ISSN 2474-8978

TOXICOLOGY AND FORENSIC MEDICINE

OpenJournal

Openventio PUBLISHERS

Original Research

Function of the Human Dopamine Transporter After Heavy Metal and Psychostimulant Exposure

David R. Wallace, PhD[1,2*]; Amber N. Hood, MS[1]

[1]School of Forensic Science, Oklahoma State University Center for Health Sciences, 1111 West 17th Street, Tulsa, Oklahoma, 74107-1898, USA
[2]School of Biomedical Science, Oklahoma State University Center for Health Sciences, 1111 West 17th Street, Tulsa, Oklahoma, 74107-1898, USA

Corresponding author
David R. Wallace, PhD
Professor, Department of Pharmacology and Physiology, Oklahoma State University Center for Health Sciences, Room E-367, 1111 West 17th Street, Tulsa, Oklahoma, 74107-1898, USA; Tel. +1-918-561-1407; Fax: +1-918-561-5729; E-mail: david.wallace@okstate.edu

Article information
Received: May 9th, 2023; **Published:** June 2nd, 2023

Cite this article
Wallace DR, Hood AN. Function of the Human Dopamine Transporter After Heavy Metal and Psychostimulant Exposure. *Toxicol Forensic Med Open J*. 2023; 8(1): 1-13.

ABSTRACT

Background
Psychostimulant use combined with environmental exposure to heavy metals may present a special risk to a user's neurological health at the level of dopamine transporter (DAT) function.

Goals
These investigations looked into how human DAT density and function were affected by low-concentration heavy metal and psychostimulant (co)exposure.

Materials and Procedures
PbCl2 (Pb), HgCl2 (Hg), cocaine (COC), and methamphetamine (MA) were administered to Neuro2A neuroblastoma cells (N2A) in order to measure changes in cell survival and the density and function of the human dopamine transporter (hDAT). The assays comprised [3H] dopamine (DA) absorption (hDAT functionality), [3H] GBR12935 binding (hDAT density), and Lactate Dehydrogenase activity (cell viability). The concentration employed in combination exposure experiments (10 μM for Hg and Pb; 100 nM for COC and MA) with an ideal exposure duration of 72 hours is the threshold for changes in cell viability of a metal or stimulant.

Findings
Pb and Hg both decreased cell viability in relation to concentration and exposure duration. N2A cells showed increased resistance to the cytotoxicity caused by MA and COC. After being exposed to Hg, Pb, COC, or MA, the density of hDAT rose (from 115 to 175%) in comparison to the vehicle values. In comparison to control values, hDAT density rose 161-288% following exposure to metal-psychostimulant combinations. While COC groups hindered uptake (17–20%), we saw the greatest increase in [3H] DA uptake in the MA group (35-81%). Exposure to each drug, either alone or in combination, raised the density of hDAT. Since changes in [3H] DA absorption did not coincide with changes in density, our findings imply that increased protein content did not correspond to greater functioning.

Conclusion
The ability to remove [3H] DA from the extracellular space is compromised due to decreased function, even if there is more hDAT protein at the cell surface. Therefore, after using psychostimulants, exposure to low levels of heavy metals may raise the risk of altered DA neurotransmission/turnover, which could lead to an exaggerated reaction or an elevated risk of toxicity.

Keywords
Mercury; Lead; Cocaine; Methamphetamine; Dopamine uptake.

Abbreviations
ANOVA: Analysis of Variance; CNS: Central Nervous System; COC: Cocaine; CPM: Counts per minute; DA: Dopamine; DAT: dopamine transporter; DMSO: Dimethyl sulfoxide; DPM: disintegrations per minute; GBR 12909 - 1-(2-[bis(4-Fluorophenyl)methoxy]ethyl)-4-(3-phenylpropyl)piperazine; GBR 12935 - 1-[2-(Diphenylmethoxy)ethyl]-4-(3-phenylpropyl)piperazine; Hg: Mercury.

Toxicol Forensic Med Open J. 2018; 3(1): 1-13. doi: 10.17140/TFMOJ-3-124

INTRODUCTION

Over the past three to four decades, a large number of papers have detailed the processes by which cocaine (COC) and methamphetamine (MA) work in the central nervous system (CNS). It is evident that dopamine plays a significant role in the processes of addiction, euphoria, and dependency in both COC and MA.1–3 Drug-taking risk variables may be influenced by changes in drug responsiveness caused by the extracellular chemical environment of the neuron synapse. Because certain metals cannot pass across the blood-brain barrier, the mechanisms by which heavy metals affect the central nervous system are less understood. The majority of our knowledge is based on in vivo research, and our understanding is growing.4-6 Lead's (Pb) effects on dopaminergic function have been well documented.7-9 Pb is known to affect the mesolimbic dopamine (DA) pathway, namely the projection neurons that run from the nucleus accumbens to the ventral tegmental region.7.

Functional disruptions in mesolimbic DA processes brought on by prenatal or postnatal Pb exposure may result in permanently elevated sensitivity to COC since DA activity in this circuit plays a crucial role in determining COC responsiveness. Both Pb and COC have an impact on DA absorption and release.8,9,11,12 Other metal/drug interactions, such those between MA and mercury (Hg), which are known to impact the DAT, needed to be investigated.13–16 Because of the elevated levels of DA seen after exposure to mercury ($HgCl_2$), previous research has also revealed that Hg has gained access to striatal tissue.14 Prior research has demonstrated that mercury inhibits the breakdown route of catecholamines, including DA. Long-lasting reductions in DA levels, tyrosine hydroxylase activity, and DA absorption sites in the brain have been shown in a substantial body of research investigating the effects of MA on the dopaminergic system.19–22 Numerous investigations have revealed alterations in DAT function, which could be linked to any or both of the changed DA uptake and release.19–25 The degeneration of DA neurons may be the cause of the long-term alterations seen after MA exposure. After being exposed to greater concentrations or doses for extended periods of time, MA has been demonstrated to be a neurotoxic.20, 22, and 23 Interest in the toxicological research of toxicant/toxin mixtures has increased due to the idea that these mixtures can produce additive or synergistic toxicity.In the US, COC and MA are both psychostimulants that fall under Schedule II substances (C-II). This list includes medications that still have medical applications but have significant addiction, dependence, and health risks. COC can be injected, smoked, or snorted; the method of administration will determine how strong the "high" is and how long it lasts.

Because COC is a stimulant, using it excessively will cause physiological reactions such elevated blood pressure, elevated heart rate, anxiety, paranoia, and irritability.26 While the precise method by which MA acts in the central nervous system is different, the broad symptoms of MA and COC are comparable.27 The simplistic theory of psychostimulant action in the past has been that COC increases DA transmission by blocking DA absorption, which results in the physiological and behavioral alterations that are seen after COC injection. In addition to decreasing DA uptake in vivo and in vitro, COC treatment also raises DA in the synapse via non-antagonist pathways (2, 28, 29).3.30 It is believed that reverse transport of DA through the DAT is the cause of increased synaptic DA.3. Since neither the frequency nor the amplitude of DA release is altered, in vivo study shows that enhanced synaptic DA is not caused by increased firing rates of dopaminergic neurons.2 A competitive interaction with DA in the binding pocket on DAT is thought to be the cause of COC interference with DA absorption.29, 31 The reduction in DA uptake that follows COC injection is a result of a decrease in the Km, or affinity, of DA for the DAT, which happens after prolonged exposure to COC.28 The ability of DAT inhibitors to raise DA clearance rates can be partially explained by their immediate effects on DAT, which show that a higher clearance happens in response to elevated extracellular DA.32 Imaging studies showed that human abusers of COC had more DAT molecules on their cell surfaces, which is probably an adaptive response to increased synaptic DA.11. In vivo findings of enhanced DAT mRNA expression, increased DAT protein synthesis, and increased trafficking or migration to the cell surface have all been supported by molecular research.29, 30, 33, and 34 Daws et al.12 showed an increase in DA uptake after exposure to COC, which is in contrast to other studies. They came to the conclusion that COC had caused a rise in DAT insertion into the cell membrane following exposure and removal from the culture. The capacity of COC to block DA binding to DAT is reduced when the COC is removed prior to evaluating DAT function. The behavioral results following COC treatment, when combined with neurochemical and molecular research, indicate that COC is functioning through more than only DA and other brain systems.2,29Compared to COC, MA has a more intricate effect on the dopaminergic system. Without affecting Vmax or Bmax, MA exposure lowers the Km of DA for DAT in primary striatal cultures.23 Variations in DAT kinetics after exposure to MA show that the striatum varies regionally and is more sensitive to MA's effects.22, 24 The effects of COC and MA after one or more injections were compared by Fleckenstein et al.15,35. The effects on DA uptake were strong, although the IC50 values for COC (337 nM) and MA (291 nM) differed slightly. While MA decreased dopamine uptake to 63% of control levels after a single injection and 30% of control after multiple injections, COC barely significantly changed DA uptake following a single injection. The authors hypothesized that MA modifies DA uptake and release via a variety of pathways, including reverse transport, exchange diffusion, phosphorylation, and protein trafficking. According to earlier research, exposure to MA causes DAT to internalize, which lowers DA uptake, while COC enhances DAT trafficking to the cell surface.34, 36

It is evident that COC's role in the addictive process and how it works through DAT are far more intricate than previously thought, and they may be influenced by a variety of different neurotransmitter systems.Compared to COC and MA, the effect of heavy metal-mediated alterations in CNS receptor or transporter function is less clear. According to the first reports, Pb, Hg, and other divalent metals may have a competing effect on muscarinic receptor binding sites.37, 38 The behavioral and learning abnormalities that occur after exposure to Pb7,39–41 or Hg4,18,42 have been described in numerous papers, but no specific neurotransmitter system has been identified. The neurotransmitter system impacted by lead exposure determines the behavioral outcomes.7. It is still unknown

how the DA system is compromised. Although Pb injection in vivo causes a decrease in DA uptake, exposure to Pb has been shown to enhance messenger ribonucleic acid (mRNA) for both tyrosine hydroxylase and DAT.9. These seemingly contradictory findings can be explained by the presence of more mRNA to compensate for the decrease in DA uptake. To find out if the Pb-mediated rise in DAT mRNA corresponds to an increase in DAT protein synthesis and trafficking to the cell membrane, more research is required. The Hg-mediated effects imply DA hyperfunction in contrast to the Pb-mediated impact of DA hypofunction. The effects of mercury (both organic and inorganic) on the DA system have been thoroughly investigated by Faro et al.17,43–46, and they have consistently documented a substantial increase in DA release. A) enhanced DA release, B) inhibition of DA metabolism, and C) inhibition of DA uptake are the three mechanisms they propose for increasing synaptic DA. The processes behind the effects of inorganic and organic mercury are distinct.46 A net increase in synaptic DA results from organic mercury's inhibition of absorption and increase in DA release. Changing the DAT's kinetic profile is one way that mercury can change uptake. Both [3H] WIN35,428 and [3H] mazindol bound to DAT more readily when Hg ions were included in a binding buffer.13. Either a rise in the quantity of DAT or an increase in DAT's affinity for the radioligands could be the cause of this binding increase. A biphasic response was seen in the ability of inorganic Hg to modify [3H] methylphenidate binding to DAT.47 Increased binding, the first phase, is characterized by a sharp rise in affinity (Kd), which is followed by a decrease in binding as a result of a decline in both affinity (Kd) and density (Bmax).

