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Lauren Jonas Date

Environmental & Pharmaceutical Exposures alter the function of VMAT2 and Promote Neurodegeneration in Parkinson's Disease

By

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Degree to be awarded: MPH

Environmental Health

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By

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Thesis Committee Chair: Gary W. Miller, PhD

An abstract of
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Abstract

Environmental & Pharmaceutical Exposures alter the function of VMAT2 and Promote Neurodegeneration in Parkinson's Disease
By Lauren Jonas

Parkinson's disease (PD) is characterized by the gradual loss of dopaminergic neurons in the substantia nigra (SNc). Dopaminergic neurons located within the SNc innervate the dorsal striatum in both the direct and indirect striatal pathways within the basal ganglia and allow for balanced and smooth muscle movements. A breakdown of this pathway leads to many of the features associated with PD. While there are several genetic mutations associated with PD, both genetic and environmental factors are thought to contribute to the pathogenesis of Parkinson's disease. Dopamine is one of the main neurotransmitters involved in the mediation of balanced motor movements in a normal patient. Altered regulation of dopamine is involved in the disturbed motor movements in Parkinson's patients. This altered regulation can come in many forms; however, this thesis assesses environmental and pharmaceutical exposures that may alter the packaging and storage of dopamine by the vesicular monoamine transporter 2 (VMAT2) into small synaptic vesicles of the dopaminergic nerve terminals. Many studies have linked the disruption of VMAT2 to PD and this research serves to screen a number of compounds including PCBs, pesticides and drugs thought to alter dopamine storage. FFN206 was used as a VMAT2 substrate to perform high throughput screens of VMAT2-tranfected HEK-293 cell uptake activity. By employing this assay we were able to visualize significant decreased VMAT2 uptake activity in the compounds paraquat, rotenone, deltamethrin, Aroclor 1260, methylphenidate and methamphetamine. These data furthermore reveal a pathway that must be further analyzed in regards to toxicant exposure and PD. Additional investigation of these compounds and other compounds are necessary to fully understand the mechanisms of these toxicants; however, in light of these findings, we propose that further study of the role of environmental and pharmaceutical exposures on VMAT2 activity and the pathogenesis of PD is warranted.

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Table of Contents

Background

Parkinson's disease (PD) is characterized by the gradual loss of dopaminergic neurons in the substantia nigra (SNc) as well as the increased formation of alpha-synuclein aggregates in the SNc ultimately leading to the dysfunction of the dopaminergic nigrostriatal system.[1] Dopaminergic neurons located within the SNc (Figure 1.) innervate the dorsal striatum in both the direct and indirect striatal pathways within the basal ganglia and allow for balanced and smooth muscle movements. A breakdown of this pathway leads to many of the features associated with PD. Major symptoms of PD are tremor at rest, bradykinesia, akinesia, and rigidity, as well as other Parkinsonian features such as hypomimia, speech disturbance, shuffling, dystonia, and respiratory difficulties.[2] Bradykinesia is a slowness of movements or the inability to initiate a movement. Rigidity can be visualized in the hurried short steps of a Parkinsonian patient, caused by increased muscle tone during passive motion. Regular and oscillatory movements can characterize a tremor.

There are several genetic mutations associated with Parkinson's disease (i.e. PARK1:SNCA, PARK2:Parkin, PARK8:LRRK2) but these alone cannot account for the majority of cases, leaving a large amount still unknown about its disease pathology.[3] Both genetic and

environmental factors are thought to contribute to the pathogenesis of Parkinson's disease.[45]

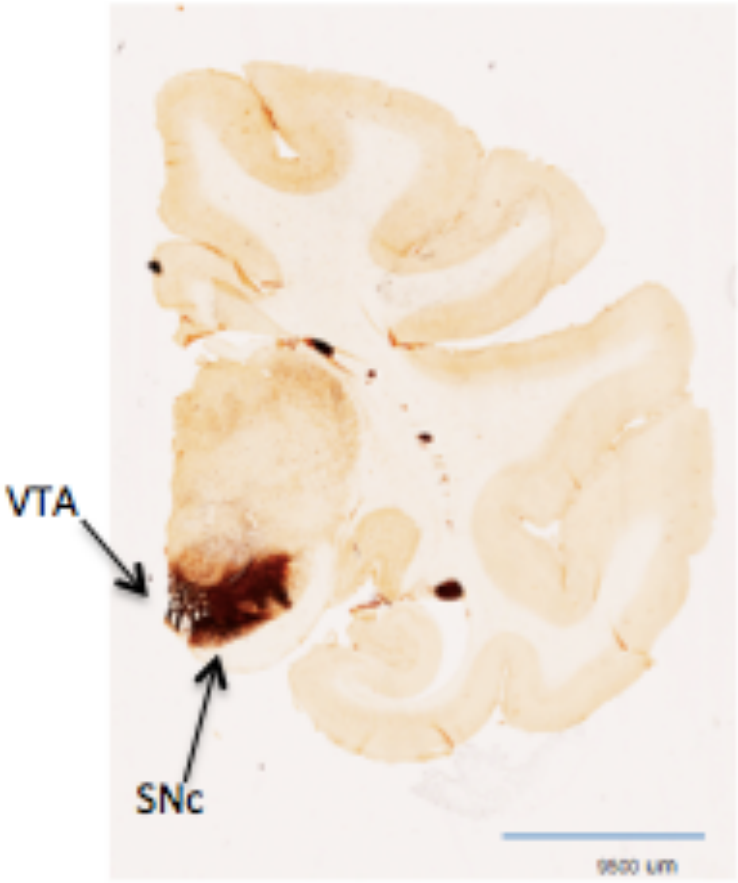

Figure 1: Tyrosine Hydroxylase (TH) 3,3'-Diaminobenzidine staining of non-human primate striatal section. Tyrosine hydroxylase is the rate-limiting enzyme of dopamine biosynthesis, using tetrahydrobiopterin and molecular oxygen to convert tyrosine to DOPA, a precursor of dopamine. Dense TH staining is found in the basal ganglia, specifically in the ventral tegmental area (VTA) and the substantia nigra pars compacta (SNc). TH staining illustrates where these dopaminergic neurons are located, and consequently where they degenerate in PD.

