EFFECTS OF HEAVY METALS AND

PSYCHOSTIMULANTS ON DOPAMINE

TRANSPORTER FUNCTION

By

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NOMENCLATURE

^{3}H	Tritium
AADC	DOPA decarboxylase
AD	Alzheimer's disease
AMPH	Amphetamine
ANOVA	Analysis of variance
Ca^{2+}	Calcium ion
cDNA	Complementary DNA
Cl^-	Chloride ion
CNS	Central nervous system
Coc	Cocaine
DA	Dopamine
DAT	Dopamine transporter
DNA	Deoxyribonucleic acid
ECF	Extracellular fluid
GBR12909	1-[2-[Bis(4-fluorophenyl)methoxy]ethyl]-4-(3-phenylpropyl) piperazine
GBR12935	1-[2-(diphenylmethoxy) ethyl]-4-(3-phenylpropyl) piperazine
hDAT	Human dopamine transporter
Hg	Mercury
LDH	Lactate dehydrogenase
L-DOPA	L-dihydroxyphenylalanine
MA	Methamphetamine
N2A	Neuro2A
Na^+	Sodium ion
*Not*I	*Not*I restriction endonuclease
NT	Neurotransmitter
Pb	Lead
TH	Tyrosine hydroxylase
VTA	Ventral tegmental Area
*Xma*I	*Xma*I restriction endonuclease

CHAPTER I

INTRODUCTION

The interactive effects associated with the administration of multiple drugs have long been a focus of pharmacologic research. When used in combination, drugs can interact in an additive, synergistic, or antagonistic manner. Recently, the potential interaction among toxic agents and drugs possessing abuse liability has attracted the attention of the scientific community. Consideration of adverse effects caused by co-exposure of lead (Pb) and cocaine must be an integral part of protecting public health.

Animal models have indicated that adult dietary Pb exposure, at clinically relevant concentrations, produced changes in cocaine-induced behavior (Burkey et al., 1997). Adult exposure to Pb delayed the development of locomotor sensitization, and ultimately, the magnitude of elevated responding to repeated cocaine challenges was less in metal-exposed than in control animals (Grover et al., 1993; Nation et al., 1996). Sensitization refers to the progressive activation of a pharmacodynamic response at a lower dose than the initial dose. Alternatively, sensitization is described as a shift to the left in the dose-response curve. The dose-response curve for rodents begins with a mild arousal, then hyperactivity with locomotion, then more intense sniffing or oral stereotypies gradually leading to extremely constricted stereotypies.

It was reported that developmental exposure to Pb produces an alteration in cocaine sensitization. Perinatal exposure to Pb attenuated the locomotor-activating

properties of an *acute* dose of cocaine relative to non-exposed animals. With *repeated* drug administration, perinatally Pb-exposed animals developed greater locomotor sensitization than the control animals (Nation et al., 2000), a pattern opposite that reported with adult Pb exposure (Nation et al., 1996).

The most recent literature on Pb/cocaine interactions showed that developmental Pb exposure increased sensitivity to the behavioral effects of repeated cocaine administration. It produced a displacement to the left in the cocaine dose-response curve (Nation et al., 2004; Valles et al., 2005) and increased drug seeking in a reinstatement (relapse) paradigm (Nation et al., 2003). In the latest cases, behavioral results in developmentally Pb-exposed animals expressed an amplified response to cocaine relative to controls.

Significance of the Study

These behavioral findings associated with metal/drug interactions are significant because of their clinical relevance to the human population. Heavy metals may enhance the drug-induced behavior of cocaine. It is possible that the interaction of metal exposure and drugs may similarly influence drug seeking and use/abuse (Nation et al., 1997). The interaction of metal contaminants and drugs of abuse may contribute to the commonly reported high levels of drug abuse. Despite the increasing awareness that environmental contaminants may increase drug-related risks, the neurochemical mechanisms responsible for the observed metal and drug interactive effects remain undetermined.

Purpose of the Study

The purpose of this study was to gain mechanistic insight into the interaction of heavy metals and drugs of abuse. It is increasingly apparent that the extracellular chemical environment of the nerve synapse may alter drug responsiveness and therein influence risk factors associated with drug taking. Pb is known to target the mesolimbic dopamine (DA) system, most conspicuously projection neurons from the ventral tegmental area to the nucleus accumbens (Cory-Slechta, 1995; Tavakoli-Nezhad et al., 2001). Since DA activity along this circuit is critically involved in determining cocaine responsiveness (Ranaldi & Wise, 2001), functional disturbances in mesolimbic DA operations resulting from perinatal/postnatal Pb presence may translate into an enduring increased sensitivity to cocaine. Both Pb and cocaine affect the release and uptake of DA. There was a need to pursue accounts of other metal/drug interactions, such as mercury (Hg) and methamphetamine (MA). This study was the initial step in explaining the interaction of metals and drugs. Further studies will need to be performed to associate the neurochemical mechanism of the interaction of metals/drugs to the cognitive functions and/or behavioral effects of the interaction.

Assumptions

It was assumed that other prevalent divalent metals and psychostimulants have similar effects on the DA system. Therefore, Hg and MA were investigated in addition to Pb and cocaine. Most metal/drug interaction studies have been performed *in vivo*, or a combination of *in vivo* and *in vitro*, with brain tissue slices. Thus far, no *in vitro* studies have investigated metal/drug interactions in neuronal cell lines. In using SK-N-SH, COS-

7, and Neuro2A cells in this study, it was assumed that the DA activity correlates with the metal/drug interaction responses in previous animal models.

Limitations

There are several limitations to *in vitro* toxicity testing. The general side effects and pharmacokinetic effects of substances cannot be assessed. Most importantly, chronic effects cannot be tested. Thus, the ability to expose cells to heavy metals for long periods of time is lacking.

Objective of the Study

The topic to be investigated is the possible interactive effects of heavy metals (Pb and Hg) and psychostimulants (cocaine and MA) on the dopaminergic system *in vitro*. Specifically, does low dose heavy metal exposure in combination with psychostimulant use alter functionality of the dopamine transporter? Altered function in the presence of heavy metals and psychostimulants may indicate interactive effects. The detection of interaction is useful in illuminating mechanisms of drug action and in the development of new pharmacological theories.

CHAPTER II

REVIEW OF LITERATURE

II. A. Cell Cultures

Developed from neuroblastomas, human SK-N-SH and mouse Neuro2A (N2A) are two cell lines that were used in this study. Neuroblastomas are malignant tumors that develop from nerve cells. Developed by J.L. Biedler, SK-N-SH cells are human neuroblastoma cells established from a bone marrow metastasis in a four-year old female (www.atcc.org) (Figure 1). The clone N2A was established by R.J. Klebe and F.H. Ruddle from a spontaneous neuroblastoma tumor of a strain-A albino mouse (Figure 2). Neuroblastoma cells possess neuronal and amoeboid stem cell morphology. Because of potential differences in regulation and function between neuronal and non-neuronal cells, a non-neuronally derived model system, COS-7, was also evaluated (Figure 3). Established in the 1980s by Yakov Gluzman, the COS-7 cell line was developed from the standard CV-1 African green monkey kidney line by transforming normal cells with an origin defective mutant of simian virus 40 that codes for the wild-type virus T-antigen. COS-7 cells exhibit typical fibroblast morphology and are often used in transfection experiments with recombinant plasmids. Adherent/semi-adherent monolayer growth to both glass and plastic surfaces is characteristic of all three cell cultures used in this study.

Figure 1. SK-N-SH cells at low and high density (www.atcc.org)
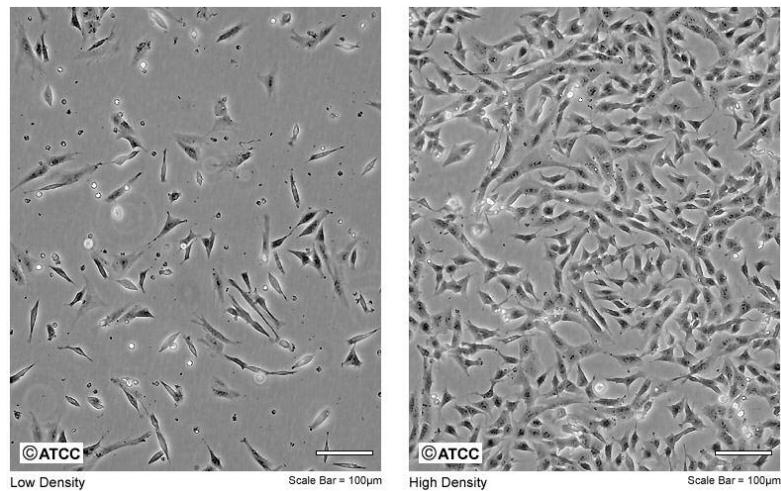
Figure 2. Neuro2A cells at low and high density (www.atcc.org)
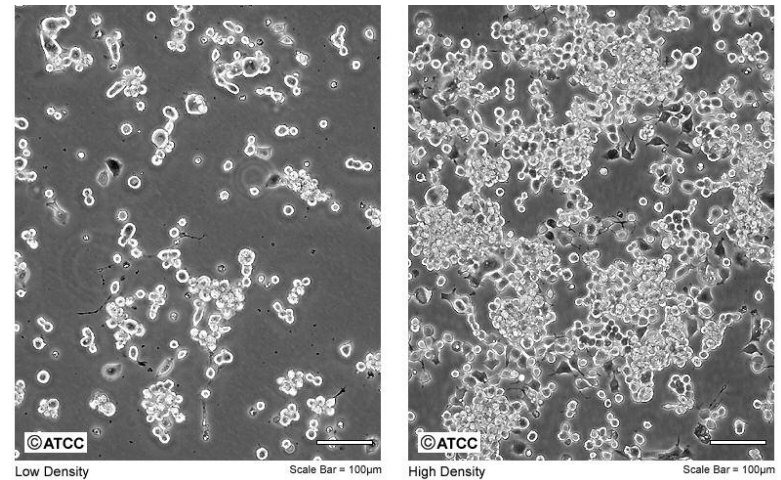
Figure 3. COS-7 cells at low and high density (www.atcc.org)
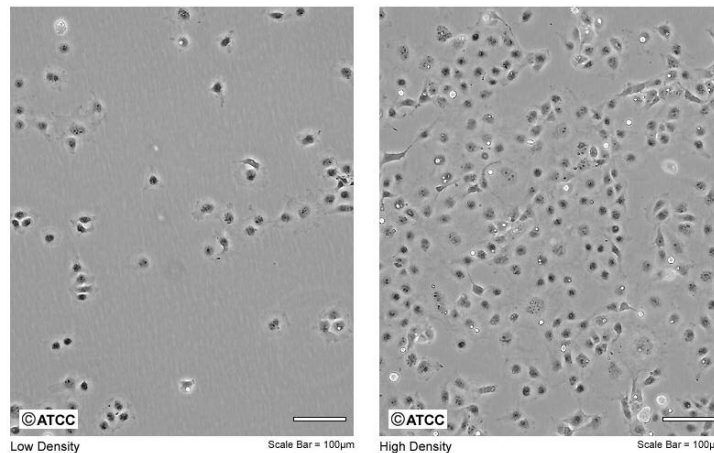
The Central Nervous System (CNS) consists of two types of cells: neurons and glia. The SK-N-SH and N2A cells are neuronal. Neurons communicate with one another across synapses (Figure 4). This communication is usually chemically mediated by rapid secretion of neurotransmitter molecules. The term neurotransmitter (NT) is used here to denote the chemical compound released from the pre-synaptic terminal at a synapse, and which produces a direct response in the post-synaptic cell. Pre-synaptic neurons produce in the post-synaptic neurons an electrical stimulation which spreads to the axon hillock, generating an action potential. Arrival of an action potential at the tip of an axon triggers the release of NTs at the synaptic gap. NTs either stimulate or inhibit the electrical excitability of a target cell. An action potential will only be triggered in the target cell if NT molecules, acting on their post-synaptic receptors, cause the cell to reach its threshold potential.

Figure 4. A neuron and transmission of a nerve signal by neurotransmitters.
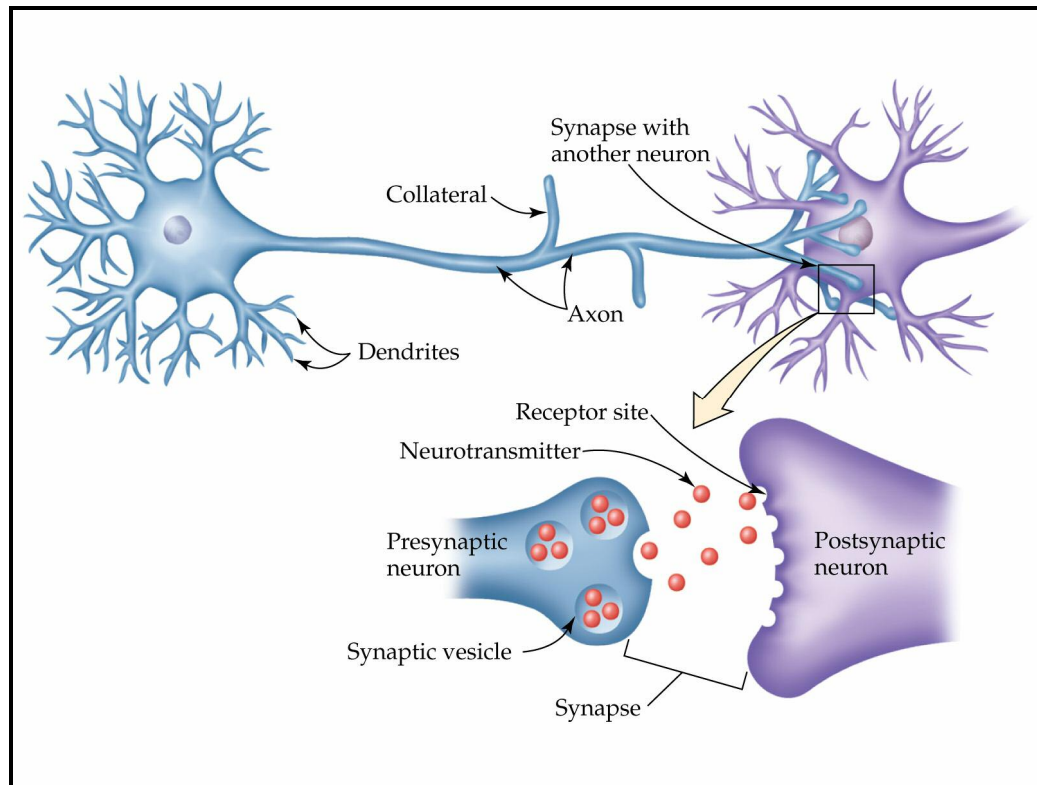
Immortalized cell lines offer advantages over other model systems in that they provide a homogenous population of cells that grow almost indefinitely in culture. Cell lines which express neuronal properties are useful model systems for studying the nervous system at the single cell and molecular levels. Neuronal cell cultures are widely used as a model system to investigate catecholamine metabolism and neurotoxicity. Neurons are capable of producing, releasing and taking up catecholamines. Catecholamines are NTs that have an aromatic portion (catechol) with an attached amine, or nitrogen-containing group.

II. B. Dopaminergic System

Dopamine (DA) is a catecholamine NT found predominately in the CNS. It is synthesized from the amino acid tyrosine, which is converted to L-dihydroxyphenylalanine (L-DOPA) by the enzyme tyrosine hydroxylase (TH) (Figure 5). L-DOPA is then converted to DA by the enzyme DOPA decarboxylase (AADC), which is found in the cytoplasm.

Figure 5. Synaptic terminal: Dopamine production.
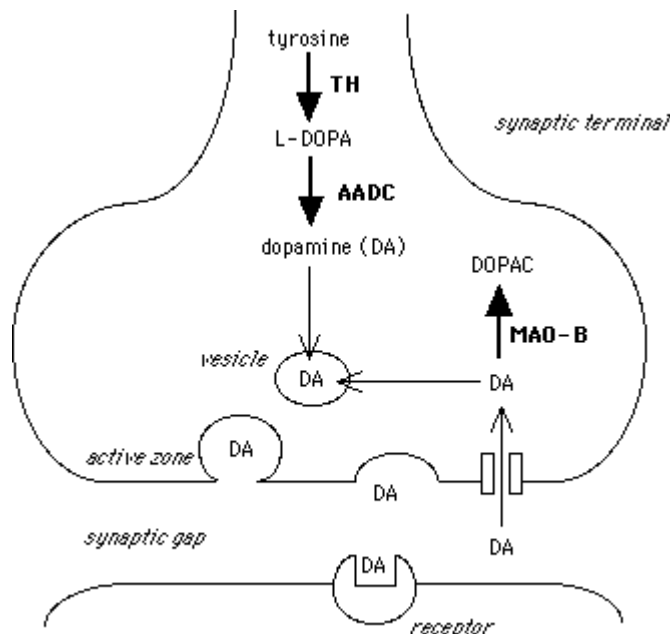
After synthesis, DA is packaged into vesicles, which are then quantally released in response to a pre-synaptic action potential. The release of DA is calcium-dependent. There is a high concentration of DA in synaptic vesicles and a relatively low concentration of DA in the cytosol. There is a normal background leak of DA out of the vesicles, but the balance is very much in favor of vesicular storage.

The inactivation mechanisms of neurotransmission are 1) uptake via a specific transporter; 2) enzymatic breakdown; and 3) diffusion. Uptake into the pre-synaptic

neuron via the DAT is the major role in the inactivation of DA neurotransmission. The recycled DA can face breakdown by enzymes such as dopamine-β-hydroxylase, monoamine oxidase and catechol-O-methyltransferase. Recycled DA can also be re-packaged into vesicles and reused.