This biphasic reaction implies that a detailed investigation of a time-course would be required and that timing is crucial. According to a more recent study, there is a more intricate model for how first-row transition metals interact with DAT, which lowers DAT's affinity for DA.48 This decrease in affinity may not apply to Pb and Hg because Pb is a post-transition metal and Hg is a third-row transition metal, but it does provide an intriguing viewpoint on possible metal-mediated effects on DAT. It is clear that Pb and Hg behave differently on the DA system in terms of mechanism, much like what has been observed with COC and MA. Heavy metals and psychostimulants are more likely to interact because of their intricacy and the involvement of other neurotransmitter systems, like glutamate.Humans have attempted to limit their exposure as our knowledge of metal-induced toxicity has grown. The capacity of heavy metals to linger in the environment for long periods of time has resulted in a persistent health risk, which has not been resolved by immediate decreases in exposure. Over the past 20 years, there has been a notable decrease in the usage of psychostimulants like COC and MA; nonetheless, their use has plateaued and remained at a steady level of consumption. The activities of COC and MA in the central nervous system involve the augmentation of DA transmission, notwithstanding the complexity of the mechanism.

One There is a chance that exposure to heavy metals could change how DAT works, making using COC or MA cause an increased reaction and raising the risk of misuse.5. Gaining understanding of the relationship between heavy metals and drugs of abuse at DAT was the aim of this investigation. According to binding and uptake investigations, the current study's premise is that exposure to single drugs (or mixtures of agents) will substantially change DAT function. We did plan to investigate how DAT function in N2A cells is affected by Pb, Hg, COC, and MA. We will gain a better knowledge of how heavy metals and psychostimulants affect DAT and how this affects human health by looking at various time points and using combinations of these agents. Moreover, past exposure to environmental toxins such heavy metals may influence the strength of the psychostimulant response.

MATERIALS AND METHODS

Cell Culture

The American Type Culture Collection (CCL-131; ATCC, Manassas, VA, USA) supplied the Neuro2A (N2A; mouse neuroblastoma cells) cell line, which was utilized. Cells were quickly thawed upon arrival and introduced to Minimum Eagles Media (MEM), which was already heated and supplemented with sodium pyruvate, Earle's balanced salt solution, and nonessential amino acids. 10% heat-inactivated fetal bovine serum (FBS), 1.5 g/L NaHCO3, 2 mM L-glutamine, and 1% penicillin/streptomycin (10,000 I.U./mL and 10,000 µg/mL, respectively) were added to the media by Cellgro (MediaTech Inc., Herndon, VA, USA and Hyclone, Logan, UT, USA). The cultures were kept at 37 °C in a humidified environment with 5% CO2 in 25 cm^2 cell culture flasks with vented covers (Corning Inc., Corning, NY, USA). Every other day, the culture media was traded. Trypsin (0.25%) was introduced to the flask when the cells were nearly confluent, which generally happened within a week. After the cells were detached, aliquots were moved to other flasks containing medium. Depending on their development, N2A cells were subcultured at a ratio of 1:4 to 1:6. Each well of a 24-well plastic cell culture plate (Costar® 3599, Corning, Inc., Corning, NY, USA) contained 1×105 cells for the purposes of cell viability, binding, and uptake tests. Before being tested, the plates were put back in the incubator (37°C/5% CO2) and the cells were left to grow normally. Only cells in the passage range of 4–14 were utilized to assess the impacts of heavy metal/psychostimulant effects, and cells were typically >80% confluent at the time of experiment.

N2A Neuroblastoma Cell Transfection with hDAT

One Shot® Top10 Chemically Competent E. coli (Invitrogen) was created from the vectors pCMV6-XL5 with the hDAT-cDNA insert (Origene) and pCMV6-Neo (Origene). Overnight, cells were cultured on LB-agar/ampicillin (100 µg/mL) plates. Six separate colonies were chosen the next day to inoculate 2 mL of LB/ampicillin broth overnight. The Wizard® Plus SV Minipreps DNA Purification System kit (Promega) was used to isolate plasmid DNA. Using Promega's Not I endonuclease, both plasmids were broken down. The StrataPrep® DNA Gel Extraction kit (Stratagene) was used to extract the fragments after they had been separated by 0.7% agarose gel electrophoresis. Using spectrophotometric analysis, DNA was quantified. T4 DNA ligase (Promega) was used to ligate the pCMV6-Neo and the hDAT-cDNA overnight at 4 oC. The newly subcloned construct was regrown on LB-agar/ampicillin plates after being converted into Top10 cells. Selected colonies were added to LB/ampicillin broth, and Miniprep was used

to isolate the resultant pDNA. After XmaI digestion and DNA sequencing, the correct insertion was verified. The Qiagen Plasmid Maxi kit was used to purify the confirmed plasmids after they were plated for colonies and inoculated in 100 mL of LB/ampicillin broth. After spectrophotometric measurement, aliquots of the cloned plasmid pCMV6-Neo-(hDAT) were frozen for assay usage. The day before to transfection, 500 µL of antibiotic-free growth media was used to subculture cells at a density of 1×105 cells per well in a 24-well plate. Confluence was between 90 and 95 percent at transfection. As directed by the manufacturer, LipofectamineTM 2000 (Invitrogen) was used to transfect cells. Culture media (500 µL), plasmid DNA (0.8 µg), LipofectamineTM 2000 (2.0 µL), and Opti-MEM I® (Invitrogen) as a diluent made up the transfection mixture. A cationic liposome formulation called LipofectamineTM 2000 works by complexing with nucleic acid molecules to overcome the cell membrane's electrostatic repulsion and permit the nucleic acid to enter. Before transgene expression testing, cells were cultured for 24 hours at 37°C in an incubator with 5% CO2 and the media was replaced after 4–6 hours. Cells were transferred 1:10 into new growth media 24 hours after transfection to ensure sustained transfection. To reach a final concentration of 500 µg/mL, Geneticin was added to the culture medium the next day. The selection of transfected cells lasted for three weeks.

Using Green Fluorescent Protein (GFP) to tag hDAT

The fusion construct hDAT-GFP was created by joining the C terminus encoding region of the human synthetic DAT cDNA from pCMV6-XL5 (Origene, Rockville, MD, USA) with the N terminus encoding region of the enhanced green fluorescent protein (eGFP) cDNA from pEGFP-N3 (BD Biosciences Clontech, Palo Alto, CA, USA). This resulted in a fluorescently tagged hDAT. In order to express the synthetic hDAT from a CMV promoter and a neomycin resistance gene, this construct was subcloned into a pIRES-Neo3 expression vector. The hDAT-GFP was transfected into Neuro2A (N2A) cells using LipofectamineTM 2000 (Invitrogen, Carlsbad, CA, USA). After KpnI digestion and DNA sequencing, the correct insertion was verified. After 500 µg/mL of geneticin (Invitrogen, Carlsbad, CA, USA), a stable transfected pool of hDAT cells was decided upon. With the exception of growing them in 75 cm² cell culture flasks with vented covers (Corning Inc., Corning, NY, USA) at 37°C in a 5% CO2 humidified atmosphere, cells were cultivated in supplemented MEM as explained in the "Cell Culture" section. After being brought to confluence in standard N2A growth conditions, N2A cells bearing the hDAT-GFP construct were plated in a 24-well plate at a density of 1×105 cells/well. Prior to assaying, cells were grown to at least 80% confluence in the plate.

Lactate Dehydrogenase Assay for Cell Viability

A colorimetric test that measures LDH activity in the media (CytoTox 96® Non-Radioactive Cytotoxicity test; Promega) was used to quantify released LDH. In summary, a 30-minute coupled enzymatic assay was used to measure the quantity of a red formazan product in culture media by releasing LDH activity. The amount of lysed cells is directly correlated with the measured absorbance. The vehicle control groups' data were compared to the viability of cells in the treatment groups [0.1, 1, or 10 µM HgCl2 or PbCl2 with 10 or 100 nM psychostimulants (COC; MA) for 24, 48, 72, or 96 hours (Promega; Madison, WI, USA). To measure spontaneous LDH release, 50 µL of each sample supernatant was duplicated and put into a transparent 96-well plate (control). Aspiration was used to remove the leftover media from the 24-well plate. To determine the total amount of LDH in each well, 150 µL of lysis solution (0.9% Triton-X) was poured to each well of the 24-well plate. For forty-five minutes, the plates were put back in the incubator at 37°C. To restore the total volume to the initial 1050 µL, 900 µL of full media was then added to each well. 50 µL of each sample supernatant was once more added to the 96-well plate in duplicate. Each of the 96 wells was then filled with 50 µL of the activated substrate mix (Assay buffer = Tris-buffered tetrazolium dye, Triton-X-100, lyophilized diaphorase, lactate, and NAD+) while being shielded from the light by foil. After 30 minutes on a plate shaker with the plates covered, 50 µL of stop reagent (1M acetic acid) was added to halt the reaction. Absorbance was measured using a Synergy HT microplate reader (Bio-TEK® Instruments, Inc., Winooski, VT, USA) at 490 nm. Cell viability was determined by the following equation:

$$\%\ Viability = \frac{[Total\ Abs] - [Spontaneous\ Abs]}{[Total\ Abs]} \times 100$$

