THE EFFECTS OF SUB-TOXIC HEAVY METALS ON DOPAMINE TRANSPORTER FUNCTION

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LIST OF SYMBOLS

WHO	World Health Organization
EPA	Environmental Protection Agency
ATSDR	Agency for Toxic Substances and Disease Registry
MMT	Methylcyclopentadienyl manganese tricarbonyl
NIH	National Institute of Health
ADHD	Attention Deficit Hyperactivity Disorder
CNS	Central Nervous System
DOPAC	3,4,-Dihydroxyphenylacetic acid
HVA	Homovanillic acid
ATP	Adenosine triphosphate
NMDA	N-methyl-D-aspartate
DNA	Deoxyribonucleic acid
ROS	Reactive oxygen species
Mn	Manganese
GABA	Gamma-aminobutyric acid
Na^{+}	Sodium ion
Ca^{2+}	Calcium ion
Mg^{2+}	Magnesium ion
GSH	Glutathione

DAT	Dopamine transporter
SERT	Serotonin transporter
NET	Norepinephrine transporter
GAT	GABA transporter
GLYT	Glycine transporter
Taurt	Taurine transporter
PRO	Proline transporter
MPP^+	1-methyl-4-phenylpyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
$[^3H]DA$	Tritiated dopamine
$HgCl_2$	Mercuric chloride
$MnCl_2$	Manganese chloride
GBR-12,935	1-[2-(diphenylmethoxy) ethyl]-4-(3-phenylpropyl) piperazine
GBR-12,909	1-[2-[Bis(4-fluorophenyl)methoxy]ethyl]-4-(3-phenylpropyl) piperazine
EC_{50}	Median effective concentration

I. INTRODUCTION

Heavy metals are well known to cause adverse effects in living organisms. At both high and low concentrations, heavy metals disrupt the normal functioning of various systems including the cardiovascular, respiratory, and central nervous systems. Because of the serious overt effects of high concentrations of heavy metals to the populations, many organizations such as the World Health Organization (WHO) and the Environmental Protection Agency (EPA) have set standards, restrictions, and recommendations as to the amount of heavy metals an individual should be exposed to. These set levels while low, may not encompass the sub-toxic concentrations that can cause deleterious effects. A better understanding of how sub-toxic concentrations affect the population could either confirm the set levels or require them to be reevaluated and lowered. Therefore, the question is, "how do these metals affect the organism?" The purpose of this study is to determine how "long-term" exposure to sub-toxic concentrations of mercury or manganese affects the functioning of the dopamine transporter.

Individuals are exposed to toxic metals through a variety of ways. Naturally occurring processes, certain occupations, waste sites, and accidents can all lead to increased contact, absorption and toxicity. The extent of toxicity an individual may experience is partly dependent upon the metal, dose, and route of exposure, and partly dependent on the individual affected. Characteristics, such as age (children and the

elderly have been shown to be more susceptible to toxins), genetics, and current or developing disease states impact how an individual will react to the metal exposure.

Volcanic eruptions, erosion, occupations such as those in the thermometer and dry-cell battery industries, and dentistry are all routes of mercury exposure (Levine, 2003; Tchounwou et al., 2003). Individuals may also be exposed to mercury through dental amalgams and a diet of fish (Aschner et al., 2000; Levine, 2003). Other populations that the Agency for Toxic Substances and Disease Registry (ATSDR) consider to be at an increase risk of toxicity due to mercury exposure are those living close to a former mercury mining site, recycling facility, and municipal or medical incinerator (Patrick, 2002). Waste sites from industrial dumping and the burning of coal increases the environmental mercury content which also increase the amount of exposure to the population (Patrick, 2002; Levine, 2003).

Exposure to manganese can also occur through a variety of methods. Individuals employed in welding, mining, and steel manufacturing occupations are among those at risk for elevated manganese exposure and its harmful effects (Di Lorenzo et al., 1996; Crossgrove & Zheng, 2004; Kitazawa et al., 2005). The antiknock agent, methylcyclopentadienyl manganese tricarbonyl (MMT), and fertilizers and pesticides containing manganese increase the environment's and individual's exposure levels to manganese (Crossgrove & Zheng, 2004). Waste sites, such as Tar Creek, are also a means for increased manganese exposure and toxicity. According to a news release from the National Institute of Health in July of 2004, "Tar Creek is a residential area in Northeastern Oklahoma that is heavily contaminated with metals from mining waste. The principal pollutants are lead, cadmium, zinc, iron and manganese (NIH news release

2004)." Manganese exposure also occurs naturally through ingestion of certain food such as whole grains and nuts (Greger, 1999).

The dopamine transporter, a plasma protein, is responsible for terminating dopamine's actions (Sorkina et al., 2003). Through the regulation of the dopamine transporter, the entire dopaminergic system can be affected because of this transporter's effect on the neurotransmission of dopamine. The dopamine transporter in turn can be regulated by substrates, proteins such as alpha-synuclein, oligomerization, and transport into and out of the plasma membrane. The effects of manganese and mercury on the dopamine transporter have also been studied and reported, with manganese studies being more numerous and on going. In contrast, the most recent report on the effects of mercury on the dopamine transporter, as determined through a search of PUBMED with key words "dopamine transporter" or "dopamine uptake" and "mercury," was in 1997 by Wu, Coffey and Reith. This may reflect the finding that manganese specifically affects the dopaminergic system, while mercury's effects appear to be more general.

I.A. Objective of Study

The objective of this study is to determine the effects of long-term exposure to sub-toxic concentrations of mercury and manganese on the dopamine transporter function. Studies aimed at measuring [^{3}H]dopamine uptake and [^{3}H]GBR-12935 binding to the dopamine transporter expressed in the cell membrane will provide the opportunity to see how the functioning of the transporter is affected by low concentrations of these metals.

II. LITERATURE REVIEW

II.A. Mercury

The toxic effects of mercury have been observed over hundreds of years. During the Roman era, mercury intoxication was observed in the slaves mining the Spanish Almaden mine (Asano et al., 2000). This mine is currently still one of the dominant mines, having produced 236 tons of mercury in 2000 (Hylander & Meili, 2003). Mercury intoxication reportedly was experienced in the year 752 by the builders of the giant bronze Buddha, which is coated with a mixture of gold, tin and mercury (Asano et al., 2000). Another group of working individuals, those in the fur and felt hat-making industries in the 17th century also experienced mercury intoxication (Asano et al., 2000).

More recent outbreaks of mercury toxicity occurred at Minamata Bay, Niigata, Japan, Iraq and along the Amazon River Basin. The Minamata Bay and Niigata, Japan mercury toxicity outbreaks occurred from eating fish with high levels of mercury. Industrial dumping of wastes in the water supplies caused a bioaccumulation of mercury in the fish, which then affected the humans and animals that ate the fish (Gochfeld, 2003; Castoldi et al., 2001). These individuals were diagnosed with Minamata Bay disease, a disorder characterized by symptoms such as visual, hearing, and other sensory impairment, muscle weakness and mental deterioration (Castoldi et al., 2001). In Iraq, mercury poisoning occurred after grain that had been treated with pesticide containing mercury was made into bread and eaten (Castoldi et al., 2001). Mercury toxicity also occurred along the Amazon River Basin due to gold mining operations, deforestation and

damming of rivers (Asano et al., 2000; Gochfeld, 2003). All of these actions cause an increase in mercury levels in fish, which are then consumed by the indigenous people (Gochfeld, 2003).

II.A.i. Characteristics of Mercury

Mercury, a known toxicant, is ubiquitous in the environment and naturally exists in three forms: elemental, inorganic and organic (Tchounwou et al., 2003; Levine, 2003). Elemental mercury, also known as metallic mercury, has been used in thermometers and dental amalgams (Langford & Ferner, 1999; Patrick, 2002). It has a melting point of -38.87°C and while it is not absorbed well from the gastrointestinal tract, when the vapors are inhaled, this form of mercury easily crosses into the circulatory system from the lungs (Tchounwou et al., 2003). Inorganic mercury salts have many uses. A few of these uses include(d) some medicines, skin lightening creams among various other products such as antibacterials and explosive detonators (Langford & Ferner, 1999; Tchounwou et al., 2003). This form of mercury has a low absorption rate when ingested (7-15%) (Patrick, 2002), however extended exposure to mercury salts can lead to CNS damage (Tchounwou et al., 2003). The third form of mercury, organic mercury, is believed to be the most toxic of the three forms (Patrick, 2002). Two examples of organic mercury are ethylmercury, a component of thimerosal (used to preserve vaccines), and methylmercury.

II.A.ii. Central Nervous System

The central nervous system (CNS) is one of the main targets of mercury accumulation and damage. This metal's effects on the CNS are well known and numerous (Tchounwou et al., 2003; Gasso et al., 2001). Some of these detrimental

effects include disruption of neuronal migration (Tchounwou et al., 2003), neuronal degeneration (Castoldi et al., 2001), and demyelination (Patrick, 2002). Mercury is capable of harming the blood-brain barrier by damaging the cerebral microvascular endothelial cells that compose this important barrier (Papp et al., 2005). Ca^{2+}-channels, neurotransmitter turnover, and nerve conduction are also affected by mercury (Papp et al., 2005). High dose mercury exposure can cause the cerebellum and limbic structures to undergo morphological and biochemical changes in both adult and developing central nervous systems (Vicente et al., 2004; Costa et al., 2004). Lesions can be found in this area, as was seen in individuals diagnosed with Minamata Bay disease (Costa et al., 2004).

However, there are still questions about mercury's mechanism(s) of toxicity (Bucio et al., 1995; Shanker & Aschner, 2001). Questions still needing to be answered include: what is the primary cellular target (Bertossi et al., 2004)? While some authors report that mercury primarily affects astrocytes, others argue that the primary targets are neurons (Bertossi et al., 2004). Another question that needs to be addressed is, what is mercury's effect(s) on the differentiation of the blood brain barrier (Bertossi et al., 2004)?

II.A.iii. Mercury and the Dopaminergic System

While not a specific target of mercury, the dopaminergic system is still affected by its toxicity. Mercury increases the extracellular levels of dopamine through inhibiting synaptic uptake of this neurotransmitter (Crinnion, 2000). It is hypothesized that this action may be due to the inhibitory effects mercury has on Na^+, K^+-ATPase which is believed to play a role in catecholamine uptake (Rajanna & Hobson, 1985). Increases in dopamine release have been reported after mercury treatment (Faro et al., 2001).

Extracellular levels of dopamine metabolites, 3,4,-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), are decreased in cases of mercury exposure, which may be explained by the decrease of dopamine synthesis that occurs (Faro et al., 2001).

It has also been recognized that prenatal exposure to mercury affects the dopamine system. Altered locomotor activity was reported from studies examining the outcomes from prenatal mercury exposure (Castoldi et al., 2001). Additionally, individuals who are prenatally exposed to mercury may be at risk for developing pathological conditions such as Parkinson's disease or Alzheimer's disease later in life because of these early dopaminergic changes (Castoldi et al., 2001).

II.A.iv. Cellular Mechanism of Toxicity

Whether the primary cellular target of mercury is astrocytes or neurons, mercury can disturb cellular function through a variety of mechanisms. Altering calcium ion homeostasis (Juarez et al., 2005; Patrick, 2002), depleting antioxidants, especially glutathione (Bucio et al., 1999), and perturbing mitochondrial membrane potential (Juarez et al., 2005) have all been reported upon mercury exposure. Disturbed glutamate activity (Juarez et al., 2005) and enhanced formation of reactive oxygen species (Ercal et al., 2001) are cellular effects of mercury toxicity. Mercury has been shown to inhibit Na^+-K^+-Cl^- cotransport (Jacoby et al., 1999), and to decrease ATP levels (Fonfria et al., 2005) as well. These effects, both individually and in combination, can all lead to cell death through apoptosis and/or necrosis.

In astrocytes, mercury inhibits the uptake of the excitatory neurotransmitters glutamate and aspartate (Shanker & Aschner, 2001; Aschner et al., 2000). Mercury further increases the extracellular levels of these amino acids by inducing their release

from intracellular stores (Shanker & Aschner, 2001; Castoldi et al., 2001). Such an increase in extracellular glutamate and aspartate can cause the N-Methyl-D-Aspartate (NMDA) receptors to overactivate, which could possibly cause the cell to enter into the excitotoxic cascade (Aschner et al., 2000; Juarez et al., 2005). Neurodegeneration (Castoldi et al., 2001), neuronal lesions (Aschner et al., 2000), and DNA damage (Juarez et al., 2005) may be accelerated or develop in response to these excitotoxic effects.

Due to the high affinity, approximately, 10^{-15}-10^{-23} of mercury for thiol groups, the major chemical reaction of mercury is with this group (Castoldi et al., 2001; Aschner et al., 2000). Glutathione levels, in particular, have been reported to be decreased due to mercury. This decrease of glutathione results in a reduced cellular defense against oxidative damage (Shanker & Aschner, 2001). Decreased levels of glutathione also provides an opportunity for the formation and accumulation of reactive oxygen species (ROS) along with the potential for an increase in damage done by other toxins from the environment (Shanker & Aschner, 2001; Vicente et al., 2004; Crinnion, 2000).

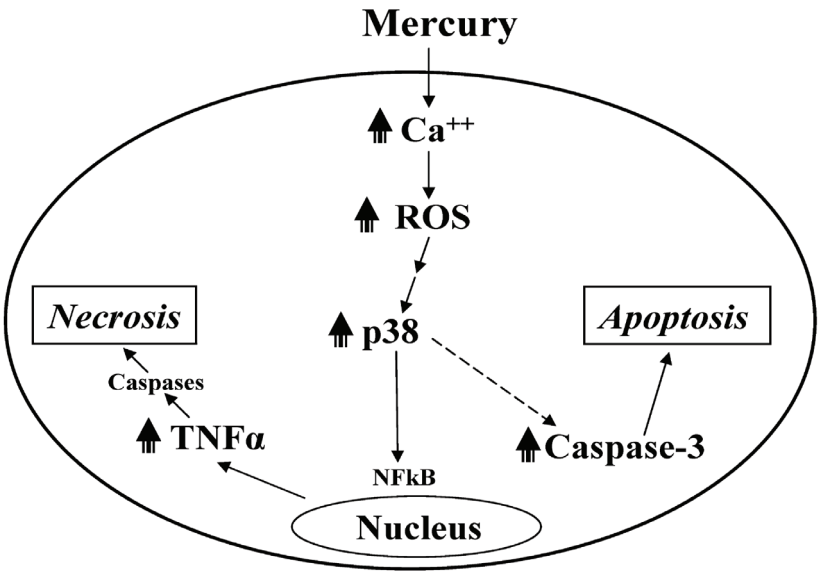

Figure 1: Mercury can cause apoptosis and necrosis. Hypothesized drawing of how mercury can cause apoptosis and necrosis (Kim and Sharma, 2004).

One action of reactive oxygen species (ROS) is to induce cell death, and at least one of mercury's effects, disrupting calcium homeostasis, can lead to an increased production of ROS (Kim & Sharma, 2004). Further depletion of glutathione (Patrick, 2002) and enhanced lipid peroxidation and DNA damage (Stohs & Bagchi, 1995) can occur due to ROS formation. Neuronal degeneration and cellular damage due to increased ROS production can lead to cell apoptosis and/or necrosis (Figure 1) (Kim & Sharma, 2004; Juarez et al., 2005; Castoldi et al., 2001).

Mercury in the nervous system can disrupt calcium homeostasis by causing an increase in intracellular calcium (Stohs & Bagchi, 1995; Castoldi et al., 2001). Depending on the mercury species, the resulting intracellular calcium concentrations may come from both an influx from extracellular spaces and intracellular stores (Gasso et al., 2001; Stohs & Bagchi, 1995). Kim and Sharma (2004) reported this change in calcium homeostasis occurred prior to an increase in reactive oxygen species production as ROS production could be eliminated by calcium antagonists.

Mercury can cause a decrease in the membrane potential of mitochondria, an organelle that accumulates mercury (Kim & Sharma, 2004; Toimela & Tahti, 2004). This decreased membrane potential allows for an efflux of Ca^{2+} out of the mitochondria along with decreased Ca^{2+} uptake into the mitochondria (Atchinson & Hare, 1994). This, together with the release of cytochrome c, can cause the cell to enter apoptosis (Kim & Sharma, 2004; Toimela & Tahti, 2004).

