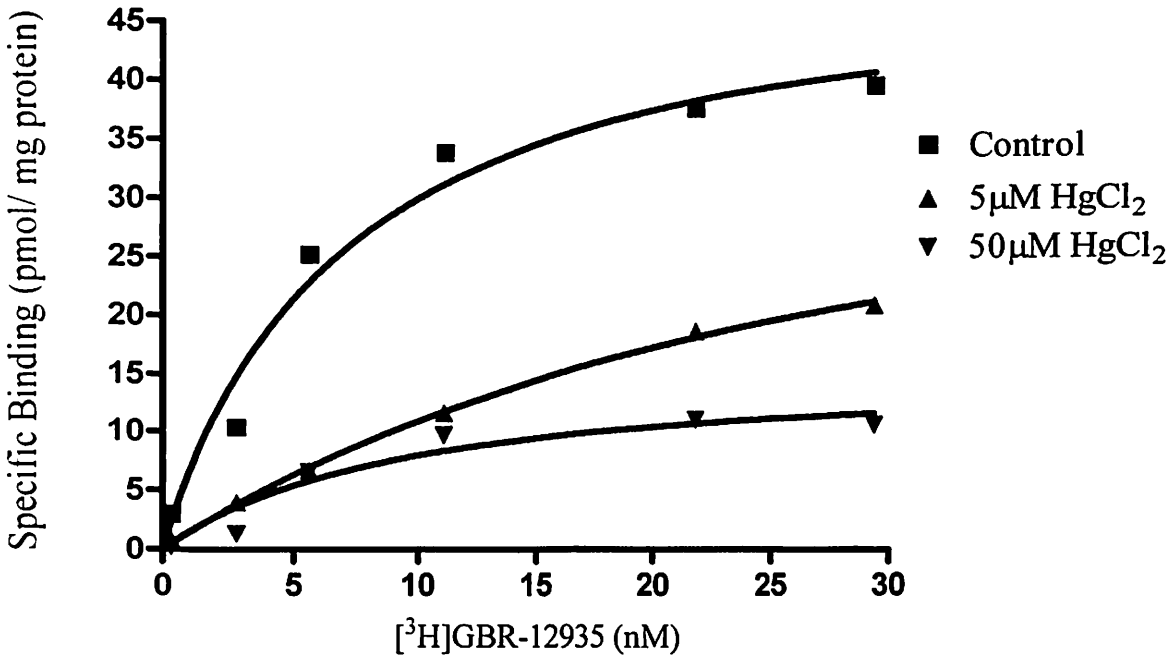

Figure 12. Saturation of [^{3}H] GBR-12935 binding to DAT in SK-N-SH cells. The cells were incubated with [^{3}H] GBR-12935 an for a final concentration of 1 nM - 30 nM for 60 minutes at room temperature. Non-specific binding was determined in the presence of 5 μM GBR-12909. Data expressed as pmol of [^{3}H] GBR-12935 bound per mg of protein from one experiment performed in duplicate. B_{max} values determined from these experiments for control, 5μM $HgCl_2$, and 50 μM $HgCl_2$ are 49.7, 41.0, and 15.4 pmol/ mg protein, respectively.

V. SUMMARY AND DISCUSSION

V.A. Summary of Research Findings

The main thrust for this research was to investigate how subtoxic concentrations of mercury affected normal functional, cellular and pharmacological aspects of neuronal cells, specifically the SK-N-SH cell line. The functional studies focused on the dopamine transporter due to the fact that it's main substrate, dopamine, has been linked to neurotoxicity through contributions to oxidative stress, mainly production of reactive metabolites. The cellular studies defined cell viability after mercury treatment by measuring the activity of lactate dehydrogenase and caspase, enzymes associated with two different methods of cell death, necrosis and apoptosis, respectively. The pharmacological studies were employed to determine the affinity of dopamine for the dopamine transporters found in SK-N-SH cells as well as the density of transporters. The overall findings from this project reveal that subtoxic concentrations of mercury do indeed produce functional, cellular and pharmacological changes in neuroblastoma cells, resulting in cell damage and/or cell death.