Dopamine

Dopamine is one of the main neurotransmitters involved in the mediation of balanced motor movements in a normal patient. Altered regulation of dopamine is involved in the disturbed motor movements in Parkinson's patients. Dopamine synthesis occurs within the neuronal terminal where tyrosine, an amino acid abundant in dietary proteins, enters the neuron and is converted to dihydroxyphenylalanine (L-dopa) by tyrosine hydroxylase (the rate-limiting step in dopamine biosynthesis). Aromatic amino acid decarboxylase (AADC, dopa decarboxylase) decarboxylates L-DOPA to dopamine. (L-DOPA, the precursor for dopamine is a therapy currently in use for the treatment of Parkinson's disease.) Once synthesized, dopamine, along with other monoamines, is transported from the cytoplasm to inside synaptic vesicles, and is packaged inside the vesicle to .1 Molar (10-1000 times the level of dopamine outside the vesicle).[6] Vesicular dopamine is then released into the synapse from the presynaptic neuronal terminal by exocytosis following stimulation.[7]

Vesicular Monoamine Transporter 2

It is important to understand the critical nature of this storage system, as without it the neuron would face serious consequences.

Dopamine can act as a double-edged sword. On the one hand inside the vesicle it can be stored, transported, and released properly to maintain a homeostatic system. Generally, greater than 90% of intracellular dopamine is sequestered into these small synaptic vesicles. If dopamine were to remain outside of these vesicles in the cytosol, dopamine accumulation could lead to its breakdown into toxic species and result in ROS generation and exacerbation and resulting mitochondrial dysfunction and mitophagy, ubiquitin-proteasome system malfunction, and subsequent neuronal degradation. [89101112]

The vesicle's membrane is thus decorated with an amalgamation of transport proteins and mediators that orchestrate the storage of synthesized dopamine and help the neuron steer clear of these potential consequences. The vesicular monoamine transporter 2 is a critical transporter found on the spherical lipid bilayer of the small synaptic vesicle. The vesicular monoamine transporter II (VMAT2; SLC18A2) predominantly localized in the central nervous system in monoaminergic brain regions, packages dopamine, serotonin, norepinephrine, epinephrine, and histamine into small synaptic vesicles.[13]

Since packaging and storage of neurotransmitters relies heavily on synaptic vesicles and VMAT2, disrupting this protein would cause increased cytosolic neurotransmitter concentration and decreased

release of the neurotransmitters, all of which would ultimately lead to decreased dopaminergic communication with adjacent cells, and intracellular neurotoxicity. Therefore, inhibition of VMAT2 would cause a major decrease in dopamine innervation to the striatum and degeneration of midbrain dopaminergic cells. This fact has lead many researchers to believe that VMAT2 disruption is associated with the breakdown of dopaminergic neurons found in the pathology of Parkinson's disease.

VMAT2 disruption leads to neurodegeneration

Genetic and pharmacologic disruption of VMAT2 has been extensively reviewed in and outside of our lab. Our lab has shown that genetic alterations of VMAT2 disrupt vesicular function and dopamine handling in VMAT2 knockout mice. These mice degenerate more quickly than control mice and display Parkinson-like phenotypes such as motor deficits and cognitive disabilities.[14] Pharmacologic alterations to VMAT2, such as vesicular inhibitors tetrabenazine and reserpine, have also been used to assess decreased vesicular function and activity. Tetrabenazine (TBZ), a reversible VMAT2 inhibitor, is used to treat chorea associated with Huntington's disease.[15] Reserpine, an irreversible VMAT2 inhibitor, was used as an antihypertensive drug as well as a first generation anti-psychotic drug.[16] In addition it has been

shown that a number of environmental toxicants and pharmaceuticals have this same ability to inhibit VMAT2 and thus disrupt the normal functioning of the dopaminergic cell.[17][18][19] If not only internal genetic manipulations but also external man-made environmental and pharmacologic exposures can affect the functioning of the neuron, this gives the scientist impetus to continue the search for other external causes for the neurodegeneration of the cell in relation to neurodegenerative disease.

MPTP

Research in toxicant exposure and Parkinson's disease was revitalized through Dr. William Langston who discovered drug addicts in Northern California had been injecting a synthetic heroine, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which gave patients parkinsonian-like symptoms.[20] MPTP, a model toxicant, is a compound that can cross the blood brain barrier, transport into dopaminergic cells and cause neurotoxicity. MPTP is selectively toxic to dopamine neurons, as the enzyme monoamine oxidase B converts MPTP to MPP+, which is then taken up by DAT. Within DA nerve terminals MPP+ blocks the mitochondrial complex I, depleting ATP and increasing reactive oxygen species (ROS).[21] The degenerative process caused by MPTP is indicative that external exposures affect the packaging, transport, and

release of dopamine and other midbrain monoamines. MPTP serves as a model toxicant, providing motivation to researchers to assess other potential neurotoxic chemicals and their possible impact on cellular components, including the neuronal vesicular transport protein VMAT2. While oxidative stress has been clearly implicated in the MPTP-induced Parkinsonism, other environmental or drug exposures may operate differently and thus these mechanisms of disturbance must be analyzed further. Human populations come into contact with these different environmental and pharmaceutical exposures on a daily basis. Many of these exposures have been studied for their association to Parkinson's disease. Because of these motivations for further research, our lab compiled a list of suspected chemicals to be further assessed in regards to VMAT2. This catalogue of compounds includes polychlorinated biphenyls, pesticides (herbicides, insecticides, and pyrethroids), methamphetamine, and methylphenidate. A brief background on the toxicological and pharmacological actions of these compounds is included below.