II.A.v. Peripheral Effects

Mercury exposure can have effects on many body systems. The renal, respiratory, cardiovascular, gastrointestinal, hepatic, immune, reproductive and dermal

systems have all been reported to have negative responses after mercury exposure. The renal system, particularly the epithelium cells of the proximal tubules, is one of the main mercury targets (Carranza-Rosales et al., 2005; Aleo et al., 2005). Signs of nephrotoxicity that have developed due to mercury exposure include reduced blood flow and glomerular filtration, and albuminuria (Van Vleet & Schnellmann, 2003; Crinnion, 2000). The respiratory system is affected when mercury particles are inhaled. Mercury in the lungs can cause respiratory distress, pneumonitis (Graeme & Pollack, 1998), and acute chemical bronchitis (Asano et al., 2000). Another system affected by mercury is the cardiovascular system. Cardiovascular effects due to mercury exposure include decreased variability of heart rate, which is associated with heart failure (Grandjean et al., 2004; De Jong & Randall, 2005), and arterial hypertension caused by vascular smooth muscle contraction (Golpon et al., 2003). An increased risk for cardiovascular disease and progression of carotid atherosclerosis has been reported in individuals with mercury exposure (Patrick, 2002). Because mercury is corrosive to the mucous membranes, oral burning and stomatitis may occur when mercury is ingested (Graeme & Pollack, 1998). Ulceration and hemorrhage can occur in the gastrointestinal tract with this route of mercury exposure (Jacoby et al., 1999; Stohs & Bagchi, 1995). Autoimmune abnormalities (Ben-Ozer et al., 2000), decreased fertility (Horsted-Bindslev, 2004), and dermatitis (Levine, 2003) have also been reported from exposure to mercury.

II.A.vi. Symptoms of Mercury Toxicity

The symptoms and effects of mercury toxicity are vast and depend upon: the nature of metal, the route of administration, length and dose of exposure, species type, gender, and age (Vicente et al., 2004; Bertossi et al., 2004; Blaxill et al., 2004). All of

the symptoms that occur with mercury toxicity are too numerous to mention here, therefore the focus will be on the central nervous system. These symptoms include tremor, insecurity, loss of memory, fatigue, excitability, insomnia, hallucinations, and irritability (Graeme & Pollack, 1998; Langford & Ferner, 1999; Levine, 2003). Seizures, decreased strength, hearing loss, constricted visual field and ataxia are other central nervous system symptoms reported from mercury exposure (Graeme & Pollack, 1998; Levine, 2003; Castoldi, et al., 2001).

II.A.vii. Diseases Associated with Mercury

Mercury exposure has been associated with autoimmune disease (McCabe et al., 2005), scleroderma (Hess, 2002), autism (Bernard et al., 2001), Alzheimer's disease and Parkinson's disease (Vicente et al., 2004). While mercury may directly cause these diseases, it may also trigger or exacerbate current but unrecognized conditions such as genetic factors (Bernard et al., 2001). It has been reported that autistic children have been exposed to elevated mercury and they have atypical mercury metabolism (Blaxill et al., 2004). The dysfunctions mercury causes in the immune, sensory, neurological, motor and behavioral systems are similar to those found in autism (Bernard et al., 2001).

Mercury levels are found to be elevated in the blood of patients with Alzheimer's disease. In one study, mercury was found to be more than two fold higher in the blood of patients with Alzheimer's disease than in the control group (Hock et al., 1998). Blood mercury levels of the individuals with Alzheimer's disease were found to be $2.64 \pm 0.38 \mu g/l$ while the control group that consisted of individuals without any psychiatric disorders levels were 1.09 ± 0.12 µg/l (Hock et al., 1998). Postmortem brain

tissue from patients with Alzheimer's disease shows elevated levels of mercury (Hock et al., 1998; Olivieri et al., 2000).

II.B. Manganese

Manganese was first recognized as a neurotoxicant in France in 1837. Workers grinding manganese ore developed symptoms such as drooling, loss of facial expression, unsteady gait and muscular weakness (Normandin & Hazell 2002; Jankovic, 2005). Since then, several more incidences of manganese poisoning have occurred. Cases of toxicity have been documented concerning workers exposed to pesticides and fungicides containing manganese, welders, miners, and individuals drinking water with high manganese content (Bowler et al., 2006; Zatta et al., 2003).

II.B.i. Characteristics and Normal Functional Role

Although also a transition metal, manganese differs from mercury in that it is an essential element important for normal brain development and functioning (Takeda, 2003). Manganese has eleven oxidation states from -3 to +7 (Takeda, 2003), with Mn^{+2}, $^{+3}$, and $^{+4}$ being physiologically relevant (Tiffany-Castiglioni & Qian, 2001). Manganese can be found in foods such as whole grains, nuts, legumes and tea (Greger, 1999). According to the FDA's daily food composite, the average adult man ingests 2.7mg of manganese while the average woman consumes 2.2mg of manganese (Greger, 1999).

Under normal conditions, manganese has many important functions. Besides being required by the brain for proper development and functioning (Takeda, 2003), manganese is important for connective tissue and bone formation as well as bone maintenance (Roth et al., 2002). Animals deficient in manganese have been shown to develop skeletal abnormalities that are thought to occur due to a reduction in

proteoglycan synthesis, impaired osteoblast and osteoclast activities and/or an alteration in insulin and insulin-like growth factor (Keen et al., 1999). The metabolism of amino acids, carbohydrates and lipids are other roles manganese performs while present in normal concentrations (Slikker et al., 2004).

Manganese functions through activating, regulating, being a co-factor for or binding to many different enzymes. A few of the enzyme families that require manganese include: oxidoreductases, transferases, hydrolases (Slikker et al., 2004) and peroxidases (Stredrick et al., 2004). For example, the enzymes that manganese associates with and functions through are mitochondrial superoxide dismutase (MnSOD), arginase, pyruvate carboxylase and glutamine synthetase. Through its participation with the mitochondrial superoxide dismutase, manganese plays a role in antioxidant activity (Zhang et al., 2003). Manganese is a co-factor for arginase, an enzyme found in the liver which plays a role in the production of urea (Crossgrove & Zheng 2004). Pyruvate carboxylase, another mitochondrial enzyme, participates in gluconeogenesis by converting pyruvate to oxaloacetate and requires manganese as a co-factor (Crossgrove & Zheng 2004; Siegel et al., 1999). Glutamine synthetase, a metalloprotein found in glia, is bound and regulated by manganese (Takeda 2003; Crossgrove & Zheng 2004). Alterations in glutaminase activity would alter the concentration of glutamine, the primary precursor for glutamate, either increasing the synthesis of glutamate or decreasing the metabolism of glutamate (Takeda, 2003). Manganese may also activate decarboxylases and kinases (Di Lorenzo et al., 1996).

Manganese actions have been shown to be bimodal. At low concentrations, manganese is not toxic and functions as mentioned previously as an essential element.

However, at higher concentrations manganese causes toxicity and cell death (Keller et al., 2005). According to the National Academies' Institute of Medicine, the adequate intake level of manganese for adult males is 2.3mg/day and for females, 1.8mg/day, while the upper intake level for manganese in the adult is 11mg. These values are adjusted with pregnancy and the different stages of childhood (Dobson et al., 2004). Manganese in water has a lowest observable adverse effect level (LOAEL) estimated to be 4.2mg Mn/day (Greger, 1998). Additionally, it has been reported that chronic manganese exposure to levels >1-5mg Mn/m^3 in air can cause adverse effects (Dobson et al., 2004).

II.B.ii. Central Nervous System

Manganese can enter the brain by crossing the choroid plexus, the blood brain barrier, and/or possibly the olfactory nerve (Normandin & Hazell, 2002; Takeda, 2003). This uptake into the brain through the blood brain barrier and choroid plexus occurs either via transferrin-mediated endocytosis and/or non-transferrin mediated process (Zatta et al., 2003). Transferrin, a protein in blood that carries iron, can also bind manganese (Verity, 1999; Zatta et al., 2003). The non-transferrin processes include: calcium channels, Na^+/Ca^{2+} exchanger, active calcium uniporter and Na^+/Mg^{2+} antiporter (Takeda, 2003). Manganese is taken up into the brain more rapidly when it is not in transferrin bound form (Zatta et al., 2003). Although not yet proven in humans, it is possible that manganese may be able to by-pass the brain barriers and enter and accumulate in the brain by crossing transneuronally through the olfactory nerve (Jankovic, 2005; Normandin & Hazell 2002). This method of manganese entry into the brain has been demonstrated in rats exposed to manganese intranasally (Yu et al., 2003).

Once manganese passes the brain barriers, regardless of route, it specifically targets and accumulates in the areas of high dopaminergic content (Andersen et al., 1999). Specifically, the nigrostriatal dopaminergic system has been found to be affected by excessive manganese (Kitazawa et al., 2005, Tomas-Camardiel et al., 2002). The caudate putamen, globus pallidus, substantia nigra, and subthalamic nuclei, all structures of the basal ganglia, accumulate the highest concentrations of manganese (Dobson et al., 2004). The choroid plexus has also been found to sequester and accumulate manganese (Ingersoll et al., 1995; Zheng, 2001).

II.B.iii. Manganese and Dopaminergic System

As mentioned above, the nigrostriatal dopaminergic system is selectively targeted by manganese (Kitazawa et al., 2005). The targeting of this area may be related to the reuptake and/or concentration of dopamine since both a dopamine reuptake inhibitor, cocaine, and a dopamine depleter, reserpine, inhibited manganese concentrating in the central nervous system (Ingersoll et al., 1999). Manganese treatment in experimental animals does not appear to cause structural changes in the nigrostriatal dopamine neurons (Normandin & Hazell 2002). However, an irreversible loss of dopaminergic neurons does occur due to chronic manganese exposure (Slikker et al., 2004; Yu et al., 2003).

The effect of manganese on dopamine content is still uncertain. Reports documenting a significant decrease, no measurable changes, or an increase in dopamine concentrations have all been published (Bonilla & Prasad, 1984; Ali et al., 1985; Eriksson et al., 1987; Baek et al., 2003). Baek et al. (2003), attribute these differences to variations in experimental design or the "multifactorial nature" of manganese. Others have reported that manganese causes biphasic changes in dopamine levels, where at low

concentrations manganese causes an increase in dopamine levels and at higher concentrations a decrease in dopamine levels (Tomas-Camardiel et al., 2002). Another study reported no changes in dopamine levels, but an increase in GABA levels with low concentration manganese treatments (Gwiazda et al., 2002). Similar to the discrepancies of manganese effects on dopamine, the dopamine metabolites DOPAC and HVA have been reported to be either unchanged (Ingersoll et al., 1995; Takeda, 2003) or reduced (Normandin & Hazell, 2002).

Manganese has been reported to effect dopamine synthesis, storage, and release. The rate limiting step of dopamine synthesis, tyrosine hydroxylation, has shown to be reduced by manganese exposure (Higashi et al. 2004; Hirata 2002). This would explain the decrease in dopamine content because a slower rate at this stage of dopamine synthesis would decrease the amount of L-DOPA formed, which is the immediate precursor of dopamine. Another explanation for the decreased dopamine content due to manganese exposure may be an alteration in dopamine storage (Ingersoll et al., 1995). Manganese has been reported to cause the release of dopamine from nerve terminals (Takeda, 2003), and to enhance the formation of dopamine reactive quinones (Stokes et al., 2000). This latter process which also produces toxic free radicals occurs through auto-oxidation of dopamine by the transfer of one electron (Takeda, 2003; Zatta et al., 2003).

II.B.iv. Mechanisms of Cellular Injury

Manganese is transported into astrocytes through a high affinity, high capacity transport system (Normandin & Hazell, 2002). Functional changes such as alteration of the glycolytic enzyme glyceraldehydes-3-phosphate dehydrogenase, production of nitric

oxide, and decreased GSH levels have been reported to occur in astrocytes due to manganese exposure (Normandin & Hazell, 2002; Desole et al., 1994). Additionally, blocking voltage-dependent calcium channels and disrupting the release and transport of the neurotransmitters glutamate and GABA have been observed in response to manganese (Takeda, 2003; Fitsanakis & Aschner, 2005; Zatta et al., 2003). Hirata et al. (1998) have reported that manganese causes internucleosomal DNA fragmentation, suggesting that part of the neurotoxic effects produced by manganese may be due to apoptosis.

Once inside the astrocytes, manganese actively concentrates in the mitochondria via the mitochondrial Ca^{+2} uniporter (Gavin et al., 1999; Gunter et al., 2005). Here manganese binds to the inner membrane of the mitochondria (Malecki, 2001; Galvani et al., 1995), and then perturbs mitochondrial function through numerous mechanisms (Kitazawa et al., 2005; Zhang 2003; Roth et al., 2002). Mitochondrial Ca^{2+} transport kinetics are affected by manganese disruption of calcium homeostasis. This can lead to decreased oxidative phosphorylation, decreased ATP and an inhibition of mitochondrial electron transfer with accumulation of reactive oxygen species (Verity, 1999; Kitazawa et al., 2005). Manganese inhibits mitochondrial respiration, causes a reduction of mitochondrial enzymatic activities (Hirata et al., 1998, Galvani et al., 1995), and a loss of mitochondrial membrane potential (Malecki, 2001). Secondary to these effects on the mitochondria, manganese causes apoptosis which is considered to be one of the mechanisms of its neurotoxicity (Malecki, 2001; Stredrick et al., 2004).

II.B.v. Peripheral Effects

There are reports of the liver and lungs being negatively affected by manganese. The liver concentrates manganese and maintains the homeostasis of manganese in the body (Zhang et al., 2003; Yu et al., 2003). When the liver is damaged, manganese elimination may be delayed or decreased which results in an increased amount of time that the body is exposed to this metal. This in turn provides manganese a longer chance to accumulate and exhibit its toxic effects (Crossgrove & Zheng 2004).

Lung damage occurs when manganese is inhaled. Manganese inhalation has caused cytotoxic effect on lung epithelial cells, and it has been connected with respiratory irritant effects (Pascal & Tessier 2004). The manganese accumulated in the lungs is characterized by slow uptake and elimination process which means lung tissue has a long exposure to manganese (Yu et al., 2003). This may result in extensive damage to the lung tissue.

II.B.vi. Symptoms of Manganese Toxicity

It may take several months or years for the symptoms of manganese toxicity to appear (Keen et al., 1999). Symptoms of manganese exposure include behavioral changes (Kitazawa et al., 2005), depressed appetite, growth depression (Greger, 1998), and a reduction in motor function (Gwizada et al., 2001). Tremors, lethargy, muscle rigidity and slowed movements are also reported by individuals affected by manganese toxicity (Ingersoll et al., 1995). Psychiatric symptoms resembling schizophrenia have been observed and documented as well in individuals who have been chronically exposed to manganese via inhalation (Greger, 1998).

II.B.vii. Diseases Associated with Manganese Toxicity

Manganism, a disorder that produces symptoms similar to idiopathic Parkinson's disease, results from chronic exposure to manganese (Di Lorenzo et al., 1996; Archibald & Tyree, 1987). Behavioral changes including anorexia, insomnia, weakness and apathy are among the first symptoms of manganism (Zatta et al., 2003; Zhang et al., 2003). The next stage of symptoms is similar to those of Parkinson's disease and may include disorientation, memory loss, anxiety, rigidity, and tremor (Zatta et al., 2003; Archibald & Tyree, 1987). Dystonia and gait disturbances appear in the last, most severe stage of manganism (Zatta et al., 2003). Even after removal from manganese exposure, once manganism has been established, this disease will continue to progress to its severest form (Zatta et al., 2003).

The data on manganese's contribution to the development of Parkinson's disease is contradictory. Some studies have reported manganese exposure as a risk factor for Parkinson's disease (Sava et al., 2004), while other studies can not make this connection (Baek et al., 2003). Other authors have proposed that pre-parkinsonian conditions such as sub-threshold neurodegeneration may be exacerbated by manganese effects (Gwiazda et al., 2002). This metal may also enhance brain physiological aging leading to an increased risk of developing Parkinson's disease (Gwiazda et al., 2002; Zatta et al., 2003).

It is recognized that other disease states can make an individual more susceptible to the effects of manganese. Individuals with an iron deficiency, Alagille syndrome, chronic liver failure and/or receiving long-term parenteral nutrition (TPN) are at an increased risk of developing manganese toxicity (Crossgrove & Zheng, 2004; Normandin

& Hazell, 2002). The hepatobilary route is the main route for manganese excretion; therefore in cases where the liver is not functioning properly such as with Alagille syndrome, chronic liver failure and TPN, manganese is not being excreted as it should. This leads to an increase in the body's manganese levels, which can then become toxic (Crossgrove & Zheng, 2004; Normandin & Hazell, 2002).