[³H]GBR-12,935 Binding

The American Type Culture Collection (CCL-131; ATCC, Manassas, VA, USA) supplied the Neuro2A (N2A; mouse neuroblastoma cells) cell line, which was utilized. Cells were quickly thawed upon arrival and introduced to Minimum Eagles Media (MEM), which was already heated and supplemented with sodium pyruvate, Earle's balanced salt solution, and nonessential amino acids. 10% heat-inactivated fetal bovine serum (FBS), 1.5 g/L NaHCO3, 2 mM L-glutamine, and 1% penicillin/streptomycin (10,000 I.U./mL and 10,000 µg/mL, respectively) were added to the media by Cellgro (MediaTech Inc., Herndon, VA, USA and Hyclone, Logan, UT, USA). The cultures were kept at 37 °C in a humidified environment with 5% CO2 in 25 cm² cell culture flasks with vented covers (Corning Inc., Corning, NY, USA). Every other day, the culture media was traded. Trypsin (0.25%) was introduced to the flask when the cells were nearly confluent, which generally happened within a week. After the cells were detached, aliquots were moved to other flasks containing medium. Depending on their development, N2A cells were subcultured at a ratio of 1:4 to 1:6. Each well of a 24-well plastic cell culture plate (Costar® 3599, Corning, Inc., Corning, NY, USA) contained 1×105 cells for the purposes of cell viability, binding, and uptake tests. Before being tested, the plates were put back in the incubator (37°C/5% CO2) and the cells were left to grow normally. Only cells in the passage range of 4–14 were utilized to assess the impacts of heavy metal/psychostimulant effects, and cells were typically >80% confluent at the time of experiment.

N2A Neuroblastoma Cell Transfection with hDAT

Openventio PUBLISHERS

One Shot® Top10 Chemically Competent E. coli (Invitrogen) was created from the vectors pCMV6-XL5 with the hDAT-cDNA insert (Origene) and pCMV6-Neo (Origene). Overnight, cells were cultured on LB-agar/ampicillin (100 μg/mL) plates. Six separate colonies were chosen the next day to inoculate 2 mL of LB/ampicillin broth overnight. The Wizard® Plus SV Minipreps DNA Purification System kit (Promega) was used to isolate plasmid DNA. Using Promega's Not I endonuclease, both plasmids were broken down. The StrataPrep® DNA Gel Extraction kit (Stratagene) was used to extract the fragments after they had been separated by 0.7% agarose gel electrophoresis. Using spectrophotometric analysis, DNA was quantified. T4 DNA ligase (Promega) was used to ligate the pCMV6-Neo and the hDAT-cDNA overnight at 4 oC. The newly subcloned construct was regrown on LB-agar/ampicillin plates after being converted into Top10 cells. Selected colonies were added to LB/ampicillin broth, and Miniprep was used to isolate the resultant pDNA. After XmaI digestion and DNA sequencing, the correct insertion was verified. The Qiagen Plasmid Maxi kit was used to purify the confirmed plasmids after they were plated for colonies and inoculated in 100 mL of LB/ampicillin broth. After spectrophotometric measurement, aliquots of the cloned plasmid pCMV6-Neo-(hDAT) were frozen for assay usage. The day before to transfection, 500 μL of antibiotic-free growth media was used to subculture cells at a density of 1×105 cells per well in a 24-well plate. Confluence was between 90 and 95 percent at transfection. As directed by the manufacturer, LipofectamineTM 2000 (Invitrogen) was used to transfect cells. Culture media (500 μL), plasmid DNA (0.8 μg), LipofectamineTM 2000 (2.0 μL), and Opti-MEM I® (Invitrogen) as a diluent made up the transfection mixture. A cationic liposome formulation called LipofectamineTM 2000 works by complexing with nucleic acid molecules to overcome the cell membrane's electrostatic repulsion and permit the nucleic acid to enter. Before transgene expression testing, cells were cultured for 24 hours at 37°C in an incubator with 5% CO2 and the media was replaced after 4–6 hours. Cells were transferred 1:10 into new growth media 24 hours after transfection to ensure sustained transfection. To reach a final concentration of 500 μg/mL, Geneticin was added to the culture medium the next day. The selection of transfected cells lasted for three weeks.

Using Green Fluorescent Protein (GFP) to tag hDAT

The fusion construct hDAT-GFP was created by joining the C terminus encoding region of the human synthetic DAT cDNA from pCMV6-XL5 (Origene, Rockville, MD, USA) with the N terminus encoding region of the enhanced green fluorescent protein (eGFP) cDNA from pEGFP-N3 (BD Biosciences Clontech, Palo Alto, CA, USA). This resulted in a fluorescently tagged hDAT. In order to express the synthetic hDAT from a CMV promoter and a neomycin resistance gene, this construct was subcloned into a pIRES-Neo3 expression vector. The hDAT-GFP was transfected into Neuro2A (N2A) cells using LipofectamineTM 2000 (Invitrogen, Carlsbad, CA, USA). After KpnI digestion and DNA sequencing, the correct insertion was verified. After 500 μg/mL of geneticin (Invitrogen, Carlsbad, CA, USA), a stable transfected pool of hDAT cells was decided upon. With the exception of growing them in 75 cm² cell culture flasks with vented covers (Corning Inc., Corning, NY, USA) at 37°C in a 5% CO2 humidified atmosphere, cells were cultivated in supplemented MEM as explained in the "Cell Culture" section. After being brought to confluence in standard N2A growth conditions, N2A cells bearing the hDAT-GFP construct were plated in a 24-well plate at a density of 1×105 cells/well. Prior to assaying, cells were grown to at least 80% confluence in the plate.

Lactate Dehydrogenase Assay for Cell Viability

A colorimetric test that measures LDH activity in the media (CytoTox 96® Non-Radioactive Cytotoxicity test; Promega) was used to quantify released LDH. In summary, a 30-minute coupled enzymatic assay was used to measure the quantity of a red formazan product in culture media by releasing LDH activity. The amount of lysed cells is directly correlated with the measured absorbance. The vehicle control groups' data were compared to the viability of cells in the treatment groups [0.1, 1, or 10 μM HgCl2 or PbCl2 with 10 or 100 nM psychostimulants (COC; MA) for 24, 48, 72, or 96 hours (Promega; Madison, WI, USA). To measure spontaneous LDH release, 50 μL of each sample supernatant was duplicated and put into a transparent 96-well plate (control). Aspiration was used to remove the leftover media from the 24-well plate. To determine the total amount of LDH in each well, 150 μL of lysis solution (0.9% Triton-X) was poured to each well of the 24-well plate. For forty-five minutes, the plates were put back in the incubator at 37ºC. To restore the total volume to the initial 1050 μL, 900 μL of full media was then added to each well. 50 μL of each sample supernatant was once more added to the 96-well plate in duplicate. Each of the 96 wells was then filled with 50 μL of the activated substrate mix (Assay buffer = Tris-buffered tetrazolium dye, Triton-X-100, lyophilized diaphorase, lactate, and NAD+) while being shielded from the light by foil. After 30 minutes on a plate shaker with the plates covered, 50 μL of stop reagent (1M acetic acid) was added to halt the reaction.

RESULTS

Development N2A cell transfection with hDAT and hDAT-GFP

Following digestion with NotI, 5.8 kb and 2.7 kb bands were produced, indicating that the development of transient clones utilizing the pCMV6-XL5 vector was successful (Figure 1A and B). hDAT was subcloned into a pCMV6-Neo vector (Figure 2A) because we wanted to produce a stable clone in addition to the transitory expression seen in Figure 1B. Over time, stable clones that were cryopreserved for eventual usage were selected thanks to the presence of the neomycin-resistant gene. When XmaI was used to digest the plasmids, pieces of 4.8 kb, 2.7 kb, and 1 kb were produced (Figure 2B), indicating a clone that was properly orientated. Stabilized N2A cells expressing hDAT were cultured in 75 cm2 flasks, and 1 mL aliquots were cryopreserved in base minimum essential medium (MEM) cryopreservation media containing 5% dimethyl sulfoxide (DMSO) (about 1 mg of cells/mL). Aliquots were kept in a liquid nitrogen cryopreservation Dewar's vapor phase.By using the hDAT-GFP fusion, we were able to examine the intracellular localization of hDAT and observe the localization of hDAT-GFP with-

Figure 1. *The pCMV6-XL5 Vector (~4.5 kb) with the hDAT cDNA (about 2.7 kb) Inserted within the Multiple Cloning Site (A). 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. We used the CMV Promoter to Express the Cloned cDNA. Plasmid Selection in E. coli is Facilitated by the Expression of the Ampicillin-resistance Gene. Plasmids were Digested with Not I and Fragments 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 5.8 kb and 2.7 kb in Size, Respectively (B). These are Successful pCMV6-Neo with the Cloned Insert.*

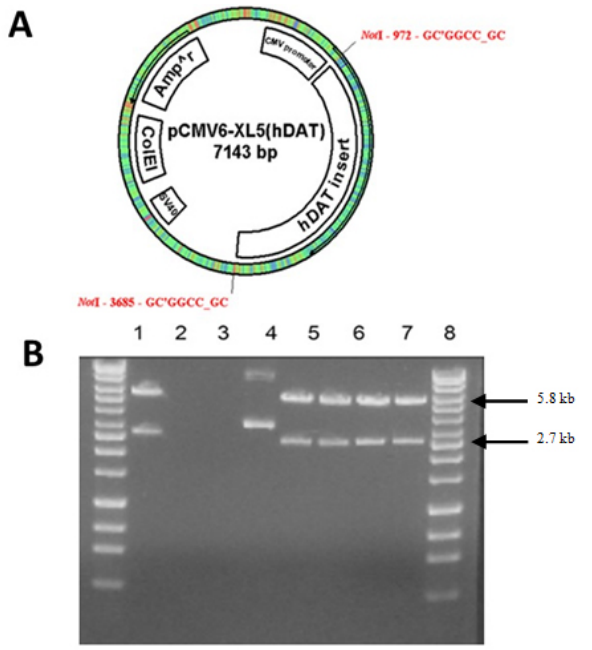


Figure 2. *The pCMV6-Neo Vector (~5.8 kb) is Similar to pCMV6-XL5 Except for the Addition of a Neomycin Resistance Gene for Establishing a Stable Clone [A]. The hDAT cDNA was Subcloned into the Vector by Not I Digestion and Ligation. Orientation of the Insert was Confirmed by Xma I Digestion. The Cloned Plasmid was Transfected into COS-7 Cells. 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 4.8 kb, 2.7 kb, and 1.0 kb in Size, Respectively [B]. These are pCMV6-Neo with the Correctly Oriented Cloned Insert. Lane 7 is the pCMV6-Neo (5.8 kb) without Insert.*