V.A.1. Functional Studies

There were three types of experiments run for the portion of the project focused on dopamine uptake: treatments incubated in assay buffer (refer to section III.A.3), treatments incubated in growth medium, and comparisons between treatments incubated in assay buffer and ascorbate-free assay buffer. For the 'in buffer' studies, SK-N-SH cells were exposed to mercury concentrations ranging from 1 nM – 100 μM $HgCl_2$ in the presence of assay buffer for 1 to 6h at room temperature. The 'in media' studies looked at cells exposed to mercury concentrations ranging from 100 nM – 1 mM $HgCl_2$ in the

presence of media for 1 to 48h at 37°C. The incubation temperature for the 'in media' studies differed in order to maintain the cells during longer treatment periods. The comparison studies that examined the effects of ascorbic acid in the assay buffer focused on 1, 10, and 100 μM $HgCl_2$ concentrations incubated for 6h also at room temperature. The rationale for different assay conditions was to assess whether the more complex complete RPMI-1640 would interfere with the actions of $HgCl_2$.

The interaction between time and concentration noted in the uptake measured at 1 and 3h may be caused by the initial action of the toxicant interacting with various cellular components, resulting in functional changes, but which are insufficient to prevent dopamine transport. The minimal effect of micromolar mercury concentrations on dopamine uptake during the 6h exposure period reflects a compensation mechanism where the toxic action has temporarily stopped. It is also important to note that the uptake data collected for experiments incubated in the assay buffer showed very little change in respect to control values, which suggests that certain components of the assay buffer may be attenuating damage to the cells or production of species that cause oxidative stress.

Prolonged exposure (> 6h) appears to allow for mercury to accumulate in the cells, resulting in cell damage and/or cell death. The uptake studies performed in the growth medium for longer periods of time demonstrate this proposed explanation. The data illustrate an extreme increase in [^{3}H] dopamine uptake when treated with 10 μM $HgCl_2$, followed by a severe drop in uptake. This may be a result of an overloaded dopamine turnover system that can no longer compensate for all the 'free' dopamine located in the synapse, which when metabolized can lead to the production of damaging

hydroxides. The fact that dopamine uptake does decrease when the cells are treated in the growth medium supports the idea that the composition of the media differs from that of the assay buffer in that it does not contain as many (if any at all) neuroprotective agents.

The experiments that compared incubation in the assay buffer to incubation in a modified assay buffer lacking ascorbic acid showed a significant difference of [^{3}H] dopamine uptake measured. Although the cells were exposed to the same concentrations of mercury (1, 10, 100 μM $HgCl_2$), those incubated in the assay buffer maintained uptake values which were similar to control while those incubated in the ascorbate-free assay buffer showed a decrease in dopamine uptake between 10 μM and 100 μM $HgCl_2$ treatments. Perhaps the severe drop in dopamine uptake represents a toxicity threshold exhibited by the cells when treated in assay buffer without ascorbate. This difference in dopamine uptake also depicts the potency of ascorbic acid as an antioxidant, which can conceivably bind to the reactive oxidative species and prevent damage to the transporter for the samples run in the unadulterated assay buffer.

V.A.2. Cellular Studies: Initiation of Necrotic or Apoptotic Mechanisms

The cellular studies, which focused on enzymatic activity, LDH and caspase assays respectively, were used to assess cytotoxicity at different mercury concentrations. SK-N-SH cells were treated with 5 and 50 μM $HgCl_2$ for a 24h period in both media and assay buffer.

The LDH assay revealed an effect of concentration; as the concentration of mercury increases, the amount of LDH release also increases. One can infer that LDH release follows a stepwise progression as the concentration of mercury increases until the

cells are completely obliterated and LDH release plateaus. The assay for caspase activity showed a decrease with the 5 μM $HgCl_2$ treatment, with no change in the data for the 50 μM $HgCl_2$ treatment. The lack of increase in caspase 3/7 activity compared to controls suggests apoptosis is not involved in the observed changes in DAT activity. Therefore mercury does not induce apoptotic cell death at these concentrations. The data from the LDH and caspase assays indicate that when cells are treated with low levels of mercury (5 and 50 μM $HgCl_2$) in the presence of media, the cells are dying, but not by apoptotic mechanisms.