II.C. Dopamine Transporter

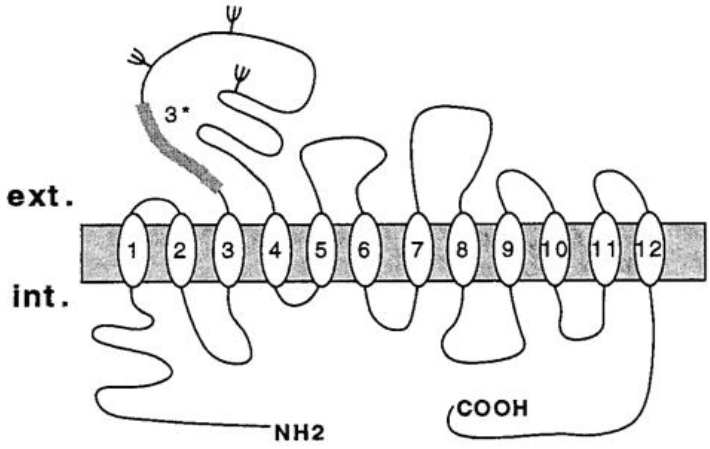

Figure 2: Schematic of human DAT. Pitchforks on extracellular loop between transmembrane domains 3 and 4 represent possible sites for glycosylation (Masson, et al., 1999).

The dopamine transporter (DAT) is an important component of the dopaminergic system. By regulating the amount of dopamine found extracellularly, the DAT has a role in all of the actions mediated by dopamine such as movement, pleasure, reinforcement and behavior (Kahlig & Galli, 2003; Mortensen & Amara, 2003; Bannon, 2005). In turn, the DAT can be regulated through a change in expression levels or an alteration in its function in response to substrates, inhibitors, psychostimulants such as cocaine and amphetamine, and interactions with other proteins. It is recognized that some of these changes in the DAT may be associated with or play a role in some disease states such as Parkinson's disease and drug addiction (Bannon, 2005).

The DAT which is solely expressed on dopamine-synthesizing neurons (Bannon, 2005) can be found on the soma and dendrites in the substantia nigra and ventral tegmental area and on axonal terminals in the striatum (Miranda et al., 2004; Maiya & Mayfield, 2004). This plasmalemmal protein is composed of 620 amino acids with twelve transmembrane domains with both the carboxy- and amino-termini located intracellularly (Reith et al., 1997). The DAT structure contains several sites intracellularly for possible phosphorylation (Melikian, 2004) and at least three sites for N-glycosylation on the extracellular loop between transmembranes 3 and 4 (Chen & Reith, 2000; Miranda et al., 2004) (Figure 2). It has been shown that glycosylation at these sites is associated with cell surface expression and important for the proper functioning of the DAT (Torres et al., 2003; Li et al., 2004). Studies have shown that the DAT becomes oligomerized via symmetrical crosslinking between cysteine residues early in its synthesis, and this formation is continued through the intracellular movement, or trafficking, of the DAT (Torres et al., 2003; Maiya & Mayfield, 2004; Mortensen & Amara, 2003). These same authors along with others have reported that oligomerization has an important role in the proper trafficking of the DAT into and out of the cell membrane (Torres et al., 2003; Maiya & Mayfield, 2004; Mortensen & Amara, 2003; Miranda et al., 2004).

II.C.i. Dopamine Transporter Function

The primary role of the DAT is to move dopamine across the cell membrane back into the cell after its release into the extracellular space. Through this re-uptake action, DAT regulates dopamine neurotransmission (Jaber et al., 1997; Uhl, 2003) and reduces the amount of dopamine synthesis needed (Kahlig & Galli, 2003). The DAT, like the

other members of the Na^+/Cl^- dependent transporter family, transports sodium and chloride ions with their substrates (Zahniser & Doolen, 2001), because they need the energy from the Na^+ gradient created by the (Na^+/K^+)-ATPase to move the substrates against their concentration gradients (Masson et al., 1999). Specifically, the DAT moves two sodium ions and one chloride ion with each dopamine molecule. At physiological pH dopamine is protonated, resulting in the net movement of two positive charges for each molecule of dopamine translocated (Maiya & Mayfield, 2004; Sonders et al., 1997).

II.C.ii. Dopamine transporter trafficking

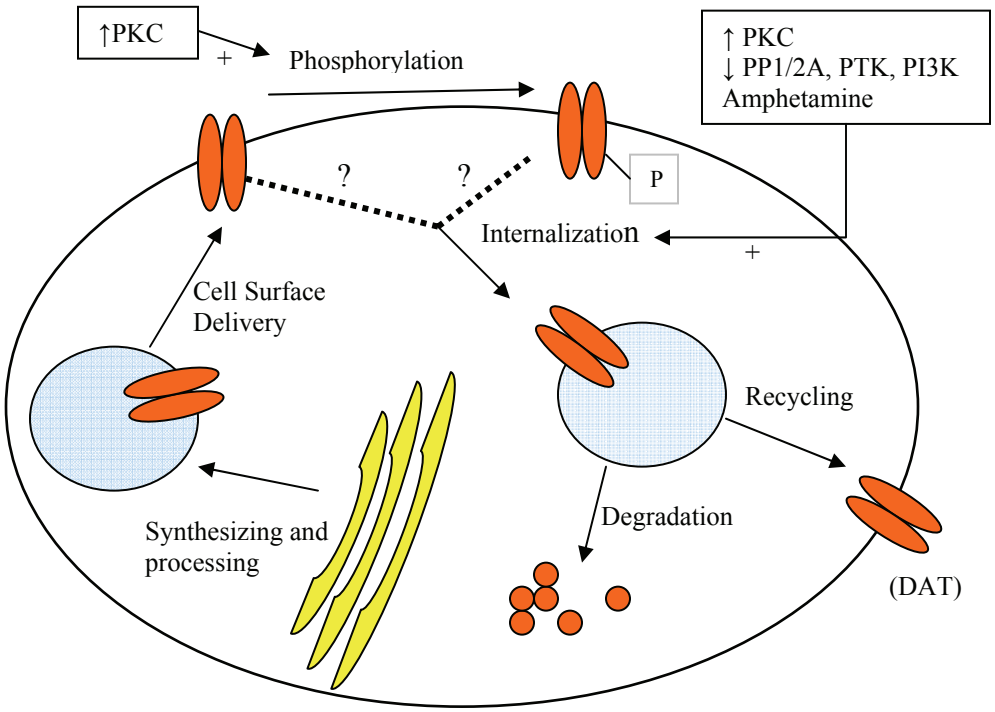

Figure 3: DAT trafficking pathway. A schematic representation of the DAT trafficking pathway (adapted from Torres et al., 2003).

The efficiency with which DAT can remove dopamine from the extracellular space depends in part on the number of DAT at the cell surface (Sorkina et al., 2003;

Kahlig et al., 2004). Therefore, the trafficking of the transporter into and out of the cell membrane plays an integral part in DAT functioning (Figure 3).

The rough endoplasmic reticulum (RER) is the site of DAT protein synthesis (Hersch et al., 1997; Kimmel et al., 2001). From here, the DAT is shuttled to the Golgi complex where it undergoes posttranslational modifications (Kimmel et al., 2001; Hersch et al., 1997). One posttranslational modification thought to occur in the Golgi complex is glycosylation (Hersch et al., 1997; Sorkina et al., 2003). The DAT is then transported to the smooth endoplasmic reticulum of the dendrites and axons before it arrives at the plasma membrane (Kimmel et al., 2001; Hersch et al., 1997). When the DAT receives a signal to re-enter the cell, it has been shown to do so through a process dependent on clathrin and dynamin (Sorkina et al., 2003; Granas et al., 2003). It first is transported to early endosomes by clathrin-coated pits (Daniels & Amara, 1999). The next step in the DAT life cycle depends on the cell system DAT is expressed in (Mortensen & Amara, 2003). Some studies have shown that after internalization DAT is recycled back to the plasma membrane in PC12 cells (Loder & Melikian, 2003), while others have shown that in the MDCK cell system DAT is degraded after internalization (Daniels & Amara, 1999).

II.C.iii. Regulating the Dopamine transporter

Regulation of the DAT can occur through altering the transporter's function or its trafficking to the plasma membrane (Figure 3). According to Mortensen and Amara (2005) and Sidhu et al., (2004), the activity of the transporter is most commonly regulated through its movement into and out of the plasma membrane. Through increasing or decreasing the number of transporters on the cell surface, the amount of

extracellular dopamine re-captured is affected along with all of the systems and reactions that dopamine interacts with. The substrates, inhibitors, and proteins that interact with DAT can regulate its function and expression.

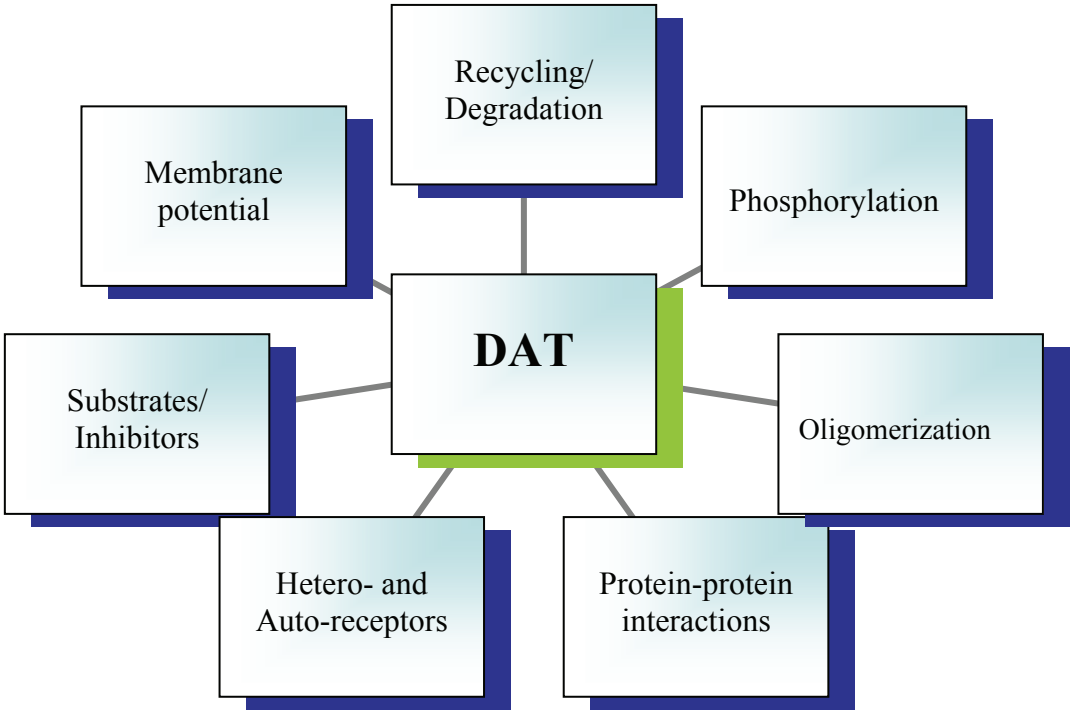

Figure 4: Possible ways the DAT may be regulated. Inhibitors, substrates, phosphorylation, hetero-and autoreceptors, along with membrane potential, oligomerization and interaction with other proteins can regulate the dopamine transporter expression on the cell surface (adapted from Mortensen and Amara, 2003).

Alpha-synuclein, a presynaptic protein, can regulate the amount of DAT located on the plasma membrane through complexing with the transporter. This complexing between these two proteins in turn affects DAT function, dopaminergic neurotransmission, and the levels of intracellular dopamine (Wersinger et al., 2003). Lee et al. (2001) have reported that alpha-synuclein causes the DAT to cluster on the cell membrane which causes an increase in dopamine uptake. This in turn enhances the chances of apoptosis due to excess dopamine (Lee et al., 2001).

Phorbol esters and kinases regulate the DAT. Phorbol esters are able to regulate the number of DAT on the cell surface by activating PKC. Upon exposure to phorbol

esters, the DAT is down-regulated through a clatherin-mediated endocytosis pathway (Chi & Reith, 2003; Daniels & Amara, 1999). This results in a decreased uptake of dopamine. Another way that DAT is regulated is through kinases. Phosphorylation and dephosporylation of the DAT by kinases controls the cycling of the transporter between internalization and the plasma membrane (Piccini, 2003).

Dopamine can affect the function of the dopamine transporter. Dopamine can directly down-regulate and indirectly upregulate the dopamine transporter function (Gulley & Zahniser, 2003). These effects of dopamine on the DAT are concentration-dependent. High concentrations have been shown to cause down-regulation of the dopamine transporter, while low concentrations of dopamine cause an up-regulation (Chi & Reith, 2003; Gulley & Zahniser, 2003).

Other stimuli that can produce DAT changes, such as alterations in kinetics, substrate transport capacity, and the number of DAT include: cocaine, amphetamine, MPP+, receptor occupation of dopamine presynaptic receptors, and second messenger signaling (Gulley & Zahniser, 2003; Batchelor & Schenk, 1998; Moron et al., 2003).

II.C.iv. Diseases Associated with DAT

Because the DAT does have such a critical role in dopaminergic neurotransmission, in disease states that are found to have an altered or improper dopamine tone, the DAT becomes one of the suspects for causing or supporting the disease state. Schizophrenia, Parkinson's disease, drug addiction (Kahlig & Galli, 2003; Moron et al., 2003), mental retardation (Loupe et al., 2002) and ADHD (Bannon, 2005) are among the disease states that have been found to, or are thought to have improper dopaminergic tone. Both in Parkinson's disease and ADHD, there have been studies

showing increases in DAT localization and functioning (Storch et al., 2004; Vernier et al., 2004; Bannon, 2005). Other diseases/disorders, such as dementia with Lewy bodies, Wilson's disease, Machado-Joseph disease, Lesch-Nyhan disease, and Gilles de la Tourette's syndrome, have observed a difference in DAT expression, but have yet to uncover the effects, if any, of these differences (Bannon, 2005).

II.D. SUMMARY

Mercury and manganese are found everywhere in the environment, exposing everyone to their effects. These effects depend on the metal, species, dose, and exposure time among many other factors. It is recognized that high doses of mercury and manganese are toxic, and because of this, agencies such as the World Health Organization and Environmental protection agency have set limits suggesting the maximum amount an individual should be exposed to. While these limits may be appropriate for acute exposure, their accurateness for long term exposures of sub-toxic concentrations is yet unknown and need further investigating.

Adding to the toxicity of mercury and manganese are their effects on the dopaminergic system. While it is recognized that these metals affect the dopamine transporter, the mechanism of how they do this has not yet been determined. The purpose of this project is to determine how long term exposure to sub-toxic concentrations of mercury and manganese affect the dopamine transporter.

II.D.i. Hypothesis

The hypothesis for this study is that mercury and manganese in their chloride forms will reduce the dopamine transporter functionality. This reduction in functioning

may possibly be due to an increase in the internalization of the dopamine transporter as compared to the control group.

II.D.ii. Aims

The aims for this project include:

● Determining the toxic threshold concentrations of mercury and manganese.

● Determining the affect of mercury and manganese on the functioning of the dopamine transporter through:

- •determining the amount of [^{3}H]DA taken up by treated cells
- •determining the amount of [^{3}H]GBR-12,935 binding to DAT on the cell surface
- •determining the kinetics of [^{3}H]GBR-12,935 binding to the DAT

III. MATERIALS and METHODS

III.A. Cell Culture

SK-N-SH cells (A.T.C.C., Manassas, VA) were grown in complete media (R.P.M.I. 1640, 1X without L-glutamine (Mediatech, Inc., Herndon, VA), supplemented with 10% Fetal Bovine Serum (Atlanta Biologicals, Norcross, GA), and 1% Penicillin-Streptomycin (Mediatech, Inc. Herndon, VA) in 25cm^2 cell culture flasks with vented caps (Corning Incorporated, Corning, NY). They were maintained in the incubator at 37°C in a humidified (5% CO_2) environment. Media was changed twice a week with 5mL of fresh complete media that had been warmed in the 37°C water bath for forty-five to sixty minutes. The flasks were then returned to the incubator.

Cells were subcultured every seven days. For this process, new flasks with 5mL of warmed complete media were placed in the incubator to allow the carbon dioxide to equilibrate in the media while the cells were being trypsinized. Trypsinization of the cells was conducted as follows. Trypsin (0.25%) + EDTA(0.1%) in HBSS without calcium, magnesium, or sodium bicarbonate (Mediatech, Inc, Herndon, VA) was placed in the warm water bath for forty-five to sixty minutes, and then 1.5mL was added to each flask of cells after the old media was carefully removed. The flasks were allowed to sit for 15-20 minutes and then if necessary gently agitated to finish removing the cells. 3.5-4.5mL of complete media was then added to these flasks to terminate the trypsinzation process and gently mixed together by pipetting the solution up and down. 1mL of this

cell suspension was then added to the new flasks containing fresh complete media, gently mixed by swirling, and placed back into the incubator.