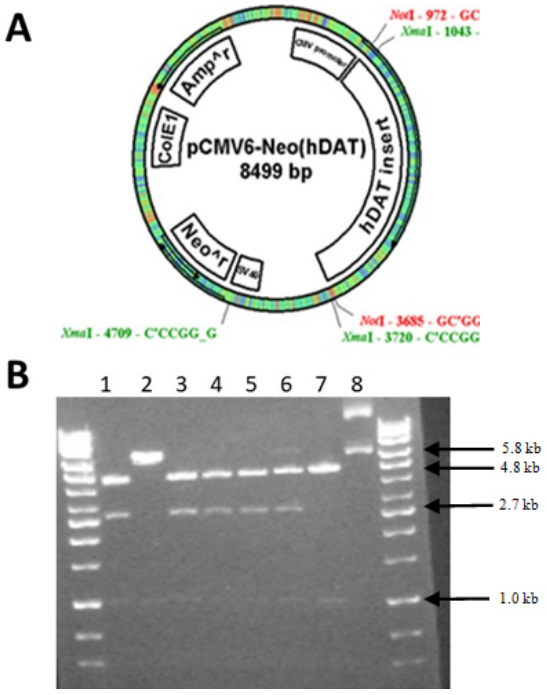


Figure 3. *pIRES-N3 Vector (About 5.2 kb in size) with hDAT-GFP (About 2.6 kb) Inserted into the Multiple Cloning Site. The hDAT-GFP Insert is Flanked by Eco RI and Not I Restriction Sites [A]. Plasmids were Digested with Kpn I. Fragments were Separated by 1.0% Agarose Gel Electrophoresis. GeneMate QuantiMarker 1 kb was Used as the DNA Ladder. Lane 2 is an Empty pIRES-N3 Vector. Lanes 3 through 8 Indicate Fragments of the Approximate Size 4.4, 2.0, and 1.5 kb [B]. These are Correctly Cloned pIRES-N3 (hDAT-eGFP)*

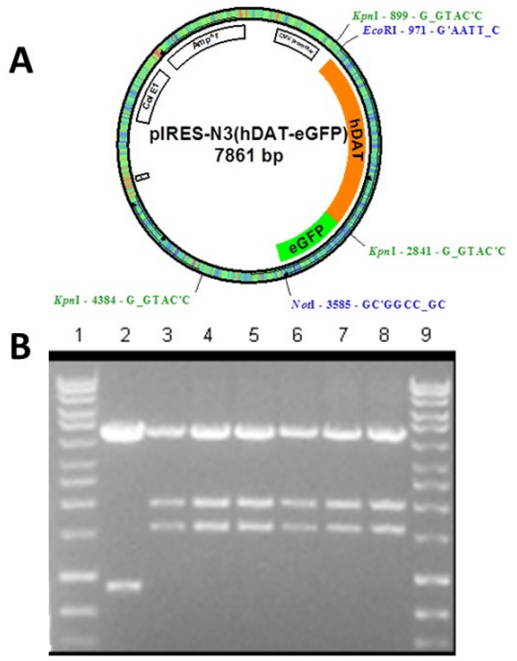


Figure 4. *Epi-Fluorescent Photomicrographs of N2A Cells Transfected with GFP-Tagged hDAT. Panels [A] and [B]: 20x Images Used to Demonstrate Transfection Efficiency. Transfection Efficiency is Near 90% Using this Procedure. Panels [C] and [D]: 40x Localization Images of GFP-Tagged hDAT. These Images Suggest that the Tagged hDAT is Expressed on the Membrane in a Delimited Manner, but will Examine this Finding by Performing [3H] Dopamine Uptake and [3H] GBR12935 Binding. It does not Appear that Tagged hDAT is Aggregating or Retained in the Endoplasmic Reticulum. Photos were Obtained using an Olympus IX71 Epi-fluorescence Microscope Fitted with a FITC Filter and CoolSnap Monochrome Camera. The Pictures were Obtained and Colored Green Using Rsimage (Roper Imaging) Version 1.7.3.*

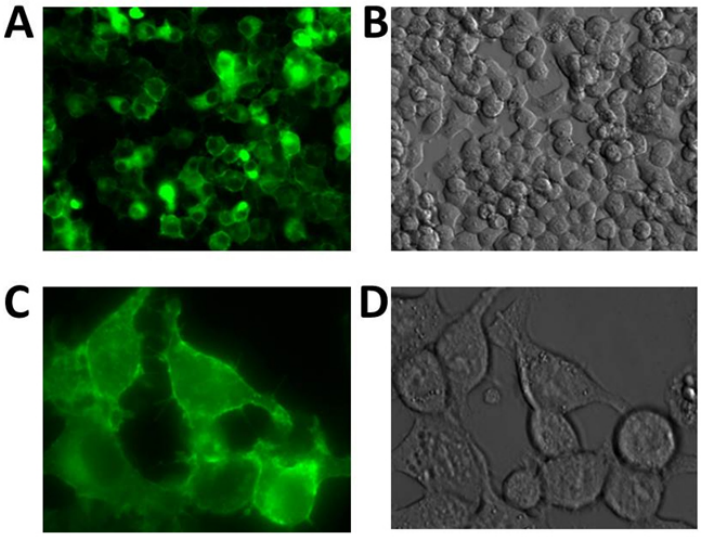


Figure 5. *Concentration- and Time-Dependent Changed in N2A Viability Following Exposure to Mercuric (Hg) and Lead (Pb) Chloride (0, 0.1, 1, or 10 µM). Exposure to Hg Resulted in Both Time- ($F_{3,48}$=7.24; p=0.0004) and Concentration- ($F_{3,48}$=14.04; p<0.0001) Dependent Reductions in Cell Viability (Figure 5A). Dunnett's Test for Posthoc Comparison to a Control (0 µM, 24 h) was Used to Determine the Threshold for Significant Reductions. The Highest Concentration of Hg Tested, 10 µM Exhibited Significant Reductions in cell Viability Compared to Control Values (*p<0.05) at 48, 72 and 96h. Exposure to Pb Resulted in Both Time- ($F_{3,47}$=19.89; p<0.0001) and Concentration- ($F_{3,47}$=4.29; p=0.003) Dependent Reductions in Cell Viability (Figure 5B). Pb (10 µM) Exhibited Significant Reductions in Cell Viability Compared to Control Values (*p<0.05) at 72 and 96 h, whereas the 1 µM Group was Significantly Reduced Compared to Controls at 96 h. After Examining the Response to Hg and Pb, it Appears that N2A Cells were more Sensitive to the Concentration of Hg, whereas Length of Exposure was a Greater Factor in Pb-Induced Reductions in Viability. Data are Expressed as the Mean±SEM of N=4 Assayed in Duplicate.*

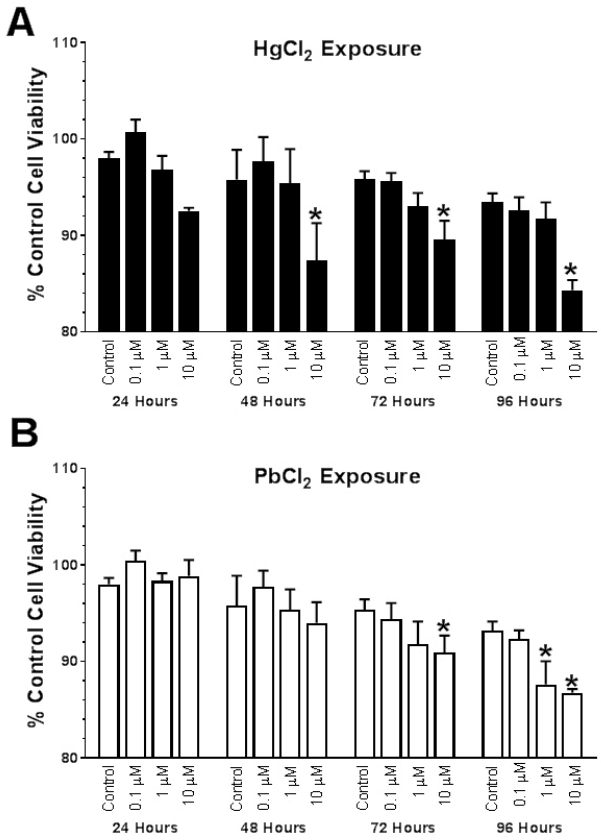
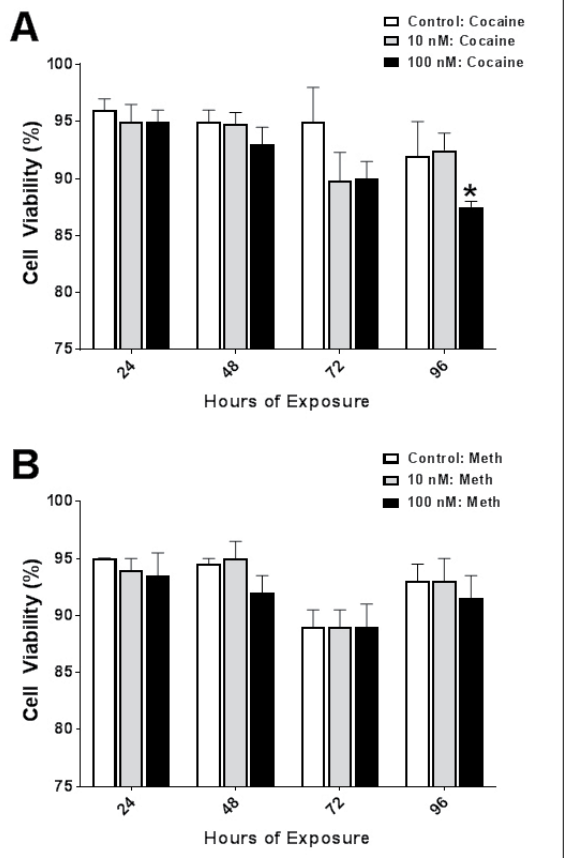


