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Chronic Amphetamine Exposure Causes Long Term Effects On Dopamine Uptake In Cultured Cells

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CHRONIC AMPHETAMINE EXPOSURE CAUSES LONG TERM EFFECTS ON DOPAMINE UPTAKE IN CULTURED CELLS

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This thesis, submitted by Nafisa Ferdous in partial fulfillment of the requirements for the Degree of Master of Science from the University of North Dakota, has been read by the Faculty Advisory Committee under whom the work has been done and is hereby approved

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This thesis is being submitted by the appointed advisory committee as having met all the requirements of the School of Graduate Studies at the University of North Dakota and is hereby approved.

Wayne Swisher
Dean of the School of Graduate Studies

December 8, 2016
Date

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Title	Chronic amphetamine exposure causes long term effects on dopamine uptake in cultured cells
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Nafisa Ferdous

13th December 2016

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ABBREVIATIONS

- AMPH — Amphetamine
- ADHD — Attention Deficit Hyperactivity Disorder
- BSA — Bovine serum albumin
- CNS — Central Nervous System
- *C.elegans* — Caenorhabditis elegans
- DMEM — Dulbecco's Modified Eagle's Medium
- DMSO — Dimethyl Sulfoxide
- DTT — Dithiothreitol
- DAT — Dopamine transporter
- Desp. — Desipramine
- ELISA — Enzyme-linked Immunosorbent Assay
- EDTA — Ethylenediaminetetraacetic acid
- FBS — Fetal Bovine Serum
- hDAT — human dopamine transporter
- IgG — Immunoglobulin
- KRH — Kreb Ringer Hepes
- LLC-PK_1 — Lewis lung carcinoma-porcine kidney
- MEM — Minimum Essential Medium
- NE — Norepinephrine
- NET — Norepinephrine Transporter
- PBS — Phosphate Buffer Saline

- Pen Strep — Penicillin and Streptomycin solution
- PVDF — Polyvinylidene fluoride
- RA — Retinoic Acid
- RIPA — Radio Immunoprecipitation Assay
- SERT — Serotonin Transporter
- SDS PAGE — Sodium dodecyl sulfate polyacrylamide gel electrophoresis
- SWIP — Swimming induced paralysis
- SLC6 — Solute carrier 6
- TH — Tyrosine Hydroxylase
- TPA — 12-O-tetradecanoyl-phorbol-13 acetate
- VMAT — Vesicular monoamine transporter
- VTA — Ventral tegmental area

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Dedicated to my parents and brother

ABSTRACT

Dopamine is a neurotransmitter which belongs to the catecholamine and phenethylamine families of organic compounds, and plays an important role in the regulation of reward, movement, attention, behavior and cognition. Dopamine neurotransmission is marked by the initiation of its release from the pre-synaptic neurons and the signal is terminated by re-uptake of dopamine from the synapse. The synaptic concentration of dopamine and therefore, the level dopamine receptor stimulation is regulated to an extent by the activity of the dopamine transporter (DAT). DAT is responsible for the uptake of dopamine back into the presynaptic neuron from the synapse. DAT is known to play a critical role in certain pharmacological or pathological conditions. For example, DAT is one of the major targets of several psychostimulant drugs like cocaine and amphetamine. Cocaine competitively inhibits dopamine uptake by blocking DAT which leads to increased dopamine in the synapse, one of the initial steps that promote addiction. DAT is also a major target of amphetamine (AMPH), another powerful stimulating drug. AMPH is used regularly in the treatment of neurodevelopment disorders such as attention deficit hyperactivity disorder (ADHD) but despite its role as an effective medication in such disorders, AMPH is more commonly known for its psychostimulant and addictive properties as a drug of abuse.

AMPH induces its rewarding and addictive properties by acting as a substrate of vesicular monoamine transporter (VMAT) and plasma membrane monoamine (dopamine, norepinephrine, and serotonin) transporters. AMPH is similar in structure to monoamines like dopamine, norepinephrine and therefore can bind and enter the presynaptic neurons via the transporters. Once in the neuron, AMPH causes an elevation in extracellular monoamine levels by inducing

vesicular depletion of monoamines and also by promoting reverse transport (efflux) of monoamines through plasma membrane transporters. Several groups have shown that acute AMPH treatments alter the function and the number of DAT on the cell membrane but the long-term effects of chronic AMPH exposure have still not been clearly identified. Previous data from our laboratory suggested that parental AMPH exposure reduces dopamine uptake in *C. elegans* dopaminergic neurons isolated from progeny.

In order to determine if the long-term effects caused by AMPH in native *C. elegans* cultured neurons is reproducible in human DAT *(*hDAT) expressing cells, we carried out our initial investigation in LLC-PK_1 porcine kidney epithelial cells stably transfected with hDAT. Cells were treated with AMPH and then analyzed for dopamine uptake, after the cells had undergone one, two or three round of division. Results from the experiments showed a significant decrease in dopamine uptake compared to untreated cells. This suggests that changes caused by AMPH were conserved up to three cell divisions. These data led us to hypothesize that long term reduction in dopamine reuptake, as a result of chronic AMPH treatment, is caused by down-regulation of DAT.

In our next approach we investigated the long term effect of AMPH in the SH-SY5Y human neuroblastoma cell lines. Previous studies have shown that the SH-SY5Y cells can be chemically differentiated into more mature neuron-like phenotype by treatment with retinoic acid (RA). Thus, we pre-treated cells with AMPH and allowed cells to cross one or more cell divisions before treating them with RA. Dopamine uptake assays revealed a significant decrease in dopamine re-accumulation in AMPH-treated cells with respect to control. Results from these

experiments also revealed NET-mediated dopamine uptake in SH-SY5Y cells. In fact, desipramine, a specific NET inhibitor, totally blocked the dopamine uptake whereas GBR12909, which is a specific DAT inhibitor, did not show inhibition. Taken together, these results suggest that parental AMPH treatment down regulates the expression or activity of catecholamine transporters such as DAT and NET in daughter cells.