V.A.3. Pharmacological Studies

To complement the characterization of the DAT inhibition by $HgCl_2$, the interaction of $HgCl_2$ with the DAT was examined using binding studies to address pharmacological changes in the treated cells. Similar to the cellular studies, SK-N-SH cells were treated with 5 and 50 μM $HgCl_2$ for a 24h period at 37°C in the growth medium.

The data from the binding studies showed changes in both the affinity and density of the dopamine transporters when SK-N-SH cells were treated with low-level mercury concentrations. When the cells were treated with 5 μM $HgCl_2$, the affinity of [^{3}H] GBR-12935 for DAT decreased four-fold, while there was no effect with 50 μM $HgCl_2$. The shift to lower affinity means the transporter is moving substrate (DA) at a high capacity and DA is able to freely diffuse into the cells, possibly reflecting the increase in DA uptake noted with 10 μM $HgCl_2$ treatments. The changes in transporter density occurred with the 50 μM $HgCl_2$ treatments but not the 5 μM $HgCl_2$ group; decreasing transporter density by over 50%. This corresponds to the differences in LDH release noted earlier;

while the transporter density decreases and transport sites are being lost, LDH release increases due to toxicity brought on by an incapacitated dopamine turnover system.

V.B. Discussion

V.B.1. Characterization of the Dopamine Transporter Using GBR-12909 and Mazindol

The data collected during the characterization studies supported the previous finding that GBR-12909 is a more selective inhibitor of the DAT. In order to differentiate whether the labeled dopamine is being taken up through the dopamine or norepinephrine transporters located on the cells, this selective inhibitor was paired up with one of the more nonselective DAT inhibitors, mazindol. The amount of dopamine uptake measured in the cells treated with GBR-12909 represents the amount of labeled dopamine that entered via DAT compared to control values while the amount of dopamine uptake measured in the cells treated with mazindol represents the amount of labeled dopamine that entered via DAT and NET compared to control values. By subtracting the uptake measured with mazindol from total uptake (control values) accounts for dopamine entering through DAT and NET. A further step of subtracting out the uptake measured with GBR-12909 from that value isolates the amount of dopamine that was taken up through DAT.

V.B.2. Measuring [^{3}H] Dopamine Uptake After Toxin (Hg^{2+}) Exposure

The data from these functional studies suggest that the toxicity of mercury appears to be more concentration-dependent than time-dependent, even at lower concentrations. The observation that as the mercury concentration increases, the amount of dopamine uptake decreases implies that exposure to low levels of mercury can result in consequential effects on dopamine transport. Since the dopamine transporter is the

primary compensatory mechanism that regulates extracellular dopamine concentrations and maintains the homeostasis of presynaptic function in dopaminergic neurons (Torres, et al., 2003), any modifications in dopamine transport has the potential to interfere with normal dopaminergic function.

The work of Chen and associates (2000) found that substrate uptake by DAT is a highly temperature-dependent, active transport process. This lends support to the "All or Nothing" phenomenon noted in the data where mercury treatments were carried out in the growth medium at 37°C, which was not apparent in the data where mercury treatments were performed in assay buffer (refer to section III.A.3) at room temperature. The sudden decrease in dopamine uptake for $HgCl_2$ concentrations greater than 100 μM for the 'in media' studies may be resultant of the higher temperature increasing the production of free radicals, possibly accelerating the dysfunction of DAT. The observed differences in dopamine uptake between the media and assay buffer experiments could also be related to the specific components of each solution and how they may react in oxidative environments.