Cells were plated following a similar process. 900µL of warmed fresh complete media was added to a clear 24 well flat bottom cell culture plate (Costar®, Corning, Inc., Corning, NY). The plate was then placed in the incubator to equilibrate the media with CO_2 while the cells were being trypsinized in the same manner as above. 5mL of complete media was added to the trypsinized cells, gently mixed by pipetting up and down, and then 100µL of this cell suspension was added to each well of the plate. The plates were then placed back into the incubator for at least 24 hours before any experimentation. All cell work was done with sterile techniques.

III.B. Metal Treatments

The metal solutions were made with RPMI, and then autoclaved before use. All of the metal solutions were made in 21X concentrations so that the final concentration in the well or flask would be the concentration desired. For example, for the 10µM manganese chloride treatment in a twenty-four well plate, a 210µM concentrated metal solution was made so when 50µL of the metal solution was added to the well with 1000µL of cells/media, the final concentrations would be 10µM (210X (50/1050)=10).

Twenty-four hours after the cells were plated or split into flasks, one of the metal solutions or control would be added either to the plate or flask of cells, depending on which experiment was being run, and then mixed by gentle oscillation.

III.C. LDH Assay

The CytoTox 96® Non-Radioactive Cytotoxicity Assay by Promega (Madison, WI) was used to determine the percent viability of the cells after treatment with the heavy

metals. This assay measures the amount of lactate dehydrogenase (LDH) present in the cell media which can then be used to determine the amount of cells that have died. Upon addition of the Substrate Mix-Assay Buffer mixture, the following chemical reactions occur in the wells during the CytoTox 96® Non-Radioactive Cytotoxicity Assay:

LDH

$$NAD^+ + \text{lactate} \rightarrow \text{pyruvate} + NADH$$

Diaphorase

$$NADH + INT \rightarrow NAD^+ + \text{formazan (red)}$$

First, the LDH released from the cells that have died catalyzes the reaction between NAD^+ and lactate from the Substrate Mix to form pyruvate and NADH. For the second reaction, this latter product, NADH, then reacts with INT via the diaphorase from the Substrate Mix to form NAD^+ and formazan red. The amount of red formazan product formed from this reaction is directly proportional to the amount of cell death, and this can be measured with a standard 96 well plate reader (CytoTox 96® Non-Radioactive Cytotoxicity Assay Technical Bulletin No. 163).

III.C.i. Method

Cells were plated as described above, allowed to sit for at least 24 hours, and then treated with $HgCl_2$ or $MnCl_2$. The sample size was four, with at least three replicates, and each well was tested in duplicate. The SK-N-SH cells were treated with 50µL of in-well final concentrations of 0.1, 1.0, 10.0 or 50.0µM mercuric chloride ($HgCl_2$), 1.0, 2.0, 5.0, or 10.0µM manganese chloride ($MnCl_2$), or RPMI (control) for four different time points (24, 48, 72, or 96 hours). At the end of the designated treatment time, the CytoTox 96® Non-Radioactive Cytotoxicity Assay was conducted essentially as described in the Promega technical bulletin. In order to determine the amount of spontaneous release,

5µL of each well was pipetted into a clear 96 well plate. To each well 45µL of complete media was added to bring the volume up to 50µL. The remaining media from the twenty-four well plate was removed with a transfer pipet and discarded. To each well, 150µL of Lysis Solution was then added in order to measure the total amount of LDH released. The plates were then placed back in the incubator for forty-five minutes. At the end of this time period, 900µL of complete media was added to each well to bring the total volume back up to the original volume of 1050µL. 5µL was again pipetted out of these wells into the remaining wells of the 96 well plate and then 45µL of complete media was added to the wells. Next, 50µL of Substrate Mix (12mL of thawed Assay buffer (Tris-buffered tetrazolium dye (INT-chloride), and Triton-X-100) mixed with one bottle of Substrate mix (lyophilized diaphorase, lactate, and NAD^+)) was added to each of the 96 wells. The plates were then protected from light and placed on a plate shaker for thirty minutes. 50µL of Stop Solution (1M acetic acid) was then added to each well and the absorbance was read with the Synergy HT Multi-Detection Microplate Reader (Bio-TEK® Instruments, Inc., Winkooski, VT) with KC4 software (Bio-TEK® Instruments, Inc., Winkooski, VT) at 490nm.

Data was calculated by dividing the spontaneous release by the spontaneous plus total release of LDH. This difference was then subtracted from one and multiplied by one hundred to obtain percent viability:

$$\left[\, 1 - \frac{\text{Spontaneous LDH release}}{\text{Spontaneous LDH release} + \text{Total LDH release}} \,\right] \times 100 = \%\ \text{Viability}$$

III.D. Caspase 3/7 Assay

Promega's Apo-ONE® Homogeneous Caspase-3/7 Assay (Madison, WI) was used to determine the amount of cells that had entered into the apoptotic pathway after treatment with the heavy metals. Because caspases play an important activator role in apoptosis through cleaving proteins that lead to cytoplasmic blebbing and DNA fragmentation, measuring the amount of caspase activity in the wells of the treated cells is an effective way to determine the degree of apoptosis in the sample. In this assay, the activities of caspase-3 and -7 are measured by detecting the amount of fluorescent product formed after the Caspase-3/7 Reagent (1:100 mixture of Caspase Substrate Z-DEVD-R110 (100X): Apo-ONE® Homogenous Caspase-3/7 Buffer) has been added and mixed in the wells. The Caspase Substrate contains the profluorescent substrate, Z-DEVD-R110, which is cleaved by the caspases present in the lysed cell contents to form a fluorescent product, rhodamine 110, upon excitation at 499nm.

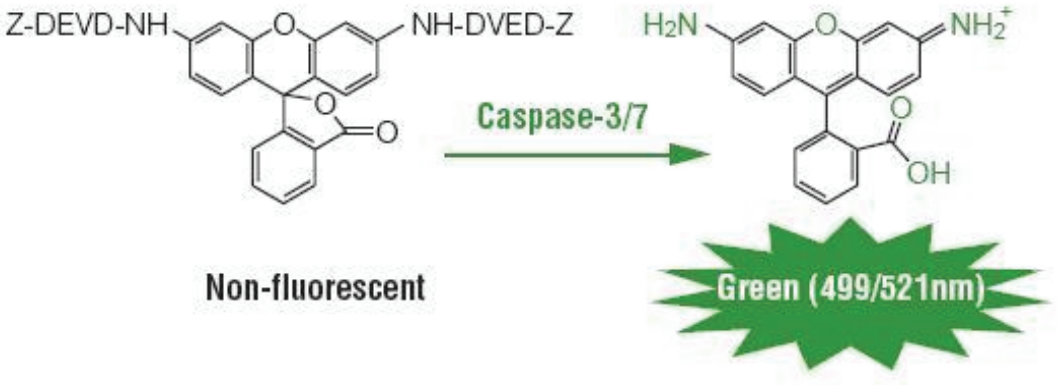

Figure 5: Caspase-3/7 Reaction. A depiction of the reaction that occurs in Promega's Apo-ONE® homogenous Caspase-3/7 Assay kit upon addition of Apo-ONE® Reagent to the sample. The caspases-3 and-7 cleave the Z-DEVD portion of the profluorescent Z-DEVD-R110 which results in the fluorescent R110 product on the right side of the reaction. The fluorescent activity can be detected by a plate reader with excitation and emission parameters set at 499 and 521, respectively.

The amount of fluorescence produced can be read by a plate reader and is in proportion to the amount of caspase-3/7 activity (Apo-ONE® homogeneous Caspase-3/7 Assay Technical Bulletin No. 295).

III.D. i. Method

The Apo-ONE® Homogeneous Caspase-3/7 Assay was carried out according to the directions supplied with the kit. The cells were plated into a Microtest 96-well black assay plate to prevent fluorescent "bleeding" into adjoining wells with a clear bottom and lid (BD Biosciences, Franklin Lakes, N.J.). Both blanks and controls were run with each treatment, and each treatment consisted of a sample size of four with replicates run in quadruplicate. Treatments consisted of 1.0μM $HgCl_2$, 10.0μM $MnCl_2$, or control treatment for 48 and 96hrs. At the end of the treatment time, 100μL of Apo-ONE® Caspase-3/7 Reagent (100μLof Caspase Substrate: 9900μL of the Apo-ONE® Caspase-3/7 Buffer®). The plate was then covered and placed on a plate shaker for thirty minutes. The plate was then read with the plate reader at an excitation wavelength of 485 ± 20nm and an emission wavelength of 530 ± 25nm.

The data was calculated by averaging the blanks and subtracting this value from both the control and treatment groups.

III.E. [^{3}H]GBR-12,935 Binding

The number of DAT in the plasma membrane affects the efficiency of dopamine clearance from the synaptic space. Therefore, the next step of this project was to determine if the treatment of SK-N-SH cells with 10μM $MnCl_2$ or 1μM $HgCl_2$ had an effect on the number of DAT in the plasma membrane. This was accomplished using [Propylene-2,3-^{3}H]GBR-12,935 and unlabeled GBR-12,909. GBR-12,935 is a selective

inhibitor of the DAT. In tritiated form, GBR-12,935 binding to the DAT can be inhibited by unlabeled GBR-12,909 (Richfield, 1991). Therefore, GBR-12,909 was used to define non-specific binding. Non-specific binding is the binding that occurs to any site other than the dopamine transporter. The values obtained from non-specific uptake were subtracted from total binding (samples without GBR-12,909) to calculate specific binding.

[^{3}H]GBR-12935 saturation binding curves were also performed to determine the B_{max} and K_D values of binding to the dopamine transporter. By obtaining the B_{max} and K_D values for the binding of [^{3}H]GBR-12,935 to the dopamine transporter, we are able to determine if there is a shift in the number of dopamine transporter sites and/or a change in the binding affinity of [^{3}H]GBR-12,935 for the dopamine transporter due to mercuric chloride or manganese chloride treatments in the SK-N-SH cell line.

III.E.i. Method

Twenty-four hours after subculturing, cells were randomly treated in the flasks with either RPMI (control), 10 μM $MnCl_2$, or 1 μM $HgCl_2$ for 48 or 96 hours. At the end of the exposure times, the treated media was removed, the cells were washed twice with 1-2mL of RPMI, and then 1mL of trypsin/EDTA was added to the flasks to detach the cells from the side of the flask. This cell suspension was then pipetted into flat top microcentrifuge tubes (Fisher Scientific, Pittsburgh, PA) and centrifuged in the Nanofuge (Hoefer Scientific Instruments, San Francisco, CA) for 15 minutes to form a cell pellet. The supernatant was decanted, and the cell pellets were placed in the -80°C freezer until the binding assays were performed.

The cell pellets were then resuspended in 2.5mL of assay buffer (50mM Tris-HCl and 120mM NaCl adjusted to pH 7.7). 400µL of this cell suspension was pipetted into round bottom 12X75mm polypropylene culture test tubes (Fisher Scientific, Pittsburgh, PA) along with either 50µL of assay buffer (total binding) or 50µL of 5µM GBR-12,909 (Sigma-RBI, St. Louis, MO) (non-specific binding) in duplicate. Next, 50µL of 40µM [^{3}H]GBR-12,935 (Perkin Elmer, Boston, MA) was added to all tubes for a final in-tube concentration of 4nM. The tubes were vortexed using the Fisher Vortex Genie 2 (Fisher Scientific, Pittsburgh, PA) followed by a sixty minute incubation period at room temperature to reach equilibrium. The binding reaction was terminated by filtration onto a Whatman GF/C filter (Whatman Paper Ltd.,) which had been pre-soaked in 0.3% polyethyleneimine (PEI) (Sigma Aldrich, St. Louis, MO) using a Brandel Tissue Harvester (Gaithersburg, MD). Filters were washed with ice cold 0.9% NaCl for 15 seconds and then carefully removed with the filter disks being placed in labeled scintillation vials (Daigger, Vernon Hills, IL). 5mL of ScintiVerse (Fisher Scientific, Pittsburgh, PA) was added to each of the vials, which were then capped and vortexed. The vials were then counted for five minutes using the Beckman Coulter LS 1801 scintillation counter (Beckman Coulter Inc., Fullerton, CA). Non-specific binding was subtracted from total binding to calculate the specific DAT-[^{3}H]GBR-12,935 binding.

For the saturation curve assays, flasks of cells were treated as mentioned above. Each one of these flasks was used for both a [^{3}H]DA uptake assay and a [^{3}H]GBR-12935 assay. At the end of the treatment time, the cells were washed twice with RPMI, detached from the side of the flask with 1mL of trypsin/EDTA, and then divided into two microcentrifuge tubes. These tubes were then centrifuged for 3-5 minutes. Once

centrifuged and the supernatant was removed, one of the tubes was used immediately for [^{3}H]DA uptake assays (method to be described below) while the other tube was stored in the -80° freezer to be used for the [^{3}H]GBR-12,935 saturation curve assays at a later date.

The pellet of cells for the [^{3}H]GBR-12,935 saturation curve assay was resuspended in 30mL of binding buffer after it was thawed. The rest of the assay was carried out as described above with the exception that instead of adding a final concentration of 4nM [^{3}H]GBR-12-935, increasing concentrations of [^{3}H]GBR-12935 (1, 2.5, 5, 10, 25, 50nM-in final concentration) was added to total and non-specific tubes. The samples were then harvested as described above.

Protein concentrations for each sample were determined using the Bradford protein assay method. The remaining sample from the above study was diluted with 500µL of assay buffer. 40µL of Dye Reagent Concentrate (Bio-Rad, Hercules, C.A.) was added to a clear 96-well plate. 160µL of the samples were then mixed in duplicate with the concentrated dye. The plate also contained eight concentrations of a protein standard and a blank sample that was used to compare the samples to. The plate was incubated at room temperature for five minutes and then read with the plate reader at an absorbance of 595nm.

The calculations for pmol/mg protein were conducted using Quick Calcs Radioactivity calculator (GraphPad Software, Inc., San Diego, CA) based on specific activity of the radioligand.

III.F. [^{3}H]DA Uptake

In order to determine if the functionality of the DAT in SK-N-SH cells changed following 48 or 96 hour exposure to either 1.0μM $HgCl_2$ or 10.0μM $MnCl_2$, [^{3}H]DA uptake assays were conducted. Two concentrations of [^{3}H]DA was used, one low (20nM) and one high (200nM), in order to detect if there was a shift in DAT functioning between these concentrations. As mentioned above, half of the flask designated for the [^{3}H]DA uptake assay was used for [^{3}H]GBR-12,935 saturation curve binding assays. This approach was used so these related aspects could be examined on cells of the same passage and from the exact same conditions, thereby making the comparison of data stronger.

III.F.i. Method

As described above, after the 48 or 96 hours, the cells were rinsed with warmed RPMI to stop the treatment. The cells were then detached from the flask using trypsin/EDTA and split into two microcentrifuge tubes (one to be used for the uptake assay and one to be used for the binding assay) and centrifuged for 3-5 minutes. After the supernatant was removed, the cell pellet was resuspended in 30mL of uptake assay buffer (25mM HEPES, 120mM NaCl, 5mM KCl, 2.5mM $CaCl_2$, 1.2mM $MgSO_4$, 300μM ascorbic acid, 1μM pargyline and 2mg/mL D-(+) glucose, supplemented with 50nM Nisoxetine to prevent uptake into NET, pH 7.4). 400μL of this cell suspension was then added to culture test tubes with either 50uL of assay buffer (total) or 50uL of 50μM GBR-12,909 (non-specific; final concentration: 5μM). 50μL of either 200 or 2000μM [7,8-^{3}H]DA (Amersham Biosciences, UK; final concentration: 20 and 200μM, respectively) was added in duplicate to the total and non-specific tubes. The tubes were

incubated for ten minutes at room temperature and then harvested by filtration under reduced pressure on a PEI soaked Whatman GF/C filter using the Brandel Tissue Harvester. The filter discs created were then placed in scintillation vials along with 5mL of scintillation cocktail. The vials were vortexed and then placed in the scintillation counter to measure counts per minute in order to determine the amount of [^{3}H]DA taken up into the cells.