INTRODUCTION

Neurotransmission

The central nervous system (CNS) in humans is an complex system responsible for processing information from all parts of the body. The CNS is made up of two kinds of specialized cells- neurons and glia. Neurons are responsible for building up the information processing network in the brain by forming interconnections in the CNS and receiving, conducting and transmitting signaling chemicals called neurotransmitters to the synaptic cleft. The synaptic cleft or synapse is the gap between two neurons where the neurotransmitters are released [1].

Neurons are electrically excitable cells capable of transmitting information through an electrochemical process. The primary components of a neuron include the soma, axon, and dendrites. The soma is the spherical part of the neuron that makes up the cell body and is connected to the axon and smaller tree-like branches called dendrites. The soma is composed of the nucleus and other important organelles like the Golgi apparatus, ribosomes, mitochondria and endoplasmic reticulum. Synaptic signals from other neurons are received by the soma and dendrites whereas signals to other neurons, muscles and glands are sent by the axons. Axons are nerve fibers which are basically long projections that extend from the soma to the terminal buttons, more commonly known as axonal terminals. Signals from axons of other neurons are primarily received by the dendrites which are smaller branched projections from the soma and are located next to the axons. The axonal terminals contain neurotransmitters and are responsible for releasing neurotransmitters into the synapse, across which impulses are sent[2].

Neuronal communication builds the foundation of the general function of the CNS where electrical events propagate a signal within a neuron and chemical processes such as

neurotransmission send signal from one neuron to another or to muscle cells (Figure 1). Neurons can communicate within the cell, referred to as ‘intracellular signaling’ and they are also capable of communicating between cells, known as ‘intercellular signaling’. The conductance of electrical signals or action potentials along axons is how a neuron achieves long distance, rapid communication with its own terminals or terminal boutons and the terminal boutons establishes communication between neurons by the process of neurotransmission[3].

Neurotransmission or synaptic transmission is communication between neurons which begins when an action potential travels along the axon of a presynaptic neuron and reaches the axon terminal. During conduction, an action potential can travel at a rate up to 150 meters or roughly 500 feet per second. At the axon terminal, the neuron sends out its output across the synapse to other neurons[4]. At electrical synapses, the ‘output’ will be an electrical signal and at chemical synapses, the ‘output’ will be neurotransmitters.

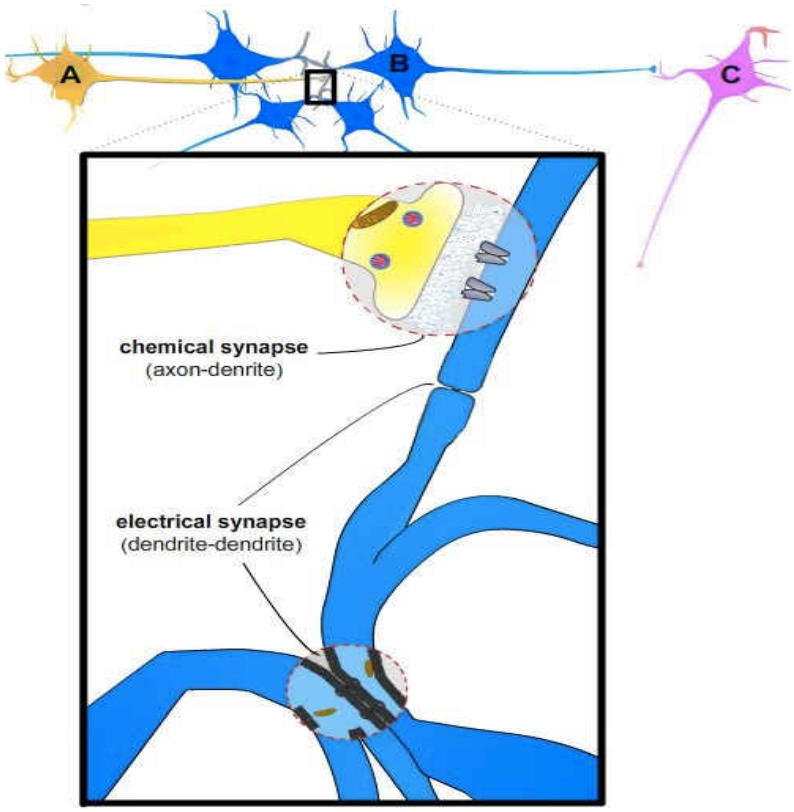

Image courtesy, http://www.mind.ilstu.edu/curriculum/neurons_intro/imgs/synapses, with permission

Figure 1: Difference between electrical and chemical synapses

Once an action potential reaches the axon terminal it cannot cross the synaptic space. At the terminal button, the action potential causes membranous sacs called vesicles to move to the membrane of the terminal. Concurrently, membrane depolarization takes place which causes the voltage-dependent calcium (Ca^{2+}) channel on the presynaptic neuron to open and allow Ca^{2+} to enter into the cell. Once Ca^{2+} enters the presynaptic neuron, it binds to the membrane of the vesicles causing vesicles containing neurotransmitters to deplete and release them into the synapse. The neurotransmitters then diffuse across the synaptic space and bind to special proteins called receptors on the post-synaptic neuron. The binding of a neurotransmitter to its receptor

can trigger an action potential in the post-synaptic neuron[4, 5]. That electrical signal then moves towards the cell body of the post-synaptic neuron. Once the post-synaptic neuron elicits an action potential, the neurotransmitter dissociates from its receptor to the synaptic space where it is either degraded by enzymes in the synapse or translocated back into the pre-synaptic neuron by transporter proteins. The brief post-synaptic potential produced by neurotransmitters is usually terminated by their re-uptake (Figure 2). For monoamine transporters like dopamine, serotonin and norepinephrine post-synaptic potentials are terminated by re-uptake.