Figure 6. *Concentration- and Time-Dependent Changed in N2A Viability Following Exposure to Cocaine (COC) or Methamphetamine (MA) (0, 10, or 100 µM). Exposure to COC Resulted in Time- ($F_{3,36}$=4.62; p=0.008) Dependent Reductions in Cell Viability (Figure 6A). Dunnett's Test for Posthoc Comparison to a Control (0 µM, 24 h) was Used to Determine the Threshold for Significant Reductions. The Highest Concentration of COC Tested, 100 µM Exhibited Significant Reductions in Cell Viability Compared to Control Values (*p<0.05) at only 96 h. Exposure to MA Resulted in Time- ($F_{3,36}$=7.05; p=0.0008) Dependent Reductions in Cell Viability (Figure 6B). There were No Differences in Cell Viability at Any Time Point or Concentration of MA Compared to Control Values. Data are Expressed as the Mean±SEM of N=4 Assayed in Duplicate*



in N2A cells. This allowed us to visualize hDAT in N2A cells using fluorescence microscopy. EcoRI and NotI restriction sites flanked the hDAT-GFP-inserted vector, pIRES-N3 (Figure 3A). KpnI was used to digest the plasmid, and fragments measuring 4.4, 2.0, and 1.5 kb were produced (Figure 3B), indicating that the insert had been successfully cloned. As previously mentioned, N2A cells that were consistently expressing hDAT-GFP were cryopreserved. We observed that hDAT was expressed on the cell membrane in a restricted manner when we visualized the expression of hDAT-GFP (Figure 4A and B). Only a little amount of aggregation occurred in intracellular organelles, and there were clear clusters and punctate forms on the cell membrane that represented hDAT localization (Figure 4C).

After Metal Exposure, Cell Viability: Time Course and Curve of Concentration

The activity of lactate dehydrogenase (LDH) was assessed at four intervals between 24 and 96 hours in cells exposed to varying doses of HgCl2 (Hg) or PbCl2 (Pb) (0–10 µM). Cell viability decreased in response to Hg exposure in both time- (F3,48=7.24; p=0.0004) and concentration- (F3,48=14.04; p<0.0001) dependent ways (Figure 5A). Utilizing Dunnett's test for posthoc comparison to a control (0 µM, 24 h), the threshold for significant decreases was established. Cell viability at 48, 72, and 96 hours was significantly lower at the highest dose of mercury examined (10 µM) than at control values (*p<0.05). Cell viability decreased in response to Pb exposure in both time- (F3,47=19.89; p<0.0001) and concentration- (F3,47=4.29; p=0.003) dependent ways (Figure 5B). At 72 and 96 hours, Pb (10 µM) showed significant decreases in cell viability when compared to control values (*p<0.05), whereas at 96 hours, the 1 µM group showed a significant decrease in cell viability when compared to controls. Upon analyzing the reaction to Pb and Hg, it seems that N2A cells were more susceptible to the concentration

Figure 7. *Effects of Metals and Psychostimulants Alone or in Combination on hDAT in N2A cells. From the Viability Studies, the Concentration of Hg and Pb was 10 μM and for COC and MA was 100 nM with an Exposure Time of 72 h. The Density of hDAT was Quantified by the Binding of [^{3}H] GBR 12935 to hDAT on the Cell Surface. Although Metals, Psychostimulants or Combinations Resulted in Shifts in hDAT by ±30% Compared to Control Values, Many of These Differences did not reach statistical significance (Figure 7A). There was a significant effect of stimulant on the hDAT Density ($F_{3,32}$=3.52; p=0.026) and a Significant Interaction between Metal- and Psychostimulant-Mediated Effects ($F_{6,32}$=3.57; p=0.008). Sidak's Test for Posthoc Multiple Comparisons Revealed Significant Increases in hDAT Density in the COC-MA Group Comparing the Hg-Pb Combination to just Hg or Pb Alone (*p<0.05). Functionality of hDAT was Determined by Measuring the Uptake of [^{3}H] Dopamine into N2A Cells via hDAT (Figure 7B). Similar to what we Observed in the hDAT Density Assay, there was a Moderate Level of Variability in the Data. There was a Significant Effect of Metal on Dopamine Uptake ($F_{2,30}$=5.63; p=0.0084). Sidak's Test for Posthoc Multiple Comparisons Revealed Significant Increases in hDAT Density in the COC-MA Group Comparing the Hg Alone to Pb Alone with the COC-MA-Hg Group being Nearly 200% of Control Values (*p<0.05). Data are Expressed as the Mean±SEM of N=4 Assayed in Duplicate.*

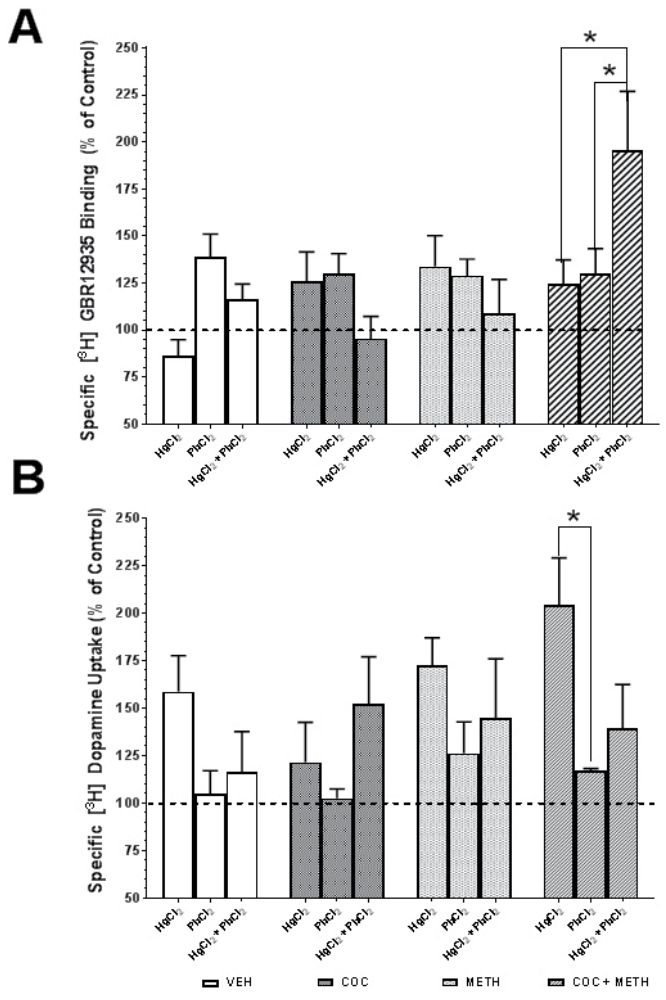
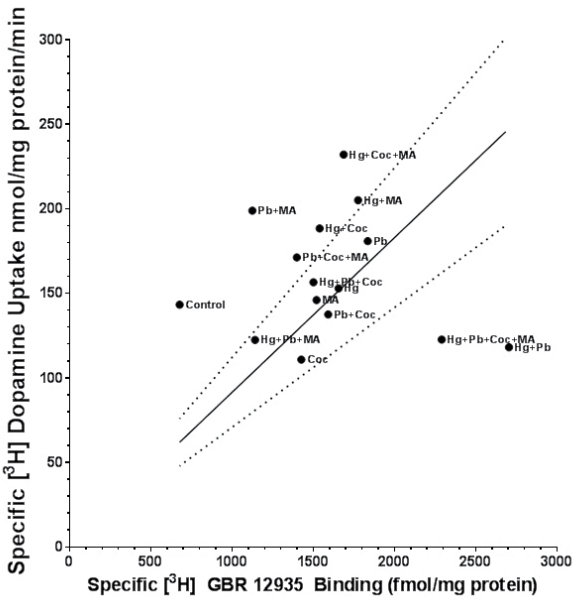


Figure 8. *Relationship Between hDAT Density ([^{3}H] GBR 12935 Binding) and Uptake ([^{3}H] Dopamine Uptake). The Basic Assumption is that Increasing Density of hDAT should Lead to an Increase in DA Uptake. Deviations from this Relationship would Suggest Changes in Protein Processing or Function. Observations Compared to Control Values Reveal a Rightward Shift in the Data (Higher Density with Minimal Changes in Uptake) whereas a Few Groups (Predominantly the Hg Groups) are Shifted to the Right as well as Upward. This Could Suggest the Increase in hDAT Density Results in Elevated DA Uptake Due to the Increased Density of a Function hDAT Protein on the Cell Surface. Data is Taken From the Binding and Uptake Data Presented in Figure 7*



of Pb, while exposure duration was a more significant element in Pb-induced viability declines.