Polychlorinated Biphenyls (PCBs)

PCBs are commercially produced compounds introduced into use in the 1930's and used for industrial purposes, more specifically marketed for their use in mechanical devices such as transformers,

ink, paint, cleaning agents, and pesticides. Their chemical makeup makes them particularly useful for electrical insulation products. Moreover, their chemical and physical stability that makes them highly lipophilic and not readily degradable has led to it's buildup in the environment and thus a growing concern of their negative long term effects on the human populations that employ them. PCBs have been released into the environment via manufacturing plant malfunctions such as spills, and improper handling, misuse, and disposal. Dr. Jensen famously reported in "The PCB Story" contamination in several populations, including that of birds and cattle.[22] Drs. Yamaguchi and Hooper have also reported findings of PCB contamination in dairy products and cooking oil. [2324] While PCBs were taken off the market in 1977 due to possible carcinogenic affects, their persistent nature offers great cause for concern. [25] While bioaccumulation is congener-dependent, PCB's still persist in indoor and outdoor air, soil, sediments, water and animals. The estimated daily human intake of PCB's have declined from 1.9 nanograms in 1978 to .7 nanograms in 1991, however there still remains high PCB levels in certain populations including Inuit populations whose traditional diet consists of fatty tissue animals (i.e. ringed seals and beluga) of in which PCB's persist. Researchers Ross Nortrum and Courtney Sandau found a PCB concentration range of 0.117-11.6 ng/g whole blood wet weight in

Inuit samples.[26] In addition, Superfund sites were found to have concentrations as high as 750 ppm. [27]

These industrial toxicants have repeatedly shown to modify the functioning of the nigrostriatal dopamine system and alter the risk for Parkinson's Disease.[2829] In addition, even with removed exposure, the chemical nature of this toxicant allows it to persist and damage dopaminergic brain regions for long periods of time.[30] Epidemiological studies have implicated the increased risk of PD due to PCB exposure. Specifically, a retrospective mortality study conducted on over 17,000 PCB-exposed workers concluded that there may be an effect of PCB's on neurodegenerative disease in women.[31]

There are many postulations as to how this may be occurring yet the exact mechanism of PCB dopamine disturbance is still widely misunderstood. Dopamine reduction can occur in a number of ways including inhibition of tyrosine hydroxylase (the rate limiting step in Dopamine synthesis) and L-aromatic acid decarboxylase (L-AADC). [32] In vitro studies have shown exposure related activation of protein kinases and alterations in intracellular calcium, which can lead to altered intracellular dopamine synthesis.[33] In addition studies have shown PCB exposure may be altering the function of the dopamine transporter (DAT)[34] and VMAT2. PCB-induced elevations in DOPAC, suggestive of increases in terminal cytosolic dopamine, indicated PCB

has a VMAT-inhibitor, which would elevate DOPAC and reduce tissue DA. [35]

Paraquat

Paraquat (N,N' -dimethyl-4,4'-bipyridinium dichloride) is a bipyridyl herbicide primarily used for weed and grass control. The US Environmental Protection Agency classifies paraquat as "restricted use" which means it can only be used by licensed applicators. First produced in 1961, Paraquat is still one of the most commonly used herbicides. The most common route of exposure is ingestion however prolonged skin exposure and inhalation could lead to negative outcomes. (CDC Paraquat)[36]

Many researchers have studied Paraquat in association with PD because of its structural similarity to MPP+. However many studies have shown that MPP+ and paraquat are mechanistically different, one example being that MPP+ and rotenone promote neurotoxicity through DAT transport and mitochondrial complex I inhibition whereas paraquat does not. [37]. While MPP+ is unable to cross the blood brain barrier (BBB), paraquat is a positively charged molecule capable of BBB penetration and neural uptake by neural amino acid transporters, making it an interesting pesticide to look at in regards to neurotoxicity. [38] Epidemiological studies have shown a link between paraquat and

Parkinson's disease and a dose-dependent relationship has been established between lifetime cumulative exposure and increased PD risk. [394041]

Based on in vivo mouse studies, nigrostriatal dopaminergic neurons were reduced following paraquat exposure. In vivo and in vitro studies have also shown that oxidative stress plays a critical role in paraquat toxicity.[42] paraquat is a well-known redox cycler and through this mechanism can create reactive oxygen species within the neuronal terminal.[43] These redox cycling reactions could be of particular consequence to the dopamine neuron as it has been proposed that nigrostriatal neurons are more vulnerable to oxidative damage.[44]

Rotenone

Rotenone is a botanical insecticide first registered in 1947 by the U.S EPA for piscicidal (fish kill) uses. It is generally applied directly to water to manage fish populations and thus can bioaccumulate in fish and drinking water. The compound is derived from natural plants found predominantly in Southeast Asia, South American and East Africa. Rotenone is classified as a Restricted Use Pesticide because of acute inhalation, acute oral, and aquatic toxicity. Rotenone's mode of

action is through inhibiting mitochondrial complex I electron transporters. [45]

This widely used pesticide has been associated with an increased risk for PD. [46] Animals injected intraperitoneally with rotenone developed characteristic Parkinsonian phenotypes including bradykinesia, postural instability and/or rigidity (reversed by the dopamine agonist, apomorphine). Rotenone injection in these animals caused a 45% loss of tyrosine hydroxylase-positive substantia nigra neurons. In addition, these same animals displayed alpha-synuclein positive aggregates in dopamine neurons, a characteristic phenotype of PD patients, as well as increased DOPAC, a possible sign of VMAT inhibition and cytosolic dopamine breakdown.[47] This study also, among others, have shown that in addition to MPTP and 6-hydroxydopamine, Rotenone can be used as an animal models of Parkinson's disease, helping scientists to recreate and further analyze particular pathogenic occurrences and behavioral results of the neurological disorder.[48] [49]

Deltamethrin

Deltamethrin first synthesized in 1974, is a widely used pyrethroid insecticide. Killing insects through dermal contact and digestion, it is used for a broad range of commercial crops including cotton (45% of the chemical's total usage), coffee, maize, and fruits. Pyrethroids are

synthesized derivatives of naturally occurring pyrethrins, extracted from the pyrethrum, the oleoresin extract of the chrysanthemum flower. Insecticidal properties of pyrethrins are derived from esters of chrysanthemic and pyrethroic acids. Strongly lipophilic, these acids attack the nervous system of insects by interfering with sodium currents and causing hyper excitation of nerve terminals. While pyrethrins are sensitive to light and display short half lives, synthetically produced pyrethroids, such as deltamethrin, have increased stability in the presence of light and increased half lives, making them a more potent insecticide in the environment. While this class of insecticides is derived from the natural pyrethrum, their potency in humans is still a cause for concern. Human exposure to deltamethrin at toxic levels (Orally, 200-250 mg/kg) have led to convulsions, ataxia, dermatitis, diarrhea, tremors, and vomiting. The EPA also lists deltamethrin as an endocrine disruptor.[5051]