The amount of protein in the sample was determined using the Bradford method as described above and used to calculate the amount of uptake per amount protein. These calculations for pmol/mg protein were conducted using Quick Calcs Radioactivity calculator based on specific activity of the radioligand. This value was then divided by 10 minutes to determine the amount of [^{3}H]DA uptake per minute (pmol/mg protein/min.).

III.G. Statistical Analysis

One-way or two-way ANOVAs were calculated on the data for the LDH, Caspase-3/7, one-point [^{3}H]GBR-12,935 binding, and two point [^{3}H]DA uptake followed by Bonferroni or Dunn's Multiple Comparison posttest, when appropriate, to determine if there was a statistical significance between the means of the various treatments. A nonparametric one-way ANOVA (Kruskal-Wallis test) followed by Dunn's Multiple Comparison posttest was conducted on K_D and B_{max} values after they were determined via nonlinear regression (curve fit) analysis. A $p<0.05$ was deemed to be statistically significant. All statistical analysis calculated for this study was done using the computer program GraphPad Prism® Version 4.0 for Windows (GraphPad Software, San Diego, CA).

IV. RESULTS

IV. A. LDH Assay

An LDH assay was used to determine the effect of $MnCl_2$ and $HgCl_2$ on cell viability. SK-N-SH cells were treated with increasing concentrations of $HgCl_2$ or $MnCl_2$ for 24, 48, 72 and 96 hours. Promega's CytoTox 96® Non-Radioactive Cytotoxicity Assay was preformed to calculate the amount of released lactate dehydrogenase in each of the samples. This colorimetric assay is directly proportional to the amount of LDH present with absorbance being read at 490nm. Percent viability values were calculated as described above.

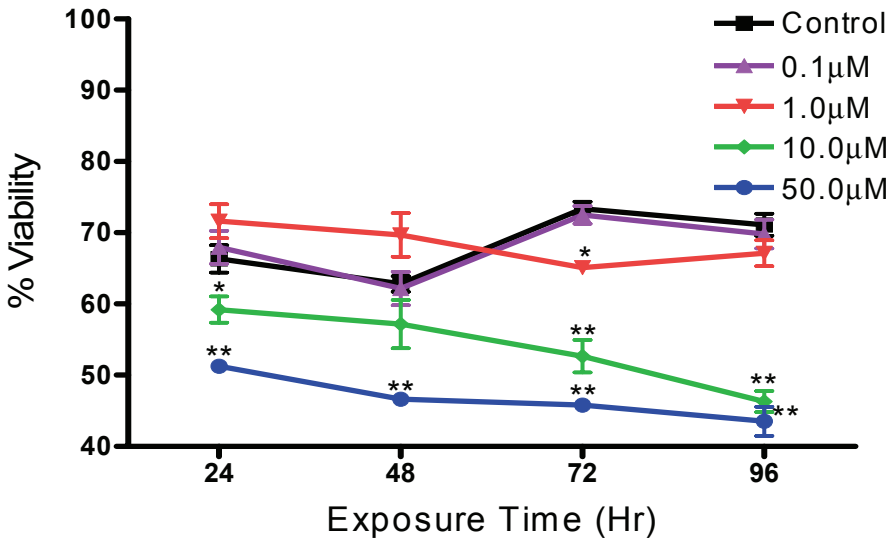

Figure 6: LDH activity in SK-N-SH cells upon various $HgCl_2$ concentrations and exposure times. Percent viability was calculated by subtracting from 1, spontaneous release divided by spontaneous plus total release, and then multiplying this value by 100%. Each value represents the mean ± SEM of a sample size of four completed in at least triplicate. The data reveals that there is a time ($p<0.0001$), concentration ($p<0.0001$), and time X concentration effect ($p=0.0069$) on the viability of SK-N-SH cells when treated with increasing concentrations of $HgCl_2$ and increasing the exposure times (*$p<0.05$; **$p<0.001$ different from control values).

Statistical analysis of the data was conducted using a two-way analysis of variance (ANOVA) in order to determine if there was a difference among the varying concentrations and exposure times. With the mercuric chloride treatments, it was observed that with increasing concentrations, differences in cell viability were observed over various time points when compared to control values (Figure 4). The results following two-way ANOVA demonstrated a significant effect of time ($F_{3,60}$=4.44; p=0.0069), concentration ($F_{4,60}$=114.36; p<0.0001) and a time X concentration interaction ($F_{12,60}$=5.33; p<0.0001) on cell viability following mercuric chloride exposure.

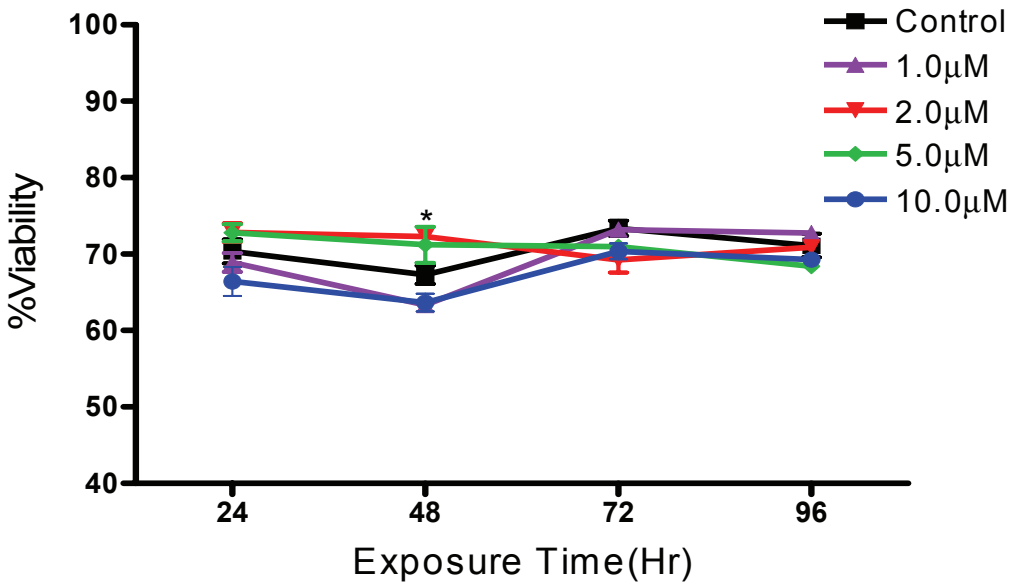

Figure 7: LDH activity in SK-N-SH cells upon various $MnCl_2$ concentrations and exposure times. Percent viability was calculated the same as for the $HgCl_2$ values, and then analyzed using a two-way ANOVA. Each value represents the mean ± SEM of a sample size of four completed in at least triplicate. The data demonstrates that there is a significant effect of time (p<0.0001), concentration (p=0.0003) and time X concentration (p<0.0001) effect on the SK-N-SH cell line (*p<0.05 different from control values).

Interestingly, the manganese chloride treatments also exhibited a significant effect of time ($F_{3,60}$=8.97; p<0.0001), concentration ($F_{4,60}$=6.22; p=0.0003) and time X concentration interaction ($F_{12,60}$=4.63; p<0.0001) on SK-N-SH viability (Figure 5). This

is likely due to the trend of the data, since the only point statistically different from control was 2.0μM $MnCl_2$ at 48 hours ($p<0.05$).

IV.B. Caspase-3/7 Assay

Based on the results of the LDH assay, it was determined that 1.0μM $HgCl_2$ and 10.0μM $MnCl_2$ exposure for 48 and 96 hours would be most appropriate. While it was determined that the SK-N-SH cells were not overtly dying at these concentrations and time points, it wasn't known if these cells may be entering into a slower programmed death process, apoptosis. Therefore, the next step was to determine if 1.0μM $HgCl_2$ or 10.0μM $MnCl_2$ exposure at 48 and 96 hours increased caspase activity, a sign that the cells were entering the apoptosis pathway. For the detection of caspase-3 and -7 activities, Promega's Apo-ONE® Homogeneous Caspase-3/7 Assay was utilized. Samples fluoresced in direct proportion to the amount of capase-3/7 activity present, which was then measured at an excitation wavelength of 485 ± 20nm and an emission wavelength of 530 ± 25nm. The average blank values were subtracted from the control and treatment groups before statistical analysis was preformed on the data.

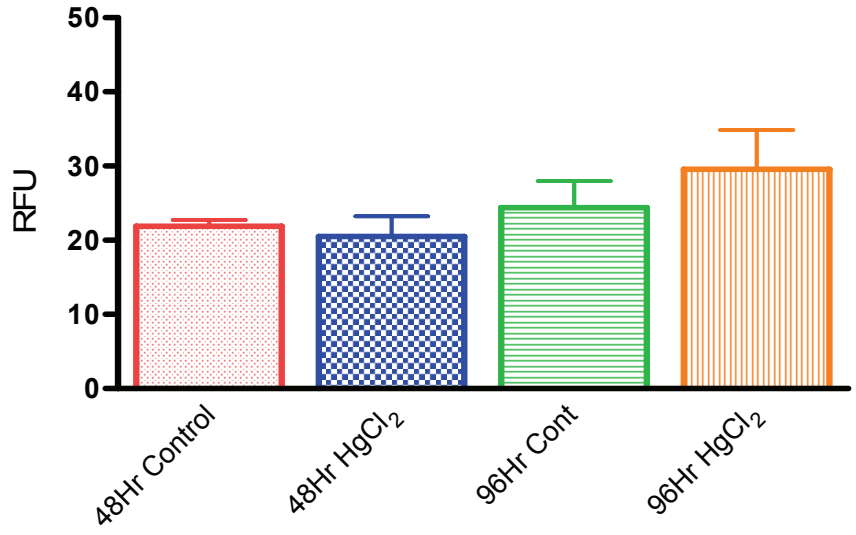

Figure 8: Caspase-3/7 activity in the SK-N-SH cell line after 1.0µM $HgCl_2$ treatment for 48 or 96 hours. Data was calculated by subtracting the average blank value (18.7188±1.7188) from the control and treatment values, and is expressed as mean absorbance values ± SEM of a sample size of four run in quadruplicate. A one-way ANOVA determined that there was not a significant effect due to 1.0µM $HgCl_2$ treatment at 48 or 96 hours (p=0.3255).

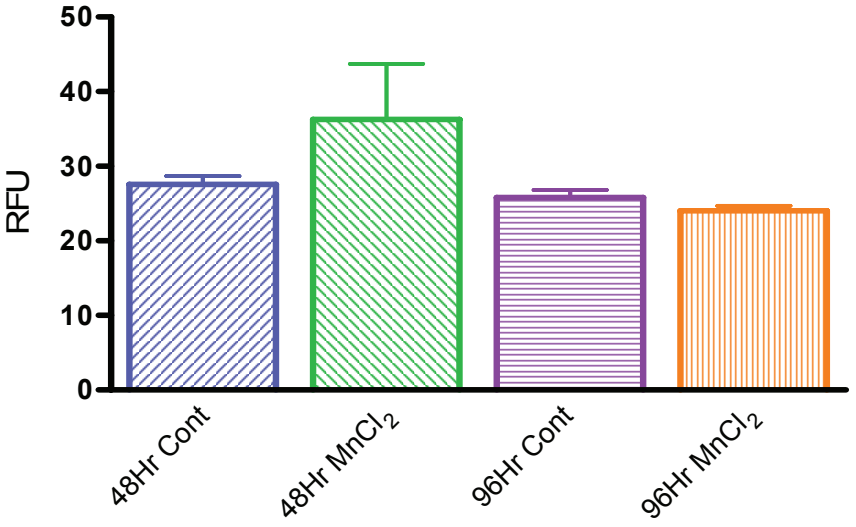

Figure 9: Caspase-3/7 activity in the SK-N-SH cell line after 10.0µM $MnCl_2$ treatment for 48 or 96 hours. Data was calculated by subtracting the average blank value (18.7188±1.7188) from the control and treatment values, and is expressed as mean absorbance values ± SEM of a sample size of four run in quadruplicate. There was not a significant effect due to 10.0µM $MnCl_2$ treatment at 48 or 96 hours as determined by a one-way ANOVA (p=0.1670).

For both the mercuric chloride and manganese chloride treatment groups, a one-way ANOVA was preformed to determine the effect of time. It was found that the 1.0µM mercuric chloride treatment at 48 and 96 hours did not have a significant effect on Caspase-3/7 activity in the SK-N-SH cell line ($F_{3,15}$=1.255; p=0.3255) (Figure 6). A similar lack of effect was observed with 10.0µM manganese chloride ($F_{3,15}$=2.006; p=0.1670)(Figure 7).

The ratio of percent viability per caspase-3/7 activity was calculated to determine if the cells that survived the metal treatment had more or less caspase-3/7 activity in comparison to the corresponding control groups (Tables 1 and 2).

Table 1: Caspase-3/7 Activity per %Viability ($HgCl_2$). 48 and 96Hr treatment with 1.0µM HgCl2 (data represents mean ± SEM for an N=4).

Treatment	48Hr	96Hr
Control	0.3480±0.0084	0.3432±0.0503
$HgCl_2$	0.2929±0.0364	0.4435±0.0869

A one-way ANOVA (Kruskal-Wallis) was calculated for the comparison of the 48 and 96 hour control and 1.0µM $HgCl_2$ groups determined there was not a significant treatment effect (p=0.4609) on the ratio of caspase-3/7 activity to percent viability.

Table 2: Caspase-3/7 Activity per % Viability ($MnCl_2$). 48 and 96Hr treatment with 10.0µM MnCl2 (data represents mean ± SEM for an N=4).

Treatment	48Hr	96Hr
Control	0.4094±0.0117	0.3642±0.0210
$MnCl_2$	0.6542±0.1087	0.3469±0.0134

One-way ANOVA (Kruskal-Wallis) analysis of the control and 10.0µM $MnCl_2$ data calculated a significant effect of treatment on the ratio of caspase-3/7 data to percent viability (p=0.0347). Because there was a significant difference, Dunn's Multiple Comparison Test was run after the one-way ANOVA. This post test determined there was not a significant difference between control and $MnCl_2$ treatment groups at 48 or 96

hours ($p>0.05$). This indicates there was no increase in the amount of apoptosis activity in the remaining live cells due to either treatment.

IV. C. [^{3}H]GBR-12,935 Binding

[^{3}H]GBR-12935 binding to the DAT was utilized to determine if the different heavy metal treatments would affect the cell surface expression of the dopamine transporter. Because GBR-12,935 binds with high affinity (5.5nM as determine in rat striatal membranes) (Andersen, 1987), and specificity, it makes it an appropriate drug to label the DAT. The binding studies were performed after cells were treated in flasks with vehicle, 1.0μM $HgCl_2$, or 10.0μM $MnCl_2$ for 48 or 96 hours. The cells were then trypsinized, and centrifuged into a pellet. The pellet was resuspended in assay buffer and incubated at room temperature to equilibrium (60 minutes) with either assay buffer (total binding) or 5μM GBR-12,909 (non-specific binding) along with the [^{3}H]GBR-12,935. Samples were then harvested on a GF/C filter followed by placement into a vial and then scintillation cocktail was added. The amount of binding was measured by liquid scintillation spectrophotometry. A protein assay was then conducted on the unused portion of the sample using the Bradford method. Data was then calculated and reported as pmol/mg protein.

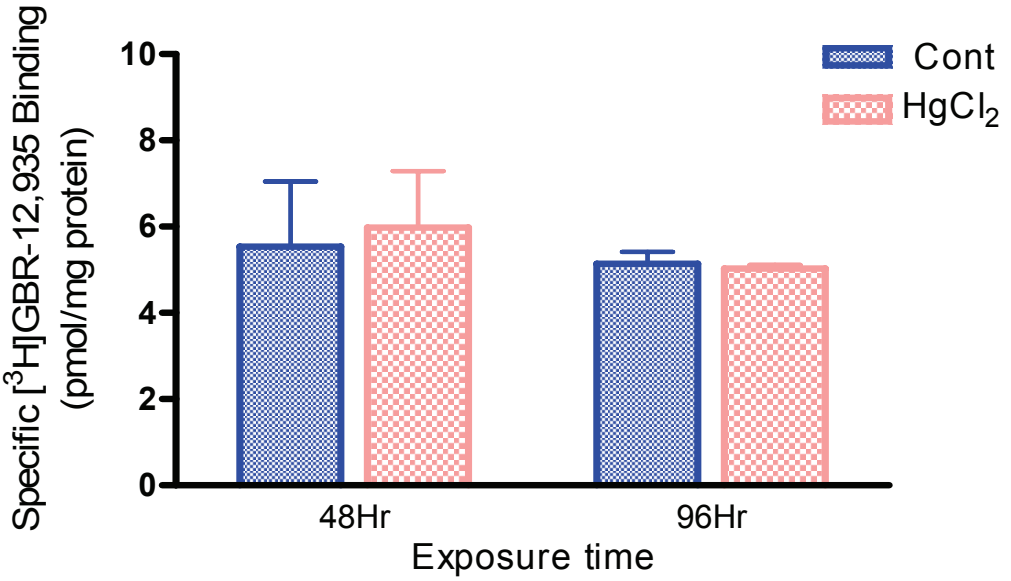

Figure 10: The effect of 1.0µM $HgCl_2$ on [^{3}H]GBR-12, 935 binding to the dopamine transporter after exposure to the SK-N-SH cell line for 48 and 96 hours. The data represents an N=4, conducted in duplicate ±SEM. Statistical analysis determined that this concentrations of $HgCl_2$ did not have a significant effect on [^{3}H]GBR-12,935 binding to the DAT over 48 and 96 hours (p=0.9053).