Time Course and Cell Viability After Psychostimulant Curve of Concentration

LDH activity was assessed at four intervals between 24 and 96 hours in cells treated with varying doses of cocaine (COC) or methamphetamine (MA) (0-100 nM) in order to assess cytotoxicity. Cell viability decreased in response to COC exposure in a time- (F3,36=4.62; p=0.008) dependent manner (Figure 6A). Utilizing Dunnett's test for posthoc comparison to a control (0 μM, 24 h), the threshold for significant decreases was established. Cell viability was significantly reduced at just 96 hours at the highest concentration of COC tested, 100 μM, as compared to control values (*p<0.05). Cell viability decreased in response to MA exposure in a time- (F3,36=7.05; p=0.0008) dependent manner (Figure 6B). At whatever time point or MA concentration, there were no variations in cell viability as compared to control values. Remarkably, there were not many changes after exposure to COC or MA. Cell viability reductions were more influenced by exposure duration than by the COC or MA concentration. Later research where a combination of time and concentration is employed that will have negligible effects on cell viability will be made easier by the detection of few concentration-dependent alterations. This method's justification is to prevent any irreparable harm to the cells, which will produce a more realistic depiction of DAT expression and function.

hDAT Density Determination by [3H] GBR12935 Binding

According to the viability studies, the concentrations of Hg and Pb were 10 μM, while the concentrations of COC and MA were 100 nM, and the exposure period was 72 hours. The binding of [3H] GBR 12935 to hDAT on the cell surface was used to measure the density of hDAT. Many of these variations did not achieve statistical significance, even when metals, psychostimulants, or combinations caused shifts in hDAT of ±30% when compared

to control values (Figure 7A). The hDAT density was significantly impacted by stimulants ($F3,32=3.52$; $p=0.026$), and the effects of metals and psychostimulants interacted significantly ($F6,32=3.57$; $p=0.008$). Increased [3H] GBR12935 binding was the outcome of exposure to treatment combinations. It's interesting to note that density rose 161% in the Hg+MA group compared to the control values. Additionally, the mean of the Hg+Pb+COC+MA treatment group rose by 227% in comparison to the control. Additionally, the mean of the Hg+Pb treatment group exceeded the control mean values by 288%. These identified Hg-containing groups seemed to affect binding in an additive manner. Significant increases in hDAT density were observed in the COC-MA group when comparing the Hg-Pb combination to either Pb or Hg alone, according to Sidak's test for posthoc multiple comparisons (*$p<0.05$). Using the following formula, we determined the fractional occupancy of [3H] GBR12935 for DAT, the norepinephrine (NET), and the serotonin (SERT) transporter:

$$\frac{[L^*]}{[L^*]+Kd}\times 100$$

where Kd (21 nM) is the reported affinity of the radioligand for the target and [L*] is the ligand concentration (50 nM) employed. [3H] GBR12935 binds to a larger number of DAT > NET > SERT using this formula. Just 18% and 0.8% of [3H] GBR12935 were attached to NET and SERT, respectively, out of the reported density of sites, with 70% of the overall number representing binding to the DAT. An accurate estimate of nonspecific binding was left after 99.99% of the [3H] GBR12935 bound was displaced by GBR 12909 at a concentration of 5 μM. This was done to make sure that we were binding mainly to DAT and that we were above the Kd value, which would only occupy 50% of the available DAT sites.

Variations in [3H] DA Uptake Indicate Changes in hDAT Function

According to the viability experiments, the concentrations of Hg and Pb were 10 μM, while the concentrations of COC and MA were 100 nM after 72 hours of exposure. The absorption of [3H] dopamine into N2A cells via hDAT was measured in order to assess the functionality of hDAT (Figure 7B). The data showed a moderate degree of variability, which is consistent with what we saw in the hDAT density assay. Dopamine uptake was significantly impacted by metal ($F2,30=5.63$; $p=0.0084$). The uptake of the COC treatment group was 17% lower than the control values. A 20% drop was also observed in the Hg+COC group. It's interesting to note that treatment groups with MA had the most shift in [3H] dopamine uptake experiments. The mean values in the MA groups were 35-81% higher than the control values. When comparing the COC-MA group to the Hg alone or Pb alone, Sidak's test for posthoc multiple comparisons showed that the COC-MA-Hg group's hDAT density was significantly higher, approaching 200% of control values (*$p<0.05$).

Connection between DA Uptake and hDAT Density in N2A Cells That Express hDAT

We plotted the results from the [3H] GBR12935 binding and [3H] dopamine uptake experiments to see whether there was a link between the two variables in order to investigate the interaction/relationship between the hDAT density and function in N2A cells (Figure 8). Remarkably, there was no significant linear relationship between density and uptake. Rather, it seems that exposure to metals or psychostimulants, either separately or in combination, caused the curve to move to the right. This change indicates a rise in density without the corresponding rise in uptake. To ascertain if this alteration represents a rise in density or perhaps an increase in the hDAT's affinity for [3H] GBR12935, more research is required.

DISCUSSION

Psychostimulants like COC and MA that are frequently abused primarily target the DAT. These drugs' affinities for the DAT are strongly correlated with their reinforcing properties, which are likely the cause of their addictive qualities.49 The primary mediator of the reinforcing and locomotor activating properties of psychostimulants is thought to be the release of DA in the nucleus accumbens.50 COC binds to the DAT's Na+ binding site and modifies the Cl-binding site, preventing the binding of both ions. An elevated extracellular DA concentration results from the inhibition of DA translocation across the pre-synaptic neuron's membrane. The DAT returns extracellular DA to the nerve terminal (i.e., uptake) under normal physiological conditions. However, non-vesicular DA efflux results from DAT reversal, which raises extracellular DA concentrations minutes after MA injection. These increases in DA uptake and cell surface expression, seen in rodents and cell lines, respectively, following acute COC administration, probably reflect attempts to maintain normal synaptic DA functions. Of interest, blockade of DAT by COC causes a rapid increase in DA uptake in synaptosomes prepared from treated rats, a preparation from which the drug has presumably been washed out.35 Perhaps this occurs via enhanced recruitment of DATs to the plasma membrane. As measured in synaptosomes from cryoprotected human brain, increased DAT function is also seen in humans who have repeatedly raised synaptic DA levels using COC.11 It is plausible that an excess of extracellular DA causes this compensatory increase in DAT activity, which in turn results in a deficit in extracellular DA, potentially contributing to drug dependence. Our curiosity about whether environmental factors (heavy metal exposure) and psychostimulant (ab) use may have overlapping processes that increase the toxicity or addictive potential of either factor stems from the in vitro and in vivo studies.

The aim of this work was to examine the effects of heavy metal exposure on psychostimulant binding at the DAT. There is evidence that metals may either promote or inhibit the binding of ligands to receptors in vitro. This is a particularly relevant concern since Pb and Hg pose a significant threat to subpopulations with higher rates of drug misuse, such as urban minorities or people from poorer socioeconomic backgrounds. A person's drug habits are determined by a variety of dispositional characteristics, including poverty, drug history, availability, and experiential factors. But it's also important to keep in mind that other kinds of environmental circumstances could increase the likelihood that selected medications would be abused. In other words, motivational characteris-

tics associated with drug seeking and taking may be redefined and affect maintenance drug use or the efficacy of specific pharmacotherapies for drug misuse to the degree that mercury, lead, or any other xenobiotic chemical modifies the impact of a specified administration of a medication.

We have demonstrated both transient and sustained expression of hDAT. We have produced a neuronal cell line in N2A cells that expresses hDAT consistently, enabling research on the control of hDAT synthesis, processing, and function. When hDAT is tagged with GFP, alterations in its position inside the cell can be seen by microscopy and anatomical references. Examining cellular alterations in hDAT regulation has been made possible by our N2A cell line that expresses hDAT. Complex and metal-dependent molecular processes underlie metal-induced neurotoxicity. The most sensitive sites or processes for each metal are those that are primarily impacted. Hg binds to the thiol groups on proteins, including the DAT, to produce its toxic effect.54 The DAT includes a disulfide bridge that is crucial to the transporter's conformational structure.54 Once Hg attaches to this bridge, DAT undergoes a conformational change that impairs its ability to function normally. These observations of enhanced DAT density and function are supported by our findings.55 Cells adjust by processing more DAT and increasing the trafficking rate to get DAT to the cell surface.13 Binding to DAT does not follow a conventional monotonic response in the presence of metal ions like Hg2+. Density is increased at lower concentrations, although [3H] WIN 35,428 binding is decreased at greater concentrations of Hg2+.13,47 Our concentrations of Hg may have been too low because we selected a value based on its lack of toxicity after 72 hours of exposure. The density of hDAT, as determined by [3H] GBR12935 binding, often increased by about 20–25%. A 25–50% increase in [3H] DA uptake occurred at the same time. In general, researchers have reported that Pb exposure leads to DA hypofunction.7-9 In vivo studies have shown that Pb exposure can result in a reduction in binding to DAT8 and a decrease in DA uptake.9 Our findings suggest that neuronal cells strive to maintain DA clearance by increasing the density of DAT at the plasma membrane. Pb competes with calcium-mediated synaptic vesicle release, resulting in an increased DA release from the synapse.

The chemical type of lead, the amount or concentration of lead employed, the exposure duration, and different model systems are some of the causes of the disparity between in vitro and in vivo investigations. Because of its better solubility, Pb-acetate is used in the majority of in vivo investigations; nevertheless, this organic form of Pb is more poisonous than the inorganic form, Pb-chloride, which is used in the current investigations. The dose or concentration employed in each study could also be a source of variance. We employed a Pb-chloride concentration of 10 μM, or roughly 2.8 ppm. Noureddine et al.9's investigation used a dosage of 1000 ppm, which is almost 400 times higher than what we used. When blood lead levels above 10 μg/dL,7 which corresponds to a concentration of 0.1 ppm, behavioral impacts of lead become apparent. The alterations in DAT density seen after Hg exposure may be followed by Pb responses. Lower Pb concentrations may enhance the density of DAT and uptake of DA, while higher concentrations (with longer exposure times) may cause DA hypofunction, as evidenced by responses that seem "biphasic" or non-monotonic. Therefore, by encouraging an increase in extracellular DA, exposure to sub-toxic levels of heavy metals causes an indirect up-regulation in DAT density. Changes in hDAT density were comparable when comparing the amount of change between Hg and Pb. When examining uptake changes, it was evident that the response magnitude of Hg-mediated alterations in DA uptake differed significantly from that of the Pb group. Compared to Pb alone, the Hg+Pb combination was marginally higher. These density and uptake differences only became statistically significant in the COC+MA group.