The principal site of action of deltamethrin, reported by Dr. Soderlund 1997, are voltage-sensitive sodium channels, as Vssc1 knockout mice (the gene encoding voltage-sensitive sodium channels in houseflies) were resistant to pyrethroids.[52] However pyrethroids are known to affect many other systems in the brain as well including that of GABA receptors, nicotinic acetylcholine receptors, excitatory glutamate receptors, and dopamine release and uptake in striatal

mouse neurons. Drs. Nian Shi and Gong-Ping Liu studied the effects of dopamine and PC12 cell survival, reporting that long term exposure decreased dopamine content, perhaps but selectively inhibiting tyrosine hydroxylase and dopamine biosynthesis.[53] Deltamethrin has also been shown to decrease dopamine uptake.[54] Furthermore, Kung and Richardson studied the effects of deltamethrin on larval zebrafish that exhibited locomotor deficits mediated by dopaminergic dysfunction.[55]

Given the known effects of the aforementioned compounds on dopamine function, we utilized our optimized vesicular monoamine uptake assay to determine the effects of these chemicals on vesicular transport of dopamine.

Hypothesis: Environmental and pharmaceutical exposures inhibit or change the function of neuronal vesicular protein VMAT2, which may ultimately promote neurological disease such as Parkinson's.

Materials and Methods

Cell Culture

HEK293 (ATCC, cat. CRL-1573) cells were cultures according to standard protocols. HEK Growth media was EDMEM (Corning 10-

0130CV) with 10% fetal bovine serum + 1% Penicillin-Streptomycin (Corning 30-002-Cl). Upon splitting cells for HEK VMAT stable cell line, Zeocin was added at 1:1000 dilution. On the day of experiments, the growth medium was replaced by experimental medium, or DMEM with out phenol red (Corning 17-205-CV, stored at 4C) with 10% fetal bovine serum + 1% L-glutamine (200mM, Life Technologies #1187509 at -20C).

VMAT2-HEK Cell Line Transfections

Human Embryonic Kidney (HEK) 293 cell lines were stably transfected with VMAT2 containing a Zeocin resistance gene. Plasmids were transfected into HEK293 cells using Lipofectamine 2000 standard protocol. Stable cell lines were created by repetitive rounds of limiting dilutions in selective media.

Immunohistochemistry DAB staining: Perfused non-human primate sections.

3,3’-Diaminobenzidine (DAB) staining was used to analyze the gold standard marker of dopaminergic neurons, Tyrosine Hydroxylase, within the striatum. Tissue sections were pre-treated with 10% H_2O_2 for 20 minutes and .05 M glycine in PBS for 60 minutes (pH 2.0) followed by blocking serum solution of 1% normal goal serum, 1%

Bovine Serum Albumin (BSA) and .3% Triton in PBS for 60 minutes. Slices were incubated overnight at 25°C in Tyrosine Hydroxylase antibody (1:1000 rabbit, Chemicon, Temecula, CA, USA) primary solution (1% normal goat serum, 1%bSA .3%Triton and PBS) followed by incubation with the corresponding secondary biotinylated horse anti-mouse After rinsing in PBS, sections were incubated with biotinylated secondary antibodies (1 : 200, Vector, Burlingame, CA, USA) solution for 90 minutes. Following another PBS rinse and peroxidase block with 10% H202 in PBS, samples were incubated in avidin-biotin peroxidase complex solution (1:1600, ABC Elite, Vector) for 90 minutes at 25°C . After rinsing in PBS and Tris buffer (pH 7.6) slices were visualized with 3, 3'-diaminobenzidine. Immunolabeled brain sections were mounted and cover slipped. The reaction product was visualized with 0.25 mg/mL 3,3'-diaminobenzidine and 0.03% H_2O_2. Additional sets of sections were stained using FD Neurosilver Kit I (Ellicott City, MD, USA). Sections were mounted on slides, stained with cresyl violet, dehydrated in graded ethanols, and coverslipped using dibutyl phthalate xylene (DPX, Sigma, St. Louis, MO, USA) mounting media.

Western Blotting

Western blots were used to identify the presence of the vesicular monoamine transporter 2 in HEK-293 cells using mCherry-VMAT2 cells, HEK-293 transfected cells, and naked HEK cells for control. Cell lysates were harvested for western blotting. Lysate pellet was resusspended in homogenization buffer and proteins were quantified using BCA protein assay (BCA Protein Assay kit; Pierce, Rockford, IL, USA). Ten micrograms of protein from each sample was exposed to polyacrylamide gel electrophoresis (NuPAGE, 10% BisTris, 1 mm thick, 12-well gels, Invitrogen, Carlsbad, CA, USA) then electrophoretically transferred to polyvinylidene difluoride membranes (Invitrolon 0.45 μm PVDF, Invitrogen). Blots were incubated in 7.5% non-fat dry milk (Carnation, Glendale, CA, USA) in Tween Tris-buffered saline (TTBS) for 1 h at 23°C. Membranes were then incubated overnight with an antibody to VMAT2 (432 VMAT2 antibody; 1 : 10; Chemicon, Temecula, CA, USA). Primary antibody binding was detected using a goat anti-rat horseradish peroxidase (HRP) secondary antibody (1 : 5,000; Jackson Immuno Research, West Grove, PA, USA) and enhanced chemiluminescence (SuperSignal; Pierce, Rockford, IL, USA). Luminescence was captured with a Fluorochem 8800 (Alpha Innotech, San Leandro, CA, USA) imaging system.