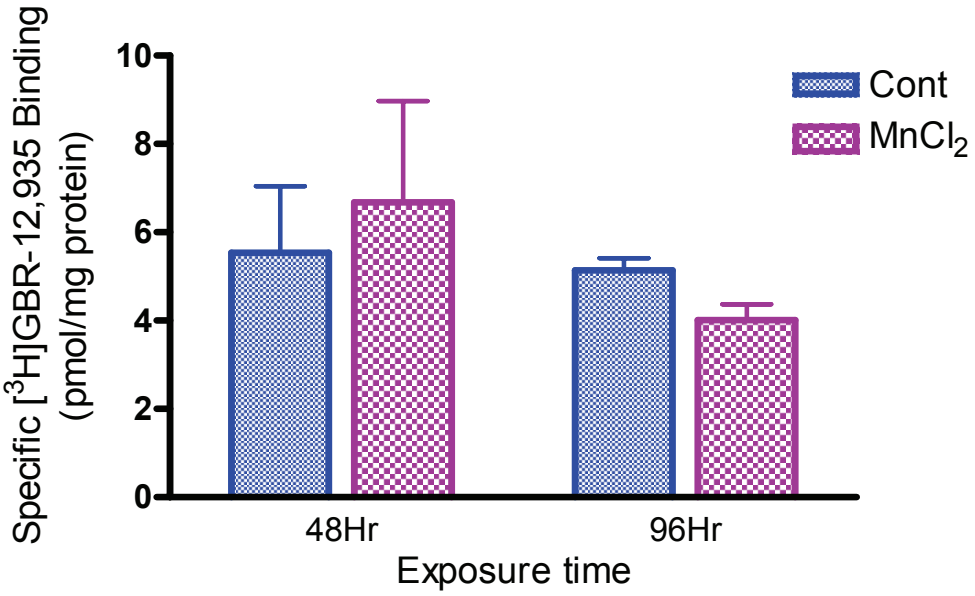

Figure 11: The effect of 10.0µM $MnCl_2$ on [^{3}H]GBR-12,935 binding to the plasma membrane bound dopamine transporter. Binding took place in assay buffer, and this reaction was stopped by washing the samples with an ice cold 0.9% NaCl. The data represents a sample size of four conducted in duplicate. Statistical analysis determined that 10.0µM manganese chloride did not have a significant effect on [^{3}H]GBR-12,935 binding to the dopamine transporter at either 48 or 96 hours (p=0.6112).

A one-way ANOVA was preformed on the data to determine if there was a significant time effect on the amount of DAT expression as when compared to the values calculated for the control groups at each time point. There were no significant effects of $HgCl_2$ on the binding of [^{3}H]GBR-12,935 binding to the DAT in the SK-N-SH cell line ($p=0.9053$) (Figure 8). The one-way ANOVA calculated on the $MnCl_2$ data also determined that 10.0μM $MnCl_2$ did not effect [^{3}H]GBR-12,935 binding to the DAT at 48 or 96 hours ($p=0.6112$) (Figure 9).

The saturation binding curve assays were carried out similarly to the one-point [^{3}H]GBR-12,935 binding described above. One difference is instead of using the whole flask for the assay, only one half of the flask was used so that the other half could be used for [^{3}H]DA uptake assays. The other difference is that instead of testing one concentration, the saturation binding curve assays utilized six increasing concentrations, 1, 2.5, 5, 10, 25, 50nM (final in tube concentrations). Specific [^{3}H]GBR-12,935 binding values were calculated as before: Total Binding-Nonspecific Binding=Specific Binding. This value was combined with the results from the protein assay on the remaining samples to calculate pmol/mg protein values for each of the data points.

Linear regression was performed on the data after it was plotted (Figures 10 and 11) using GraphPad prism in order to calculate the K_D and B_{max} values for each of the treatments (see Tables 2 & 3). Once this was conducted, a one-way ANOVA was performed to compare the K_D and B_{max} values in order to determine if the 48 or 96 hour treatments with 1.0μM $HgCl_2$ or 10.0μM $MnCl_2$ caused a change in the dopamine transporter binding affinity or capacity.

Table 3: B_{max} and K_D values for [^{3}H]GBR-12,935 binding. 48Hr treatment with 1.0µM $HgCl_2$ or 10.0µM $MnCl_2$.

	48 Hr Control	48Hr $MnCl_2$	48Hr $HgCl_2$
B_{max} (pmol/mg protein)	469.9±162	1736±993.3	1592±407
K_D (nM)	8.96±9.20	15.43±19.84	16.73±9.73

***All values represent the mean ± SEM for an N=4 completed in duplicate.**

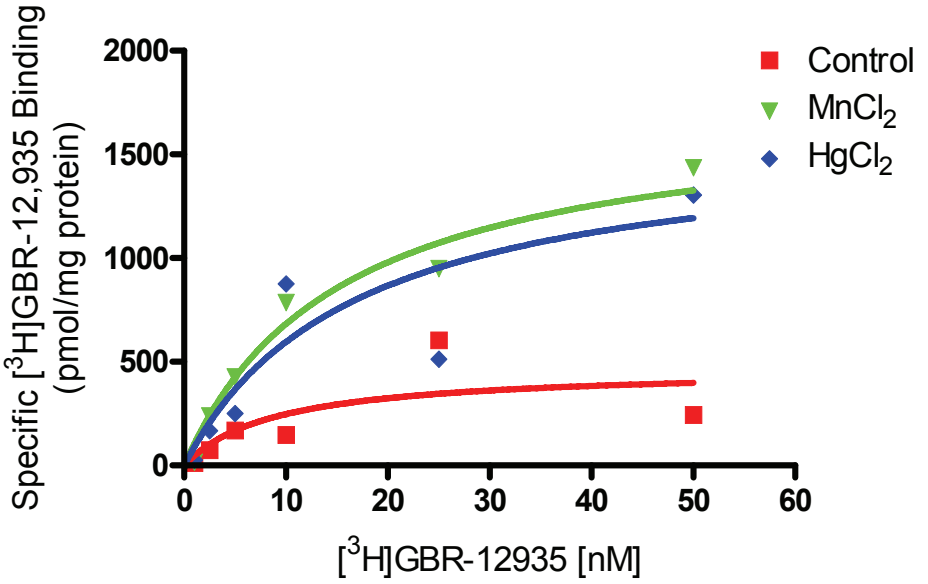

Figure 12: 48 hour saturation [^{3}H]GBR-12,935 binding curves after 1.0µM $HgCl_2$ or 10.0µM $MnCl_2$ exposure. Binding was conducted in assay buffer and incubated at room temperature for 60 minutes. The samples were harvested onto PEI soaked filters before being transferred to scintillation vials and counted. Above values represent an N=4 conducted in duplicate. Statistical analysis using a one-way ANOVA determined no significance in B_{max} (p=0.2106) or K_D (p=0.1738) among the control and two metal treatments. SEM error bars were omitted for graphical purposes.

The results from the one-way ANOVA Kruskal-Wallis test performed on the 48hour data determined neither the B_{max} nor K_D values from the treatments with 1.0µM $HgCl_2$ or 10.0µM MnCl2 were significantly different from the 48hour control values for these measures (p=0.2106, 0.1738 respectively).

Table 4: B_{max} and K_D values for [^{3}H]GBR-12,935 binding. 96Hr treatment with 1.0µM $HgCl_2$ or 10.0µM $MnCl_2$.

	96Hr Control	96Hr $MnCl_2$	96Hr $HgCl_2$
B_{max} (pmol/mg protein)	339.4±84.7	260.7±68.6	181.5±68.2
K_D (nM)	7.08±5.44	6.32±5.28	6.89±8.03

***All values represent the mean ± SEM for an N=4 completed in duplicate.**

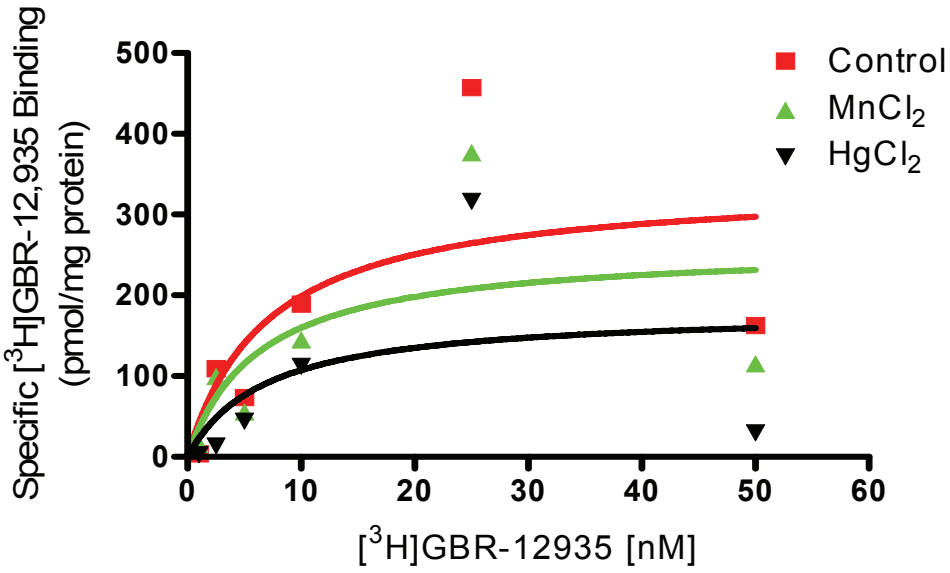

Figure 13: 96 hour saturation [^{3}H]GBR-12,935 binding curves after 1.0µM $HgCl_2$ or 10.0µM $MnCl_2$ exposure. Specific binding was determined by subtracting non-specific binding (GBR-12,909) from total binding (assay buffer). One-way ANOVA statistical analysis calculated no significance for either the Bmax or KD values due to the either of the metal treatments after 96Hr treatments (p=0.1738, 0.6677 respectively). SEM error bars were omitted for graphical purposes.

The one-way ANOVA Kruskal-Wallis test analysis calculated for the data obtained from the 96Hr treatments with 1.0µM $HgCl_2$ or 10.0µM $MnCl_2$ also determined there was no significant difference in the B_{max} (p=0.1738) or K_D (p=0.6677) values at this time point.

Additional one-way ANOVA calculations were conducted to see if treatment time (48Hr vs. 96Hr) had an effect on B_{max} and K_D values. Analysis of the $HgCl_2$ data determined while there was not a significant treatment difference for the K_D value (p=0.8137), there was a significant treatment effect of B_{max} (p=0.0110) calculated using the Kruskal-Wallis test. A Dunn's Multiple Comparison test following the one-way ANOVA calculated a significant difference between the 48 and 96 hour $HgCl_2$ treated groups ($p<0.01$). Analysis of 10.0µM $MnCl_2$ and the control groups found no significant difference in Bmax (p=0.0877) or K_D (p=0.3256) from 48 to 96 hours.

IV.D. [^{3}H]DA Uptake

Two point [^{3}H]DA uptake assays were conducted to determine if the functionality of the DAT had changed due to 48 or 96 hour exposure to 1.0μM $HgCl_2$ or 10.0μM $MnCl_2$ and/or if there was a change in function from a low to high concentration of [^{3}H]DA. For the uptake assay, the other half of the flask used for [^{3}H]GBR-12,935 saturation curve binding assays was used. The treatment was stopped with two washes of warmed RPMI and the cells were detached from the side of the flask using trypsin/EDTA. The cells were then centrifuged for three to five minutes, the supernatant removed, and then the cell pellet was resuspended in 30mL of assay buffer. 400μL of the cell suspension was then incubated at room temperature with either assay buffer (total uptake) or 5μM GBR-12,909 (non-specific uptake) and 20 or 200nM [^{3}H]DA (final in tube concentrations) for ten minutes. At the end of the incubation period, the samples were harvested onto GF/C filters and transferred to a vial. Scintillation cocktail was added; the vial was vortexed, and then placed into the scintillation counter. The Bradford method was used to determine the amount of protein in the samples. All of the data was then combined and calculated so values could be reported as pmol/mg protein/min.

For statistical analysis, a two-way ANOVA was calculated on the data to determine if there was an effect on [^{3}H]DA uptake due to metal treatment, [^{3}H]DA concentration, and/or interaction between these variables. The Bonferroni post test was also calculated, so the means of each group analyzed in the two-way ANOVA could be compared to each other.

The two-way ANOVA calculated on the 48 and 96Hr control and 1.0μM $HgCl_2$ [^{3}H]DA uptake data determined there was a significant effect of treatment ($F_{3,24}$=9.11;

p=0.0003), [^{3}H]DA concentration ($F_{1,24}$=19.86; p=0.0002), and interaction of treatment X [^{3}H]DA concentration ($F_{3,24}$=6.16; p=0.0029) in SK-N-SH cells. The Bonferroni posttest calculated no significant difference established between 48 and 96 hour $HgCl_2$ values at 20 or 200nM [^{3}H]DA (p>0.05 for both concentrations).

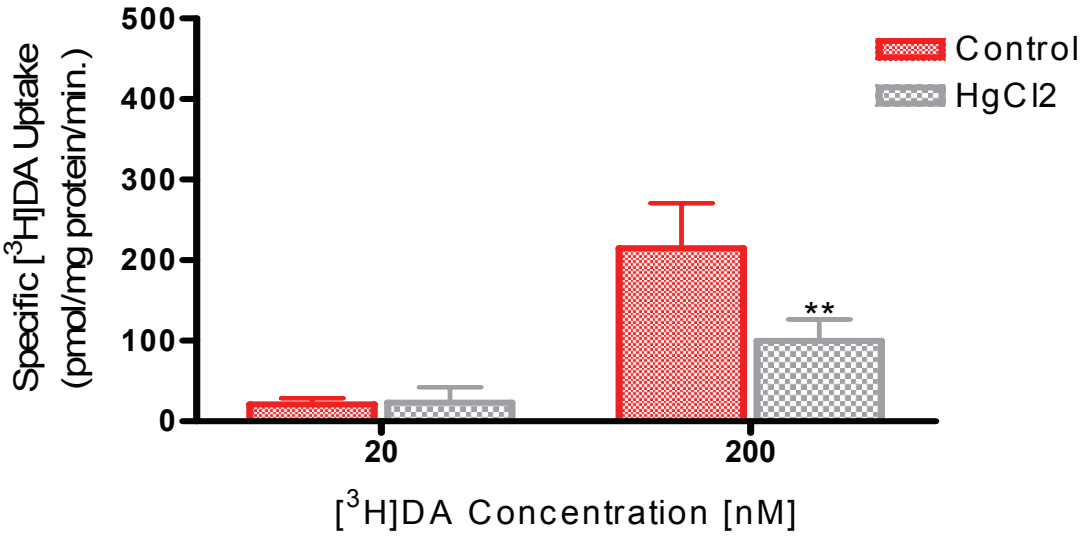

Figure 14: 20 and 200nM [^{3}H]DA uptake in SK-N-SH cells after 48 hour exposure to 1.0µM $HgCl_2$. Specific uptake was determined by subtracting non-specific uptake from total uptake. The Bradford method was used to calculate protein values so data could be reported as pmol/mg protein/min. The Bonferroni posttest calculated no significant difference between control and $HgCl_2$ [^{3}H]DA uptake values at 20nM [^{3}H]DA. There was a significant difference in [^{3}H]DA concentration uptake between control and $HgCl_2$ groups at 200nM (p<0.01) detected in the SK-N-SH cell line (p<0.01 different from control values).**

The results from the Bonferroni posttest indicated that after 48hour treatment with $HgCl_2$ there was a not a significant difference between the control and $HgCl_2$ groups at 20nM (p>0.05), but a significant difference was detected at 200nM [^{3}H]DA (p<0.01) (Figure 14).