The majority of DAT protein is normally located at the cell surface. One essential mechanism for controlling DAT homeostasis and function is the translocation of DAT from the membrane to the cytosolic region. Cloned DAT-expressing cell cultures show that psychostimulant treatment controls DAT expression in the plasma membrane.36,55,57,58 Psychostimulants may work by encouraging the exocytosis of internalized DAT or by reducing constitutive internalization of DAT, which increases plasma membrane DAT by changing the equilibrium between internalization and surface recycling. One mechanism that helps increase extracellular DA levels in response to psychostimulants is the redistribution of DAT on the cell surface. The means of the COC and Hg+COC treatment groups indicated a tendency toward lower uptake as compared to the control. However, in cells treated with COC, binding was increased. Since COC is a DAT inhibitor, it blocks [3H] DA uptake, which raises postsynaptic stimulation and synaptic DA. The up-regulation of the DAT may be a homeostatic response to the weakened dopaminergic system following COC exposure, whereby a greater ability to absorb DA would keep neurotransmission at more normal levels. These findings imply that the DAT may actively contribute to regulating the behavioral effects of long-term exposure to COC. However, there are differing accounts of how DAT function changes as a process that leads to addiction. Other groups have reported changes consistent with increased activity of DAT, including increased DA uptake35,60,61 and an attenuation of COC-induced increases in ECF levels of DA.62 The variable effect of COC on uptake likely reflects the use of different dosing regimens, routes of administration, brain regions, and the ECF concentration of DA at the time of COC administration, as well as the techniques to quantify DAT function.2,32,60 [3H]DA uptake studies showed the largest shift in treatment groups containing MA. Both DAT binding and uptake were markedly up-regulated upon exposure to MA. Given that MA stimulates DA release, this is to be expected.15 The presence of MA may start a compensatory mechanism that increases DAT density to improve DA removal from the synaptic cleft.

Our research shows that metal/drug treatments increase the distribution of DAT on the cell surface, and that this redistribution may be linked to an increase in extracellular DA. A significant interaction between metal and psychostimulant was seen in Figure 7A ($F_{6,32}=3.57$; $p=0.008$). Drug-related trafficking of DAT may be a significant factor in the development of its misuse since psychostimulants alter DA. Reduced DA uptake or increased GBR

binding without any change could indicate that DAT is not functioning as well. This will therefore lead to a decrease in DA uptake and an increase in synaptic DA. Over time, excessive synaptic DA levels can cause cell injury and additional alterations in DAT density and function if they are not appropriately eliminated. It has been demonstrated that heavy metals like mercury and lead are directly harmful through a variety of routes, such as oxidative stress, or that they might have indirect harmful effects through interactions with proteins. According to the evidence presented in this work, metal poisoning may have an indirect impact on synaptic DA levels and DAT function.

CONCLUSION

Our findings' impact takes into consideration possible environmental factors, like exposure to heavy metals, that may make psychostimulant usage easier or increase toxicity. By altering the density or absorption of DA, we discovered that Hg, Pb, COC, and MA all had an impact on hDAT. It's interesting to note that there is no straightforward antagonistic, synergistic, or additive mechanism underlying the effects. In most cases, we would have anticipated synergistic or additive reactions. The lack of observation points to a more intricate pathway that is both direct and indirect and is implicated in the modification of DAT function caused by heavy metals or psychostimulants. In response to elevated DA in the synapse, a generalization of the data did reveal an increase in both density and uptake. When transfected N2A cells were treated to combinations of heavy metals and psychostimulants, no significant connections were seen, nor were there any clear correlations between individual exposures to Hg, Pb, COC, or MA. From the standpoint of forensics, the pre-exposure and DA changes linked to heavy metals may cause an exaggerated response to lesser dosages of a psychostimulant. Future in vitro research will focus on assessing DAT turnover and the impact of therapy on N-linked glycosylation of DAT. More molecular research is being done to examine epigenetic modifications brought on by prolonged exposure to Pb and Hg. The reactivity to psychostimulants would be permanently impacted if genetic alterations took place. In in vivo research, metals are added to drinking water for a few weeks, and DAT alterations are measured months later. It has been demonstrated that long-term neurochemical alterations in neurotransmitter systems brought on by chronic exposure to psychostimulants might result in tolerance or epigenetic modifications. The limitation of in vitro research is that only "acute" exposure may be investigated.

ACKNOWLEDGEMENTS

The authors would like to acknowledge the expertise and assistance of Dr. Greg Sawyer and Crystal Shults who aided in the construction of the transfected N2A cell lines. The work supported in part by subcontract flow-thru funds from NIH DA13137 and through intramural funding by the Oklahoma State University Center for Health Sciences.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

REFERENCES

1. Dela Peña I, Gevorkiana R, Shi WX. Psychostimulants affect dopamine transmission through both dopamine transporter-dependent and independent mechanisms. *Eur J Pharmacol.* 2015; 764: 562-570. doi: 10.1016/j.ejphar.2015.07.044

2. Cass WA, Gerhardt GA, Gillespie K, Curella P, Mayfield RD, Zahniser NR. Reduced clearance of exogenous dopamine in rat nucleus accumbens, but not in dorsal striatum, following cocaine challenge in rats withdrawn from repeated cocaine administration. *J Neurochem.* 1993; 61(1): 273-283. doi: 10.1111/j.1471-4159.1993.tb03565.x

3. Heal DJ, Gosden J, Smith SL. Dopamine reuptake transporter (DAT) "inverse agonism" - A novel hypothesis to explain the enigmatic pharmacology of cocaine. *Neuropharmacology.* 2014; 87: 19-40. doi: 10.1016/j.neuropharm.2014.06.012

4. Curtis JT, Hood AN, Chen Y, Cobb GP, Wallace DR. Chronic metals ingestion by prairie voles produces sex-specific deficits in social behavior: An animal model of autism. *Behav Brain Res.* 2010; 213(1): 42-49. doi: 10.1016/j.bbr.2010.04.028

5. Jones DC, Miller GW. The effects of environmental neurotoxicants on the dopaminergic system: A possible role in drug addiction. *Biochem Pharmacol.* 2008; 76(5): 569-581. doi: 10.1016/j.bcp.2008.05.010

6. Chang J, Kueon C, Kim J. Influence of lead on repetitive behavior and dopamine metabolism in a mouse model of iron overload. *Toxicol Res.* 2014; 30(4): 267-276. doi: 10.5487/TR.2014.30.4.267

7. Cory-Slechta D. Relationships between lead-induced learning impairments and changes in dopaminergic, cholinergic and glutamatergic neurotransmitter system functions. *Annu Rev Pharmacol Toxicol.* 1995; 35: 391-415. doi: 10.1146/annurev.pa.35.040195.002135

8. Pokora MJ, Richfield EK, Cory-Slechta DA. Preferential vulnerability of nucleus accumbens dopamine binding sites to low-level lead exposure: Time course of effects and interactions with chronic dopamine agonist treatments. *J Neurochem.* 1996; 67(4): 1540-1550. doi: 10.1046/j.1471-4159.1996.67041540.x

9. Noureddine D, Miloud S, Abdelkader A. Effect of lead exposure on dopaminergic transmission in the rat brain. *Int J Child Neuropsychiatry.* 2004; 1(1): 97-105. doi: 10.1016/j.tox.2004.10.016

10. Ranaldi R, Wise RA. Blockade of D1 dopamine receptors in the ventral tegmental area decreases cocaine reward: Possible role for dendritically released dopamine. *J Neurosci.* 2001; 21(15): 5841-5846. doi: 10.1523/JNEUROSCI.21-15-05841.2001

11. Mash DC, Pablo J, Ouyang Q, Hearn WL, Izenwasse S. Dopamine transport function is elevated in cocaine users. *J Neurochem.* 2002; 81(2): 292-300. doi: 10.1046/j.1471-4159.2002.00820.x

12. Daws LC, Callaghan PD, Morón JA, et al. Cocaine increases dopamine uptake and cell surface expression of dopamine transporters. *Biochem Biophys Res Commun*. 2002; 290(5): 1545-1550. doi: 10.1006/bbrc.2002.6384

13. Wu Q, Coffey LL, Reith ME. Cations affect [3H]mazindol and [3H]WIN 35,428 binding to the human dopamine transporter in a similar fashion. *J Neurochem*. 1997; 69(3): 1106-1118. doi: 10.1046/j.1471-4159.1997.69031106.x

14. Vidal L, Alfonso M, Faro LF, Campos F, Cervantes R, Durán R. Evaluation of the effects and mechanisms of action of mercuric chloride on striatal dopamine release by using *in vivo* microdialysis in freely moving rats. *Toxicology*. 2007; 236(1-2): 42-49. doi: 10.1016/j.tox.2007.03.023

15. Fleckenstein AE, Volz TJ, Riddle EL, Gibb JW, Hanson GR. New insights into the mechanism of action of amphetamines. *Annu Rev Pharmacol Toxicol*. 2007; 47(1): 681-698. doi: 10.1146/annurev.pharmtox.47.120505.105140

16. Mortensen OV, Amara SG. Dynamic regulation of the dopamine transporter. *Eur J Pharmacol*. 2003; 479(1-3): 159-170. doi: 10.1016/j.ejphar.2003.08.066

17. Faro LRF, Do Nascimento JLM, Alfonso M, Durán R. *In vivo* effects of inorganic mercury ($HgCl_2$) on striatal dopaminergic system. *Ecotoxicol Environ Saf*. 2001; 48(3): 263-267. doi: 10.1006/eesa.2000.2024

18. Weinstein M, Bernstein S. Pink ladies: Mercury poisoning in twin girls. *Can Med Assoc J*. 2003; 168(2): 201.

19. Ricaurte G, Guillery R, Seiden L, Schuster C, Moore R. Dopamine nerve terminal degeneration produced by high doses of methylamphetamine in the rat brain. *Brain Res*. 1982; 235(1): 93-103. doi: 10.1016/0006-8993(82)90198-6

20. Seiden L, Ricaurte G. Neurotoxicity of methamphetamine and related drugs. In: Meltzer H, ed. *Psychopharmacology: The Third Generation of Progress*. New York, USA: Raven Press; 1987: 359-366.

21. Gibb J, Hanson G, Johnson M. Neurochemical mechanisms of toxicity. In: Cho A, Segal D, eds. *Amphetamine and Its Analogues: Psychopharmacology, Toxicology and Abuse*. San Diego, CA, USA: Academic Press; 1994: 269-295.