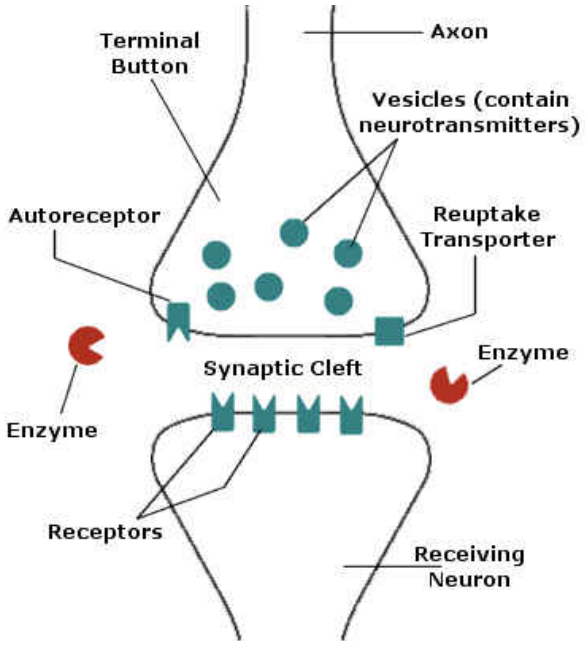

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Figure 2: Illustration of chemical neurotransmission

Monoamine neurotransmitters

The monoamine family of neurotransmitters includes- dopamine, norepinephrine and serotonin. Their respective transporters- the dopamine transporter (DAT), the norepinephrine transporter (NET) and the serotonin transporter (SERT), which are transmembrane proteins are located in plasma membranes of monaminergic neurons. The monoamine transporters are a major target of psychostimulant drugs. Structurally, monoamines contain an amino group which is connected to an aromatic ring by a two carbon chain. Besides being referred to as monoamines, dopamine and norepinephrine are also addressed as catecholamines as they consist of a catechol group (Figure 3)[6].

Catecholamine is an amine derived from the amino acid tyrosine, examples include epinephrine, adrenaline, norepinephrine, and dopamine that act as hormones or neurotransmitters. The amino acid tyrosine is created from phenylalanine by hydroxylation of the enzyme phenylalanine hydroxylase. Catecholamine-secreting neurons via several reactions convert tyrosine to L-DOPA and then to dopamine and then based on the cell type dopamine is sometimes further converted to norepinephrine and eventually to epinephrine[6, 7].

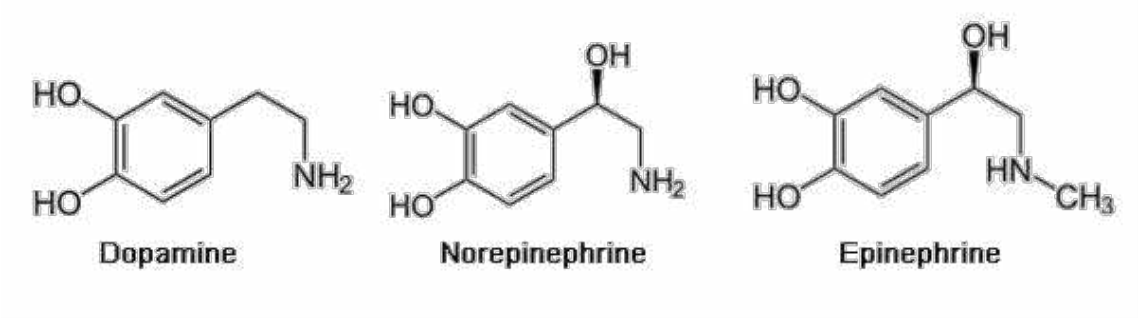


Image courtesy, http://mybrainnotes.com/brain-neurotransmitters-catecholamines

Figure 3: Types of catecholamines

Dopaminergic system and Noradrenergic system

The dopaminergic system plays an integral role in the regulation of the reward system. When we are exposed to a rewarding stimulus, the brain responds by increasing release of the neurotransmitter dopamine. The pathway most often associated with reward is the mesolimbic dopaminergic pathway which originates in the ventral tegmental area (VTA). As a part of the reward pathway, dopamine is principally produced in the ventral tegmental area which is connected to the nucleus accumbens by the mesolimbic dopaminergic pathway. The nucleus accumbens is situated in the part of the brain that is strongly associated with motivational reward called the ventral striatum. When we experience something rewarding or use an addictive drug, dopamine neurons in the VTA are activated. These neurons project to the nucleus accumbens via the mesolimbic dopamine pathway and their activation causes extracellular dopamine levels in the nucleus accumbens to rise[8]. Another major dopamine pathway is the mesocortical pathway which also originates in the VTA and travels to the cerebral cortex specifically to the frontal lobe. This dopamine pathway is also activated during rewarding experiences and is considered a part of the reward system[9].

Dopamine neurotransmission is also involved in the regulation of movement and emotional responses. Deficiency in dopamine can lead to delayed and uncoordinated movement, a clinical symptom seen in the neurological disease Parkinson's. The dopaminergic system has been associated with psychiatric disorders like schizophrenia and attention-deficit hyperactivity disorder (ADHD) and also mediates extrapyramidal side effects in pathological conditions like dysphagia[10]. The dopaminergic system is a primary target of drugs of abuse and dopamine plays an integral role in drug abuse, dependence and addiction[11].

Dopamine synthesis in the brain involves two enzymes- tyrosine hydroxylase (TH) and amino acid decarboxylase. Tyrosine is converted to the direct precursor of dopamine, L-DOPA, by TH and then L-DOPA is converted into dopamine by amino acid decarboxylase. Once synthesized, dopamine is transported from the cytosol into storage vesicles by vesicular monoamine transporter (VMAT). Dopamine is stored in these vesicles where it is protected from degradation by enzymes like monoamine oxidase and catechol-O-methyl transferase. Dopamine is stored in these storage vesicles until it is released into the synapse in response to physiological stimuli. Once in the synapse, dopamine binds to and activates dopamine receptors. All of the dopamine receptors are G-protein coupled receptors and their signaling is primarily mediated by interaction with and activation of G-proteins. The action in response to dopamine released is terminated by its reuptake into the presynaptic neuron which is principally regulated by the dopamine transporter (DAT)[12].