As previously mentioned, when uptake assays were conducted in the media, dopamine uptake was virtually abolished following a 3-fold increase in uptake observed at 10 μM $HgCl_2$. It has been suggested that one reason levels of dopamine (quantitated by [^{3}H] DA uptake) have been shown to increase after administration of $HgCl_2$ may be due to the toxic metal's mechanism of action on various aspects of neuronal function; including release of neurotransmitter, reuptake and transporter systems (Faro, et al., 2001). The fact that amounts of dopamine uptake did not fluctuate over time (1, 6, and 24 h) with 10 μM treatments, but only changed as the concentration of mercury increased

provides evidence that the SK-N-SH cell line may exhibit a toxicity threshold between 10 and 100 μM $HgCl_2$. Kim and Sharma (2004) found an EC_{50} value of approximately 70 μM of $HgCl_2$ in their work with BALB/c macrophage cells, which means this concentration of mercury resulted in 50% cell death. This also lends support to the severe change in dopamine uptake between 10 – 100 μM $HgCl_2$ noted in the data collected.

The increase in dopamine uptake at 10 μM $HgCl_2$ treatments performed in the growth medium may be indicative of an upregulation of the dopamine transporter. This could be due to insertion of reserve or "spare" DAT and not necessarily new transporter proteins, which would take longer than 24 hours to synthesize and insert into the membrane. Our data suggests that upregulation did not occur as indicated by no changed in B_{max}. Increased uptake may reflect an increase in dopamine release; in order to compensate for the higher levels of extracellular dopamine in the synaptic space, the cells may increase, or upregulate the number of transporters to take up more dopamine. Wu, Coffey and Reith (1997) observed Hg^{2+} to have a biphasic effect on both [^{3}H] mazindol and [^{3}H] WIN 35,428 binding to the dopamine transporter; with a stimulatory component in the low micromolar range and a strongly inhibitory component at 30-100 μM in glioma cells expressing human DAT. If the number of binding sites decreases, then the number of transporters must also decrease. The data from the experiments performed in the growth medium show a similar biphasic pattern with a decrease in dopamine uptake at higher mercury concentrations. Perhaps the decrease is due to depletion in the number of DAT, a suggestion that is supported by this data, which demonstrated a 70% reduction in the B_{max} of DAT.

Due to interaction of metal ions with sulfhydryl groups in the dopamine transporter, both dopamine uptake and binding of uptake inhibitors are changed when exposed to metal ions (Ferrer and Javitch, 1998), especially with mercury because of its high affinity for sulfhydryl groups. Previous research has suggested sulfhydryl groups play a key role in binding to the dopamine uptake complex (Richfield, 1993). If reduced sulfhydryl groups (bound to electrophiles) have the ability to alter the affinity states of uptake inhibitor drugs or transmitter for DAT (Richfield, 1993), the acute decrease in uptake noted in the data for higher mercury concentrations could be attributed to a reduced affinity of dopamine for the DAT.

Sulfhydryl groups have also been associated with conformational changes in the dopamine transporter protein, which has been shown to affect both inward and outward DA transport (Chen, et al., 2000). The human dopamine transporter contains 13 cysteine residues (Torres, et al., 2003); therefore providing 13 potential sites for mercury to bind (refer to Figure 1) and alter the conformation of the transporter. In reference to the bimodal pattern observed, the stimulatory effect of Hg^{2+} has been attributed to a conformational change in the DAT caused by creating disulfide linkages between two intra- or intermolecular cysteine sulfhydryl groups (Schweri, 1994), whereas the inhibitory effect probably involves recognition of cysteine –SH groups intimately linked with the binding sites for uptake blockers (Wu, et al., 1997). According to Torres, Gainetdinov and Caron (2003), a cysteine residue located in the third intracellular loop of DAT was found to be more reactive to thiol-modifying agents like mercury during uptake, implying that this residue participates in a conformational reorganization of DAT during substrate translocation, ultimately impeding DAT function. Perhaps the extreme

increase in dopamine uptake noted with 10μM treatment is resultant from mercury binding to sulfhydryl groups, altering the conformation of DAT such that inward transport of extracellular dopamine has stopped and in order to remove excess dopamine from the synapse, the cells must upregulate DAT expression. The severe decrease in dopamine uptake observed at higher concentrations could be a consequence of the dopamine uptake system simply becoming overloaded by excess dopamine and causing complete cessation of transporter function. Mercury's ability to alter DAT conformation substantiates the biphasic response demonstrated by the data.