Figure 6. Dopaminergic Pathways.
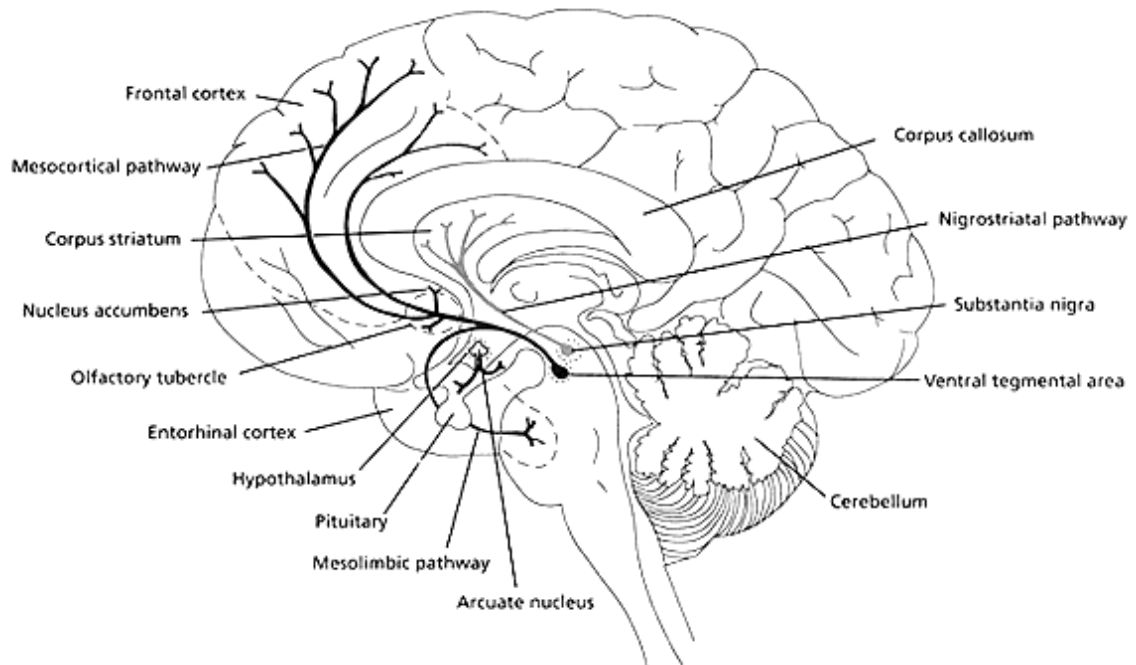
DA is the principal NT in three major neural systems in the midbrain : 1) the *nigrostriatal pathway* which originates from DA-synthesizing neurons of the midbrain substantia nigra complex and innervates the dorsal striatum (caudate-putamen), and whose degeneration leads to Parkinson's disease; 2) the *mesolimbic system* which arises in the midbrain ventral tegmental area (VTA) and innervates the ventral striatum (nucleus accumbens and olfactory tubercle) and part of the limbic system - this system influences motivated behavior, including activity related to reward; 3) the VTA also gives rise to the smaller *mesocortical pathway*, which innervates part of the frontal cortex and may be involved in certain aspects of learning and memory. The substantia nigra and the VTA

play key roles in the generation of pleasure and in the development of psychological drug addiction.

DA does not directly produce reward or motor activity, but instead modulates inputs and adjusts the state of an organism in order to redirect the stimulus response output to achieve the most effective behavioral outcome.

II.C. Dopamine Transporter

The dopamine transporter (DAT) belongs to the Na^+/Cl^--dependent family of neurotransmitter transporters. This family includes the transporters for biogenic amines (norepinephrine, serotonin and DA). A 620 (human) or 619 (rat) amino acid DAT sequence is predicted from cloned cDNAs (Kilty et al., 1991; Giros et al., 1992) with an apparent molecular mass of approximately 80,000 daltons (Patel et al., 1994). DAT is composed of 12 transmembrane domains (Figure 7) connected by alternating intracellular and extracellular loops, with the N- and C-termini located on the intracellular side of the membrane (Edvardsen & Dahl, 1994; Nirenberg et al., 1996; Hersch et al., 1997). It has a large second extracellular loop that has potential N-glycosylation sites (Vandenbergh et al., 1992; reviewed in Surratt et al., 1993). Stability and plasma membrane trafficking of the DAT is strongly influenced by glycosylation (Reith et al., 1997).
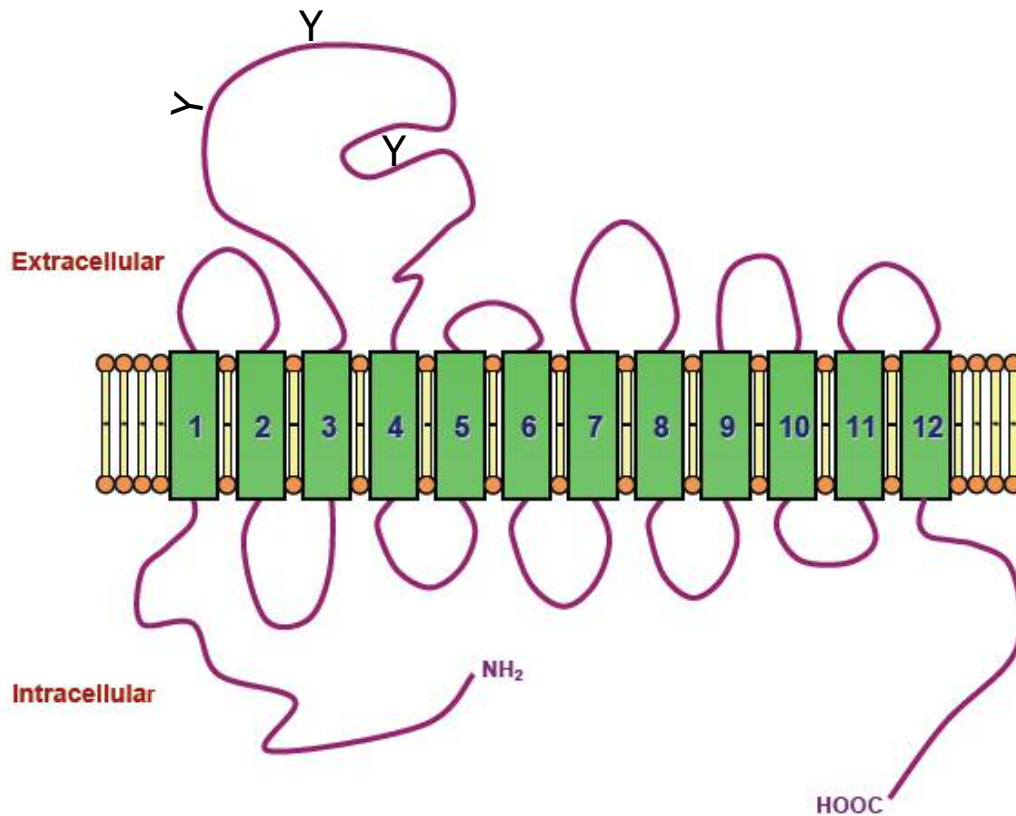
Figure 7. Topology of the dopamine transporter. The dopamine transporter has 12 transmembrane domains with the N- and C-termini located on the intracellular side of the membrane. The large second extracellular loop has several sites for glycosylation.

DA nerve terminals possess high-affinity DA uptake sites, which are important in terminating NT action and maintaining NT homeostasis. Uptake is accomplished by the DAT which can transport DA into and out of the terminal. The DAT utilizes an inward-directed Na^+ ion gradient across the plasma membrane as the driving force for intracellular accumulation of NT (Singh et al., 1990). The DAT exhibits ion dependence distinct from other transporters with an apparent stoichiometry of transport of $2Na^+:1Cl^-$:1DA (Figure 8). The DAT is important for neural function because it removes excess DA from the synapse, effectively ending the signaling properties of the NT. DA efflux is elicited by diminishing or reversing the Na^+ gradient. The DAT is a major target for

various pharmacologically active drugs and environmental toxins. DAT is densely

concentrated in the substantia nigra pars compacta and scattered throughout the VTA

(Freed et al., 1995).

Extracellular

Intracellular
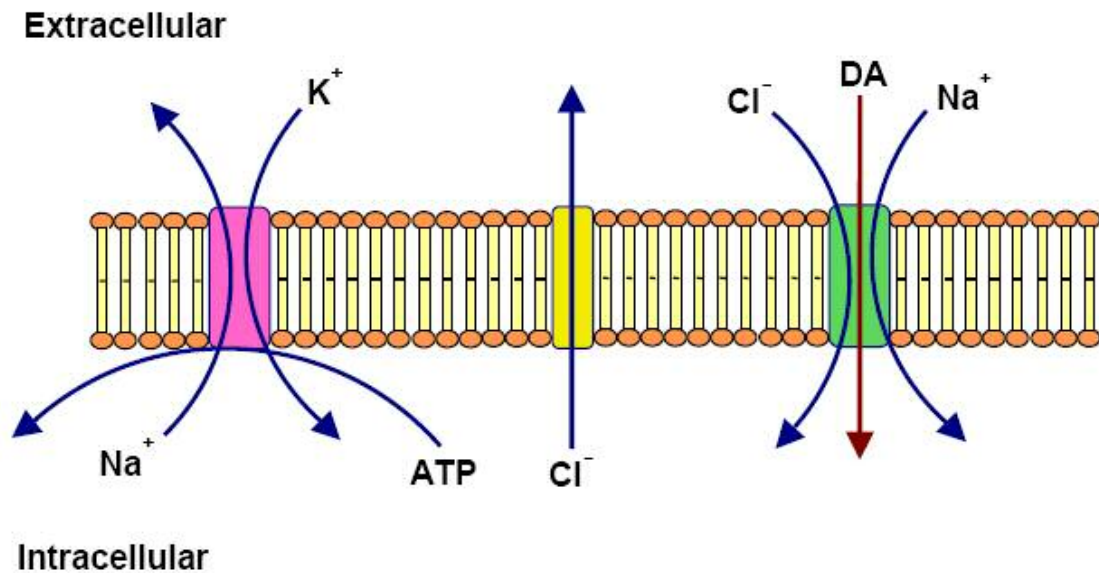
Figure 8. Dopamine transport via the DAT. The Na^+K^+-ATPase (pink) generates the Na^+ and K^+ gradients across the plasma membrane by moving three Na^+ ions out of, for every two K^+ ions into, the cell. The resultant transmembrane electric potential (negative inside) leads to Cl^- redistribution. The DAT (green) translocates one dopamine molecule along with two Na^+ ions and one Cl^- ion as co-substrates in one transport cycle.

The DAT seems to be implicated in the etiology of various neurological or

psychiatric syndromes. Thus, as expected of the marked degeneration of dopaminergic

neurons, a decrease of DAT is regularly observed in Parkinson's disease (Boja et al.,

1994; Miller et al., 1997). Aberrant dopaminergic neurotransmission is also associated

with disorders of the schizophrenic spectrum and Tourette's syndrome (Pearce et al.,

1990; Singer et al., 1991).

Because reuptake plays an important role in clearing DA from the extracellular

fluid, drugs and toxic agents that inhibit reuptake cause increased extracellular DA levels

and enhance the various functions mediated by these NTs. The administration of heavy

metals and psychostimulants *in vitro* was investigated in this study to gain understanding of their neurotoxicity on the dopaminergic system.

II. D. Heavy Metals

The molecular mechanisms of the neurotoxicity induced by metals and organometals are not well understood. Metals appear to be somewhat nonselective. Yet for each metal there are sites or processes which are the most vulnerable and primarily affected. Since metals bind to various sites at cell membranes, they are likely to change the charges and conformations of surface proteins. This can be expected to alter membrane-related functions in the cell. Mercury and lead are toxic metals affecting mainly the CNS.

Being glycoproteins, the receptors for NTs are a possible target for metals. There is already some evidence that metals may inhibit or increase the binding of ligands to receptors *in vitro* (Aronstam et al., 1978; Aronstam & Eldefrawi, 1979; Von Burg et al., 1980; Peterson & Bartfai, 1983; Mizuno et al., 1983). The sensitivity of different receptors for metals varies however (Bondy & Agrawal, 1980).

II.D.1. Mercury

A heavy, silvery, transition metal, mercury (Hg) is one of five elements that are liquid at or near room temperature. Hg occurs in the Earth's crust, mainly in the form of sulfides. The red sulfide, cinnabar, is the main component of the Hg ores. Hg is also released into the environment by human activities, for example, combustion of fossil fuels, waste disposal and industrial activities. If heated, it is a colorless, odorless gas.

Hg exists in elemental, ionic, and organic forms, and each of these forms has a unique toxicity related to its differential accumulation in sensitive tissues. Hg combines with other elements, such as chlorine, sulfur, or oxygen, to form inorganic mercury salts, which are usually white powders or crystals. Hg also combines with carbon to make organic Hg compounds, such as methylmercury. The predominant source of methylmercury is atmospheric Hg deposited on the surfaces of bodies of water that is then biomethylated by microorganisms and subsequently biomagnified as it ascends the food chain. Metallic Hg is used to produce chlorine gas and caustic soda, and is also used in thermometers, dental fillings, and batteries. Hg salts are sometimes used in skin lightening creams and as antiseptic creams and ointments. Other products containing Hg include auto parts, fluorescent bulbs, medical products, vaccines, and thermostats. There are many more sources of Hg that could potentially cause harmful exposure to organisms (Table 1).

Table 1. Sources of Hg exposure.

ELEMENTAL	INORGANIC	ORGANIC
Amalgam preparation	Disinfectant making	Bactericide preparation
Barometer manufacture	Dye making	Drug manufacture
Bronzing	Explosive production	Embalming/Cremation
Dentistry	Fur processing	Insecticide production
Photography	Tannery work	Histology
Hg refining	Laboratory research	Farming
Paint manufacture	Taxidermy	Seed handling

The biological half-life for inorganic Hg is about 40 days. For elemental mercury or mercury vapor the biological half-life is linear with a range of values from 35 to 90 days. The biological half-life is different for different organs. A fraction of the absorbed Hg will remain in the body for a longer time (e.g. years in the brain and bones; International Programme on Chemical Safety, 1991). It is biologically nonessential and toxic to all organisms.

Human exposure to inorganic Hg is mainly occupational, most commonly associated with Hg vapor. It is often related to specific working conditions, for example, mining, spillage of Hg compounds on work clothes or in the working environment, and handling of Hg salts in the chemical industry and laboratories (Bluhm et al., 1992). Due to the health effects of Hg exposure, industrial and commercial uses are broadly regulated in Western countries. The World Health Organization, Occupational Safety and Health Administration, and National Institute for Occupational Safety and Health all agree that Hg is an occupational hazard and have established specific occupational exposure limits. Environmental releases and disposal of Hg is regulated in the U.S. primarily by the Environmental Protection Agency. In recent years, governments have issued warnings that certain fish in excess quantities are unsafe due to methylmercury levels. Such warnings especially target pregnant women because all forms of Hg cross the placenta to the fetus.

The risk of Hg to human health has been the subject of several reviews (Goering et al., 1992; Fung & Molvar, 1992; Enwonwu, 1987; Mjor, 1994; Halbach, 1994; Aposhian et al., 1992). One of the most important points raised by these reviews is that the effect of chronic low dose Hg exposure on humans is not known. There are claims

that long-term exposure to low concentrations of Hg vapor either cause or exacerbate degenerative diseases such as amyotrophic lateral sclerosis, Alzheimer's disease (AD), multiple sclerosis, and Parkinson's disease. Speculation has been most intense concerning AD after a report that Hg levels were higher in autopsy brains of AD patients than in brains of members of a control group (Thompson et al., 1988).

One of the worst industrial disasters in history was caused by the dumping of Hg compounds into Minamata Bay, Japan. The Chisso Corporation, a fertilizer and later petrochemical company, was found responsible for polluting the bay from 1932 to 1968. It is estimated that over 3,000 people suffered various deformities, severe Hg poisoning symptoms, or death from what became known as Minamata disease. The neurotoxic signs include ataxia, speech impairment, constriction of visual fields, hypoesthesia, dysarthria, hearing impairment, and sensory disturbances. These neurological problems persisted and were found in other areas of Japan as the Hg contamination spread (Ninomiya et al., 1995). Follow-up studies in the Minamata area 40 years after the spill and 30 years since a fishing ban was enacted revealed continued problems. In 1995, male residents of fishing villages in the area reported significantly higher prevalences than "town-resident-controls" for the following complaints: stiffness, dysesthesia, hand tremor, dizziness, loss of pain sensation, cramping, atrophy of the upper arm musculature, arthralgia, insomnia, and lumbago.

Hg compounds are highly potent, but are non-specific cellular poisons that influence many vital processes involving proteins. Hg ions are protein precipitants and, as a result, cause severe necrosis on direct contact with tissue. They have affinity for a number of cellular components essential for the function and survival of the cell such as

enzymes, membrane proteins, antioxidants, nucleic acids and mitotic apparatus. Toxicity is related to the covalent binding of Hg to sulfhydryl groups, as well as to carboxy, amide, amine, and phosphoryl groups. As a result, specific membrane transport is blocked and selective permeability of the membrane is altered. Because Hg-containing compounds have a high propensity for interacting with sulfhydryl groups, the cysteine residues in the DAT molecule may play an important role in the regulation of psychostimulant binding to the uptake complex. It also affects the uptake and release of neurotransmitters from presynaptic nerve terminals. This may be due to its ability to change the intracellular concentration of calcium (Ca^{2+}) by disrupting regulation of Ca^{2+} from intracellular pools and increasing the permeability of plasma membranes to Ca^{2+}. There is still undoubtedly much more to learn about the specific mechanisms of Hg-induced neurotoxicity.

II.D.2. Lead

Lead (Pb) has a bright luster and is a dense, ductile, very soft, highly malleable, bluish-white metal that has poor electrical conductivity. This metal is highly resistant to corrosion. Because of this property, it is used to contain corrosive liquids such as sulfuric acid.

The historical use of Pb acetate (also known as sugar of lead) by the Roman Empire as a sweetener for wine is considered by some to be the cause of the dementia which affected many of the Roman Emperors. Pb is a toxic metal that can damage nerve connections and can cause blood and brain disorders in children and in adults. Long term exposure to Pb or its salts (especially soluble salts or the strong oxidant PbO_2) can cause

nephropathy, and colic-like abdominal pains. There are numerous sources by which humans can be exposed to Pb (Table 2).