In noradrenergic neurons dopamine is converted to norepinephrine by the action of the enzyme dopamine-β-hydroxylase and this reaction takes place within the synaptic vesicles. Norepinephrine, also referred to as noradrenaline, works as both a hormone and a neurotransmitter, and is predominantly released from the ends of sympathetic nerve fibers (noradrenergic neurons). Neurons that produce norepinephrine are distributed throughout the brain stem, most notably in the locus coeruleus. The output of the noradrenergic locus coeruleus cells projects further throughout the cerebrum, cerebral cortex, thalamic nuclei, cerebellum and the spinal cord. Due to this broad range of projection paths, norepinephrine is involved in mediating many behavioral and physiological processes like mood, attention, stress, overall arousal and sexual behavior[13]. Like dopamine, norepinephrine has also been found to play a

large role in attention and focus (Figure 4). In neurodevelopment disorders like ADHD, psychostimulant medications are prescribed to help elevate levels of dopamine and norepinephrine.

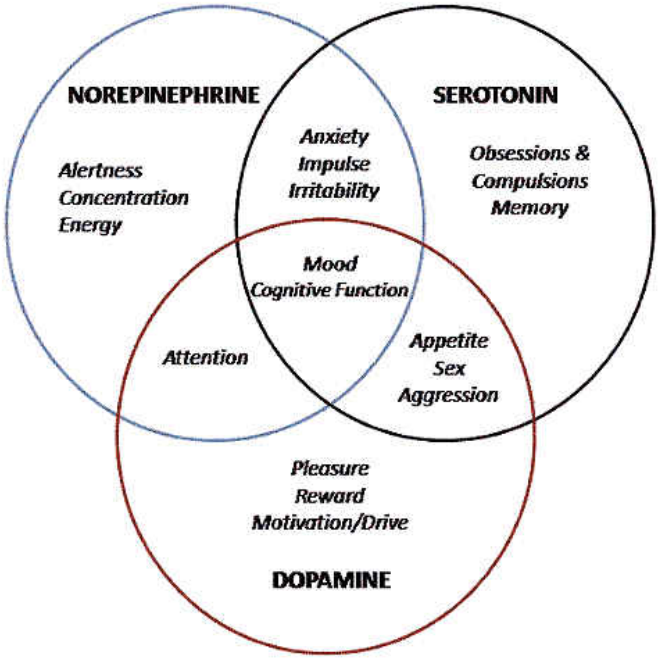


Figure 4: Monoamines and the functions they share[14]

Monoamine Transporters

Transporters like NET, SERT, GABA and DAT belong to the SLC6A gene family. NET, DAT and SERT are monoamine transporters responsible for regulating the synaptic concentration of monoamines (norepinephrine, dopamine and serotonin). DAT is a membrane spanning protein that pumps dopamine out of the synapse and back into cytosol where other vesicle membrane transporters like VMAT sequester dopamine into vesicles for storage and later release (Figure 5). Dopamine uptake by DAT provides the primary mechanism in which dopamine is cleared from synapse although there may be an exception in the frontal cortex where evidence suggests a

possibly larger role of NET in regulating dopamine uptake[15]. Transporters belonging to SLC6A gene family translocate neurotransmitters into cells by coupling transport to ion gradients, for example- DAT is a symporter that moves dopamine across the cell membrane by coupling the movement to the energetically favorable movement of sodium ions moving from high to low concentration into the cell. DAT function requires binding and co-transport of $2Na^+$ ions and one Cl^- ion with dopamine substrate. The binding of dopamine and the ions induces DAT to undergo a conformational change which allows dopamine to unbind on the intracellular side of the membrane[16].

The activity of DAT promotes regulation of dopamine mediated signals which are involved in cognition , behavior and also reward. DAT localization and distribution has been found in areas of the brain which include nigrostriatal, mesolimbic and mesocortical pathways. Previous studies have shown that functional regulation of DAT is largely accomplished by phosphorylation, palmitoylation and internalization of the transporter by the kinases, substrate pretreatment and interaction with presynaptic receptors[17, 18]. DAT is implicated in a number of dopamine related disorders including ADHD, bipolar disorder, clinical depression, addiction and alcoholism[19]. Since DAT is largely responsible for the termination of dopamine neurotransmission, it has a pivotal role in psychostimulant actions induced by stimulating drugs like cocaine and AMPH. The mechanism behind cocaine mediated inhibition is simple where the drug binds to the transporter and blocks dopamine uptake but AMPH causes inhibition at the transporter by binding to the transporter and entering the presynaptic neuron and inducing efflux of dopamine through DAT.

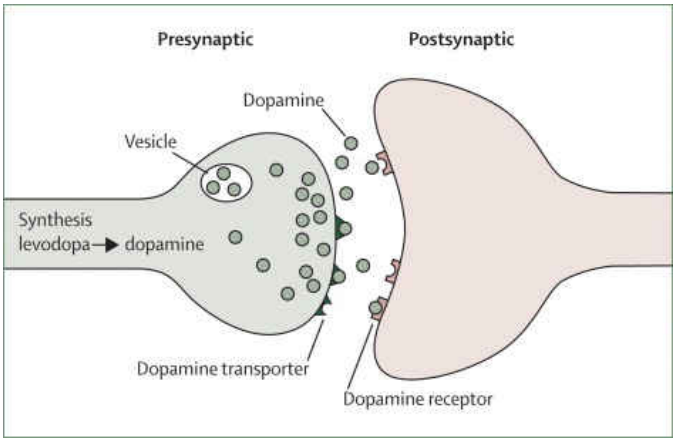


Image courtesy, Daniela. B et al, *Transcranial sonography in movement disorders*. The Lancet Neurology, 2008. Volume 7, No. 11, p1044–1055.