22. Cass WA. Decreases in evoked overflow of dopamine in rat striatum after neurotoxic doses of methamphetamine. *J Pharmacol Exp Ther*. 1997; 280(1): 105-113.

23. Bennett BA, Hollingsworth CK, Martin RS, Harp JJ. Methamphetamine-induced alterations in dopamine transporter function. *Brain Res*. 1998; 782(1-2): 219-227. doi: 10.1016/S0006-8993(97)01281-X

24. Kokoshka JM, Vaughan RA, Hanson GR, Fleckenstein AE. Nature of methamphetamine-induced rapid and reversible changes in dopamine transporters. *Eur J Pharmacol*. 1998; 361(2-3): 269-275. doi: 10.1016/S0014-2999(98)00741-9

25. Kimmel HL, Carroll FI, Kuhar MJ. Dopamine transporter synthesis and degradation rate in rat striatum and nucleus accumbens using RTI-76. *Neuropharmacology*. 2000; 39(4): 578-585. doi: 10.1016/S0028-3908(99)00160-4

26. NIDA. Cocaine. 2016. Web site. https://d14rmgtrwzf5a.cloudfront.net/sites/default/files/drugfacts-cocaine.pdf. Accessed April 19, 2018.

27. NIDA. Methamphetamine. 2017. Web site. https://d14rmgtrwzf5a.cloudfront.net/sites/default/files/df_methamphetamine_february2017_1.pdf. Accessed April 19, 2018.

28. Izenwasser S, Cox B. Daily cocaine treatment produces a persistent reduction of [3H]dopamine uptake in vitro in rat nucleus accumbens but not in striatum. *Brain Res*. 1990; 531(1-2): 338-341. doi: 10.1016/0006-8993(90)90797-F

29. Verma V. Classic studies on the interaction of cocaine and the dopamine transporter. *Clin Psychopharmacol Neurosci*. 2015; 13(3): 227-238. doi: 10.9758/cpn.2015.13.3.227

30. Kalivas PW, Duffy P. Effect of acute and daily cocaine treatment on extracellular dopamine in the nucleus accumbens. *Synapse*. 1990; 5(1): 48-58. doi: 10.1002/syn.890050104

31. Beuming T, Kniazeff J, Bergmann ML, et al. The binding sites for cocaine and dopamine in the dopamine transporter overlap. *Nat Neurosci*. 2008; 11(7): 780-789. doi: 10.1038/nn.2146

32. Zahniser NR, Larson GA, Gerhardt GA. In vivo dopamine clearance rate in rat striatum: Regulation by extracellular dopamine concentration and dopamine transporter inhibitors. *J Pharmacol Exp Ther*. 1999; 289(1): 266-277. Web site. http://jpet.aspetjournals.org/content/289/1/266.long. Accessed April 19, 2018.

33. Kahlig KM, Galli A. Regulation of dopamine transporter function and plasma membrane expression by dopamine, amphetamine, and cocaine. *Eur J Pharmacol*. 2003; 479(1-3): 153-158. doi: 10.1016/j.ejphar.2003.08.065

34. Zahniser NR, Sorkin A. Rapid regulation of the dopamine transporter: Role in stimulant addiction? *Neuropharmacology*. 2004; 47(Suppl. 1): 80-91. doi: 10.1016/j.neuropharm.2004.07.010

35. Fleckenstein AE, Haughey HM, Metzger RR, et al. Differential effects of psychostimulants and related agents on dopaminergic and serotonergic transporter function. *Eur J Pharmacol*. 1999; 382(1): 45-49. doi: 10.1016/S0014-2999(99)00588-9

36. Saunders C, Ferrer J V., Shi L, et al. Amphetamine-induced loss of human dopamine transporter activity: An internalization-dependent and cocaine-sensitive mechanism. *Proc Natl Acad Sci*. 2000; 97(12): 6850-6855. doi: 10.1073/pnas.110035297

37. Aronstam RS, Mohyee EE. Transition and heavy metal inhibition of ligand binding to muscarinic acetylcholine receptors

from rat brain. *Toxicol Appl Pharmacol.* 1979; 48(3): 489-496. doi: 10.1016/0041-008X(79)90432-0

38. Von Berg R, Northington F, Shamoo A. Methylmercury inhibition of rat brain muscarinic receptors. *Toxicol Appl Pharmacol.* 1980; 53(2): 285-292. doi: 10.1016/0041-008X(80)90428-7

39. Min MO, Singer LT, Kirchner HL, et al. Cognitive development and low-level lead exposure in poly-drug exposed children. *Neurotoxicol Teratol.* 2009; 31(4): 225-231. doi: 10.1016/j.ntt.2009.03.002

40. Lidsky TI, Schneider JS. Lead neurotoxicity in children: Basic mechanisms and clinical correlates. *Brain.* 2003; 126(1): 5-19. doi: 10.1093/brain/awg014

41. Yoon JH, Ahn YS. The association between blood lead level and clinical mental disorders in fifty thousand lead-exposed male workers. *J Affect Disord.* 2016; 190: 41-46. doi: 10.1016/j.jad.2015.09.030

42. Lin C, Liou S, Hsiech C, Ku M, Tsai S. Dose-response relationship between cumulative mercury exposure index and specific uptake ratio in the striatum on Tc-99m TRODAT SPECT. *Clincial Nucl Med.* 2011; 36(8): 689-693. doi: 10.1097/RLU.0b013e3181e9fa93

43. Faro LRF, do Nascimento JLM, Alfonso M, Durán R. Protection of methylmercury effects on the in vivo dopamine release by NMDA receptor antagonists and nitric oxide synthase inhibitors. *Neuropharmacology.* 2002; 42(5): 612-618. doi: 10.1016/S0028-3908(02)00009-6

44. Faro LRF, Do Nascimento JLM, Alfonso M, Durán R. Mechanism of action of methylmercury on in vivo striatal dopamine release: Possible involvement of dopamine transporter. *Neurochem Int.* 2002; 40(5): 455-465. doi: 10.1016/S0197-0186(01)00098-5

45. Faro LRF, Durán R, Do Nascimento JLM, Perez-Vences D, Alfonso M. Effects of successive intrastriatal methylmercury administrations on dopaminergic system. *Ecotoxicol Environ Saf.* 2003; 55(2): 173-177. doi: 10.1016/S0147-6513(02)00127-6

46. Faro LRF, Rodrigues KJA, Santana MB, Vidal L, Alfonso M, Durán R. Comparative effects of organic and inorganic mercury on in vivo dopamine release in freely moving rats. *Brazilian J Med Biol Res.* 2007; 40: 1361-1365. doi: 10.1590/S0100-879X2006005000157

47. Schweri M. Mercuric chloride and p-chloromercuriphenylsulfonate exert a biphasic effect on the binding of the stimulant [3H] methylphenidate to the dopamine transporter. *Synapse.* 1994; 16(3): 188-194. doi: 10.1002/syn.890160304

48. Li Y, Mayer FP, Hasenhuetl PS, et al. Occupancy of the zinc-binding site by transition metals decreases the substrate affinity of the human dopamine transporter by an allosteric mechanism. *J Biol Chem.* 2017; 292(10): 4235-4243. doi: 10.1074/jbc.M116.760140

49. Ritz M, Lamb R, Goldberg S, Kuhar M. Cocaine receptors on dopamine transporters are related to self-administration of cocaine. *Science.* 1987; 237(4819): 1219-1223. doi: 10.1126/science.2820058

50. Jones SR, Gainetdinov RR, Wightman RM, Caron MG. Mechanisms of amphetamine action revealed in mice lacking the dopamine transporter. *J Neurosci.* 1998; 18(6): 1979-1986.

51. Aronstam RS, Abood LG, Hoss W. Influence of sulfhydryl reagents and heavy metals on the functional state of the muscarinic acetylcholine receptor in rat brain. *Mol Pharmacol.* 1978; 14: 575-586.

52. Peterson L Lou, Bartfai T. In vitro and in vivo inhibition of [3H]imipramine binding by cadmium. *Eur J Pharmacol.* 1983; 90(2-3): 289-292. doi: 10.1016/0014-2999(83)90252-2

53. Mizuno S, Ogawa N, Mori A. Differential effects of some transition metal cations on the binding of beta-carboline-3-carboxylate and diazepam. *Neurochem Res.* 1983; 8(7): 873-880. doi: 10.1007/BF00964548

54. Kniazeff J, Loland CJ, Goldberg N, et al. Intramolecular cross-linking in a bacterial homolog of mammalian SLC6 neurotransmitter transporters suggests an evolutionary conserved role of transmembrane segments 7 and 8. *Neuropharmacology.* 2005;49(6): 715-723. doi: 10.1016/j.neuropharm.2005.07.003

55. Daniels GM, Amara SG. Regulated trafficking of the human dopamine transporter. *J Biol Chem.* 1999; 274(50): 35794-35801.

56. Struzynska L, Rafalowska U. The effect of lead on dopamine, GABA and histidine spontaneous and KCl-dependent releases from rat brain synaptosomes. *Acta Neurobiol Exp (Wars).* 1994; 54(3): 201-207.

57. Zhang L, Elmer LW, Little KY. Expression and regulation of the human dopamine transporter in a neuronal cell line. *Brain Res Mol Brain Res.* 1998; 59(1): 66-73. doi: 10.1016/S0169-328X(98)00138-7

58. Little KY, Elmer LW, Zhong H, Scheys JO, Zhang L. Cocaine induction of dopamine transporter trafficking to the plasma membrane. *Mol Pharmacol.* 2002; 61(2): 436-445. doi: 10.1124/mol.61.2.436

59. Sharpe L, Pilotte N, Mitchell W, DeSouza E. Withdrawal of repeated cocaine decreases autoradlographic [3H]mazindol-labelling of dopamine transporter in rat nucleus accumbens. *Eur J Pharmacol.* 1991; 203(1): 141-144. doi: 10.1016/0014-2999(91)90804-Y

60. David DJ, Zahniser NR, Hoffer BJ, Gerhardt GA. In vivo electrochemical studies of dopamine clearance in subregions of rat nucleus accumbens: Differential properties of the core and shell. *Exp Neurol.* 1998; 153(2): 277-286. doi: 10.1006/exnr.1998.6898