FFN206 Uptake Assay with TBZ

Trypsinized confluent cells grown on 10 cm plates were diluted 1:4 in experimental media. 100 uL VMAT2-HEK cells were plated onto PDL coated clear-bottom96-well plates (Grenier Bio One 675090). Cells were grown at 37°C in 5% CO2. Following about 3-4 days of growth cells appeared 80-90% confluent. Cells were plated into inner 60 wells and PBS was plated into the outer ring of wells to minimize evaporative stress. Plates were incubated overnight and 90% confluent after 24 hours. Post-incubation, culture media was aspirated off and 90 uL of experimental media (DMEM without phenol red) was pipetted to each well. 5 uL of 200 uM tetrabenazine was added to negative control wells (stored at -20C; final working TBZ concentration is 10 uM). 5 uL of tested compounds (Aroclor 1260, paraquat, rotenone, deltamethrin, methamphetamine, methylphenidate, reserpine) were added at this step, in a range of 0.001 uM-100 uM in order to analyze VMAT2 activity in the presence of these chemical compounds. Cells were incubated for 30 minutes at 37°C. FFN206 (Abcam; ab144554) dilution was prepared 5 minutes prior to the end of plate incubation (2 uM, prepared from a 10 mM stock solution in DMSO with a final fluorescent probe concentration of 1 uM in uptake assay: .2% FFN + 99.8% eDMEM) 5 uL FFN dilution was added to each

well and plates were incubated for 1 hour at 37°C. After 1 hour incubation experimental media was aspirated off and 180 uL warm PBS was pipetted into each well. PBS was aspirated off and 90 uL of warm PBS was added to each well. Plates were read in a microplate reader (Perkin Elmer 2030) to assess fluorescence (FFN206 Excitation @269 nm; FFN206 Emission @ 464 nm).

Results

Characterization of HEK293 stable cell line

To characterize HEK293 cell line and confirm the presence of transfected VMAT2 we performed a western blot analysis (Figure 3). Western blots were used to identify the presence of the vesicular monoamine transporter 2 in HEK-293 cells using mCherry-VMAT2 (Figure 2.) cells, HEK-293 transfected cells, and naked HEK cells for control. To further demonstrate the presence of VMAT2 in HEK293 cells, an uptake assay was used in combination with tetrabenazine and reserpine, known VMAT2 inhibitors (Figure 4.). Tetrabenazine and reserpine both inhibited VMAT2's uptake capability as evidenced by a decrease in fluorescence (Figure 5.). This assay verified that the cells contain functional VMAT2 and that they respond to appropriate inhibitors.

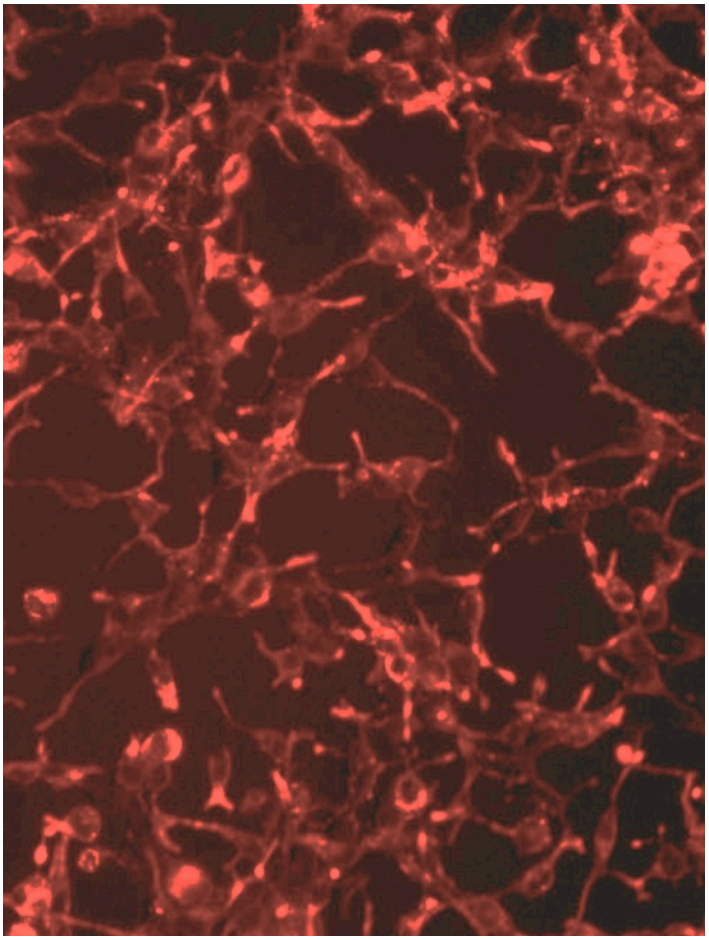

Figure 2. mCherry-VMAT2 cell line: mCherry VMAT cell line was used as a control in western analysis to confirm the presence of VMAT2 in HEK-293 cell line**.**

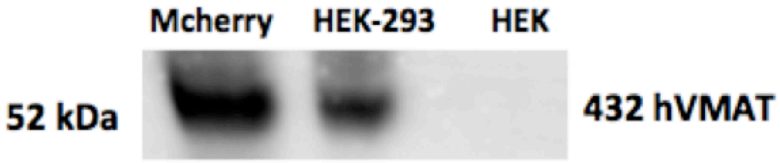

Figure 3. HEK-293 cell line characterization. Western blots were used to identify the presence of the vesicular monoamine transporter 2 in HEK-293 cells using mCherry-VMAT2 cells, HEK-293 transfected cells, and naked HEK cells for control. Cell lysates were harvested for western blotting. Antibody 432 hVMAT recognized VMAT in mCherry-VMAT2 and HEK-293 cells but not in naked HEK cells confirming the presence of VMAT2 in HEK-293 cell line