Figure 5: Dopamine neurotransmission and uptake of dopamine by DAT

Like DAT, NET is responsible for clearing off extracellular norepinephrine (NE) from the synapse and pumping it back into the presynaptic neurons. NET mediated reuptake of NE is crucial in preventing excess concentration of the neurotransmitter in the synaptic cleft; it is also pivotal in the removal of norepinephrine from the heart and other peripheral organs like lungs, liver, kidney and muscles[20]. NE is hypothesized to play a role in psychiatric disorders like depression and ADHD. Psychostimulants like cocaine and AMPH, and antidepressants like desipramine, reboxetine and various others, impede the reuptake of norepinephrine via NET and elevate NE extracellular concentration and potentiate the activation of postsynaptic receptors[21]. AMPH acts as a substrate of monoamine transporters like NET and induces a reversal in the direction of neurotransmitter transport (efflux) which results in large accumulation of synaptic NE. Another important feature of NET is it can transport dopamine as well as NE. In fact, NET exhibits greater affinity for dopamine than DAT.

Psychostimulant Drugs

Psychostimulants are drugs that stimulate the brain mostly by causing activation of the dopaminergic system; besides dopamine, stimulants also may cause release of tremendous amount of norepinephrine and serotonin. The effect of stimulating drugs on dopamine and norepinephrine neurotransmission is primarily mediated by these drugs interfering with neurotransmitter transporter function. DAT and NET are often associated with addiction as they are high affinity molecular targets of powerful stimulating drugs like cocaine and AMPH[22]. Psychostimulants have varying affinity for different monoamine transporters (DAT, NET, SERT), for example AMPH and methylphenidate have much lower affinity for SERT compared to their affinity for DAT and NET. Cocaine has earned the reputation of being one of the most addictive and dangerous illicit drugs. Cocaine is deemed as a simple inhibitor as it inhibits monoamine uptake by blocking plasma membrane monoamine transporters-DAT, NET and SERT[23].

AMPH and methamphetamine are also CNS stimulant drugs that fall under the category of substrate-type releasers. Releasers like AMPH are more effective in increasing synaptic concentration of monoamine neurotransmitters as they not only cause competitive inhibition of reuptake by competing with substrate binding to the transporters but also increase the pool of neurotransmitters available for release by transporter-mediated exchange. AMPH acts as substrate of DAT and NET and previous studies have reported the transport of AMPH into the presynaptic neuron via DAT results in more number of transporters in the inward-facing conformation which increases the probability of cytosolic dopamine to bind to DAT and exit the

neuron via reverse transport. Beside AMPH- induced reverse transport, AMPH analogues may affect monoamine transporters through phosphorylation and transporter trafficking[24, 25].

AMPH is commonly used to treat several disorders, including ADHD, narcolepsy, and obesity. Prescription AMPHs like Adderall are used to treat ADHD patients. AMPH has proven to be very effective in treating children, adolescents, and adults diagnosed with ADHD, with responsiveness rates in the range of 70-80%. Prescription AMPHs are known to induce a calming and 'focusing' effect on individuals diagnosed with ADHD since ADHD patients face difficulty to pay attention and they are more hyperactive or impulsive than people of their age.

Despite being an effective medication, concerns have been raised about possible cardiovascular effects with the daily use of prescription AMPH. There have been consistent reports of increase in mean heart rate and blood pressure following AMPH treatment. This could be due to peripheral functions of dopamine and norepinephrine. A substantial amount of dopamine circulates in the bloodstream which is produced by the sympathetic nervous system and is independent of dopamine synthesis and function in the brain. There are some dopamine receptors on the walls of arteries and the AMPH mediated increase in dopamine may act through the sympathetic nervous system to increase heart muscle contraction force and heart rate, thereby increasing cardiac output and blood pressure.

Addiction to AMPH is also a matter of serious consideration for anyone taking this drug without medical supervision. When stimulants are prescribed by doctors they begin with low doses and gradually increase the dose until the therapeutic effect of the stimulant is achieved. The gradual

increase in dose enables slow and steady increases of dopamine, which are similar to the way dopamine is naturally produced in the brain. Addiction most likely occurs when stimulants are taken in doses and via routes other than those prescribed. Unsupervised use of prescription AMPH can increase brain dopamine in a rapid and highly amplified manner. This rapid rise in dopamine in the brain disrupts normal communication between brain cells and produce euphoric like sensation which leads to drug seeking and dependence and increases the risk of addiction[24]. There is in fact ongoing concern regarding use of stimulants to treat children or adolescents with ADHD. Stimulants prescribed to treat a child's or adolescent's ADHD could affect an individual's vulnerability to developing drug and psychiatric problems at a later stage in life. Recent statistics have shown that the longer the use and abuse of Adderall continues, the stronger an addiction can become. For example, in the year 2012, almost 16 million prescriptions were written for Adderall and over 116,000 people were admitted to rehab for an addiction to Adderall.

Taking these incidences of AMPH abuse and addiction into account we decided to analyze long term effect of AMPH. Previous studies done in our lab in the model organism *C.elegans* have shown that AMPH exposure during embryogenesis induces behavioral changes in adult worms suggesting that the presence of AMPH during development has a long term effect and we see consequences at a much later stage in the worm's life.

Also previous studies have demonstrated that AMPH specifically targets the DAT to promote dopamine release consequently engaging different classes of dopamine receptors (*e.g.* the D2 like receptors).