Table 2. Sources of lead exposure.

OCCUPATIONAL	ENVIRONMENTAL	ADVOCATIONAL
Plumbing	Lead paint	Pottery making
Auto repairs	Lead-painted homes	Target shooting
Glass making	Leach from plumbing	Soldering of electronics
Printing	Leaded gasoline	Fishing sinkers
Steel welding	Ceramics	Car repair

Inhalation and ingestion are the main routes of exposure. Adults are primarily exposed occupationally (ATSDR, 2005), and this occurs by inhalation with 35-40% of inhaled lead dust or fumes deposited in the lungs with extensive blood absorption (Leggett, 1993). Children are primarily exposed by ingestion and absorb 50% of an ingested dose through the gastrointestinal tract. Lead also readily crosses the placenta to the fetus (Roels et al., 1978). Unlike Hg, Pb is not known to be biologically transformed into chemical forms that may enhance its absorption or retention.

The mechanism by which Pb disrupts normal physiological processes is based on the similarity of ionized lead (Pb^{2+}) to calcium (Ca^{2+}). Both are divalent cations; however, Pb can disrupt the physiological effects of calcium at concentrations several orders of magnitude lower than the concentration of calcium (Silbergeld & Adler, 1978). Pb and calcium are used interchangeably by bone. Pb has an affinity for bone and acts by replacing calcium. In the developing brain, Pb causes an inappropriate release of NT at

20

rest and competes with calcium to interfere with evoked NT release (Bressler &

Goldstein, 1991). This increase in basal release and decrease in evoked release may

interfere with selective pruning of synaptic connections in the brain during the first few

years of brain development. Overall, Pb is a calcium antagonist that competes with

calcium-mediated intracellular signaling and activates calcium-mediated synaptic vesicle

release mechanisms.

Both biochemical and behavioral studies implicate dopaminergic NT systems in

the neurotoxicity of Pb. Reported effects are consistent with the hypothesis that Pb

exposure, by some as yet undetermined mechanism, depletes DA availability. Pb

exposure decreases DA turnover (Jason & Kellogg, 1981; Lasley et al., 1984),

synaptosomal DA release (Minnema et al., 1986), and synaptic transmission in peripheral

nerve (Cooper et al., 1984). It also impairs autoreceptor-mediated regulation of DA

release, an effect accompanied by decreased levels of DA metabolites (Lasley & Lane,

1988; Lasley, 1992).

Both Hg and Pb toxicity will be investigated in this study because both remain a

significant public health issue because of their global pervasiveness and because of their

adverse effects on the dopaminergic system.

II.E. Psychostimulants

Psychostimulants promote increased extracellular DA concentrations. There are

two primary mechanisms by which these agents affect the DAT. Psychostimulants can be

separated into "uptake blockers" and "releasers" based on the mechanism of their acute

effects on NT flux through the DAT. Although uptake blockers can have releasing

properties and releasers may also have some uptake blocking ability, the general separation of drugs into these two classes helps to functionally distinguish the pharmacological profiles of some of the most commonly used psychostimulants.

The DAT is the principle target for widely abused psychostimulants such as cocaine, amphetamine and methamphetamine. The reinforcing properties of these drugs (which likely underlie their addictive properties) are strongly correlated with their affinities for the DAT (Ritz et al., 1987). Dopamine (DA) release in the nucleus accumbens is believed to be the main mediator of the reinforcing and locomotor activating properties of psychostimulants (Jones et al., 1998).

II.E.1. Cocaine

Cocaine is classified as a DA uptake blocker because its primary mechanism of action is by directly binding and inhibiting the transport of DA through the DAT (Ritz et al., 1987). This DA reuptake, mediated by Na^+, Cl^-, and ATP-dependent active transport, is inhibited when cocaine binds to the Na^+ binding site on the transporter and alters the Cl^- binding site, thus preventing the binding of both ions. Because translocation of DA across the membrane of the pre-synaptic neuron is inhibited, increased extracellular DA concentrations result in chronic stimulation of the DA receptor in the post-synaptic neuron. Clinically, cocaine's most important mechanism of action lies in its ability to block sodium channel conductance and thereby increase the threshold required to generate an action potential. Thus, like lidocaine and novocaine, it is used medicinally as a local anesthetic.

Of interest, blockade of DAT by cocaine leads to a rapid increase in DA uptake in synaptosomes prepared from treated rats, a preparation from which the drug has been presumably been washed out (Fleckenstein et al., 1999). Perhaps this occurs via enhanced recruitment of DATs to the plasma membrane (Daws et al., 2002). These acute increases in DA uptake and plasmalemmal surface expression, observed in rodents and cell lines, respectively, after cocaine administration likely represent efforts to maintain normal synaptic DA functions. In humans who have repeatedly increased synaptic DA levels through the use of cocaine, increased DAT function is also observed, as assessed in synaptosomes from cryoprotected human brain (Mash et al., 2002). The combination of an initial DAT blockade and a subsequent increase in DA uptake could contribute to the development and expression of cocaine addiction. It is possible that an overabundance of extracellular DA during DAT blockade triggers this compensatory increase in DAT activity, which would ultimately produce a deficit in extracellular DA, perhaps contributing to drug dependence.

After cocaine is introduced to the body it travels to reward areas of the brain: the VTA, the nucleus accumbens, the amygdala, and the prefrontal cortex (Figure 9). These areas are saturated with DA synapses.

Figure 9. The main structures that make up the reward pathway are the ventral tegmental area, the nucleus accumbens (both shown in purple), the amygdala (in green), and the prefrontal cortex (in grey).
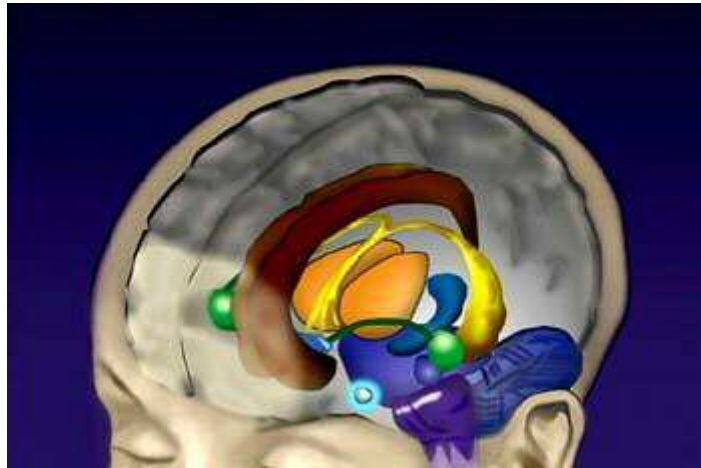
Repeated administration of cocaine results in both diminished effects (tolerance) and increased effects (sensitization). The initial signs of stimulation are hyperactivity, restlessness, increased blood pressure, increased heart rate and euphoria. The euphoria is quickly followed by feelings of discomfort and depression and a craving to re-experience the drug. Side effects can include twitching and paranoia, which usually increase with frequent usage. With excessive dosage the drug can produce hallucinations, paranoid delusions, tachycardia, itching, and delusional parasitosis. Toxicity results in seizures, followed by respiratory and circulatory depression of medullar origin. This may lead to death from respiratory failure, stroke, cerebral hemorrhage, or heart failure.

II.E.2. Methamphetamine

Methamphetamine (MA) is structurally similar to amphetamine (AMPH), and it may be produced from ephedrine or pseudoephedrine by chemical reduction. Most of the necessary chemicals are readily available in household products or over-the-counter

medicines. This makes MA appear unusually easy to make, unlike cannabis and cocaine, which are both harvested directly from plants.

It is often asked if amphetamine and MA differ in effect. The two drugs show no differences in terms of changes in DA release in the striatum, elimination rates, or other pharmacokinetic properties (Melega et al., 1995), and equal doses of the two drugs are not distinguished in human discrimination studies (Lamb & Henningfield, 1994). There is, however, a subtly greater DA release by AMPH than MA in the prefrontal cortex, and likely as a result, some subtle differences in effects of the drugs on working memory and behavioral tolerance (Shoblock et al., 2003a, 2003b). AMPH can elicit somewhat more locomotor activity in rodents than MA, perhaps due to indirect effects (Shoblock et al., 2003b). The occasional statement in the literature that MA is more addictive, favored by drug addicts, a more potent psychostimulant, or has diminished peripheral activity appears to be unfounded according to Shoblock (2003a). By tradition, studies on mechanisms of action are generally on AMPH, and studies of neurodegeneration on MA. This may be because MA is more readily available on the illicit market due to its easier synthesis, which uses either a one-step reduction of ephedrine or pseudoephedrine, drugs that at this writing remain readily available, or a condensation of phenylacetone and methylamine (Cho, 1990).

MA is classified as a Schedule II substance by the Drug Enforcement Agency in the U.S. meaning that it is illegal to buy, sell, or possess without a prescription. It is legally controlled in most countries, although it has medical uses, so it is also available by prescription in many places. MA is legally marketed in the U.S. under the trade name

Desoxyn, manufactured by Abbott Laboratories. Generic formulations of the drug are also available.

MA can be swallowed, snorted, smoked, dissolved in water and injected, or inserted anally. MA causes significant tolerance, as well as psychological dependence. This combination can be particularly bad because the user is likely to have strong cravings for more MA, while at the same time being unable to reach a satisfactory high. Withdrawal from high doses can produce severe depression.

MA is commonly abused for its rewarding effects, which are thought to result from DA release in the nucleus accumbens (Wise & Hoffman, 1992). Acute administration of MA causes release of DA *in vivo* (O'Dell et al., 1991). Repeated administration of MA to laboratory animals causes long-term reduction in DA and in the activity of its synthesizing enzyme tyrosine hydroxylase (Kogan et al., 1976; Wagner et al., 1980; Cass & Manning, 1999). In findings reminiscent of data seen in chronic cocaine users, MA abusers had substantially lower DA and DAT levels (39-55% and 25-53%, respectively), as well as slightly (20%) lower TH levels in caudate, putamen and nucleus accumbens (Wilson et al., 1996). Another study found reduced (23-25%) striatal DAT density in chronic MA abusers abstinent for months to years (McCann et al., 1998). Although there are other possible interpretations of the data obtained from chronic cocaine and MA abusers, it seems plausible that stimulant-induced down-regulation of DAT gene expression underlies the observed changes in DA and TH levels.

Although the toxic effects of MA on the central dopaminergic systems have been well documented *in vivo*, the exact mechanisms underlying the neurotoxicity have yet to be identified. Studies have indicated that increased extracellular DA due to enhanced

release or uptake inhibition may become harmful and consequently result in cell death (Choi et al., 2002; Gibb et al., 1994). Also, DA may oxidize to form superoxide and hydrogen peroxide, which in turn become hydroxyl radicals in the presence of transition metals (LaVoie & Hastings, 1999). Therefore, the increase in oxidative stress by MA may be due to DA release and subsequent oxidation. Evidence suggests that *in vivo* free radical formation within the dopaminergic neuron may be responsible in the MA-induced dopaminergic cell death (Cubells et al., 1994; Hirata et al., 1995; Maragos et al., 2000; Imam et al., 2001). In addition, the mechanism of MA may involve excitatory amino acid receptors (Sonsalla et al., 1989), glial cell activation (Stadlin et al., 1998) and cytokines (Asanuma & Cadet, 1998; Ladenheim et al., 2000). Elucidation of the mechanism underlying the MA toxicity would benefit from the availability of *in vitro* model systems, because more controlled and isolated cellular phenomena may be observed.

II.F. Interaction of Heavy Metals and Psychostimulants

The interaction between two or more compounds can play an important role in neurotoxicity. Interaction can result in either an increase or decrease in the toxic effects of a compound. A toxic effect is additive if after simultaneous administration of two or more substances, it is the sum of the individual effects. Potentiation occurs when a compound showing little or no toxicity markedly increase the toxicity of a second compound. A synergistic effect will produce more severe symptoms than would be expected from the toxicities of the individual compounds. The purpose of this study was to gain mechanistic insight into the interaction of heavy metals and drugs of abuse.

CHAPTER III

METHODOLOGY

III.A. Cell Cultures

III.A.1. SK-N-SH, COS-7, and N2A Cells

Cell lines used were SK-N-SH (human neuroblastoma cells), COS-7 (monkey kidney cells) and N2A (mouse neuroblastoma cells). All cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). SK-N-SH cells were cultured in RPMI 1640 without L-glutamine (Cellgro, MediaTech Inc., Herndon, VA). COS-7 cells were cultured in DMEM with 4.5 g/L glucose supplemented with 4 mM L-glutamine and 1.5 g/L $NaHCO_3$ (Cellgro, MediaTech Inc., Herndon, VA). N2A cells were cultured in MEM with Earle's balanced salt solution, nonessential amino acids, and sodium pyruvate. The media was supplemented with 2 mM L-glutamine and 1.5 g/L $NaHCO_3$ (Cellgro, MediaTech Inc., Herndon, VA). All media was supplemented with 10% heat inactivated fetal bovine serum (FBS; Hyclone, Logan, UT) and 1% penicillin/streptomycin solution – 10,000 I.U./mL and 10,000 µg/mL (Cellgro, MediaTech Inc., Herndon, VA). All cultures were maintained in vented 25 cm² cell culture flasks (Corning Inc., Corning, NY) at 37°C in a 5% CO_2 humidified atmosphere. As recommended by the ATCC, the culture medium was changed twice a week in a sterilized vented hood. To change the media, the old media was decanted; careful not to

disturb the monolayer of cells. Without creating bubbles, 5 mL of warmed complete media (heated in 37°C water bath for 20-30 minutes) was added to the 25 cm^2 flasks.

Upon achieving confluence, within 7 days, the cells were split and subcultured to 1:4 ratio. Subculturing took place once a week. A solution of 0.25% Trypsin, 1.0 mM ethylenediaminetetraacetic acid (EDTA) in Hank's Balanced Salt Solution (HBSS) without calcium and magnesium salts (Atlanta Biologicals, Lawrenceville, GA). 1.5 mL of trypsin was added to the flask to allow cells to detach. Typically, cells loosened from the culture surface in 10-15 minutes. The flasks were also returned to the incubator if the cells began to clump or did not appear to be detaching from the plastic. Gentle agitation was used as a last resort. Once the cells were detached from the surface, 3.5 mL of complete media was added to each flask to stop the action of the trypsin. Pipetting the solution up and down broke up any clumps and dispersed the cells in the suspension. Again avoiding bubbles, 1 mL of the cell suspension was transferred to each new sterile 25 cm^2 culture flask. By adding 4 mL of fresh media, each new flask maintained a 5 mL final volume. After the splitting procedures were complete, all culture flasks were recapped and returned to the incubator. The leftover cell suspension (approximately 1 mL) was discarded.

In 24-well plastic cell culture plates (Costar® 3599, Corning, Inc., Corning, NY), 1×10^5 cells were seeded and maintained in the complete growth medium. After plating, the cells were allowed at least 24 h to adhere to the surface of the well before experiments were performed. The cells from passages 4 to 14 were used to perform the studies.

III.A.2. hDAT-Transfected COS-7 Cells

Vectors, pCMV6-XL5 with the hDAT-cDNA insert (Origene) and pCMV6-Neo (Origene), were transformed into One Shot® Top10 Chemically Competent *E. coli* (Invitrogen). Cells were grown on LB-agar/ampicillin (100µg/ml) plates overnight. The following day six independent colonies were selected to inoculate 2 mL LB/ampicillin broth overnight. Plasmid DNA was isolated using the Wizard® *Plus* SV Minipreps DNA Purification System kit (Promega). Both plasmids were digested with *Not*I endonuclease (Promega). Fragments were separated by 0.7% agarose gel electrophoresis, cut out of the gel, and extracted using the StrataPrep® DNA Gel Extraction kit (Stratagene). DNA was quantified by spectrophotometric analysis. The hDAT-cDNA and the pCMV6-Neo were ligated with T4 DNA ligase (Promega) at 4ºC overnight. The new subcloned construct was transformed in Top10 cells and grown again on LB-agar/ampicillin plates. LB/ampicillin broth was inoculated with selected colonies, and the resulting pDNA was isolated by Miniprep. Correct insertion was confirmed by *Xma*I digestion and DNA sequencing. Confirmed plasmids were replated for colonies, inoculated in 100 mL LB/ampicillin broth, and purified using the Qiagen Plasmid Maxi kit. The cloned plasmid pCMV6-Neo(hDAT) was quantified by spectrophotometric analysis, and aliquots were frozen back for later use.
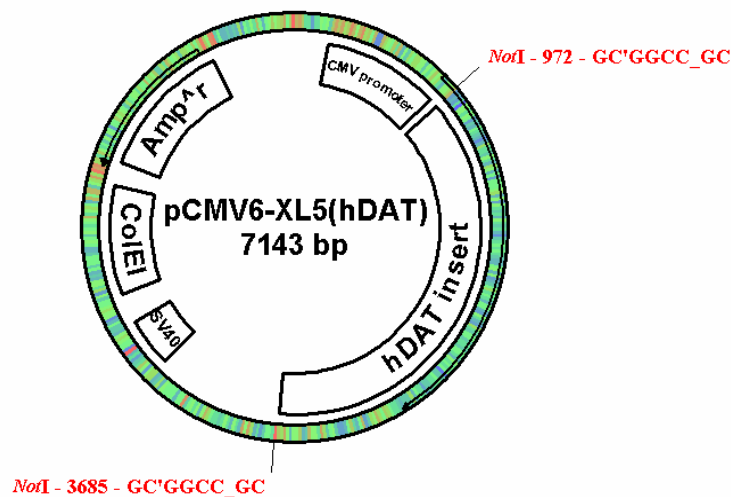
Figure 10. The pCMV6-XL5 vector (about 4.5 kb in size) with the hDAT cDNA (about 2.7 kb) inserted within the multiple cloning site. The hDAT insert is flanked by two *Not*I restriction sites. ColE1 is the bacterial origin of replication and SV40 allows for replication in mammalian cells. The CMV promoter is used to express the cloned cDNA. Selection of the plasmid in *E. coli* is conferred by the ampicillin resistance gene.
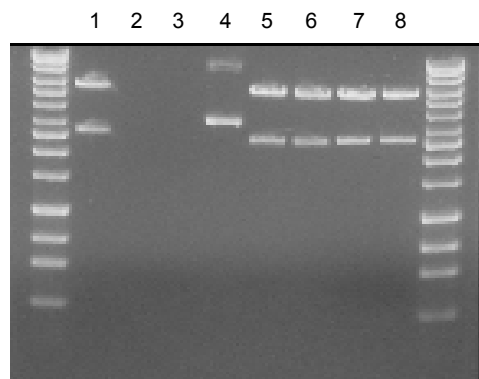
Figure 11. Plasmids were digested with *Not*I. Fragments were separated by 0.7% agarose gel electrophoresis. GeneMate QuantiMarker 1 kb was used as the DNA ladder. Lanes 1, 5, 6, 7, and 8 show fragments of the approximate size of 5.8 kb and 2.7 kb. These are successful pCMV6-Neo with the cloned insert.