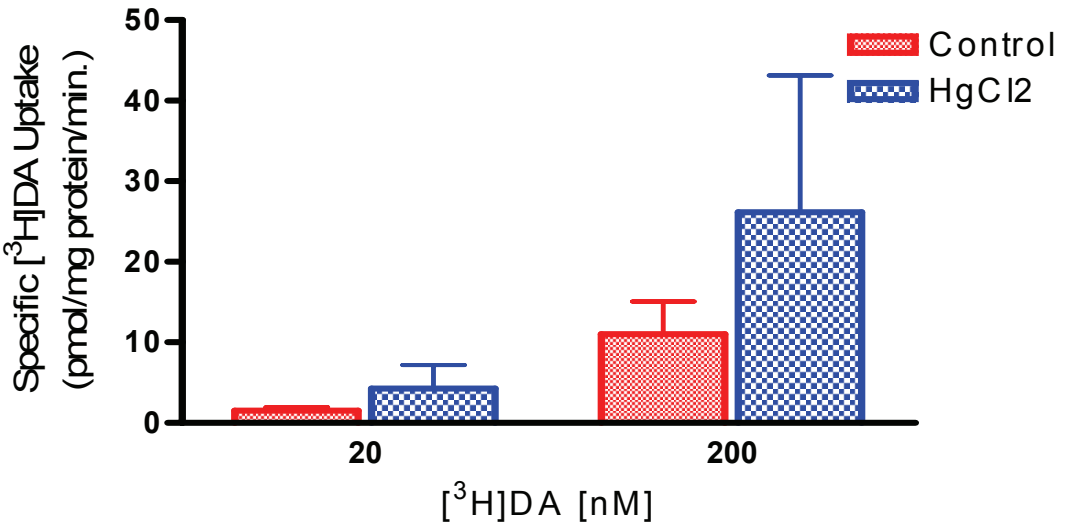

Figure 15: 20 and 200nM [^{3}H]DA uptake in SK-N-SH cells after 96 hour exposure to 1.0µM $HgCl_2$. Specific uptake was determined by subtracting nonspecific uptake (GBR-12909) from total uptake (assay buffer). The Bradford method was used to determine the amount of protein in each sample so values could be reported as pmol/mg protein/min. A Bonferroni posttest determined there was no difference between the control and 96 hour 1.0µM $HgCl_2$ group at concentration of 20nM [^{3}H]DA ($p>0.05$) or 200nM [^{3}H]DA($p>0.05$).

No significant difference was observed between the 96 hour control and $HgCl_2$ groups in SK-N-SH cells when means from the [^{3}H]DA uptake assay were compared using the Bonferroni posttest ($p>0.05$ for both 20 and 200nM [^{3}H]DA) (Figure 13).

Table 5: Summary of Uptake Findings after treatment with 1.0uM $HgCl_2$ in SK-N-SH cells

	20nM	200nM
48 Control vs. 48 $HgCl_2$	no significant difference	**significant difference ($p<0.01$)**
96 Control vs. 96 $HgCl_2$	no significant difference	no significant difference

Similar statistical calculations were also performed on the $MnCl_2$ data. Results from the two-way ANOVA conducted on the combined 48 and 96 hour data comparing the control data to that of 10.0µM $MnCl_2$ treatment determined there was a significant treatment ($F_{3,24}=9.95$; $p=0.0002$), [^{3}H]DA concentration ($F_{1,24}=22.58$; $p<0.0001$), and treatment X [^{3}H]DA concentration ($F_{3,24}=6.98$; $p=0.0015$) effect on [^{3}H]DA uptake in SK-N-SH cells. The Bonferroni posttest determined there was also a significant

difference between 48 and 96 hour MnCl2 values at 200nM ($p<0.001$), but not at 20nM ($p>0.05$).

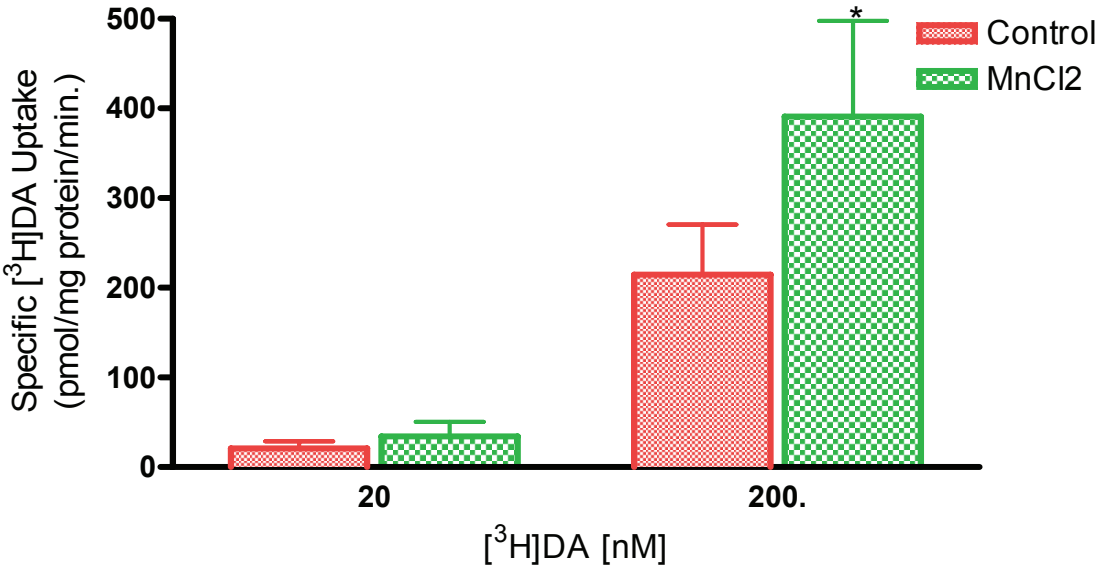

Figure 16: 20 and 200nM [^{3}H]DA uptake in SK-N-SH cells after 48 hour exposure to 10.0µM $MnCl_2$. Pmol/mg protein/min values were calculated using specific uptake values (total uptake-nonspecific uptake) and protein concentrations as determined by the Bradford method. The Bonferroni posttest conducted on this data established there was not a significant treatment effect at 20nM [^{3}H]DA ($p>0.05$), but there is a significant treatment effect at 200nM [^{3}H]DA ($p<0.05$) (*$p<0.05$ different from control values).

Statistical analysis on the 48 hour control vs. 10.0µM $MnCl_2$ data using a Bonferroni posttest determined there was not a significant difference between means at 20nM [^{3}H]DA ($p>0.05$). The same test did determine a significant difference between control and $MnCl_2$ values with 200nM [^{3}H]DA uptake ($p<0.05$) (Figure 14).

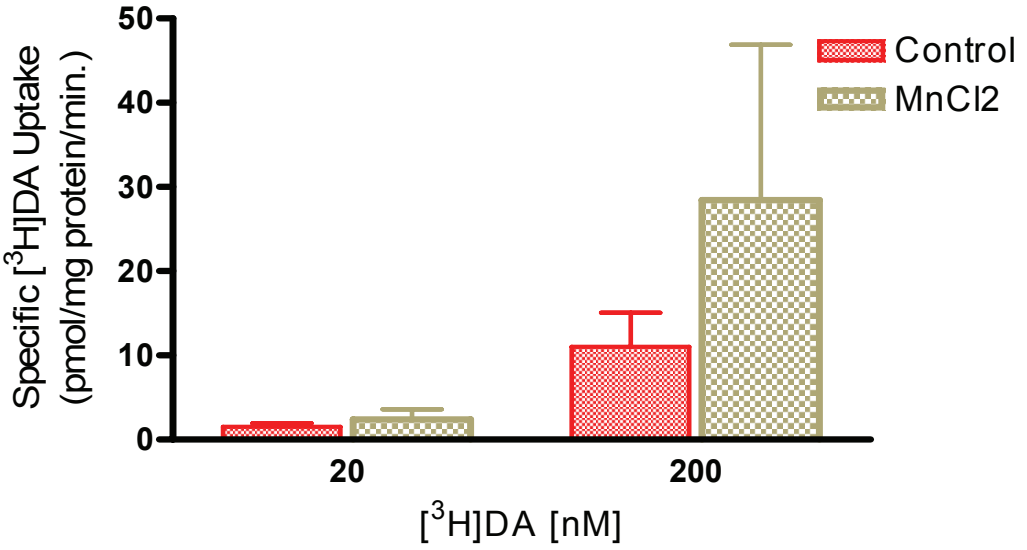

Figure 17: 20 and 200nM [^{3}H]DA uptake in SK-N-SH cells after 96 hour exposure to 10.0µM $MnCl_2$. Above values represent mean ± SEM of an N=4 run in duplicate. The Bradford method was used to determine protein concentration so values could be reported as pmol/mg protein/ min. A Bonferroni posttest calculated no significant treatment effects at either 20 or 200nM [^{3}H]DA (p>0.05 for both concentrations).

The Bonferroni post test conducted on the 96 hour uptake data determined there was not a significant difference due to treatment at either [^{3}H]DA concentration examined (p>0.05 for both 20 and 200nM [^{3}H]DA) (Figure 15).

Table 6: Summary of Uptake Findings after treatment with 10.0uM $MnCl_2$ in SK-N-SH cells

	20nM	200nM
48 Control vs. 48 $MnCl_2$	no significant difference	**significant difference (p<0.05)**
96 Control vs. 96 $MnCl_2$	no significant difference	no significant difference

V. DISCUSSION

The heavy metals mercury and manganese can be found throughout the environment. The acute toxic affects of these metals have been recognized for a long time, and therefore recommendations and precautions have been taken to limit or avoid exposure to these metals. Because of these efforts, acute toxicity due to manganese or mercury is relatively rare in today's world. However, what is not as well documented or recognized are the possible effects of these metals when exposure occurs at low concentrations for extended time periods. Because these metals are ever present with increasing exposure to the population, this study was designed to look at the effects of extended exposure to low concentrations of mercuric and manganese chlorides on dopamine transporter function. The dopamine transporter is suspected to play a role in disease states affecting the dopaminergic system. Because the dopaminergic system is responsible for the reward and locomotor responses among others, its proper functioning and how it is affected by exogenous toxins is important to understand.

The purpose of this study was to determine what effects extended, low dose mercuric and manganese chloride treatments have on the DAT functionality in the SK-N-SH cell line. Initial studies were aimed at finding the greatest concentration of metal that did not result in a reduction in cell viability. These concentrations and time points were then analyzed for caspase-3/7 activity to determine if the cells were entering a slower death process, apoptosis, as this could factor into any effects detected from the metal treatments. Subsequent assays were then more focused on the specific effects of

mercuric and manganese chloride on the dopamine transporter. These studies measured cell expression, saturation binding, and uptake as specifically related to the dopamine transporter.

The overall findings from this study are as follows: sub-lethal concentrations for mercuric and manganese chlorides in the SK-N-SH cell line were identified that did not cause the cells to enter into apoptosis at either 48 or 96 hour time points. Expression and functionality studies, [^{3}H]GBR-12,935 binding and [^{3}H]DA uptake assays indicated that low concentrations of mercuric chloride exerted an effect on the binding of [^{3}H]GBR-12,935 to the dopamine transporter and the [^{3}H]DA uptake capabilities of this protein. Low levels of manganese chloride also caused changes on the ability of [^{3}H]GBR-12,935 to bind to the dopamine transporter as well as alterations in this transporter's ability to uptake [^{3}H]DA.

V.A. LDH and Caspase-3/7 Assays

In order to determine how long term exposure to low concentrations of heavy metals affects the dopamine transporter, it was first necessary to determine the threshold mercuric and manganese chloride concentrations that would not reduce viability in the SK-N-SH cells when treatment times were increased up to 96 hours. The initial concentrations chosen were selected from a review of the literature, and overt toxicity was determined using a lactate dehydrogenase (LDH) assay from Promega (Madison, WI).

LDH is an enzyme found in the cytosol and released when cells undergo lysis. Promega's Cytotoxicity Assay kit detects the presence of LDH as indicated by a color change that is proportional to the amount of cell death that occurred in the sample. In this

study, the amounts of LDH activity present in the samples were measured immediately at the end of the treatment time (spontaneous release) and after the remaining viable cells in the sample were lysed (total release). By measuring both the spontaneous release and total release, calculations could be made to negate any difference in the number of cells per sample or between plates.

The concentrations chosen for analysis for mercuric chloride were 0.1, 1.0, 10.0, and 50.0μM. A review of the literature uncovered only two studies reporting effects of mercury on SK-N-SH cells (Abdulla et al., 1995; Humphrey et al., 2005). However, both studies looked at different mercury reagents (thimerosal or mercuric oxide) compared to this study. Reported median effective concentrations (EC_{50}), the concentration that caused an effect in half of the cells, from mercuric chloride cytotoxicity studies ranged from 3.49μM in SH-SY5Y neuroblastoma cells (Toimela & Tahti, 2004) to 70μM in BALB/c macrophages (Kim & Sharma, 2004). The concentrations tested in this study either fell below or within this range.

From the LDH assay performed on the cells treated with mercuric chloride, an effect on cell viability was found that is dependent on concentration and time, as well as an interaction of the two. In other words, not only was there a decrease in viability due to increasing exposure times and increasing metal concentrations individually, but when the effects of these variables were combined there was an even greater increase in cell death. While other studies have reported observing a concentration effect on cell viability (Parran et al., 2001; Olivieri et al., 2000), few studies have reported the effects of mercuric chloride on viability at time points extending beyond 48 hours. Three studies were found reporting viability effects after 48 hour exposure times (Ben-Ozer et al.,

2000; Kim & Sharma, 2004; Aleo et al., 2005). The two studies reporting results up to 72 hours had different findings. In the study by Ben-Ozer et al., (2000) with U-937 human monocyte-like cells, concentrations of 0.1 to 100μM mercuric chloride over 24-72 hours were examined. A decrease in viability due to concentration was observed, and a trend of decreased viability over time at 1μM was pointed out, however this later observation was slight and not statistically significant (Ben-Ozer et al., 2000). The other study examining varying concentrations (3, 10 and 30μM) of mercuric chloride up to 72 hours reported no effect on cell viability in Madin-Darby canine kidney cells at any of the concentrations or times tested (Aleo et al., 2005). The third study reported no effect on viability after 96 hour exposure times with concentrations up to 20μM in macrophages (Kim & Sharma, 2004). These different effects of mercury on cell viability emphasize the point that the physiological response to exposure depends on several factors, including the cell line being used.

For the viability assays using manganese chloride, concentrations of 1, 2.0, 5.0 and 10.0μM were chosen for their effects on the SK-N-SH cell line. A significant effect of concentration or time, and a synergistic effect of concentration and time were observed. However, since statistical analysis determined only one point to be significantly different from control values (48hr exposure to 2μM manganese chloride), the effect is likely to be representative of the observed trends. Therefore, 10.0μM manganese chloride at 48 and 96 hour treatment times was chosen to be examined for the remaining studies.

Unlike mercuric chloride, there are some studies reporting the effects of manganese chloride on the SK-N-SH cell line. Two of these studies examined the effect

of increasing concentrations of manganese chloride on cell viability. Keller et al., (2005) reported a concentration-dependent biphasic response after treating SK-N-SH cells for 48 hours with manganese chloride, in concentrations ranging from 10.0μM to 3mM, with lower concentrations increasing viability and higher concentrations resulting in increased cell death. The other study examining the effects of manganese chloride on SK-N-SH cells calculated an EC_{50} = 200μM after treating the cells with increasing concentrations from 10μM-1mM for 24 hours (Stredrick et al., 2004). While it is not discussed in their report, the data showed a concentration effect on cell viability; as the concentration of manganese chloride exposure increased, cell viability decreased. Both of the trends mentioned above, are more obvious trends compared to the current data, which is likely due to the larger range of concentrations examined in previous studies.

Before the specific effects of these metals on the dopamine transporter were examined, an assay was conducted to see if sub-toxic concentrations of the two metals caused the cells to enter apoptosis. Promega's Caspase-3/7 Assay was utilized to detect any changes in the activity of caspases-3 and -7 after 1μM $HgCl_2$ and 10μM $MnCl_2$ treatment for 48 and 96 hours. These caspases are activated during the "effector phase" of apoptosis which is immediately before the "cell degradation phase" and cell death (Honing and Rosenberg, 2000). Therefore, measuring the activity of these caspases not only is an indication of cells in apoptosis, but also indicates that cell death is imminent.