We investigated how chronic AMPH exposure causes long term effect on dopamine uptake in cultured cells. For our investigation we used human DAT (hDAT) transfected cell line, the LLC-PK_1 , and the human neuroblastoma cells, SH-SY5Y, which endogenously expresses hDAT, in order to test our hypothesis that the 'long term reduction in DA reuptake, as a result of chronic AMPH treatment, is caused by down-regulation of DAT.

EXPERIMENTAL METHODS

Cell Cultures

Lewis lung carcinoma-porcine kidney (LLC-PK_1) cells

The LLC-PK_1 cells are derived from the kidney of a normal, healthy male pig and the cell line exhibits typical epithelial –like morphology. These pig kidney epithelial cells are widely used in pharmacologic and metabolic research investigations worldwide.

For our experiments we obtained LLC-PK_1 cells which were stably transfected with hDAT (courtesy of Vaughan and Foster labs). Transfected LLC-PK_1 cells were maintained in α-modified Eagle's medium (AMEM) containing 2 mM L-glutamine, 5% fetal bovine serum (FBS), 200 μg/mL G418 sulfate and 1X penicillin/streptomycin. Cells were grown in a 5% CO_2 incubator at 37°C.

After obtaining hDAT transfected LLC-PK_1 cells, we maintained and propagated the cell line in Minimum Essential Medium (MEM) supplemented with L-Glutamine, Antibacterial and

Antimycotic (AA) solution, 5% Fetal Bovine Serum (FBS) and 200μg/ml G418. Cells were grown at 37°C in a 5% CO_2 incubator throughout the course of experiments.

SH-SY5Y cells

The human neuroblastoma cell line, SH-SY5Y was originally derived from a bone marrow biopsy of a neuroblastoma patient. The cell culture contains both adherent and floating cells where both types are viable. The SH-SY5Y has two morphologically distinct phenotypes- the neuroblast-like cells (N type) and the epithelial-like cells (S type). Cells with neuroblast-like phenotype are positive for the presence of catecholaminergic markers as they express tyrosine hydroxylase and dopamine-β-hydroxylase. Another distinct feature of SH-SY5Y cells is that they can be chemically differentiated into a more mature neuron-like phenotype using Retinoic Acid (RA) or phorbol esters such as 12-O-tetradecanoyl-phorbol-13 acetate (TPA). In our experiments, cells were subjected to only RA-induced differentiation[26].

SH-SY5Y cells were grown in Dulbecco's Modified Eagle's Medium (DMEM). Medium was prepared by adding 50% Fetal bovine serum (FBS) and 1% Penicillin and Streptomycin solution (Pen Strep) in 500ml DMEM. Cells were grown at 37°C in a 5% CO_2 incubator.

During the course of the project, both LLC-PK_1 and SH-SY5Y cells were passaged according to the following protocol where after removing media, cells were washed twice with 10ml sterile 1X phosphate buffer saline (1X PBS). After 1X PBS wash, 2ml of 1X trypsin solution was added to promote cell detachment. Trypsin was removed by aspiration and the cells were incubated in

37°C incubators for 5 mins to promote cell detachment from the flask. Once cells detached, the cell suspension was gently mixed with 10 ml DMEM and collected in a 15 ml tube. Cell pellets were collected by centrifugation (at 1500 rpm and 4°C for 5 mins). Cells pellets were re-suspended in 1ml fresh DMEM media and then equally divided in T75 flasks containing 10-15 ml media. Cells were then grown in CO_2 incubator till 75-90% confluent.

Methods

Radiolabeled ([^{3}H]) dopamine uptake assay

In order to determine if AMPH pre-treatment has an effect on the dopamine uptake and if the effect persists in daughter cells after many cell divisions we performed [^{3}H] dopamine uptake assays.

Uptake Assays in LLC-PK_1 cells

For each experiment a certain number of cells (150,000-300,000) were plated in 24wells/12wells/6wells plates. After 6 or 7 hours once the cells have adhered to the wells, one set of cells were treated with 1μM or 50μM AMPH for 15 hours and the others served as control (without treatment). After 15 hours, the drug was washed off with 1X PBS and after three washes the cells were either taken to measure dopamine uptake after 15 hours of chronic AMPH treatment or they were allowed to grow in fresh media and cross one/two/three cell divisions in order to perform uptake assay in daughter cells. Uptake assay begins with washes with Krebs-Ringer HEPES (KRH) buffer. After removing media from the cells, each well is washed two times with 1 ml of warm KRH buffer. After the washes, KRH buffer containing antioxidants and monoamine oxidase inhibitors- 0.1mMTropolone, 0.1mM Ascorbic Acid and 0.1mM Pargyline

(KRH+TAP), was added to wells to inhibit substrate degradation and the cells were incubated with 20nM [^{3}H] DA for 5 minutes. Following the incubation, the cells were washed with cold KRH+TAP 3 times and lysed with 1%Triton. The lysates were collected in vials and disintegration per minute (DPM) of [^{3}H] dopamine in the lysates was counted using the β-counter.

Uptake Assays in SH-SY5Y cells

The experimental paradigm is similar to the experiments with LLC-PK$_1$ cells where it starts with plating a certain number of cells in 24wells/12wells/6wells plates and treating them with 1μM or 50μM AMPH for 15 hours after 6 or 7 hours once they have adhered to the wells. After 15 hours, the drug was washed off with 1X PBS and after three washes the cells were either taken for uptake assay to analyze dopamine uptake after 15 hours of chronic AMPH treatment or they were allowed to grow in fresh media and cross one/two/three cell divisions, in order perform uptake assay in daughter cells. The experiments with the SH-SY5Y cells involved one extra step where we differentiated the cells with 10 μM RA in low serum media (DMEM containing 1% FBS). After the cells crossed one/two/three cell divisions, they were subjected to RA induced differentiation for 5 days. Cells were treated with 10μM RA twice over 5 days before performing the [^{3}H] dopamine uptake assay. Uptake assay begins with washes with Krebs-Ringer HEPES (KRH) buffer. After removing media from the cells, each well is washed two times with 1 ml of warm KRH buffer. After the washes, KRH buffer containing antioxidants and monoamine oxidase inhibitors- 0.1mMTropolone, 0.1mM Ascorbic Acid and 0.1mM Pargyline (KRH+TAP), was added to wells to inhibit substrate degradation and the cells were incubated with 20nM [^{3}H] dopamine for 5 minutes. Following the incubation, the cells were washed with cold KRH+TAP

3 times and lysed with 1%Triton. The lysates were collected and disintegration per minute (DPM) of [^{3}H] dopamine in the lysates was counted using the β-counter.