One day prior to transfection, cells were split into a 24-well plate at a density of 1×10^5 cells per well in 500 μl of growth medium without antibiotics. Confluence was 90–95% at the time of transfection. Cells were transfected with Lipofectamine™ 2000 (Invitrogen) according to manufacturer's instructions: volume of culture medium (500 μl), quantity of pDNA (0.8 μg), volume of Lipofectamine™ 2000 (2 μl) and Opti-MEM

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I® Reduced Serum Medium (Invitrogen) as solution to dilute the transfection agent. For successful transfection a nucleic acid, which carries a net negative charge under normal physiological conditions, must come into contact with a cell membrane that also carries a net negative charge. Lipofectamine™ 2000 is a cationic liposome formulation that functions by complexing with nucleic acid molecules, allowing them to overcome the electrostatic repulsion of the cell membrane and to be taken up by the cell. Cells were incubated at 37°C in a 5% CO_2 incubator for 24 h prior to testing for transgene expression. Medium was changed after 4-6 h. For stable transfection, cells were passed 1:10 into fresh growth medium 24 h after transfection. The following day Geneticin was added to the culture medium to give a final concentration of 500 μg/ml. Selection was continued for three weeks.
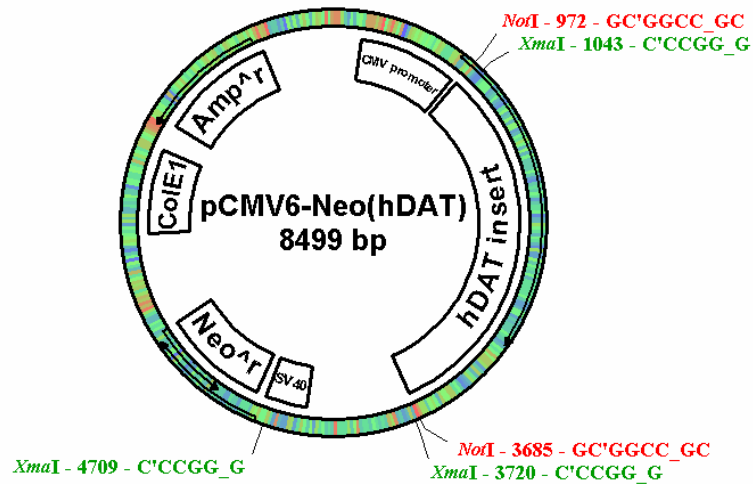
Figure 12. The pCMV6-Neo vector (about 5.8 kb in size) is similar to pCMV6-XL5 except a neomycin resistance gene is added for establishing a stable clone. The hDAT cDNA was subcloned into the vector by *Not*I digestion and ligation. Orientation of the insert was confirmed by *Xma*I digestion. The cloned plasmid was transfected into COS-7 cells.
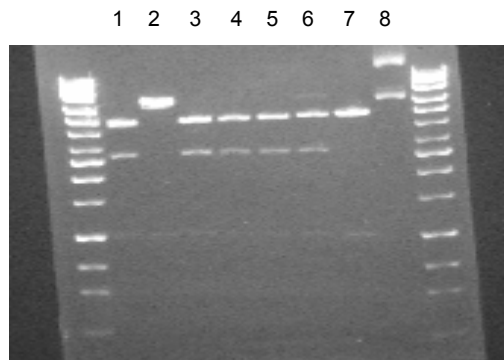
Figure 13. Successfully cloned plasmids were digested with *Xma*I. Fragments were separated by 0.7% agarose gel electrophoresis. GeneMate Quanti-Marker 1 kb was used as the DNA ladder. Lanes 1, 3, 4, 5, and 6 show fragments of the approximate size of 4.8 kb, 2.7 kb, and 1.0 kb. These are pCMV6-Neo with the correctly oriented cloned insert. Lane 7 is the pCMV6-Neo (5.8 kb) without insert.

III.A.3. hDAT-Transfected N2A Cells

The transfected N2A(hDAT) cells were a generous gift from Dr. Karley Little (University of Michigan, Ann Arbor, MI). As previously described (Zhang et al., 1998), N2A cells were obtained from American Type Culture Collection (Manassas, VA). Briefly, the cells were grown in Opti-MEM I (Invitrogen, Carlsbad, CA) supplemented with 10% FBS and 1% penicillin/streptomycin in 75 cm^2 flasks. After the cells reached confluence (about 3 days of growth), they were trypsinized. Cells were then transfected with a pcDNA3 plasmid cloned with an hDAT-cDNA insert. cDNA was provided by Zdenek Pristupa (University of Toronto, Toronto, ON, Canada). Electroporation employing a BTX Electroporation System 600 (Biotechnologies and Experimental Research, Inc., San Diego, CA) was used to transfect the cells. Cells were stably selected with geneticin (G418) over several weeks. After reaching confluence, cells were seeded in 24-well plates and allowed at least 24 h to adhere to the surface of the well before experiments were performed.

III.B. Lactate Dehydrogenase Assay

Lactate dehydrogenase (LDH) is a stable cytoplasmic enzyme which is present in most cells. It is released into the cell culture supernatant upon damage of the cytoplasmic membrane. The CytoTox 96® Assay is a colorimetric assay that quantitatively measures LDH activity released from the damaged cells into the supernatant. SK-N-SH cells were exposed to multiple concentrations of metals, drugs and combinations of both. To determine which concentrations were optimal to use, lactate dehydrogenase (LDH) assays were performed to assess cell viability. The CytoTox 96® Non-Radioactive Cytotoxicity Assay kit was performed according to manufacturer's instructions (CytoTox 96® Non-Radioactive Cytotoxicity Assay Technical Bulletin No. 163). Released LDH in culture supernatants was measured with a 30-minute coupled enzymatic assay, which results in the conversion of a tetrazolium salt (INT) into a red formazan product. The amount of red color formed is proportional to the number of lysed cells.
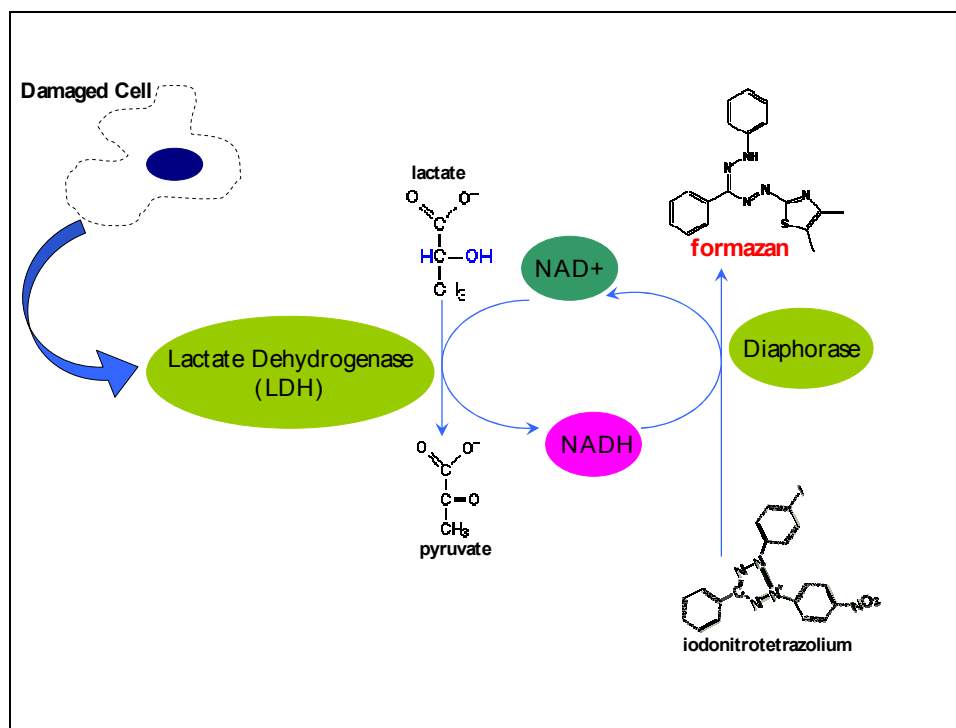
Figure 14. LDH is a cytoplasmic enzyme that is released into the cytoplasm upon cell lysis. The LDH assay, therefore, is a measure of membrane integrity. The basis of the LDH assay: (1) LDH oxidizes lactate to pyruvate, (2) Pyruvate reacts with the tetrazolium salt INT to form formazan, and (3) the water-soluble formazan dye is detected spectrophotometrically.

The assay was performed as described in the technical bulletin. For determining the amount of spontaneous release, 50 µL of each sample supernatant was transferred in duplicate into a clear 96-well plate. The remaining media from the 24-well plate was aspirated. To each well of the 24-well plate, 150 µL of Lysis Solution was added in order to measure the total amount of LDH released. Plates were then placed in the 37°C incubator for 45 minutes. Afterward, 900 µL of complete media was added to each well to bring the total volume back up to the original volume of 1050 µL. Again, 50 µL of each sample supernatant was transferred in duplicate into the 96-well plate.

Next, 50 µL of Substrate Mix (12 mL 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. Protected from light with aluminum foil, the plates were placed on a plate shaker for 30 minutes. 50 µL of Stop Solution (1M acetic acid) was then added to each well.

Visible wavelength absorbance data were collected using the Synergy HT Multi-Detection Microplate Reader with KC4 software (Bio-TEK® Instruments, Inc., Winkooski, VT) at 490 nm. 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 cell viability. The LDH assay indicates a concentration- and time-dependent agent-induced toxicity.

III.C. Treatments

III.C.1. Metal Treatments

Cells were plated as described above and exposed to multiple concentrations (0.1, 1, and 10.0 µM) of Mercury (II) Chloride ($HgCl_2$; Sigma Aldrich, St. Louis, MO) and Lead (II) Chloride ($PbCl_2$; Sigma Aldrich, St. Louis, MO). Cells were exposed to the metals over different periods of time (24, 48, 72, and 96 h).

Hg was accurately dissolved in RPMI 1640 to make a 1 mM stock solution. Pb was accurately dissolved in ddH_2O to make a 1 mM stock solution. Then, aliquots of the original stock solutions were diluted with RPMI 1640 to make working solution concentrations (2.1, 21, and 210 µM) used for the various treatments. A 1:21 in-well to working solution ratio was used.

Once the treatments were added, the LDH assays were performed four separate times in triplicate.

III.C.2. Psychostimulant Treatments

Plated cells were exposed to two concentrations (10 nM and 100 nM) of Cocaine HCl (Coc; Sigma Aldrich, St. Louis, MO) and Methamphetamine HCl (MA; Sigma Aldrich, St. Louis, MO). Cells were exposed to the drugs over different periods of time (24, 48, 72, and 96 h).

The drugs were accurately dissolved in RPMI 1640 to make stock solutions. Then, aliquots of the original stock solution were diluted with RPMI 1640 to make working solution concentrations (210 and 2100 nM). A 1:21 in-well to working solution ratio was used.

LDH assays were performed four separate times in triplicate.

III.C.3. Combinations of Metal and Psychostimulant Treatments

From the LDH assay results of the individual metal and drug treatments, sub-lethal concentrations were chosen for combination treatments. A final in-well concentration of 10 μM was chosen for both Hg and Pb. And, a final in-well concentration of 100 nM was chosen for both cocaine and MA. A single time point of 72 h was chosen for all subsequent LDH assays. Eleven treatment groups were identified and are listed in Figure 15.

Figure 15. Treatment groups used in studies.

TREATMENT GROUPS
Control
Hg + Coc
Hg + MA
Hg + Coc + MA
Pb + Coc
Pb + MA
Pb + Coc + MA
Hg + Pb
Hg + Pb + Coc
Hg + Pb + MA
Hg + Pb + Coc + MA
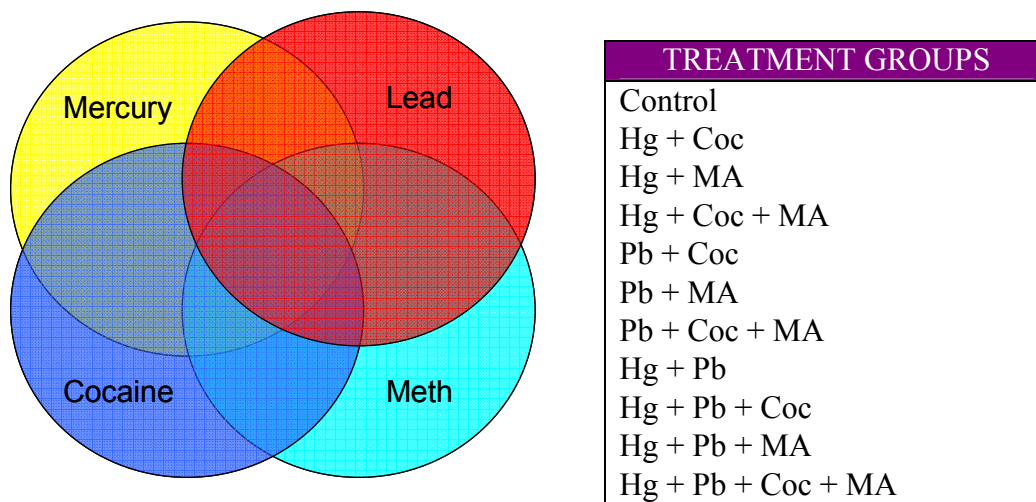
Working solutions of each treatment group were made from aliquots of stock solutions of all metals and drugs. Then, these solutions were added to plated cells as done previously in the individual metal and drug treatments.

LDH assays were performed four separate times in triplicate.

III. D. [^{3}H]GBR12935 Binding

The efficiency of DA clearance from the synaptic space depends on the number of DAT in the plasma membrane. Therefore, the next step of this project was to determine if the treatment of N2A(hDAT) and COS-7(hDAT) cells with sublethal metal/drug concentrations had an effect on the number of DATs. The DAT is selectively inhibited by GBR12935. In tritiated form, GBR12935 binding to the DAT can be inhibited by unlabeled GBR12909 (Richfield, 1991). Therefore, GBR12909 was used to define non-specific binding. Non-specific binding is the binding that occurs to any site other than the

DAT. The values obtained from non-specific binding were subtracted from total binding to calculate specific DAT binding.

Twenty-four hours after subculturing into 24-well plates, cells were treated with multiple combinations of metals (10 μM) and drugs (100 nM) for 72 h. At the end of the exposure time, the treated media was aspirated. The cells were washed once with 1 mL of buffer, and then 200 μL of trypsin/EDTA was added to the flasks to detach the cells. 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 5 minutes to form a cell pellet. The supernatant was aspirated, and the cell pellets were resuspended in 1.25 mL Tris-HCl buffer (50 mM Tris-HCl and 120 mM NaCl, adjusted to pH 7.7). 400 μL of this cell suspension was transferred into round bottom 12x75 mm polypropylene culture test tubes (Fisher Scientific, Pittsburgh, PA). Either 50 μL of assay buffer (total binding) or 50 μL of 5 μM GBR12909 (Sigma-RBI, St. Louis, MO) (non-specific binding) was then added in duplicate. Next, 50 μL of 500 nM [^{3}H]GBR12935 (43.0 mCi/mmol; Perkin Elmer, Boston, MA) was added to all tubes for a final in-tube concentration of 50 nM. 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. Using a Brandel Tissue Harvester (Gaithersburg, MD), the binding reaction was terminated by filtration onto a Whatman GF/C filter (Whatman Paper Ltd.) that had been pre-soaked in 0.3% polyethyleneimine (PEI) (Sigma Aldrich, St. Louis, MO). Filters were washed with ice cold 0.9% NaCl for 15 seconds and then carefully removed with the filter disks being placed in scintillation vials (Daigger, Vernon Hills, IL). 5 mL 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 three 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-[³H]GBR12935 binding. Remaining cell lysates were placed in -20°C until protein content could later be measured.

III. E. [³H]Dopamine Uptake

The DAT is responsible for clearing DA from the synaptic space to maintain neurotransmitter homeostasis. Therefore, the next step of this project was to determine if the treatment of N2A(hDAT) and COS-7(hDAT) cells with sublethal metal/drug concentrations had an effect on the function of DAT. Two different protocols for [³H]DA uptake through the DAT were conducted: (1) in a 24-well plastic cell culture plate and (2) on a tissue harvester.