The results from this study demonstrated that treatment of SK-N-SH cells with 1.0μM $HgCl_2$ for 48 hours did not result in an increase in the number of cells entering apoptosis as compared to the control group. No significant difference in caspase activity was observed after the 96 hour exposure time either. There was a slight increase in the

amount of fluorescence in both the control and mercury treated groups at 96 hours as compared to the 48 hours groups, however this difference was not significant. It may be that the increase in fluorescence is due to a slight increase in caspase activity in the viable cells. This would be expected and probably reflects increases in basal levels of caspase with increasing time. To test this idea, the data from the caspase-3/7 assay was combined with the data from the LDH assay to analyze the ratio of unit of caspase-3/7 activity per viability (as reflected in LDH activity). Statistical analysis of the ratios determined there was not a difference in the means of the control and mercury treatment groups at either 48 or 96 hours, indicating there is no change in the amount of caspase-3/7 activity in the viable cells present in the sample.

For the cells treated with 10.0μM $MnCl_2$, little difference was observed in caspase-3/7 activity between the control and treatment groups at either the 48 or 96 hour exposure times. Again, the ratio of caspase-3/7 activity was paired to the corresponding values of viability after manganese chloride treatment and statistically analyzed for any differences from control values. When control values were compared to manganese chloride treatments, there was a significant difference detected in the unit caspase-3/7 activity per viability, suggesting there may be an increase in the activities of caspases-3 and -7 in the remaining cells. Further statistical analysis, however, did not calculate a difference between control and manganese chloride groups at 48 or 96 hours.

It was concluded from the LDH and Caspase-3/7 assays that mercuric chloride is a more robust toxin compared to manganese chloride at the concentrations and times examined. This conclusion supports the idea that mercuric chloride would be able to exert its effects more quickly than manganese chloride after exposure, predicting that

neurological symptoms would appear sooner in patients exposed to mercury as opposed to those exposed to manganese.

V.B. DAT Functional Assays

Because the number of dopamine transporters present on the cell plays a part in how well dopamine is taken up into the cell, analyzing how its presence is affected by toxins is just as important as studying the actual function of substrate movement by this protein. The selectivity of [^{3}H]GBR-12,935 binding to the dopamine transporter makes it a useful tool for labeling and studying the presence and functioning of the transporter. [^{3}H]GBR-12,935 binding to the dopamine transporter was used to determine if the heavy metal treatments had an effect on (1) the amount of DAT being expressed in cells (B_{max}) and (2) the binding affinity (K_D) of the transporter. Alterations in the amount of the DAT being expressed by the cells exposed to heavy metal was detected using a one-point binding assay with 4nM [^{3}H]GBR-12,935, while DAT affinity and capacity kinetics were evaluated using increasing concentrations (1, 2.5, 5, 10, 25, 50nM) of [^{3}H]GBR-12,935.

Another aspect of dopamine transporter function is the actual uptake of dopamine. This was measured after mercuric and manganese chloride exposure. Both low (20nM) and high (200nM) concentrations of [^{3}H]DA were utilized in the uptake studies so that any shift in uptake due to these metals could be detected. To reduce variability, half of the flask of cells treated for the uptake assay were set aside and used for the saturation binding curve assays mentioned in the preceding paragraph.

V.B.i. Mercuric chloride effects on DAT functionality

The results from the single-point (4nM) [^{3}H]GBR-12,935 binding assay indicated 1.0μM mercuric chloride treatment does not affect the expression of DAT on SK-N-SH

cells when compared to control values. Neither was there a change in DAT expression from 48 to 96 hour treatment after exposure to this metal. Statistical analysis of the data generated from the saturation binding curve assays revealed that there was no difference in the binding affinity of [^{3}H]GBR-12,935 for the DAT after 48 or 96 hour exposure to mercuric chloride when compared to corresponding control values. There was also no significant difference detected for binding capacity after mercuric chloride treatment at 48 and 96 hours. However, there was a trend of increased binding capacity when compared to control values after 48 hour exposure, and a slight decrease in binding capacity after 96 hour exposure when compared to the corresponding control values. There was a significant difference in binding capacity due to mercuric chloride treatment between the 48 and 96 hours exposure times. These results demonstrate that the number of DAT binding sites decrease from 48 to 96 hours after mercuric chloride treatment.

Previous studies have reported effects on DAT binding and DA uptake after acute exposure to mercury. Cao et al. (1989) reported an inhibition of [^{3}H]cocaine binding and [^{3}H]DA uptake by the DAT following inorganic mercury exposure in bovine and rat striata. From their results, they hypothesized the existence of one or more thiol groups on the dopamine transporter are important for binding and uptake functions (Cao et al., 1989). Richfield (1993) also reported Hg^{2+} ions reduced binding of [^{3}H]GBR-12,935 to the DAT. Short term mercury exposure (ten minutes to an hour) effects on the DAT resulted in low (3μM) concentrations stimulating the binding of two other dopamine uptake inhibitors, [^{3}H]mazindol and [^{3}H]WIN-35,428, while higher concentrations (30-100μM) inhibited binding of these radioligands to the DAT (Wu et al., 1997). Another study also found this biphasic effect on DAT binding after mercuric chloride exposure

with [^{3}H]methylphenidate binding in striatal tissue exposed to increasing concentrations (0.05-1000μM) for thirty minutes (Schweri, 1994). Schweri (1994) observed both the affinity and B_{max} of [^{3}H]methylphenidate binding to be altered at the higher mercury concentrations (both reduced), while only the affinity was affected (increased) at lower concentrations.

Mercury was also reported to inhibit [^{3}H]DA uptake after acute exposure. Cao et al., (1989) reported in the same study as their binding experiments, an inhibition of [^{3}H]DA uptake following inorganic mercury exposure in bovine and rat striata. Another study, however, determined there was no effect of $HgCl_2$ on synaptosomal [^{3}H]DA uptake (Schweri, 1994; Komulainen & Tuomisto, 1981). Rajanna and Hobson (1985) reported a concentration-dependent reduction in [^{3}H]DA uptake when compared to control following exposure to mercuric chloride, with an initial significant decrease occurring after a five minute incubation with 1.0μM.

The current study noted an effect of exposure time, [^{3}H]DA concentration, and a synergistic interaction of exposure time and [^{3}H]DA concentration on the uptake of [^{3}H]DA through the dopamine transporter. After 48 hour exposure to mercuric chloride, a statistically significant decrease in uptake was observed in comparison to the control groups. 96 Hour exposure to mercuric chloride did not cause a significant effect on [^{3}H]DA uptake, however a increase in uptake when compared to control values was observed.

Combining the [^{3}H]DA uptake results with the [^{3}H]GBR-12,935 saturation binding studies would suggest that $HgCl_2$ at low concentrations and longer exposure times does affect the DAT functioning. As was seen after 48 hour mercuric chloride

exposure, an increase in binding capacity with a decrease in uptake through the DAT was observed. This was followed by a decrease in binding capacity and an increase in uptake when compared to the control groups after 96 hour exposures. These effects could possibly be due to a direct interaction of mercury with the cysteine residues on the transporter since mercury does have a high affinity for thiol groups. Another possible explanation is that mercury may interfere with or alter the N-glycosylation sites on the large extracellular loop of DAT. As mentioned earlier in the DAT section of the literature review, previous studies have reported the importance of glycosylation of the DAT. Torres et al. (2003) and Li et al. (2004), looking at the effects of the removal of one or more of the glycosylation sites have reported an affect on the binding capacity of DAT with no (Torres et al., (2003)) or little (Li et al., (2004)) effect on the affinity of this transporter. In other words, this transporter while still expressed in the cells has decreased ability for DA uptake. Results from [3H]GBR-12,935 binding and [3H]DA uptake as described above, suggests this may be a possible mechanism of mercury on the DAT.

V.B.ii. Manganese chloride effects on DAT functionality

In this study, it was determined 10.0μM manganese chloride treatment, when compared to corresponding control values, had no effect on the single-point (4nM) [^{3}H]GBR-12,935 binding to the DAT, nor did this metal significantly affect the binding affinity or capacity of the DAT as determined through [^{3}H]GBR-12,935 saturation curve binding. There was a decrease in binding capacity from the 48 to 96 hour exposure times; however, this difference was not statistically significant from control values.

Increased [^{3}H]DA uptake was observed after manganese chloride exposure in comparison to control groups. This increase occurred at 200nM [^{3}H]DA after 48 and 96 exposure times, however the increase was determined statistically significant only after 48 hour exposure times. As was detected with mercuric chloride, there was an overall decrease in uptake experienced by control and groups treated with manganese chloride from the 48 to 96 hour time points.

In vivo effects of manganese on DAT binding has been reported in several studies. Reichel et al. (2006) reported that early exposure of rats to manganese altered DAT function once the rat reached adulthood. They found a decrease in [^{3}H]GBR-12,935 striatal DAT binding in the rats without an effect on DAT affinity, but a significant decrease in binding capacity (Reichel et al., 2006). Huang et al. (2003), also found that DAT binding was decreased in patients after chronic manganese exposure when compared to control patients. Chronic manganese exposure in monkeys also has been reported to cause a reduction in [^{3}H]mazindol binding to DAT in the caudate nucleus and putamen after chronic exposure (Eriksson et al., 1992).

While it appears there is supportive data that chronic manganese exposure reduces DAT binding, the effect of dopamine uptake via the DAT after chronic manganese exposure is not as clear. Results from [^{3}H]DA uptake studies have reported both increases and decreases in DAT functioning from animal studies (Reichel et al., 2006). Lai et al., (1982) reported a decrease in [^{3}H]DA uptake after 1mg $MnCl_2 \cdot H_2O$/ml for 70 to 90 days. This is in contrast to the increase in [^{3}H]DA uptake reported by Leung et al (1982) after 10 mg $MnCl_2 \cdot H_2O$/ml for 80 days. The differences seen between these two studies are likely due to the increase in $MnCl_2$ concentration. Higher concentrations may

result in upregulation of DAT as a compensatory safety mechanism. The concentrations in these *in vivo* studies are higher than the ones used in the current study. However, caution is needed in comparing the manganese concentrations between animal and *in vitro* studies. It is difficult to extrapolate the concentrations from *in vitro* synaptic to corresponding *in vivo* whole tissue studies. It is unknown to what extent the metals concentrate in the synaptic regions in the animal studies.

The combination of uptake and binding results after manganese chloride exposure found in this study suggest that manganese at low concentrations over 48-96 hours does affect the DAT. It was seen that uptake was increased after 48 hour exposure time with no difference seen in binding capacity in comparison with control values. 96 Hour exposure to manganese when compared to control also demonstrated an increase in [^{3}H]DA uptake with a little decrease in binding capacity. It is believed that manganese exerts much of its toxic effects through oxidative stress (Migheli et al., 1999). The results in this study would support this theory as oxidation from manganese could have been reduced due to the antioxidant substrates in the solvent used as a vehicle for manganese chloride.

V.VI. Summary

Because the dopamine transporter is specifically targeted by certain neurotoxins, it is already considered to be highly susceptible to alterations in the dopaminergic system. The chances of an individual developing Alzheimer's disease or Parkinson's disease increases when this transporter is affected by long term exposure to low concentrations of heavy metals such as mercury and manganese. Pifl et al. (2004) suggested the idea of a second noxious factor causing a pre-condition to develop into an overt symptomology. It

is possible that while these low concentrations of mercury or manganese by themselves are not detrimental, there may be pre-disposing factors such as additional neurotoxin exposure or a compromised dopaminergic system that will result in additive or synergistic effects, thereby allowing these low levels of metals to result in a full-fledged disease state.

Mercury and manganese are both ubiquitous in the environment, and therefore exposure is inevitable. Exposure routes to these metals can occur through many routes including natural, occupational, and accidental. Advisories and regulations have been suggested to limit population exposure to these metals, however it is unknown if these levels are low enough to protect individuals from developing toxicity after extended exposure. Therefore, further research into the effects of extended exposure to low concentrations of mercury and manganese should be conducted.

V.V. Future Studies

Further studies to be conducted will examine if and how these concentrations of mercuric chloride or manganese chloride at the 96 hour time point affects the intracellular trafficking of the DAT and whether exposure to these metals results in the transporter to be more rapidly degraded or recycled after leaving the cell surface. This study could also be replicated in an *in vivo* model to determine if [^{3}H]DA uptake, DAT binding, and DAT trafficking are affected the same way by these metal concentrations and time points.

Another aspect that should be examined is the effects of low levels and long term exposure to methyl mercury and manganese sulfate, more potent and damaging forms of mercury and manganese than the metal salts used in this study. The effects of the combination of these two metals on the DAT would also be an interesting and pertinent

study to undertake. Since both metals are ubiquitous in nature, it is unlikely an individual is only exposed to one or the other of these metals.

V.IV. Summary of Conclusions

• [^{3}H]GBR-12,935 saturation binding studies indicated there was a change in the binding capacity (B_{max}) of DAT after extended exposure to 1.0μM mercuric chloride treatment, with no statistically significant change in DAT affinity (K_D). 10.0μM manganese chloride did not statistically affect B_{max} or K_D values at these same exposure times.

• [^{3}H]DA uptake studies determined there was a significant change in uptake in SK-N-SH cells due to exposure with 1.0μM $HgCl_2$ or 10.0μM $MnCl_2$ after 48 hours, with $HgCl_2$ decreasing uptake and $MnCl_2$ increasing uptake in comparison to control groups. [^{3}H]DA uptake after 96 hour exposure to these metals did not result in a difference in uptake in comparison to respective control values.

• $HgCl_2$ may result in direct alteration of DAT and DA function. This is most likely due to interactions at thiol groups and could involve changes in DAT glycosylation or other intracellular changes.

•$MnCl_2$ appears to affect DAT and DA function indirectly. In vivo studies have shown a more robust change in DAT and this could be due to effects on other systems. Upregulation of DAT would result in increased dopamine uptake and metabolism by MAO leading to formation of H_2O_2 and free radicals, thus slowly damaging the cell from the inside.

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VITA

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Candidate for the Degree of

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Thesis: THE EFFECTS OF SUB-TOXIC HEAVY METALS ON DOPAMINE TRANSPORTER FUNCTION

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Scope and Method of Study: The purpose of this study was to investigate the effects of low concentrations of mercuric (II) and manganese (II) chloride on the dopamine transporter (DAT) functioning through radioligand uptake and binding studies. SK-N-SH cells were utilized for this study, and the effect of low concentrations on cell viability was examined using LDH assays and caspase-3/7 activity to establish cell death or apoptotic activity, respectively. [^{3}H]Dopamine uptake and [^{3}H]GBR-12,935 binding saturation curve studies were conducted to establish the effects low concentrations had on DAT functioning.

Findings and Conclusions: The results of the LDH assay determined that there was a significant effect of time ($p=0.0069$), concentration ($p<0.0001$), and time X concentration interaction ($p<0.0001$) on cell viability when SK-N-SH cells were treated for 24-96 hours with $HgCl_2$ (0.1-50µM). Treatments of $MnCl_2$ at concentrations of 1.0-10.0µM for 24-96 hours also resulted in a significant effect of time ($p<0.0001$), concentration ($p=0.0003$) and time X concentration interaction ($p<0.0001$) on the SK-N-SH cell line viability. Caspase-3/7 activity was not significantly different in either the 1.0µM $HgCl_2$ ($p=0.3255$) or 10.0µM $MnCl_2$ ($p=0.1670$) groups compared to controls. [^{3}H]GBR-12,935 binding also was not significantly different in the 1.0µM $HgCl_2$ ($p=0.7761$) or 10.0µM $MnCl_2$ ($p=0.9966$) treatments when compared to control. [^{3}H]GBR-12,935 saturation binding curve studies revealed there was no significant difference in B_{max} or K_D values following 48 or 96 hour $HgCl_2$ ($p>0.05$) or $MnCl_2$ ($p>0.05$) exposure when compared to control values. However, there was a trend of increased B_{max} after 48 hour exposure to $HgCl_2$. [^{3}H]DA uptake assays used to measure functionality determined there was a significant difference between $HgCl_2$ (decreased uptake, $p<0.01$) and $MnCl_2$ (increased uptake; $p<0.05$) in comparison to control values after 48 hours. Uptake values were not different between treatment and control groups at 96 hours. From these studies, it is concluded that DAT functionality is affected by 1.0µM $HgCl_2$ or 10.0µM $MnCl_2$ treatment in SK-N-SH cells. These effects may be due to direct or indirect effects of the metals on the DAT.

ADVISOR'S APPROVAL: Dr. David Wallace