Enzyme-linked Immunosorbent Assay (ELISA)

To ensure that AMPH was completely washed out after the 1hour treatment, an ELISA kit was used to measure AMPH concentration in the cells prior to the uptake experiments.

The AMPH Direst ELISA Kit (Abnova, TW) consists of micro-wells coated with polyclonal anti-d-AMPH, and d-AMPH conjugated to horseradish peroxidase (HRP). The principle of the assay is based on the competitive binding of AMPH and AMPH-HRP in proportion to their concentration in the reaction mixture. 10μl of the experimental sample is incubated with 100μl dilution of enzyme (Horseradish peroxidase) labeled d-AMPH derivative in micro-plate wells which are coated with fixed amounts of oriented high affinity purified polyclonal antibody. Since it is a colorimetric assay, after removing the enzyme conjugate and washing the wells, a chromogenic substrate is added. A dilute acid stop solution is added to cease the color produced from the substrate and the absorbance in each well is read at 450 nm. The intensity of the color developed is inversely proportional to the concentration of drug in the sample.

Before the assay cells were first detached with 1X trypsin and collected by centrifugation. Then cells were washed with cold 1X PBS three times and re-suspended in PBS. The samples were then sonicated (5 pulses for 5 seconds and then 10 pulses for 10 seconds) and subjected to centrifugation at 1500Xg (4000 rpm) for 10 mins at 2-8º C to remove cellular debris. Supernatants were collected and stored at -20°C or -80°C to avoid loss of bioactivity and

contamination. When performing the assay, samples were brought to room temperature.

After collecting the samples, the assay was carried out. First 10µL of appropriately diluted standards were added to wells (coated with polyclonal anti-d-amphetamine) in duplicate in a 96-well plate. Then 10µL of cell lysates were added in duplicate in the 96-well plate. 100µL of the AMPH-HRP conjugate was added to each well. To ensure proper mixing the sides of the plate holder was tapped few times. The 96-well plate was incubated for 60 minutes at room temperature in the dark (18-26°C), after addition of enzyme conjugate to the last well. The wells were then washed 6 times with 200-350µL distilled water using either a suitable plate washer or wash bottle.

After each wash the wells were inverted and dried on absorbent paper to ensure all residual moisture is removed. This step is critical to ensure that residual enzyme conjugate does not skew results. After that 100µL of substrate reagent was added to each well and properly mixed by tapping the sides of the plate holder. After adding the substrate reagent, the plate was incubated for 30 minutes at room temperature, preferably in the dark. After 30 minutes 100µL of Stop Solution was added to each well, to change the blue color to yellow. The absorbance was then measured at a wavelength of 450 nm within 1 hour of yellow color development.

Immunoblotting

In parallel to the uptake assays, we also investigated whether chronic exposure to AMPH in progenitor cells alters the amount of DAT in daughter cells. We performed western blots in SH-SY5Y cells to measure the total amount of DAT expressed in cells treated with AMPH with

respect to control cells. As we proceeded with our investigation in the SH-SY5Y cells, we found that the dopamine uptake was completely blocked by NET-specific inhibitor desipramine whereas the DAT specific inhibitor GBR12935 failed to inhibit [^{3}H] dopamine uptake at low concentration (100nm). This suggested NET- mediated dopamine uptake in these cells and also revealed that AMPH can induce its effect via NET. This prompted us to blot for NET protein in daughter cells to determine any possible alteration in expression of NET in AMPH treated cells vs control.

To see the effect of AMPH pretreatment in daughter cells, we allowed AMPH treated cells and control cells to cross one/two/three cell divisions, before differentiating them with 10μM RA for 5 days. Following differentiation, the cells were lysed in 100μl - 400μl RIPA lysis buffer containing protease inhibitors. The lysates were then centrifuged at 14,000 rpm for 10 minutes at 4°C to remove cellular debris. Supernatants were collected and then stored in -20°C. Prior to western blots, the total amount of protein in the supernatants were quantified using BCA Protein Assay Kit. The Thermo Scientific™ Pierce™ BCA Protein Assay is a detergent-compatible formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantitation of total protein.

Western Blots

Lysates of experimental samples were mixed with sample loading buffer (4:1 ratio) containing 5% 2-mercaptoethanol and performed SDS-PAGE in a 12% polyacrylamide gel. Proteins were run in 1X tris-glycine running buffer at 120 Volts for 1.45 hours. Proteins were transferred in 1X

transfer buffer at 4°C and 35 Volts for 1.45 hours to polyvinylidene difluoride (PVDF) membranes. After transfer, membranes were blocked overnight by incubation with 3% bovine serum albumin (BSA) in PBS-T (PBS plus 0.1% tween-20) at 4°C. Subsequent to blocking, membranes were probed for 1 hour at RT with DAT specific goat polyclonal antibody (DAT antibody, C-20) raised against amino acids 601 to 620 of the C-terminus of hDAT or NET specific mouse monoclonal antibody (NET-human, NET17-1) raised against amino acids 17 to 33 of the N-terminus of hNET (1:1000 dilution in 3% BSA/PBS). The bound primary antibodies were detected by incubating the membranes for 1 hour at room temperature with anti-goat or anti-mouse IgG secondary (2°) antibody linked to alkaline phosphatase (1:5000 dilution in 3% BSA/PBS). After each antibody treatment, membranes were washed 5 times with PBS-T. In order to develop the blot, membranes were incubated in 3 ml alkaline phosphatase substrate for 5 minutes. The membranes were sandwiched between plastic film and imaged on Omega Lum™ G Imaging System and quantified using Adobe Photoshop software.