III. E.1. [³H]Dopamine Uptake: 24-well Plate Protocol

In 24-well plastic cell culture plates (Costar® 3599, Corning, Inc., Corning, NY), 1×10^5 cells were seeded and maintained in the complete growth medium. After plating, the cells were allowed at least 24 h to adhere to the surface of the well before treatments were added. After 72 h of exposure in an incubator, the growth medium/assay buffer/ treatment was aspirated from all wells. Cells were washed with 1 mL Krebs-HEPES uptake buffer. Then 400 µL of uptake buffer was added to the control and treatment wells. Made to a pH of 7.4, the uptake buffer consisted of 25 mM HEPES, 120 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 300 µM ascorbic acid, 1 µM

pargyline and 2 mg/mL D-(+) glucose, as done previously (Johnson et al., 1998). For total binding, an additional 50 µL of uptake buffer was added to half of wells. For nonspecific binding, 50 µL of 50 µM GBR12909 (5 µM, final concentration; Sigma Aldrich, St. Louis, MO) solution was added to the other half of the wells. Tritium (^{3}H) is the radioactive form of hydrogen and is used to trace dopamine. The [^{3}H]DA (48.0 Ci/mmol; Amersham Biosciences, UK) required for this portion of the experiment was prepared by making 1.2X concentration solution diluted with the assay buffer, yielding a final in-well concentration of 20 nM. Uptake was initiated by the addition of 50 µL of the [^{3}H]DA solution to all wells. The plate was incubated for ten minutes at room temperature, and occasionally rotated by hand. DA uptake was terminated by aspirating the [^{3}H]DA solution and washing the cells twice with ice cold 0.9% NaCl. Cells were detached from the plate upon addition of 150 µL Trypsin-EDTA. Protein content of cell lysates was measured using a 50 µL aliquot from each well (Bio-Rad, Hercules, CA). The remaining trypsinized cells were transferred to scintillation vials (Fisher Scientific, Pittsburgh, PA), and 5 mL scintillation cocktail (ScintiVerse®, Fisher Scientific, Pittsburgh, PA) was added to each vial. Each vial was then capped and vortexed. Uptake was determined by liquid scintillation spectrometry using a Beckman Coulter LS 1801 (Beckman Coulter, Inc., Fullerton, CA). The number of counts per minute (cpm) was measured in order to determine the concentration of [^{3}H]DA in each sample. Non-specific binding was subtracted from total binding to calculate specific [^{3}H]DA uptake through the DAT. Remaining cell lysates were placed in -20°C until protein content could later be measured.

III. E.2. [³H]Dopamine Uptake: Tissue Harvester Protocol

Twenty-four hours after subculturing into 24-well plates, cells were treated with multiple combinations of metals (10 μM) and drugs (100 nM) for 72 h. At the end of the exposure time, the treated media was aspirated. Cells were washed with 1 mL Krebs-HEPES uptake buffer. Made to a pH of 7.4, the uptake buffer consisted of 25 mM HEPES, 120 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 300 μM Ascorbic Acid, 1 μM pargyline and 2 mg/mL D-(+) glucose, as done previously (Johnson et al., 1998). The cells were then detached upon addition of 200 μL trypsin/EDTA. This cell suspension was then transferred into flat top microcentrifuge tubes (Fisher Scientific, Pittsburgh, PA) and centrifuged in the Nanofuge (Hoefer Scientific Instruments, San Francisco, CA) for 5 minutes to form a cell pellet. The supernatant was aspirated, and the cell pellets were resuspended in 1.25 mL uptake buffer. 400 μL of this cell suspension was transferred into round bottom 12x75 mm polypropylene culture test tubes (Fisher Scientific, Pittsburgh, PA). For total binding, 50 μL of uptake buffer (total binding) was added to test tubes. For nonspecific binding, 50 μL of 50 μM GBR12909 (5 μM, final concentration; Sigma Aldrich, St. Louis, MO) solution was added to parallel tubes. Tritium ([³H]) is the radioactive form of hydrogen and is used to trace dopamine. The [³H]DA (48.0 Ci/mmol; Amersham Biosciences, UK) required for this portion of the experiment was prepared by making 1.2X concentration solution diluted with the assay buffer, yielding a final in-well concentration of 100 nM. Uptake was initiated by the addition of 50 μL of the [³H]DA solution to all tubes. The tubes were vortexed using the Fisher Vortex Genie 2 (Fisher Scientific, Pittsburgh, PA) followed by a ten minute incubation period at room temperature to reach saturation. Using a Brandel Tissue

Harvester (Gaithersburg, MD), the reaction was terminated by filtration onto a Whatman GF/C filter (Whatman Paper Ltd.) that had been pre-soaked in 0.3% polyethyleneimine (PEI) (Sigma Aldrich, St. Louis, MO). Filters were washed with ice cold 0.9% NaCl for 15 seconds and then carefully removed with the filter disks being placed in scintillation vials (Daigger, Vernon Hills, IL). 5 mL 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 three minutes using the Beckman Coulter LS 1801 scintillation counter (Beckman Coulter Inc., Fullerton, CA). Non-specific binding was subtracted from total binding to calculate specific [^{3}H]DA uptake. Unused cell lysates were placed in -20°C until protein content could later be measured.

III.G. Bradford Protein Assay

The Bradford Protein Assay is a spectroscopic analytical procedure used to measure the concentration of protein in a solution. The Bradford assay is based on an absorbance shift in the dye Coomassie when bound to arginine and hydrophobic amino acid residues present in protein.

The anionic (bound) form of the dye is blue and has an absorption spectrum maximum at 595 nm. The cationic (unbound) forms are green and red. The increase of absorbance at 595 nm is proportional to the amount of bound dye, and thus to the amount (concentration) of protein present in the sample.

Using the Bradford protein assay method, protein concentrations for each remaining sample from the above studies were measured. Dye Reagent Concentrate (40 μL; Bio-Rad, Hercules, CA), containing Coomassie blue dye, was added to each well of a

clear 96-well plate. Then, 160 μL of the samples were then mixed in duplicate with the concentrated dye. Using serial dilutions, the plate also contained eight known concentrations of a bovine serum albumin (Bio-Rad, Hercules, CA) protein standard and a blank sample that was used to generate a standard curve. The standard curve was then used to interpolate the protein content of the unknown samples. The plate was incubated at room temperature for five minutes and then read with the plate reader at an absorbance of 595 nm. Based on specific activity of [³H]GBR12935 and [³H]DA, the calculations for fmol/mg and fmol/mg/min, respectively, were performed.

III.H. Data Analysis and Statistics

For LDH assays, data was collected as means of absorbance values read at 490 nm. 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.

Binding of [³H]GBR12935 and uptake of [³H]DA was calculated using total and non-specific results of liquid scintillation counting and protein determination to be expressed as cpm/mg protein. The mean of the results of the two duplicate tubes for each treatment were used for calculations. Specific binding (or uptake) was calculated as the difference between binding (or uptake) in the absence and presence of the specific binding (or uptake) inhibitor of DAT. The specific cpm/mg protein was then divided by the specific activity of the radioligand. The results were then expressed as fmol/mg protein for binding. For uptake, the fmol/mg protein was then divided by the ten minute incubation period. The results for uptake were expressed as fmol/mg/min.

LDH assays involving multiple conditions, both concentration and time of treatment, were analyzed with two-way analysis of variance (ANOVA), followed by Bonferroni's correction for multiple comparisons as appropriate. LDH assays, [^{3}H]GBR12935 binding assays, and [^{3}H]DA uptake assays involving one treatment time with multiple treatments were analyzed using one-way analysis of variance (ANOVA), followed by Bonferroni's correction for multiple comparisons as appropriate.

All computer analyses of data were performed using Prism v5.0 (GraphPAD Software, San Diego, CA). Statistical significance for all experiments was considered if $p \leq 0.05$. The number of repetitive experiments (n) is stated in figure captions.

CHAPTER IV

RESULTS

IV.A. LDH Assay

Effects of metals on cell viability To evaluate cytotoxicity, LDH activity was measured at 24, 48, 72, and 96 h in SK-N-SH cells treated with a range of $HgCl_2$ and $PbCl_2$ concentrations (0-10 μM). Promega's CytoTox 96® Non-Radioactive Cytotoxicity Assay, a colorimetric assay, was performed to quantitate the amount of released lactate dehydrogenase in each of the samples. The amount of LDH present is directly proportional to the amount of lysed cells. LDH increased significantly in a concentration-dependent manner following exposure to $HgCl_2$ [$F_{3,48}$=14.04; $p<0.0001$] or $PbCl_2$ [$F_{3,47}$=4.29; p=0.0093]. LDH activity was significantly increased in a time-dependent manner following exposure to $HgCl_2$ [$F_{3,48}$=7.24; p=0.0004] or $PbCl_2$ [$F_{3,47}$=19.89; $p<0.0001$]. LDH activity was significantly higher in 10 μM $HgCl_2$ treatments compared to control at 48 h ($p<0.05$) and 96 h ($p<0.01$) (Figure 16). LDH activity was significantly enhanced following 10 μM $PbCl_2$ exposure at 96 h compared to that of untreated control cells ($p<0.05$) (Figure 17).

LDH Mercury

Figure 16. LDH activity was measured at 24, 48, 72, and 96 h in SK-N-SH cells treated with a range of $HgCl_2$ concentrations (0-10 μM). Percent viability was calculated by subtracting from 1, spontaneous release divided by spontaneous plus total release, and then multiplying this value by 100%. Data were analyzed with two-way ANOVA, followed by Bonferroni's correction for multiple comparisons. LDH increased significantly in a concentration-dependent ($p<0.0001$) and time-dependent ($p=0.0004$) manner following exposure to $HgCl_2$. LDH activity significantly increased at 10 μM $HgCl_2$ at timepoints 48 h and 96 h compared to control cells. Results are expressed as means ± S.E.M. ($n=4$), [+]$p<0.05$, [*]$p<0.01$.

LDH Lead

Figure 17. LDH activity was measured at 24, 48, 72, and 96 h in SK-N-SH cells treated with a range of $PbCl_2$ concentrations (0-10 µM). Percent viability was calculated by subtracting from 1, spontaneous release divided by spontaneous plus total release, and then multiplying this value by 100%. Data were analyzed with two-way ANOVA, followed by Bonferroni's correction for multiple comparisons. LDH increased significantly in a concentration-dependent ($p=0.0093$) and time-dependent ($p<0.0001$) manner following exposure to $PbCl_2$. LDH significantly increased at 10 µM $PbCl_2$ at 96 h compared to control cells. Results are expressed as means ± S.E.M. ($n=4$), $^*p<0.05$.

Effects of psychostimulants on cell viability To assess cytotoxicity of psychostimulants, LDH activity was measured again at 24, 48, 72, and 96 h, however, different concentrations (0-100 nM) of cocaine or MA were used. LDH increased significantly in a concentration-dependent manner following exposure to cocaine [$F_{3,33}=3.34$; $p=0.0477$], but not to MA. LDH activity was significant increased in a time-dependent manner following exposure of cocaine [$F_{3,33}=7.1$; $p=0.0008$] or MA [$F_{3,33}=8.36$; $p=0.0003$]. LDH activity was significantly higher following 100 nM cocaine treatment at 96 h compared to that of untreated control cells ($p<0.05$). No significant LDH activity was observed for MA treated cells compared to untreated control cells.

LDH Cocaine
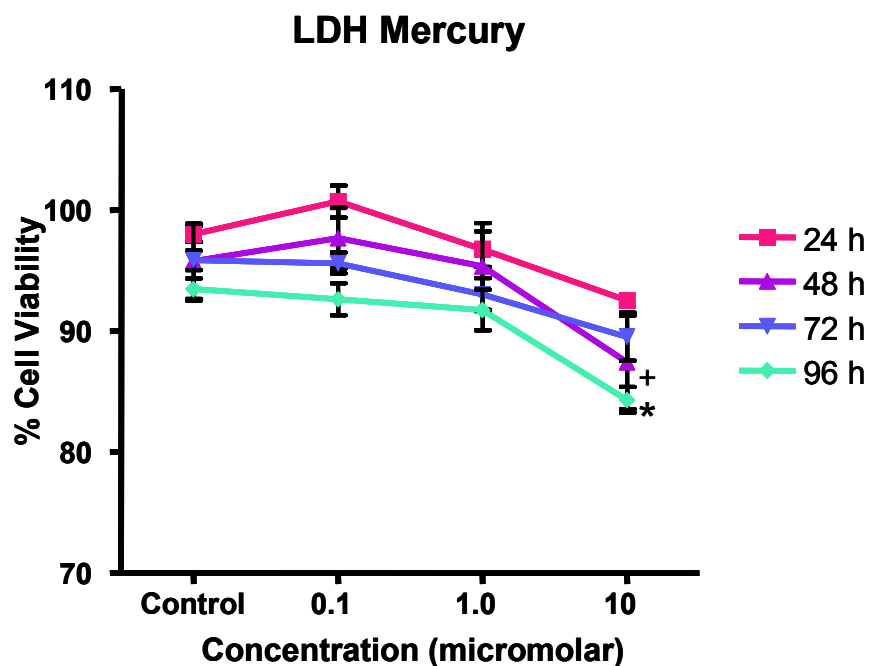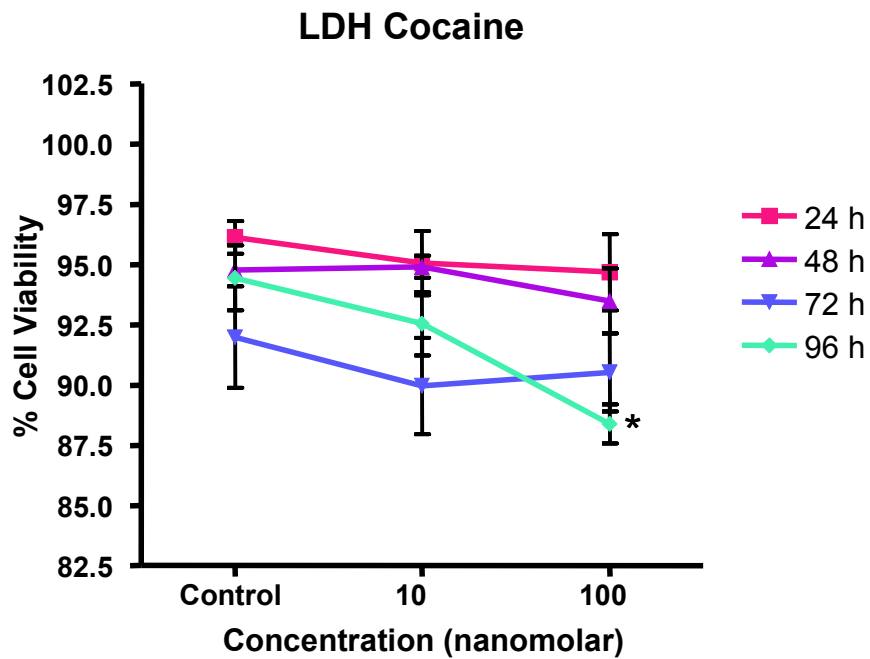

Figure 18. LDH was measured at 24, 48, 72, and 96 h in SK-N-SH cells treated with a range of cocaine concentrations (0-100 nM). Percent viability was calculated by subtracting from 1, spontaneous release divided by spontaneous plus total release, and then multiplying this value by 100%. Data were analyzed with two-way ANOVA, followed by Bonferroni's correction for multiple comparisons. LDH increased significantly in a concentration-dependent (p=0.0477) and time-dependent (p=0.0008) manner following exposure to cocaine. LDH activity was significantly higher following 100 nM cocaine treatment at 96 h compared to that of control cells. Results are expressed as means ± S.E.M. (n=4), *p<0.05.
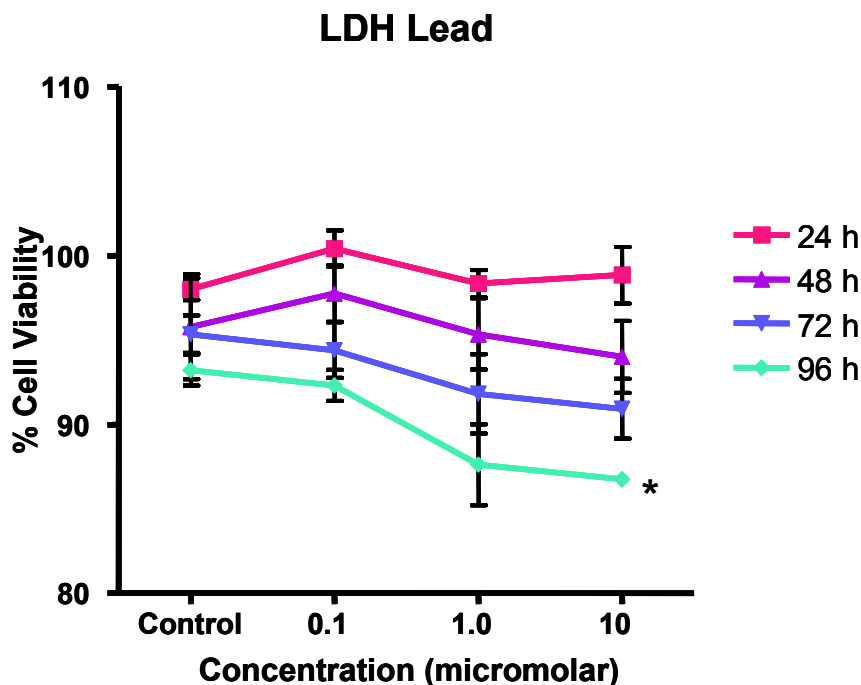
LDH Methamphetamine
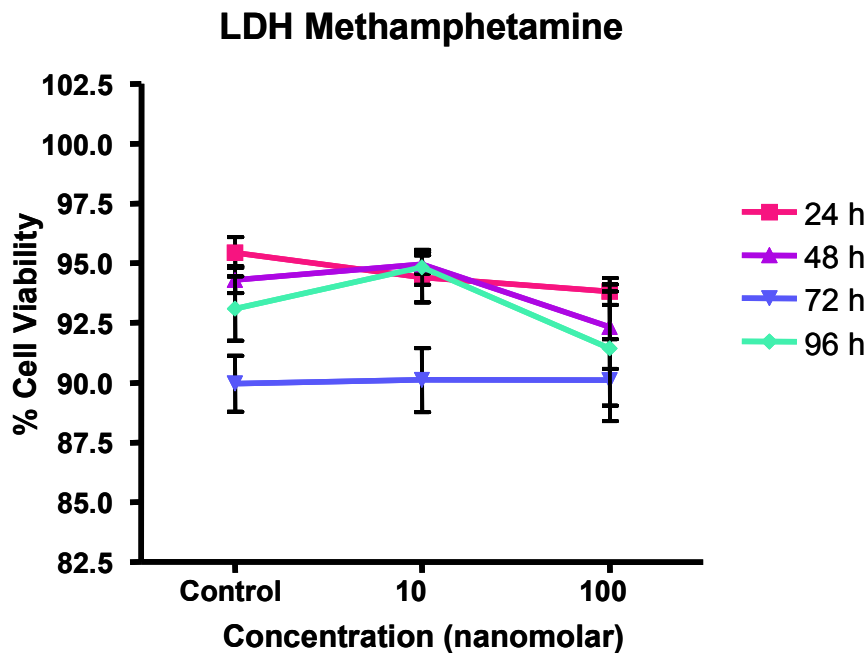
Figure 19. LDH was measured at 24, 48, 72, and 96 h in SK-N-SH cells treated with a range of methamphetamine concentrations (0-100 nM). Percent viability was calculated by subtracting from 1, spontaneous release divided by spontaneous plus total release, and then multiplying this value by 100%. Data were analyzed with two-way ANOVA, followed by Bonferroni's correction for multiple comparisons. LDH increased significantly in only a time-dependent (*p*=0.0003) manner following exposure to methamphetamine. There was no significant increase in LDH activity after methamphetamine treatments. Results are expressed as means ± S.E.M. (*n*=4).