The work of Hussain and colleagues (1997) concluded that exposure to mercury ($HgCl_2$) decreased SOD, Cu/Zn-SOD and Mn-SOD activity; all of which play a role in the cellular defense mechanisms that work to prevent accumulation of reactive oxygen species brought on by oxidative stress. The differences in dopamine uptake between experiments run in assay buffer and experiments run in ascorbate-free assay buffer (as seen in Figure 8) suggests that the presence of ascorbic acid, a potent antioxidant, may attenuate the effects of mercury-induced oxidative stress. Similarly, previous research found that other antioxidants, DPPD and Trolox, were not effective in the prevention of $HgCl_2$-induced cell death (Lee, et al., 2001). Although antioxidants may prolong the life of the cell by binding to free radicals and alleviating oxidative stress, they have not been proven to ultimately prevent cell death.

V.B.3. Cytotoxicity and Cell Viability

In order to relate the changes observed in dopamine uptake to overall cellular function, the concentrations of mercury (5 μM and 50 μM) used for the LDH and caspase experiments were chosen because these concentrations surround the increase in

dopamine uptake noted with the 10 μM $HgCl_2$ treatments. The cells were incubated for 24h in the growth medium, similar to conditions in the uptake studies, but also in the assay buffer to examine any variation in cellular function when exposed to mercury in different solutions.

Lactate dehydrogenase is a cytosolic enzyme often used as an indicator of toxicity, specifically defining cell death (Hodgson and Smart, 2001). LDH release has been closely associated with several cellular properties, including membrane stability, transporter channel function and other metabolic activities. According to Allen and Rushton (1994), *in vitro* release of LDH from cells provides an accurate measure of cell membrane integrity and cell viability. Through colorimetric or radioactive assays, the measurement of lactate dehydrogenase released from cells into the medium has been established as a useful parameter affected after toxic cellular events (Reichl, et al., 2001), such as incubation with a toxic oxidative metal, like mercury. As purported by previous research, an increase in the LDH release may reflect a wide spectrum of intracellular or membrane influences (Reichl, et al., 2001). For these experiments, an increase in LDH (compared to control values) is indicative of cell death subsequent to mercury treatment.

The data from the LDH experiments where the growth medium was the solution used for the incubation period showed a similar relationship concerning cellular function between absorbance and concentration that was observed between dopamine uptake and concentration. As the concentration of mercury treatment increased, the amount of absorbance increased, which equates to the amount of LDH increased, indicating a loss of plasma membrane integrity and/or cell death. A loss of cellular function was suggested by a decrease in dopamine uptake as the concentration of mercury treatments increased.

In their research, Lee, Ha and Kim (2002) found $HgCl_2$ had concentration-dependent effects on cytotoxicity in glioma cells. Reichl and colleagues (2001) observed a comparable phenomenon in their work with lung cells; finding an increase in LDH release after exposure to $HgCl_2$ when compared to control values.

The data from this assay demonstrated a significant increase in LDH release for SK-N-SH cells exposed to 50 μM $HgCl_2$ for 24h compared to control values, but statistical analysis (Dunnett's Multiple Comparison Test) failed to find a significant difference between the LDH release measured from cells treated with 5 μM $HgCl_2$ for 24h and control values. In previous work, mercury compounds, including $HgCl_2$, were found to increase LDH release, at concentrations greater than 10 μM for 8 and 24 hours exposure periods in lung cells (Walther, et al., 2002), again suggesting that the cells may exhibit a toxicity threshold, and once surpassed, results in cell death. This conclusion corroborates the data gathered from the LDH assay; finding significant LDH release at higher concentrations (50 μM $HgCl_2$) but not at lower concentrations (5 μM $HgCl_2$) compared to control levels.