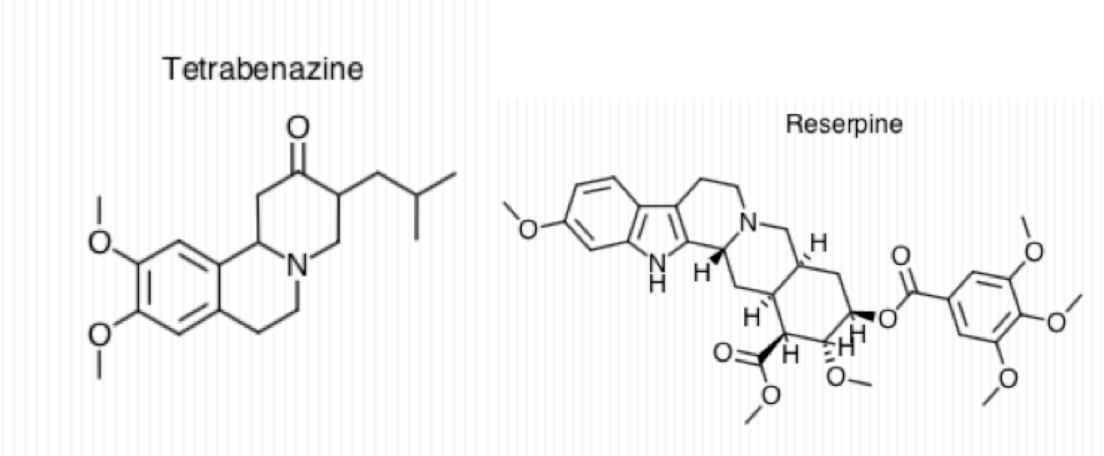

Figure 4. Tetrabenazine and Reserpine: Molecular structure of known VMAT2 inhibitors.

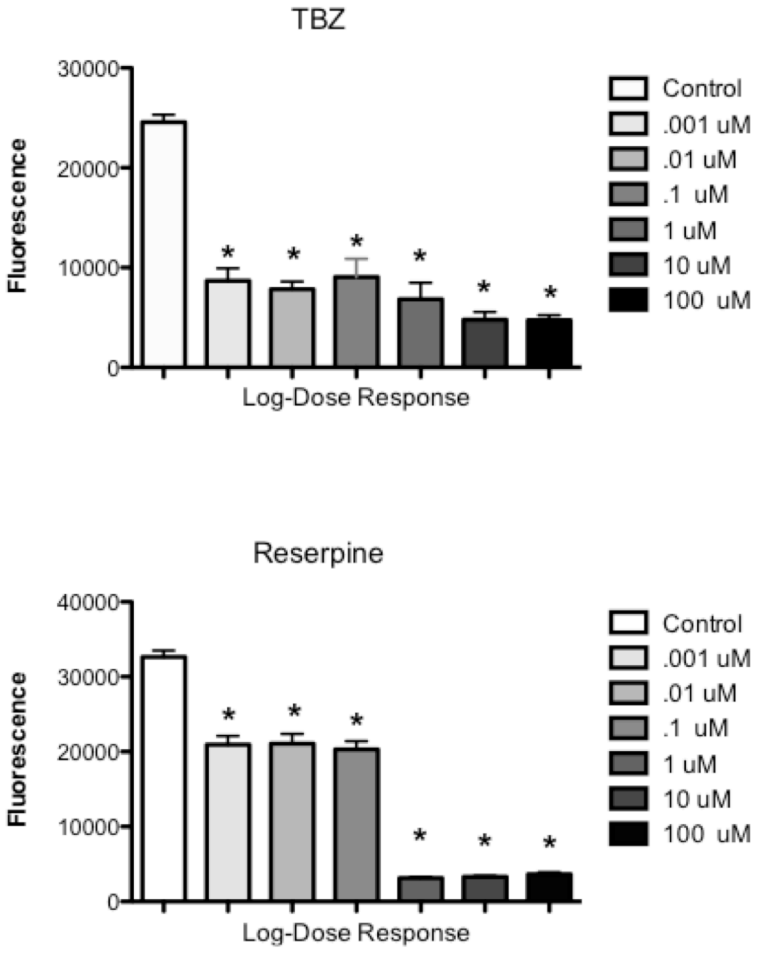

Figure 5. Tetrabenazine and Reserpine used as negative controls. 5 uL TBZ and reserpine for added to 96 well plate at subsequent dilutions to display inhibitory properties of these compounds. Significant decreases in fluorescence were observed at the lowest dose of .001 uM. Tetrabenazine was used as a negative control on all HTS 96-well plates at 10 uM in every experiment.

Experimental Screen of Environmental and Pharmaceutical Exposures

FFN206 is a false fluorescent ph-sensitive neurotransmitter optimized for the use in VMAT2-transfected cell lines. In order to examine FFN206 as a VMAT2 substrate we used the HEK-293 cell line. These cells express VMAT2 on acidic cellular organelles where a vacuolar-type H+-ATPase provides the appropriate mechanisms for substrate transport. In order to perform a robust analysis examining multiple chemical compounds, HEK-293 cells were analyzed for their uptake capabilities in a high throughput screening procedure.

Once the utility of this assay was assessed, we performed an experimental screen of environmental and pharmaceutical exposures. Potential inhibitors and substrates were screened at several concentrations ranging from 0.001 uM to 100 uM in order to look at a broad range of dose responses on uptake activity. This range includes clinically relevant doses that would be applicable to exposure levels. Positive and negative controls were employed on each 96 well plate to demonstrate proper technique. Positive controls included wells without FFN or TBZ and negative controls included 5 uL of 10 uM TBZ. The experimental Z'-factor was determined from the assay controls of n=9 to be 0.64 indicating a robust assay in practice above the minimum Z'-

value of 0.5. Figure 5 illustrates a wide display of inhibitory affect using the several environmental and pharmaceutical exposures.

Examination of Environmental and Occupational Exposures

Using Analysis of Variance (ANOVA) and Tukey's multiple comparison test, all screened environmental and occupational exposures showed significant inhibitory properties at 10 uM ($P < .05$) versus control. Paraquat has an inhibitory effect at 10 uM and 100 uM, displaying a 62% and 68% reduction versus control respectively. Deltamethrin exhibited an insignificant increase of uptake activity at 1 uM of 20% versus control, however at 10 and 100 uM the compound displayed a 36% and 75% inhibition versus control. Aroclor 1260 exhibited a 63% and 66% decrease in uptake versus control at 10 and 100 uM respectively. At its highest dose of 100 uM Rotenone inhibited VMAT uptake activity by 36%.