Combined effects of metals and psychostimulants on cell viability After

analyzing the LDH assay results of the individual metal and drug treatments, sublethal

concentrations were chosen for combination treatments. A concentration of 10 μM was

chosen for both Hg and Pb, and a concentration of 100 nM was chosen for both cocaine

and MA. A single time point of 72 h was chosen for all subsequent LDH assays. After

performing LDH assays four separate times, no significance was observed in any of the

combined treatment groups.

LDH Combination of Metals & Psychostimulants
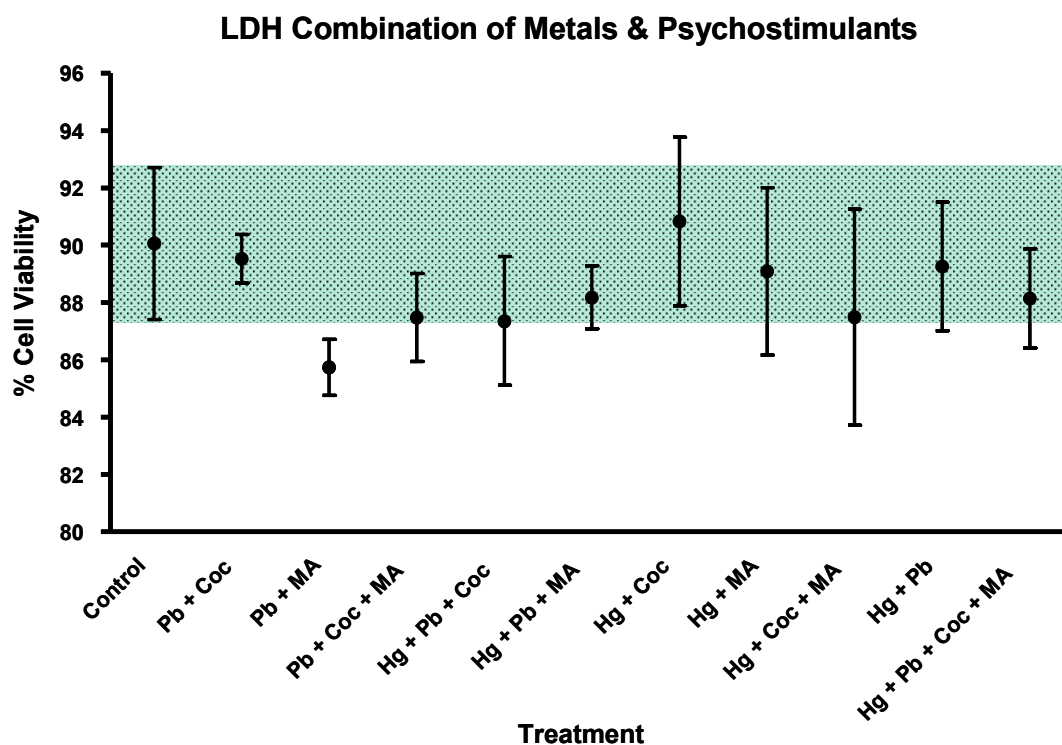
Figure 20. The effect of the combination of metals and psychostimulants on LDH activity was studied. LDH was measured at 72 h in SK-N-SH cells treated with various combinations of Hg (10 μM), Pb (10 μM), cocaine (100 nM), and methamphetamine (100 nM). Percent viability was calculated by subtracting from 1, spontaneous release divided by spontaneous plus total release, and then multiplying this value by 100%. Using one-way ANOVA, no significance was observed in any treatment group compared with the untreated control. Data represents means ± S.E.M. (n=4).

IV.B. Preliminary studies

Initial studies focused on fractional DA release on a superfusion. A superfusion chamber is used to provide a temperature-controlled environment for the tissue preparation, a constant superfusion flow rate, a system for chemical or electrical stimulation of the tissue and a fraction collector for superfusates. The first release studies used SK-N-SH cells exposed to vehicle, metals and drugs for 72 h. A consistent washout of [^{3}H]DA with no KCl-stimulated release was observed. Monoamine oxidases, present in the serum of the media, could have prematurely degraded DA. The protocol was

modified to include incubation with non-serum RPMI vehicle. Also, pargyline was added to the release buffer to inhibit monoamine oxidases present intracellularly. Yet, no KCl-stimulated release was detected (data not shown). The SK-N-SH cell line used was at passage number fourteen and fifteen, respectively, for these studies. Perhaps the cells had been passed too many times, and the DAT was less functional. A new batch of SK-N-SH cells was used. Studies were repeated at passage four of the new batch. An inconsistent washout of [^{3}H]DA occurred with KCl-evoked release between 0.04 - 12.97% among treatment groups. Perhaps not enough [^{3}H]DA uptake was occurring, accounting for the rather low release percentages. The protocol was modified again to include incubation with 200 nM [^{3}H]DA, instead of 50 nM. However, no increase in DA release was observed (data not shown). [^{3}H]DA uptake and release was occurring, but at very low levels. It was possible that SK-N-SH cells expressed DAT levels that were too low for our interests.

Because SK-N-SH cells were not a good model of dopaminergic neurons, a new homogenous cell population was sought. The search was narrowed with these parameters: (1) continuous, (2) neuronal-like, (3) biogenic monoamine transport, and (4) adherent/semi-adherent growth. Cath.a cells possessed these parameters with semi-adherent growth properties. With the new cell line, it was important to know if the DAT was functioning first, before studying DA release. Thus, [^{3}H]DA studies were performed on the Cath.a cells. Very low levels of DAT were observed. Studies showed [^{3}H]DA was transporting through norepinephrine transporters. Nisoxetine, an inhibitor of norepinephrine transporters, was used to represent non-specific uptake of [^{3}H]DA. It was concluded that Cath.a cells had extremely low levels of DAT expression. Thus, these

cells were not a good model for studying treatment effects on DAT expression and function.

A literature review suggested transfecting a cell line with cDNA of a gene of interest. This would allow an abundant amount of DAT to be expressed in a cellular population. Several journal articles showed high levels of DAT expression when cDNA of the human DAT was transfected into COS-7 cells (Bryan-Lluka et al., 1999; Pristupa et al., 1998; Lee et al., 1997; Dar et al., 2006). This project embarked on a new journey. COS-7 cells were transfected with hDAT (all obtained commercially) to develop a good DAT-expressing model system. Midway through development, another cell line, N2A(hDAT), was obtained as a gift through collaboration with Dr. Karley Little of the University of Michigan. Dr. Little has published a study characterizing N2A(hDAT) cells (2002). Most importantly the study has revealed high DAT expression levels in the cell line. This project conducted parallel studies in both cell lines, COS-7(hDAT) and N2A(hDAT).

IV.C. [³H]GBR12935 Binding

[³H]GBR12935 binding to the DAT was utilized to determine if the different metal/drug treatments would affect the cell surface expression of the dopamine transporter. Studies by Andersen have shown that GBR12935 binds with high affinity (5.5 nM as determined in rat striatal membranes) and specificity, therefore making it an appropriate drug to label the DAT (1987). [³H]GBR12935 binding was performed in COS-7(hDAT) and N2A(hDAT) cells following 72 h exposure to metals (Hg, Pb; 10 μM), drugs (Coc, MA; 100 nM), and combinations thereof. The cells were then

trypsinized, and centrifuged into a pellet. The pellet was resuspended in Tris-HCl buffer and incubated at room temperature to equilibrium with either buffer (total binding) or GBR12909 (non-specific binding) along with the [^{3}H]GBR12935. Samples were then harvested on a GF/C filter followed by placement into a vial. Once scintillation cocktail was added, the amount of radioligand 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 fmol/mg/min.

The control group means were lower in COS-7(hDAT) cells (848.9 ± 114.0) than N2A(hDAT) cells (1066.5 ± 789.1).

Effect of [^{3}H]GBR12935 binding in COS-7(hDAT) cells A one-way ANOVA was performed on data from both COS-7(hDAT) and N2A(hDAT) cells to determine if there was a significant treatment effect on the amount of DAT expression as compared to control groups. No significant effect of treatment exposure on the binding of [^{3}H]GBR12935 to the DAT was observed in COS-7(hDAT) cells (Figure 21).

The individual Hg and Pb groups had the highest increases (48.6% and 48.3%, respectively) in specific [^{3}H]GBR12935 binding. The MA group showed a 31.4% decrease in specific binding. Treatment groups containing MA caused a negative binding effect: Hg+MA (-27.1%) and Pb+MA (-6.5%).
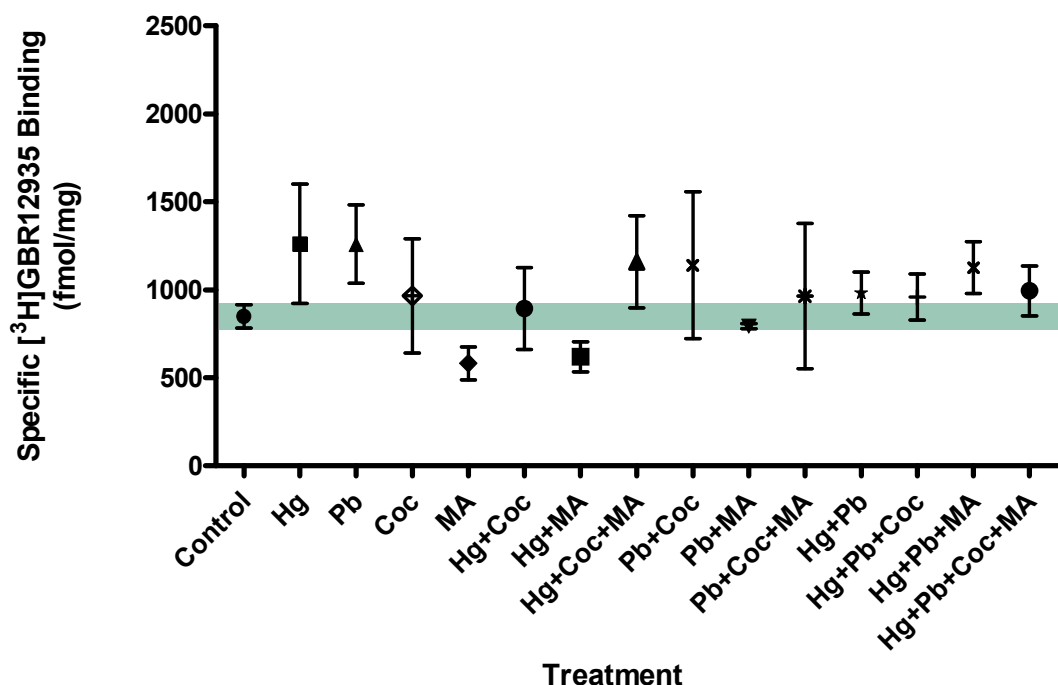
Figure 21. Specific binding of [³H]GBR12935 to DAT in COS-7(hDAT) cells. Data represents means ± S.E.M. (*n*=4). Control group (848.9 ± 114.0). Using one-way ANOVA, these treatment groups did not have a significant effect on [³H]GBR12935 binding to the DAT over 72 hours.

Effect of [³H]GBR12935 binding in N2A(hDAT) cells For all treatment groups,

an increase in binding was observed. However, statistical analysis of the effect on

binding of [³H]GBR12935 to the DAT in N2A(hDAT) cells revealed no significance. All

but one treatment group means resided outside the standard error of measurement of the

control group (Figure 22). The individual treatment groups (Hg, Pb, Coc, or MA) had

very high increases over control (146%, 175%, 115%, and 131%, respectively). When

treatments were combined, some binding increased while others showed very little

change. Interestingly, the Hg+MA group increased in binding by 161% over the control.

Also, the Hg+Pb+Coc+MA treatment group mean showed an increase in binding

exceeding 227% compared to control. In addition, the Hg+Pb treatment group mean

showed a 288% increase in binding over the control mean. These noted Hg-containing groups appear to have an additive treatment effect on binding. Because these percentages were robust, an unpaired t-test was run on each treatment group compared to the control group. A t-test compares two data sets, whereas a one-way ANOVA compares three or more groups. Analysis revealed that these treatment groups, all containing Hg, were significantly different from control values. A $p<0.05$ was deemed to be statistically significant.
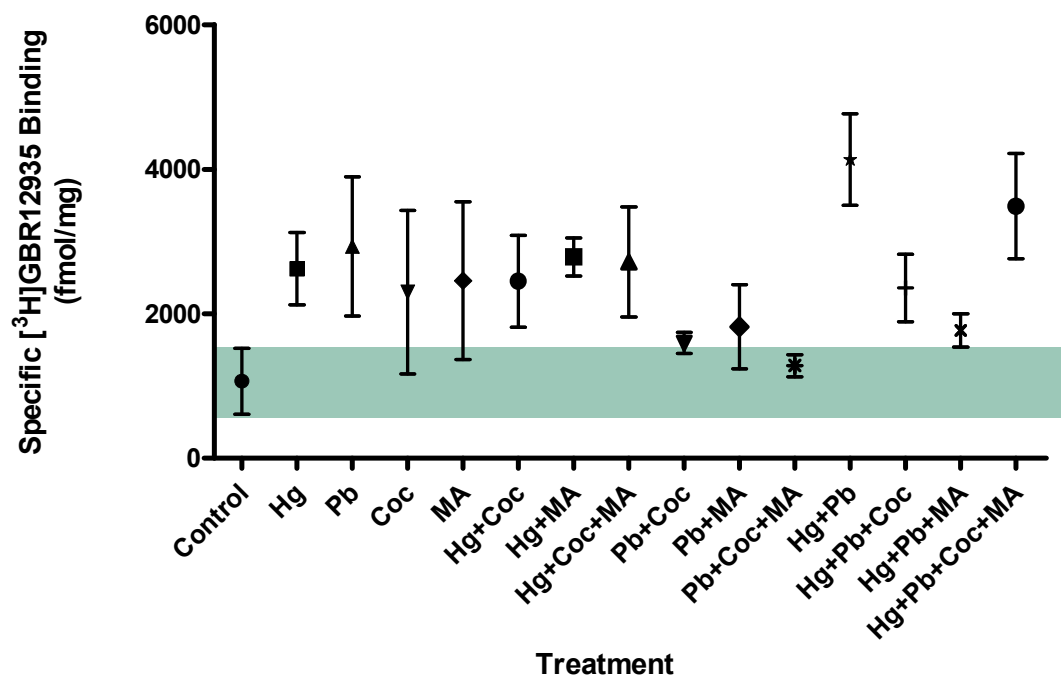
Figure 22. Specific binding of [³H]GBR12935 to DAT in N2A(hDAT) cells. Data represents means ± S.E.M. (*n*=4). Control group (1066.5 ± 789.1). Using one-way ANOVA, these treatment groups did not have a significant effect on [³H]GBR12935 binding to the DAT over 72 hours.

IV.D. [³H]Dopamine Uptake

[³H]DA uptake studies were conducted on COS-7 and N2A cells expressing the human DAT (hDAT) to establish the effects of sublethal metal/drug concentrations on

DAT functioning. First, [^{3}H]DA uptake assays were conducted in 24-well plates.

However, specific [^{3}H]DA uptake values were low and variable (data not shown). Thus,

the protocol was modified to be performed on the tissue harvester. Much higher values

were observed. The low uptake seen with the 24-well plates protocol was attributed to a

decreased number of cells. Both cell lines are semi-adherent, and through the washing

steps, cells were being aspirated out of the wells. Therefore, when the protocol was

changed to washing cells through a filter on the tissue harvester, an increase in specific

[^{3}H]DA uptake was observed.

The control group means were much higher in N2A(hDAT) cells (470.2 ± 141.34)

than COS-7(hDAT) cells (-4 ± 152.9).