V.B.4. Initiation of Apoptotic Mechanisms: Caspase-3 and -7 Detection

The various stages associated with chemically induced apoptosis are triggered by caspase cascades (Hodgson and Smart, 2001), which initiate the sequential dysregulation of mitochondrial function, resulting in cell shrinkage and nuclear fragmentation; ultimately leading to the 'systematic dismantling' of the cell. Caspases have been classified as a family of aspartate-specific cysteine proteases, which are key effectors responsible for many morphological and biochemical changes in apoptosis (Kim and Sharma, 2004). Due to the fact that activated caspase enzymes participate in the

proteolytic cleavage events found in dying cells, caspase-3 activation has been deemed an essential step to actuate the cascade of apoptotic processes (Kim and Sharma, 2004). This enzyme is also responsible for activating DNA fragmentation factor, resulting in fractured DNA (Kim and Sharma, 2004), another aspect leading to cellular dysfunction.

Although statistical analysis showed a significant difference between relative fluorescence measured for cells treated with 5 μM $HgCl_2$ and control values, there was not a marked difference between the average RFU values for both groups. Therefore, mercury-induced cytotoxicity for these concentrations does not appear to be from apoptotic mechanisms. The data presented by Kim and Sharma (2004) reported an EC_{50} value of 81.1 μM $HgCl_2$ for caspase-3 activity when murine macrophage cells were incubated with mercury concentrations (20 – 150 μM) for 24h. Since the mercury concentrations examined during this study did not exceed the EC_{50} value, this may serve as an explanation as to why little increase in relative fluorescence was noted. This suggests that larger concentrations of mercury are required to induce apoptotic cell death.

The work of Yang and colleagues (2002) found that SK-N-SH cells cultured in a similar medium (RPMI 1640 supplemented with 10% heat-inactivated FBS) for longer periods of time (5 days vs. 1 day) exhibited a 6-fold increase in caspase-3 activity. For the LDH and caspase studies, a span of four days passed from when the cells were plated out to when they were treated with mercury, followed by an additional 24h incubation period and another 8h required by the modified caspase 3/7 protocol. Perhaps the lack of change in RFU between control values and 50 μM $HgCl_2$ treatments is due to the different culture periods; since there is normally a low level of caspase activity in the cells, and a prolonged culture period may also increase caspase activity, any further

caspase activity resultant of mercury-induced apoptosis may not be detectable due to fluorescence saturation.

V.B.5. Binding Studies: Affinity and Density of the Dopamine Transporter

These data represent the first partial characterization of [^{3}H] GBR12935 binding to DAT in SK-N-SH cells. The affinity of [^{3}H] GBR12935 (6.5 nM) resembled the affinity of [^{3}H] GBR12935 (5.5 nM) previously reported in mammalian tissue (Andersen, 1987). Surprisingly, this is contrary to previous beliefs that the predominant catecholamine transport system in SK-N-SH is NET. This would suggest that prevalence of DAT is actually considerable higher than what was first determined using transporter message expression. The changes that were observed in transporter density paralleled the changes that were observed in LDH release and loss of [^{3}H] dopamine uptake in the 50 μM $HgCl_2$ group following 24 hour exposure; i.e., cells die, leading to loss of DAT. The uptake of dopamine was previously determined to be unchanged in the 5 μM $HgCl_2$ group at 24 hours, a finding substantiated by the saturation data with minimal changes in the B_{max} of [^{3}H] GBR12935 binding. Interestingly, there is a reduction in affinity in this group. Collectively, these data suggest that a reduction in affinity with no observed loss in dopamine uptake may be a prelude to the loss of dopamine uptake in functional studies and loss of [^{3}H] GBR12935 binding sites in the saturation analysis. Additional work is needed to further the pharmacological characterization of dopamine uptake sites in SK-N-SH cells.

V.C. Further Research

Future work includes examination of intra- and extracellular events that may lead to a decline in neuronal function following mercury exposure. These events could be

oxidative stress, changes in intracellular signaling systems and potential damage to cell surface proteins and lipids.