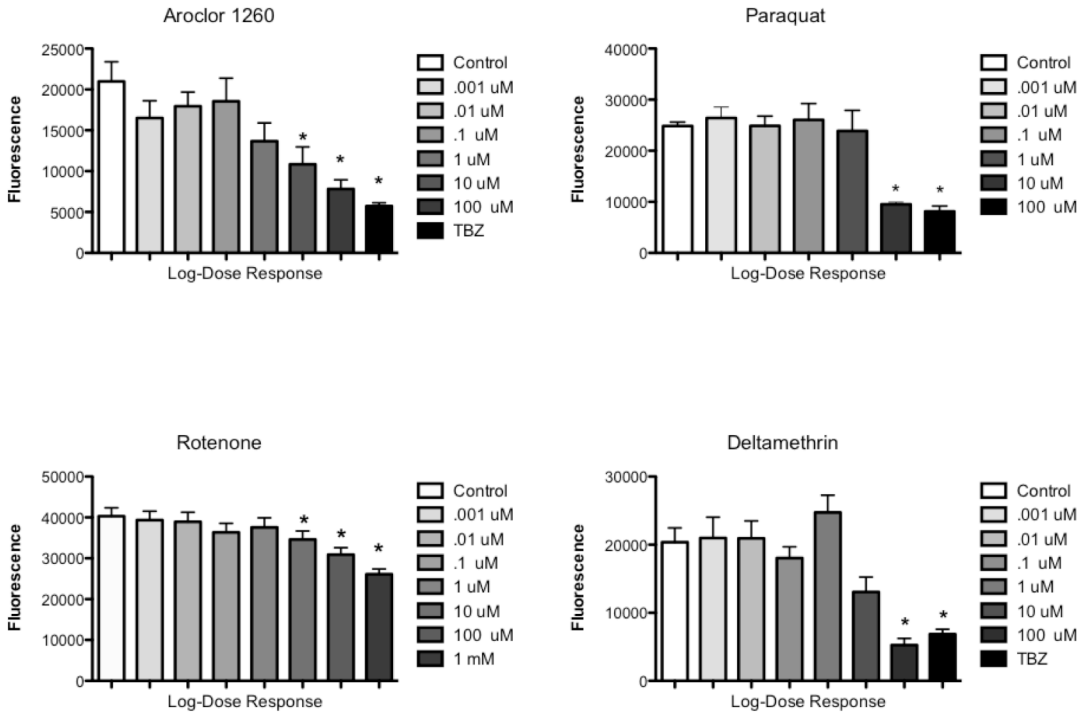

Figure 5. Effects of environmental toxicants on VMAT2 uptake activity in FFN205 assay. These effects were quantitatively analyzed in a high throughput design, allowing for a robust analysis of potential VMAT inhibitors. 5 uL of 200 uM Tetrabenazine was added to negative control wells (stored at -20C; final working TBZ concentration is 10 uM). Cells were incubated for 30 minutes at 37°C. FFN206 dilution was prepared 5 minutes prior to the end of plate incubation (2 uM, prepared from a 10 mM stock solution in DMSO with a final fluorescent probe concentration of 1 uM in uptake assay: .2% FFN + 99.8% eDMEM) 5 uL FFN dilution was added to each well and plates were incubated for 1 hour at 37°C. Uptake was terminated with two PBS buffer washes. Error bars are standard error (SE) from 3 independent trials. Each experiment was run in triplicate per plate and 3 plates total.

Examination of Pharmaceutical Exposures

Using Analysis of Variance (ANOVA) and Tukey's multiple comparison test, all screened exposures except methylphenidate showed significant inhibitory properties at 10 uM ($P < .05$) versus control. Cells exposed to methamphetamine showed a 64% inhibition of control at 10 uM followed by 51% and 25% versus control. Methamphetamines mechanism of action is heavily researched; increasing extracellular dopamine by interacting at VMAT2, Methamphetamine inhibits dopamine uptake, promotes dopamine release from synaptic vesicles, and blocks monoamine oxidase activity thereby increasing cytosolic dopamine and making it available for reverse transport by DAT.[56] It is a VMAT2 substrate and these results align with previous studies of methamphetamine's ability to alter VMAT activity.

Methylphenidate (MPD) exposure resulted in a significant increased fluorescence at 1 uM followed by a significant decreased fluorescence at 1 mM highlighting an area of further examination. These results align with previous studies on MPD's interaction with VMAT2, as it has been shown that MPD inhibits dopamine transporter function and increases vesicular uptake and binding at lower

concentrations, followed by decreased vesicular functioning at higher concentrations in vivo. [57]