Effect of [^{3}H]DA uptake in COS-7(hDAT) cells A one-way ANOVA was

performed on data from both COS-7(hDAT) and N2A(hDAT) cells to determine if there

was a significant treatment effect on the amount of DAT expression as compared to

control groups. No significant effect on the uptake of [^{3}H]DA was observed in COS-

7(hDAT) cells. All treatment group means fell within the standard error of measurement

of the control group (Figure 23). Virtually no uptake was observed.
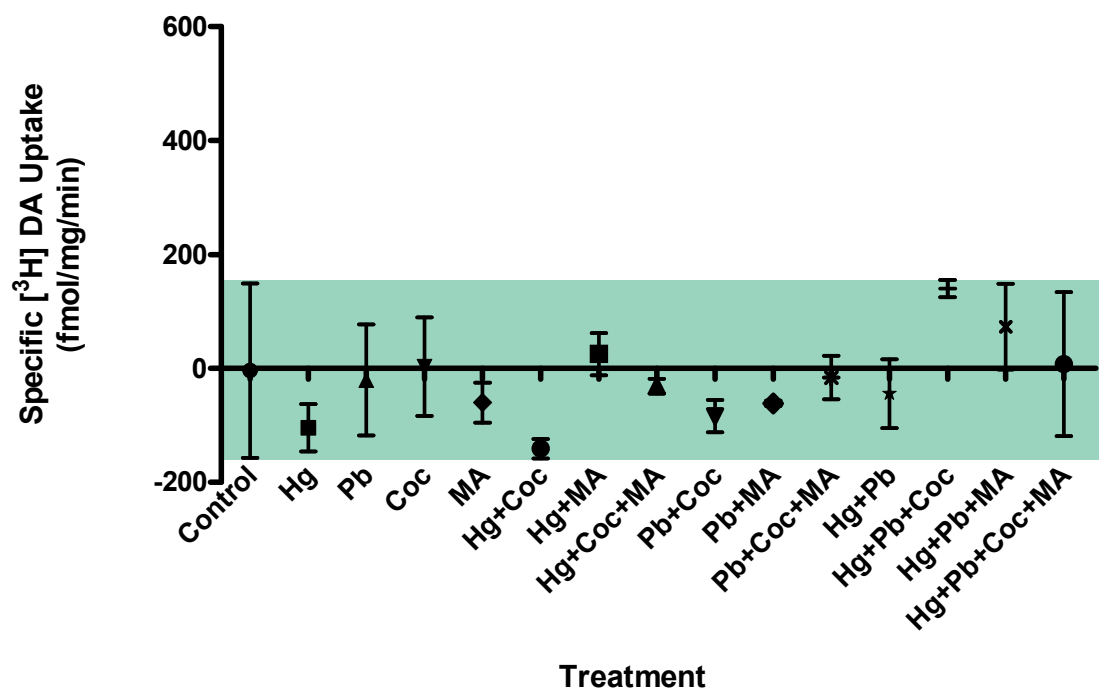
Figure 23. Specific [^{3}H]DA uptake in COS-7(hDAT) cells. Data represents means ± S.E.M. (*n*=4). Control group (-4 ± 152.9). Using one-way ANOVA, these treatment groups did not have a significant effect on [^{3}H]DA uptake through the DAT over 72 hours.

Effect of [^{3}H]DA uptake in N2A(hDAT) cells Statistical analysis of the effect on [^{3}H]DA uptake in N2A(hDAT) cells revealed no significance. However, most of the treatment group means resided outside the standard error of measurement of the control group (Figure 24). The cocaine treatment group mean showed a decrease in uptake of 17% compared to control. The Hg+Coc group also showed a decrease of 20%.

Interestingly, [^{3}H]DA uptake studies showed the largest shift in treatment groups containing MA. Mean values in MA groups were increased 35-81% compared to control values.
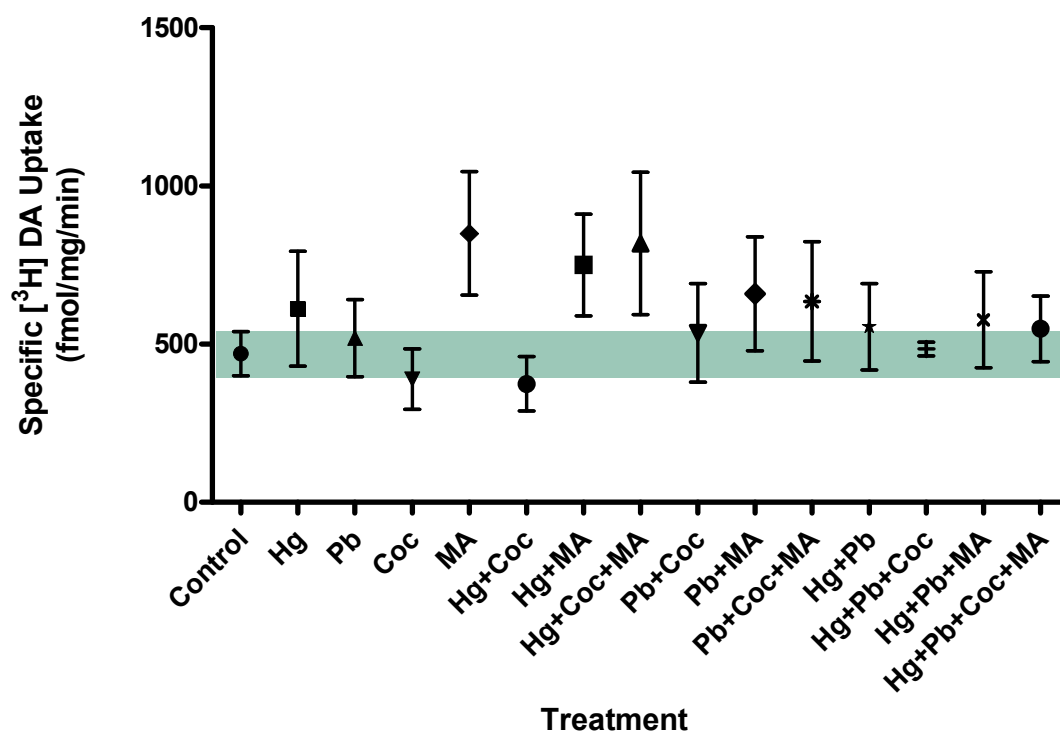
Figure 24. Specific [^{3}H]DA uptake in N2A(hDAT) cells. Data represents means ± S.E.M. (n=4). Control group (470.2 ± 141.34). Using one-way ANOVA, these treatment groups did not have a significant effect on [^{3}H]DA uptake through the DAT over 72 hours.

IV.E. Comparison of [^{3}H]GBR12935 Binding and [^{3}H]Dopamine Uptake

Data means collected from the above [^{3}H]GBR12935 binding and [^{3}H]DA uptake studies were combined and plotted on the same graph. Linear regression was performed to find the best-fit slope of the data.

Linear regression in COS-7(hDAT) cells The theoretical control line was established near y=0. Linear regression generated a best-fit line that had a slightly decreased slope compared to the theoretical control line (Figure 25). No [^{3}H]DA uptake was observed.
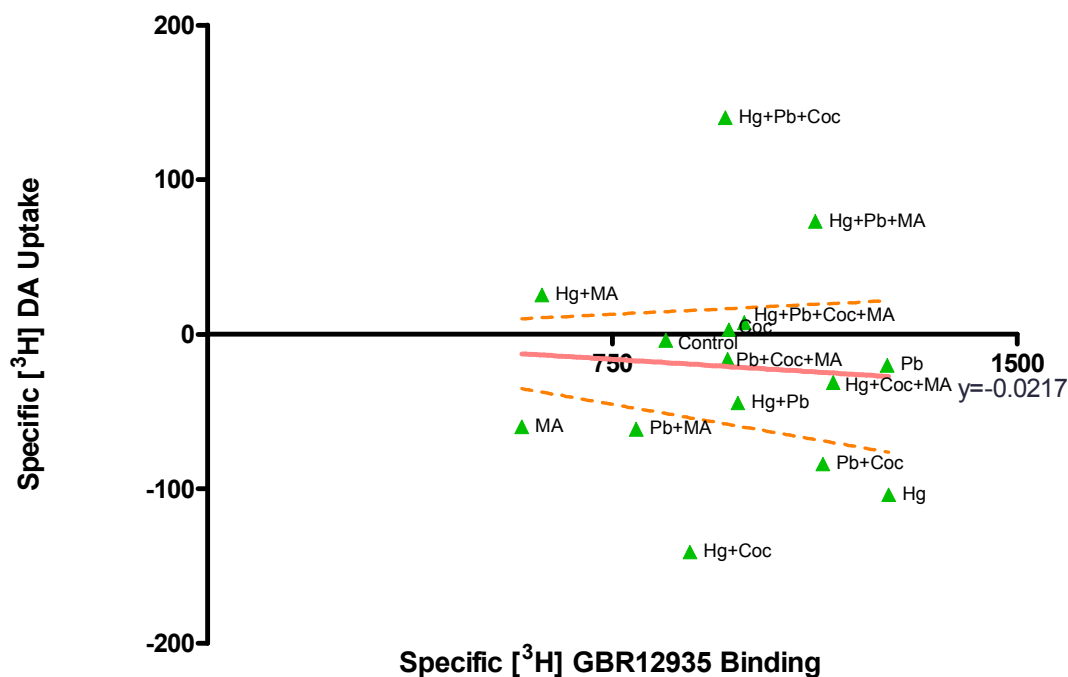
Figure 25. [³H]GBR12935 binding versus [³H]DA uptake in COS-7(hDAT) cells. Data collected from [³H]GBR12935 binding and [³H]DA uptake studies were plotted together. Using linear regression analysis, the best-fit line (y=-0.0217 ± 0.01811) did not significantly deviate from zero. The graph shows the 95% confidence interval band (y=-0.0605 and y=-0.0172). The line (y=0) through the untreated control group represents the theoretical binding v. uptake relationship.

Linear regression in N2A(hDAT) cells Linear regression generated a best-fit line shifted to the right compared to the theoretical control line (Figure 26). The shift indicates that the treatment exposure increases [³H]GBR12935 binding. The flattening slope suggests that the treatment exposure reduces [³H]DA uptake.
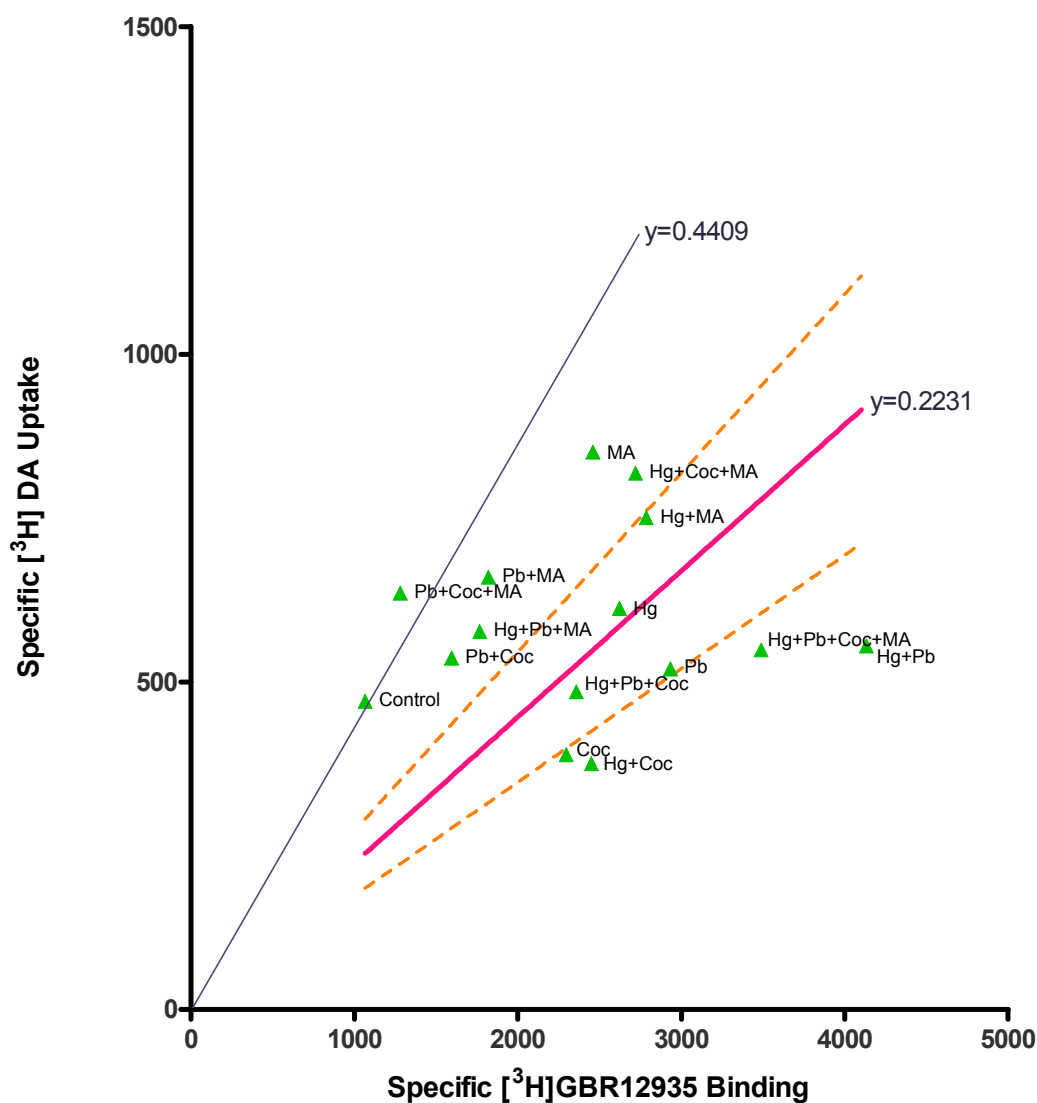
Figure 26. [3H]GBR12935 binding versus [3H]DA uptake in N2A(hDAT) cells. Data collected from [3H]GBR12935 binding and [3H]DA uptake studies were plotted together. Using linear regression analysis, the best-fit line (y=0.2231 ± 0.0232) significantly deviated from zero (*p*<0.0001). The graph shows the 95% confidence interval band (y=0.1733 and y=0.2728). The line (y=0.4409) through the untreated control group represents the theoretical binding v. uptake relationship. Data shows a shift to the right and downward for treated cells compared to control.

The MA groups appear to be concentrated in the upper half of the graph. There is an increase in specific [3H]GBR12935 binding, and more [3H]DA was taken up into the cell. This is what is expected because MA is a DA releaser. The presence of MA could

stimulate a compensatory mechanism for the up-regulation of DAT and for more DA to be taken up into the cell.

The Coc treatments are concentrated in the lower half of the graph. An increase in specific [³H]GBR12935 binding; however, less [³H]DA was taken up into the cell. Because cocaine is a DAT inhibitor, [³H]DA uptake is blocked.

The groups containing Pb are located nearest to the theoretical control line. Only slight increases in [³H]GBR12935 binding and [³H]DA uptake are seen.

Located between 2000 to 3000 fmol/mg [³H]GBR12935 binding, primarily Hg containing groups are seen with variability in [³H]DA uptake.

CHAPTER V

DISCUSSION

The objective of this study was to investigate interactions between heavy metal exposure and psychostimulant drug action at DAT. This is an especially pertinent issue given that Hg and Pb uniquely threaten sub-populations where drug abuse is more common, i.e., urban minorities (Brody et al., 1994). Clearly, experiential elements, availability, drug history, poverty, etc., must be considered in the list of dispositional factors that determine drug habits in humans. But it also must be considered that other types of environmental events may contribute to the abuse potential of selective drugs. That is, to the extent that Hg, Pb, or any other xenobiotic chemical alters the impact of a set delivery of a drug, motivational features related to drug seeking and taking may be redefined and therein influence maintenance responding and/or the effectiveness of certain pharmacotherapies for drug abuse (Mello et al., 1995).

The purpose of this study was to determine what effects low dose heavy metal treatment exposure, in combination with psychostimulants, have on the DAT functionality *in vitro*. Initial studies were aimed at finding the greatest concentration of metals/drugs that did not result in a reduction in cell viability. Subsequent assays were then more focused on the specific effects of metals and psychostimulants on the dopamine transporter. These studies measured cell expression and uptake as specifically related to DAT function.

The overall findings from this study are as follows: sub-lethal concentrations for mercury, lead, cocaine, and methamphetamine in the SK-N-SH cell line were identified that did not reduce viability at 72 hour time point. COS-7(hDAT) cells revealed high expression of DAT, but no DA uptake through the DAT. N2A(hDAT) cells showed high expression of functioning DAT, thus making it a better dopaminergic model for future studies. In COS-7(hDAT) cells, the heavy metal exposure resulted in an increase in mean DAT density, while methamphetamine exposure caused a decrease in mean DAT density. Expression studies in N2A(hDAT) revealed that DAT density increased when exposed to individual (Hg, Pb, cocaine, or methamphetamine) treatments. Interestingly, an additive effect on the binding of [^{3}H]GBR12935 to the DAT is suggested in treatment groups containing Hg. Functional studies in N2A(hDAT) showed the largest increase in [^{3}H]DA uptake through the DAT when exposed to treatment groups containing methamphetamine, whereas cocaine groups inhibited uptake.

V.A. LDH Assay

In order to determine how long term exposure to low concentrations of heavy metals and psychostimulants affects the dopamine transporter, it was first necessary to determine the threshold 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.