Oxidative stress can be quantified using dichlorofluorescein assay (Wang and Joseph, 1999); where reactive oxygen species present in the sample oxidize nonfluorescent dichlorofluorescein to produce the extremely fluorescent dichlorofluorescein. By employing this experiment, one can determine whether the cytotoxicity induced by mercury is associated with oxidative stress or due to other cell damaging mechanisms. Microscopic examination of the cells post-mercury treatment may allow for specific cellular changes to the membrane and nuclear material to be charted over time, noting especially chromatin and cell condensation, blebbing, DNA fragmentation, lysosomal degradation, lysis, etc. This information may provide support to the data from the uptake studies, which suggest subtle cellular changes may be occurring before DAT function is affected.

Previous research using neuroblastoma and glial cells concluded that lower and intermediate metal concentrations led to inhibition of neurospecific endpoints, defined by neuron-specific enolase (NSE) as a neuronal marker and glia-specific beta-S100 protein as a glial marker, while no changes were noted concerning LDH release (Huang, et al., 1993). These findings suggest that although release of LDH is indicative of overall cytotoxic effects, these nerve-specific changes may serve as a more sensitive index of neurotoxicity in similar cell lines. According to Huang and colleagues (1993), in a neural cell line, the most sensitive endpoints to neurotoxic insults are neurospecific, because while non-specific cellular endpoints only reflect the generic functions required for cell viability, by definition, tissue- or organ-specific endpoints hold special relevance to that

particular cell or tissue type. Focusing on neurospecific biomarkers may provide a more complete understanding of how mercury affects neuronal function with respect to the dopaminergic neurons in the substantia nigra and what intermediary changes are occurring to initiate toxic mechanisms in these cells.

Supplementary studies to examine potential temperature- and growth medium/assay buffer (refer to section III.A.3) composition-dependent effects on dopamine uptake could be performed to more closely reflect what would be seen *in vivo*. Parallel *in vivo* studies using striatal synaptosomes from rats where mercury is administered via injection or implantation of a mini-pump would also offer data concerning functional, cellular and pharmacological effects comparing mercury treatment and control values. These experiments may help to better gauge the threat of mercury contributing to the progression of neurodegenerative disease. *In vivo* studies could additionally be used to directly target the possible link between mercury and Alzheimer's disease. After exposing the rats to low concentrations of mercury, tests could be performed to measure the quantities of β-amyloid peptide or phosphorylated tau protein in conjunction with apolipoprotein genotyping to investigate whether the combination of homozygous ε_4 phenotype and exposure to mercury increases the animal's susceptibility to AD. *In vivo* experiments would also provide an opportunity to evaluate the potential protective action of antioxidants and possible chelators after mercury treatments.

V.D. Conclusions

- The data suggest that $HgCl_2$ effects on dopamine uptake are concentration-dependent and not time-dependent. The observation that reductions in dopamine uptake are concentration-dependent could indicate that exposure to low amounts

of mercury could result in robust effects on DA transport. Any number of changes in DA transport may have the capacity to initiate a cascade of biological events leading to cell damage and/or cell death.

- Examination of LDH and caspase enzymatic activity revealed that lower concentrations of mercury do indeed result in cytotoxicity. The increase in LDH release noted with the 50 μM $HgCl_2$ treatment and lack of increase in caspase activity suggests that apoptosis does not play a role in the observed changes in DA transport. Instead, the data demonstrate that mercury-induced cytotoxicity is more a result of necrotic mechanisms, due the overall trauma sustained by treated cells.
- Although previous research has suggested that the SK-N-SH cell line expresses more NET, the data from the binding studies indicate these cells have a high density of dopaminergic sites (49.7 pmol/ mg protein). The density of DAT decreases as the concentration of mercury increases; the data reveal a 70% decrease in density for the higher concentration (50 μM $HgCl_2$).
- Mercury treatment also resulted in pharmacological changes in radioligand binding to the DAT. The shift to lower affinity upon treatment with 5 μM $HgCl_2$ switches the DAT to a high-capacity transport system, allowing more DA to cross into the cell. The shift back to high affinity observed with 50 μM $HgCl_2$ may be explained by the significant reduction in transporter density, leaving only the resilient cells, which express the high affinity transporter.

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