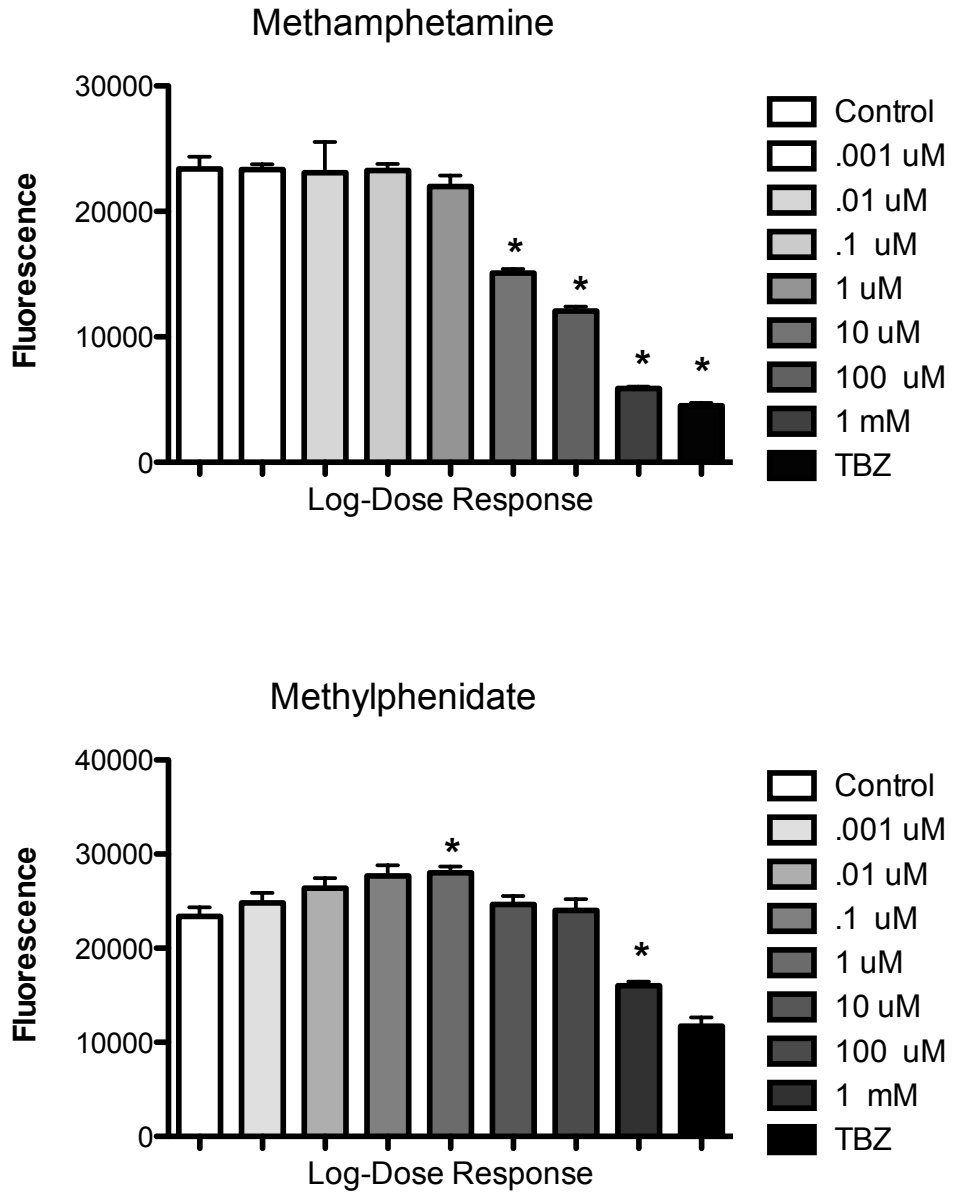

Figure 6: Effects of methamphetamine and methylphenidate exposure of vesicular uptake of dopamine: These effects were quantitatively analyzed in a high throughput design, allowing for a robust analysis of potential VMAT inhibitors. 5 uL of 200 uM Tetrabenazine was added to negative control wells (stored at -20C; final working TBZ concentration is 10 uM). Cells were incubated for 30 minutes at 37°C. FFN206 dilution was prepared 5 minutes prior to the end of plate incubation (2 uM, prepared from a 10 mM stock solution in DMSO with a final fluorescent probe concentration of 1 uM in uptake assay: .2% FFN + 99.8% eDMEM) 5 uL FFN dilution was added to each well and plates were incubated for 1 hour at 37°C. Uptake was terminated with two PBS buffer washes. Error bars are standard error (SE) from 3 independent trials. Each experiment was run in triplicate per plate and 3 plates total.

Discussion

Individuals are exposed to a variety of chemicals during leisure and occupational activities, including herbicides, insecticides, PCBs or pharmaceutical agents. Many of these exposures once perceived as helpful are now being considered detrimental. Pesticides first created to combat agricultural problems, such as pest infestation, are now being researched as potential endocrine, organ, and protein disruptors. Chemical compounds intended to assist technical or automotive operations are now being researched for their negative neurological effects. Drugs designed to cure ailments such as ADHD might now be assessed for their potentially detrimental properties. These compounds that were initially created to help mankind must be assessed as potential toxicants. Many studies have implicated the potential risk that these exposures pose on human disease, specifically neurological diseases such as Parkinson's.

Recent implications have pointed towards the vesicular monoamine transporter 2 as being a target of many of these toxicants. Over and over again vesicular integrity has been deemed of great importance to the health of the neuron. Furthermore, dopaminergic vesicles are significant targets for the study of pathogenic mechanisms of PD, since the vesicles main function is to shelter dopamine and assist with transmission of the neurotransmitter, as well as to protect

the neuron from damage by properly isolating neurotransmitters from the cytosol. [58] Thus without the proper functioning of the vesicles and vesicle transporters, the neuron could face potentially toxic consequences. Therefore it is of utmost importance to examine the exposures that humans face everyday in order to assess which of these could be involved with the pathogenesis of PD.

In observing these exposures we were able to visualize significant decreased VMAT2 uptake activity in paraquat, rotenone, deltamethrin, Aroclor 1260, methylphenidate and methamphetamine. Many of these chemicals act in a multitude of ways that are still widely misunderstood. Other compounds were assessed including Perfluorooctanoic acid (PFOA;fluorinated industrial applicant), Hexabromocyclododecane (HBCD; brominated flame retardant), Tris (1,3-dichloro-2-propyl) phosphate (TDCPP;chlorinated organophosphate), dichlorodiphenyldichloroethane (DDD; organochlorine insecticide) permethrin (pyrethoid insecticide), and chlorpyrifos (organophosphate insecticide). Of these chemicals, PFOA and HBCD showed little to no evidence of inhibition, however TDCPP, DDD, permethrin and chlorpyrifos showed inhibitory characteristics and should be assessed further. While these compounds were not included in this paper because of their small sample size (n=6), at the highest doses of 10 and 100 uM, these chemicals showed large

decreases in fluorescence and therefore should be examined for their ability to decrease VMAT2's uptake capabilities.

These data furthermore reveal a pathway that must be further analyzed in regards to toxicant exposure and PD. Additional investigation of these compounds and other compounds are necessary to fully understand the mechanisms of these toxicants; however, in light of these findings, we propose that further study of the role of environmental and pharmaceutical exposures on VMAT2 activity and the pathogenesis of PD is warranted.

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