LDH is a cytoplasmic enzyme that is released when cellular membranes are damaged. The assay 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. 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. Other studies have observed a concentration effect on cell viability (Lee et al., 2006; Walther et al., 2002). Very few studies have reported the effects of mercuric chloride on viability at time points extending beyond 48 h. Three studies were found reporting viability effects after 48 h exposure times (Ben-Ozer et al., 2000; Kim & Sharma, 2004; Aleo et al., 2005). The two studies reporting results up to 72 h had different findings. In the study by Ben-Ozer et al. (2000) with U-937 human monocyte-like cells, concentrations of 0.1-100 μM mercuric chloride over 24-72 h were examined. A concentration-dependent decrease in viability was observed, and a trend of decreased viability over time at 1 μM was pointed out, however this later observation was slight and not statistically significant. The other study examining varying concentrations (3, 10 and 30 μM) of mercuric chloride up to 72 h 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 h exposure times with concentrations up to 20 μM 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 lead chloride, concentrations of 0.1, 1, and 10 μM were chosen for their effects on the SK-N-SH cell line. A significant effect of Pb concentration or exposure duration was observed. LDH activity was significantly

65

increased at 10 μM PbCl$_2$ at 96 h compared to control cells. Few studies examined long term exposure of 48 h or more. Incubation of C6 glioma cells with Pb(II) for 48 h decreased metabolic cell viability. The reduction was by 10 and by 25% with 1 and 10 μM Pb(II) treatments, respectively (Posser et al., 2007). In another study, a rat liver-derived cell line was exposed to increasing concentrations of Pb(II) (0.1-100 μM) for 24, 48 and 72 h (Aleo et al., 2006). At 10 μM Pb(II), a significant decrease in cell density (70–75% of control values) was observed, being this effect maximal already after 48 h of treatments. In the case of 10 or 100 μM Pb-treatments, the number of cells did not change significantly from 24 to 72 h of treatment (cytostatic effect). Pb(II) intoxication is closely associated with low doses and long periods of exposure and 10 μg of Pb(II) per 100 ml of blood (0.48 μM) has been considered harmful in humans (Silbergeld, 1997; Bellinger, 2004). In the present study, the effects observed *in vitro* using the nominal concentration of Pb(II) (1–10 μM) may be considered toxicologically relevant, since it was applied in the presence of fetal bovine serum (FBS; 10%). FBS is a factor that may chelate the metal (Qian et al., 2000), thus decreasing the concentration of free Pb(II) in the incubation medium. In PC12 cells the free Pb(II) concentration is approximately 0.1% of the total Pb(II) in the presence of 10% FBS (Tiffany-Castiglioni & Qian, 2001).

To assess cytotoxicity of psychostimulants, LDH activity was measured again at 24, 48, 72, and 96 h, however, different concentrations (0-100 nM) of cocaine or MA were used. The LDH assay showed a concentration- and time-dependent effect of cocaine on SK-N-SH viability. LDH activity was significantly higher following 100 nM cocaine treatment at 96 h compared to that of untreated control cells. A study was performed in a similar neuroblastoma cell line, SK-N-AS (Feng et al., 2006). Cells were treated with

cocaine for 24 h at the concentrations of 0, 5, 25, 50, 100, and 500 μM, respectively. Cocaine concentrations higher than 5 μM all caused significant reductions in the number of living neuroblastoma cells as compared with the control group. No other studies were uncovered that conducted low-level, chronic exposure *in vitro*. Our results show that duration of exposure is as important as the concentration.

For the cytotoxicity assays using methamphetamine, concentrations of 10 and 100 nM were chosen for their effects on the SK-N-SH cell line. Our study demonstrated that the duration of exposure was important for MA toxicity. No significant LDH activity was observed for MA treated cells compared to untreated control cells. One study was found that looked at the effects of methamphetamine in SK-N-SH cells, but used much higher MA concentrations. MA dose-dependently decreased cell viability in SK-N-SH cells after exposure for 24 h (Ajjimaporn et al., 2005). The cell viability was decreased to 80, 68, 60 and 47% at 10, 100, 500 μM and 1 mM of MA, respectively. A time-dependent effect of MA on cell viability was observed when cells were treated with 1 mM of MA for 24, 48 and 72 h. Another study showed a time- and dose-dependent decrease in cell viability in Cath.a cells exposed to MA. The cells were treated with various concentrations (0.5 mM – 4 mM) of MA for 2 to 48 hours (Choi et al., 2002). At 48 h, MA exposure 1 mM and higher caused cell viability to be significantly decreased.

V.B. Preliminary studies

Initial studies focused on treatment effect on DA release. After protocol modifications, consistent [^{3}H]DA release was not obtained. It was determined that SK-N-SH expressed DAT too low for this study's interests and as such were taking up too little

[^{3}H]DA. A recent study looked at the *in vivo* effects of inorganic mercury on striatal DA release using brain microdialysis (Vidal et al., 2007). Administration of 100 μM and 1 mM mercuric chloride produced a concentration-related increase in striatal DA output. In contrast, the dose of 10 μM was without any effect on the extracellular levels of DA and metabolites. Vidal's study supports that the concentration chosen in the present study was below a level to cause significant alterations in DA release. This study also supports that treatment effects are initially a DAT-mediated event. At 10 μM mercuric chloride, changes in DAT function are known to occur. As concentrations of mercury build in an organism, changes are seen in DAT first, followed by changes in DA release. Future studies could focus on administering mercuric chloride *in vivo* for three or four weeks and measuring DAT changes two months later.

The present study attempted using another dopaminergic cellular model, Cath.a, to investigate DAT function. Cath.a is a clonal cell line of the central nervous system that produces primarily DA and, to a lesser extent, norepinephrine (Suri et al., 1993). RT-PCR analysis showed the expected PCR product size of DA transporter cDNA in Cath.a cells (Higashi et al., 2000). In another study, Bundey et al. loaded Cath.a cells with 10 nM [^{3}H]DA (2000). The investigation demonstrated that no substantial [^{3}H]DA uptake was occurring. Perhaps the Cath.a cell line does not express a functional plasma membrane DA transporter. The study also revealed that neither [^{3}H]norepinephrine nor [^{3}H]choline was taken up by the Cath.a cells. The study suggests that the cell line is of an alternative neuronal phenotype, e.g. GABA-releasing or 5-hydroxytryptamine-releasing. Thus, this model is not appropriate for studying treatment effects on DAT expression and function.

Because of these problems, a model system was developed involving transfection with DAT cDNA. Previous investigators have characterized hDAT function after expression in several types of non-neuronal cells (Eshleman et al., 1995; Giros et al., 1992; Pristupa et al., 1994; Shimada et al., 1991). This study developed a transfected non-neuronal cell line, COS-7, with hDAT. Because of potential differences in regulation and function between neuronal and non-neuronal cells, we further evaluated a neuronally-derived model system, N2A.

V.C. Expression and Function Studies

The selectivity of [^{3}H]GBR12935 binding to the DAT makes it a useful tool for labeling and studying the presence of the transporter at the plasma membrane. [^{3}H]GBR12935 binding to the DAT was utilized to determine if the different metal/drug treatments would affect the cell surface expression of the DAT. [^{3}H]GBR12935 binding was performed in COS-7(hDAT) and N2A(hDAT) cells following exposure to metals, drugs, and combinations thereof. Statistical analysis of the effect on binding of [^{3}H]GBR12935 to the DAT revealed no significance in either COS-7(hDAT) or N2A(hDAT) cell lines. However, treatment groups showed shifts in binding compared to controls in both cell lines.

[^{3}H]DA uptake studies were conducted on hDAT-transfected COS-7 and N2A cells to establish the effects of sublethal metal/drug concentrations on DAT functioning. The control group means were much higher in N2A(hDAT) cells than COS-7(hDAT) cells suggesting higher expression levels. When statistical analysis of the effect on [^{3}H]DA uptake was performed, neither COS-7(hDAT) nor N2A(hDAT) cells revealed

significant differences compared to control values. In COS-7(hDAT) cells, uptake in treatment groups was not different from control. In N2A(hDAT) cells, treatment groups showed both increases and decreases in uptake over controls.

V.C.1. Expression and Function Studies: COS-7(hDAT)

In COS-7(hDAT) cells, the individual Hg and Pb groups (48.6% and 48.3%, respectively) showed a trend where specific [^{3}H]GBR12935 binding to the DAT increased. This suggests that heavy metals are causing an up-regulation of the DAT. Hg exerts its toxic effect by binding to the thiol groups on proteins, including the DAT. The neuron attempts to compensate for the defective DAT by inserting more at the plasma membrane. Pb competes with calcium-mediated synaptic vesicle release resulting in an increased DA release from the synapse. The neuron strives to maintain DA clearance by increasing the density of DAT at the plasma membrane. Also seen in the COS-7(hDAT) cells was a decrease in specific [^{3}H]GBR12935 binding. The MA group and groups containing MA showed a reduction in binding compared to control. MA is known to stimulate DA to be released from vesicles. In a cell line with a fully functioning DAT, an increase in DAT density would be expected. However, since the COS-7 cells did not effectively take up [^{3}H]DA, the DAT was not functioning properly. Overall, it is difficult to speculate whether these treatments increased or decreased binding when the functional integrity of the DAT is questioned.

Studies in COS-7(hDAT) cells, revealed that no [^{3}H]DA uptake was observed; yet, there was a trend towards elevated [^{3}H]GBR12935 binding. This indicates that the COS-7(hDAT) cells are expressing high levels of DAT, but they are not fully functional.

Perhaps the glycosylation sites usually found on the DAT are missing. This could affect the binding of ligands and neurotransmitters to the transporter. It has been shown that glycosylation of the DAT differs between different brain regions (Lew et al., 1991; Patel et al., 1993) which may be related to regional differences in function (Garris & Wightman, 1994). Glycosylation patterns and other post-translational phenomena also likely vary between different types of cell cultures. Post-translational events can markedly influence binding and functional characteristics of receptors (Kobilka, 1990; O'Dowd et al., 1989) and recent experiments have demonstrated that protein kinase C (PKC)-mediated regulation of uptake by a related monoamine transporter—the serotonin transporter, involves changes in plasma membrane insertion (Qian et al., 1997). Specialized compartmental trafficking may depend on post translational modifications which could be critical in understanding DAT plasticity. Although it is not clear if post translational mechanisms are involved in regulating expression and function of the DAT in these cells, studies with glycosidases comparing hDAT-expressing N2A neurons and COS-7 cells might prove interesting.

V.C.2. Expression and Function Studies: N2A(hDAT)

In N2A(hDAT) cells, a trend towards elevated [^{3}H]GBR12935 binding was observed for all treatment groups. The individual treatment groups (Hg, Pb, Coc, or MA) had increase binding over control values (146%, 175%, 115%, and 131%, respectively), although these values were not statistically significant. When treatments were combined, binding results were variable. Interestingly, groups containing Hg had the greatest binding increases. This may suggest that Hg causes an up-regulation of the DAT, similar

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to that seen in the COS-7 cells. An additive treatment effect on binding is also suggested in the Hg-containing groups. A treatment effect is additive if after simultaneous exposure of two or more substances, it is the sum of the individual effects. Additional studies are needed to increase our sample size greater than 7 to determine if these changes are significantly different. Power analysis was performed on mean treatment effect (mean = 2479; SD = 1153) observed in the present study. Based on the parameters observed, a sample size in excess of 7 in each treatment group has a 95% power to detect a significance level (alpha) of 0.05 (two-tailed).

In N2A(hDAT) cells, the cocaine treatment group and the Hg + Cocaine treatment group means showed a trend towards decreased uptake compared to control. Yet, binding was elevated in cocaine treated N2A(hDAT). Because cocaine is a DAT inhibitor, [^{3}H]DA uptake is blocked. With the dopaminergic system in a compromised state during cocaine exposure, the up-regulation of the DAT could reflect a homeostatic response whereby increased capacity for DA reuptake would maintain neurotransmission at more normal levels. Thus, these data suggest that the DAT might actively participate in modulating the behavioral consequences of chronic cocaine. There are conflicting reports, however, concerning changes in DAT function as a mechanism contributing to addiction. Some studies have found changes consistent with reduced activity of DAT, including reduced DA uptake (Izenwasser & Cox, 1990), down-regulation of DAT (Sharpe et al., 1991) and elevated extracellular fluid (ECF) concentrations of DA (Kalivas & Duffy, 1990). In contrast, other groups have reported changes consistent with increased activity of DAT, including increased DA uptake (David et al., 1998; Fleckenstein et al., 1999; Ho & Segre, 2001) and an attenuation of cocaine-induced

increases in ECF levels of DA (Segal & Kuczenski, 1992). The variable effect of cocaine on uptake likely reflects the use of different dosing regimes, routes of administration, brain regions, ECF concentration of DA at the time of cocaine administration, as well as the techniques to quantify DAT function (David et al., 1998; Cass et al., 1993; Zahniser et al., 1999).

Interestingly, [^{3}H]DA uptake studies showed the largest shift in treatment groups containing MA. MA exposure in N2A(hDAT) elicited a marked up-regulation of DAT binding and uptake, which was not present in COS-7 cells. This is what is expected since MA promotes DA release. The presence of MA may initiate a compensatory mechanism whereby DAT density is increased to enhance removal of DA from the synaptic cleft.

Under normal conditions, the majority of DAT protein is found at the cell surface. The translocation of DAT from the membrane to the cytosolic space is a fundamental mechanism in the regulation of DAT homeostasis and functioning. Cell cultures expressing cloned DAT demonstrate that treatment with psychostimulants regulate DAT expression in the plasma membrane (Daniels & Amara, 1999; Saunders et al., 2000; Zhang et al., 1998; Little et al., 2002). Psychostimulants may act by promoting exocytosis of internalized DAT and/or by decreasing constitutive internalization of DAT and thus increasing plasma membrane DAT by altering the balance of internalization and recycling to the surface. Cell surface redistribution of DAT is a mechanism that contributes to the enhancement of extracellular DA levels in response to psychostimulants. Of note is that [^{3}H]GBR12935 binding is not directly reflective of the functional DA uptake process. Therefore, [^{3}H]DA uptake studies are performed to check the functional status of the DAT.

V.E. Comparison of COS-7(hDAT) and N2A(hDAT) cells

COS-7(hDAT) cells revealed high expression of DAT, but no DA uptake through the DAT. N2A(hDAT) cells showed high expression of functioning DAT, thus making it a better dopaminergic model for future studies.

Zhang et al. compared [^{3}H]DA uptake in hDAT-transfected N2A and COS-7 cells (1998). Uptake in N2A cells was of high affinity ($K_m = 0.43$ µM). DA uptake was also examined in COS-7 cells, which displayed a higher K_m of 1.6 µM. Thus the affinity for DA uptake was somewhat higher in the N2A system. Zhang et al.'s DA uptake findings were similar to Giros et al. (1992) determined a K_m of 1.2 µM in Ltk-fibroblasts. Two studies performed in COS-7 cells reported a $K_m = 1.8$ µM (Pristupa et al., 1994) and a $K_m = 1.2$ µM (Eshleman et al., 1995). These studies provide further evidence that the N2A cells may be a better dopaminergic model for our proposed studies.

When working with hDAT-transfected COS-7 cells again, stable selection will be modified. Surviving cells after G418 selection need to be put into 96-well plates so that a single cell colony is present in each well. The clonal cell lines from each well then need to be tested for their DA transport activity. One of the clones that exhibits the highest activity would then be selected for extensive studies. Future studies also include sequencing the entire hDAT-cDNA insert. The size of the insert from *Not*I digestion is 2.7 kilobases. In this study, the first 1.25 kilobases were sequenced and were confirmed a match to the validated SLC6A3 sequence (the official name of the dopamine transporter) in Genbank's database. The other 1.45 kilobases of the insert need to be sequenced to verify that the cDNA is in fact the entire hDAT gene. A point mutation could have

occurred in a critical site of the hDAT gene used in this study, resulting in a dysfunctional DAT seen in the COS-7 cells.

V.F. Conclusion

This study posed the question: does low dose heavy metal exposure in combination with psychostimulant use alter functionality of the dopamine transporter? N2A(hDAT) cells revealed a trend where the metal/drug-treatment exposures increased the density of DAT, but reduced [^{3}H]DA uptake. This study shows that metal/drug treatments could increase the cell surface distribution of DAT and that this redistribution of DAT could be associated with increases in extracellular DA. Because changes in DA neurotransmission are thought to play an important role in the addictive properties of psychostimulants, drug-related trafficking of DAT may be an important mechanism in the development of its abuse. Increased GBR binding with no change or decreased DA uptake may suggest decreased function of DAT. In turn, this will result in decreased DA uptake leading to increased synaptic DA. If not cleared properly, high levels of synaptic DA may lead to cell damage over time and further changes in DAT density and function.

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Scope and Method of Study: Heavy metals may alter the abuse liability of drugs due to actions on the dopamine transporter (DAT). This study examined the effects of extended, low-level heavy metal and psychostimulant co-exposure on DAT function. SK-N-SH cells, incubated in the presence of multiple concentrations of lead (Pb), mercury (Hg), cocaine (COC) and methamphetamine (MA), were used to measure LDH activity to determine optimum time/concentration for sublethal exposure assays. Parallel studies were conducted on non-neuronal vs. neuronal cell lines, COS-7(hDAT) and N2A(hDAT) respectively. [^{3}H]GBR12935 binding assays were performed to determine DAT expression at the plasma membrane. [^{3}H]Dopamine (DA) uptake assays were conducted to establish effects on DAT functioning.

Findings and Conclusions: LDH activity significantly increased in both a concentration- (Hg [$p<0.0001$], Pb [$p=0.0093$], COC [$p=0.0477$]) and time-dependent (Hg [$p=0.0004$], Pb [$p<0.0001$], COC [$p=0.0008$], MA [$p=0.0003$]) manner. Sublethal concentrations of drugs/metals were chosen for further studies (10 μM for $HgCl_2$ and $PbCl_2$; 100 nM for COC and MA), using a 72 h exposure. COS-7(hDAT) cells revealed expression of DAT, but no DA uptake. N2A(hDAT) cells showed higher expression of functioning DAT. Statistical analysis of the treatment effect on DAT density or DA uptake through the DAT revealed no significance in either cell line. Studies in N2A(hDAT) revealed that DAT density increased when exposed to individual treatment groups [Hg (146%), Pb (175%), COC (115%), or MA(131%)]. When treatments were combined, DAT density increased in: Hg+MA (161%), Hg+Pb+Coc+MA (227%), and Hg+Pb (288%). An interactive effect on DAT density is suggested in treatment groups containing Hg. Studies in N2A(hDAT) showed the largest increase in DA uptake when exposed to treatment groups containing MA (35-81%), whereas COC groups inhibited uptake (17-20%). Overall, a trend was observed where DAT density was increased, but cause functional decreases in DA clearance were observed. Individuals exposed to low-levels of Hg, may be at risk for increased DA neurotransmission/ turnover following psychostimulant use, resulting in an elevated addictive, or toxic, potential of these already addictive drugs.

ADVISER'S APPROVAL: David R. Wallace