Materials used

Reagents

Phosphate Buffer Saline (1.37 M Sodium chloride, 2.7 mM Potassium chloride, 100mM disodium phosphate, 18mM potassium di-hydrogen phosphate, pH 7.4). Krebs-Ringer HEPES buffer (116 mM Sodium chloride, 4mM Potassium chloride, 1mM Magnesium chloride, 1.8 mM Calcium chloride, 25 mM Glucose, 10mM Hepes, pH 7.4). RIPA lysis buffer (20 mM Tris, 150 mM NaCl, 1mM Na_3VO_4 , 10 mM NaF, 1mM EDTA , 1mM EGTA, 1% Triton, 0.1% SDS, 0.5% deoxycholate, pH-7.4). 1X Transfer buffer (25 mM Tris, 192 mM glycine, 0.1% SDS; 20%

methanol). SDS-PAGE 10X gel running buffer (248 mM Tris, 1.92 M glycine, 1% w/v SDS); the 10X buffer is diluted to 1X for running SDS-PAGE gels.

DMEM and Penicillin/Streptomycin media were from Thermo Fisher Scientific (Waltham, MA, USA); EMEM, G418 sulfate, 1X Trypsin, Antibacterial and Antimycotic and L-Glutamine were from Corning Cellgro (Manassas, VA, USA); FBS was from Atlanta Biologicals (Atlanta, GA, USA); Glucose, AMPH, and anti-mouse IgG 2° antibody linked to alkaline phosphatase were from Sigma Aldrich (St. Louis, MO, USA); DAT specific goat polyclonal antibody raised against amino acids 601 to 620 of the C-terminus of hDAT and anti-goat IgG 2° antibody linked to alkaline phosphatase were from Santa Cruz Biotechnology (Dallas, TX, US); Dimethyl Sulfoxide (DMSO), Sucrose, HEPES, EDTA, Protease Inhibitor Tablets, Tween-20, Triton X-100, Sodium Deoxycholate, Sodium Chloride, Sodium Fluoride, BSA, Sodium Phosphate, Potassium Chloride, Disodium Phosphate, Calcium Chloride, Potassium Dihydrogen Phosphate, SDS, Methanol, Glycine, 2-Mercaptoethanol, Tris-HCl, DTT, and PVDF membranes were from Fisher Scientific (Waltham, MA, USA); Alkaline phosphatase substrate (ImmunStar) was from Bio-Rad (Hercules, CA, USA).

Equipment

Transfected LLC-PK_1 cells and SH-SY5Ycells were maintained in a Nuair 2700-30 water jacketed CO_2 incubator and handled in a Nuair class II type A/B3 class II biological safety cabinet laminar flow hood. The cellular lysates for ELISA and western blots were centrifuged using an Eppendorf micro centrifuge 5424R. Cellular pellets were solubilized using a Bransonic® water bath sonicator. Cell membranes were assayed for protein content using Epoch microplate reader from Biotek. SDS-PAGE and protein transfer to PVDF membranes was

performed using Mini-Protean tetra electrophoresis apparatus and Mini trans-blot electrophoretic transfer cell respectively from Bio-Rad. The power supply used to control both the electrophoresis apparatus and electrophoresis transfer cell was also from Invitrogen. Illuminescence from PVDF membranes were imaged using an Omega Lum™ G Imaging System and quantified using Adobe Photoshop software. All statistical analyses were done using graphpad prism software. Chemical reagents were measured on analytical balance from Ohaus. Disintegration per minute (DPM) of [^{3}H] dopamine in experimental samples were counted using LS 6500 multi-purpose scintillation counter from Beckman.

RESULTS

Chronic AMPH exposure causes long term effect on dopamine uptake in hDAT expressing LLC-PK_1 cells

AMPH is known to cause a down-regualtion of DAT activity, as well as NET and SERT functions. AMPH causes an elevation in synaptic concentration of dopamine by preventing neurotransmitter uptake by competitively binding to DAT and promoting dopamine efflux via reverse activity of the transporter.

Studies have shown that AMPH targets DAT, among many other proteins, to promote elevated dopamine transmission and previous work done in our lab demonstrated that in the long run AMPH treatment reduces dopamine uptake by acting on the transporter in the model organism *C.elegans* . To find out if the effect we observed in *C.elegans* is reproducible in hDAT expressing cells and to focus on the effect of AMPH on hDAT, initial experiments were done in LLC-PK_1 cells stably expressing hDAT.

In our initial experiments with LLC-PK_1, cells were with 1μM or 50 μM AMPH for 15 hours and then dopamine uptake was measured by [^{3}H] dopamine uptake assays. We also measured dopamine uptake following 15 hrs of AMPH treatment after the cells have undergone one, two and three cell divisions.

When treated with 1μM AMPH the results show a significant reduction of 20% in dopamine uptake in treated cells with respect to control after 15 hours of treatment (Figure- 7A). However, this significant decrease in dopamine uptake was not maintained in daughter cells as we saw no significant change in dopamine uptake between AMPH treated cells and control after one, two, and three cell divisions (Figure- 7B, 7C, 7D).

When cells were treated with much higher concentration of 50 μM AMPH, the results show a significant reduction of 35-40% in dopamine uptake in AMPH treated cells with respect to control (cells without treatment) after 15hrs of AMPH treatment (Figure-8A). This effect was transmitted and maintained in daughter cells up to at least three cell divisions where we saw a similar percent reduction (~40%) in dopamine uptake in treated cells (Figure- 8B, 8C, 8D). We used GBR12935 as a selective DAT inhibitor to validate that the dopamine uptake was solely mediated by DAT.

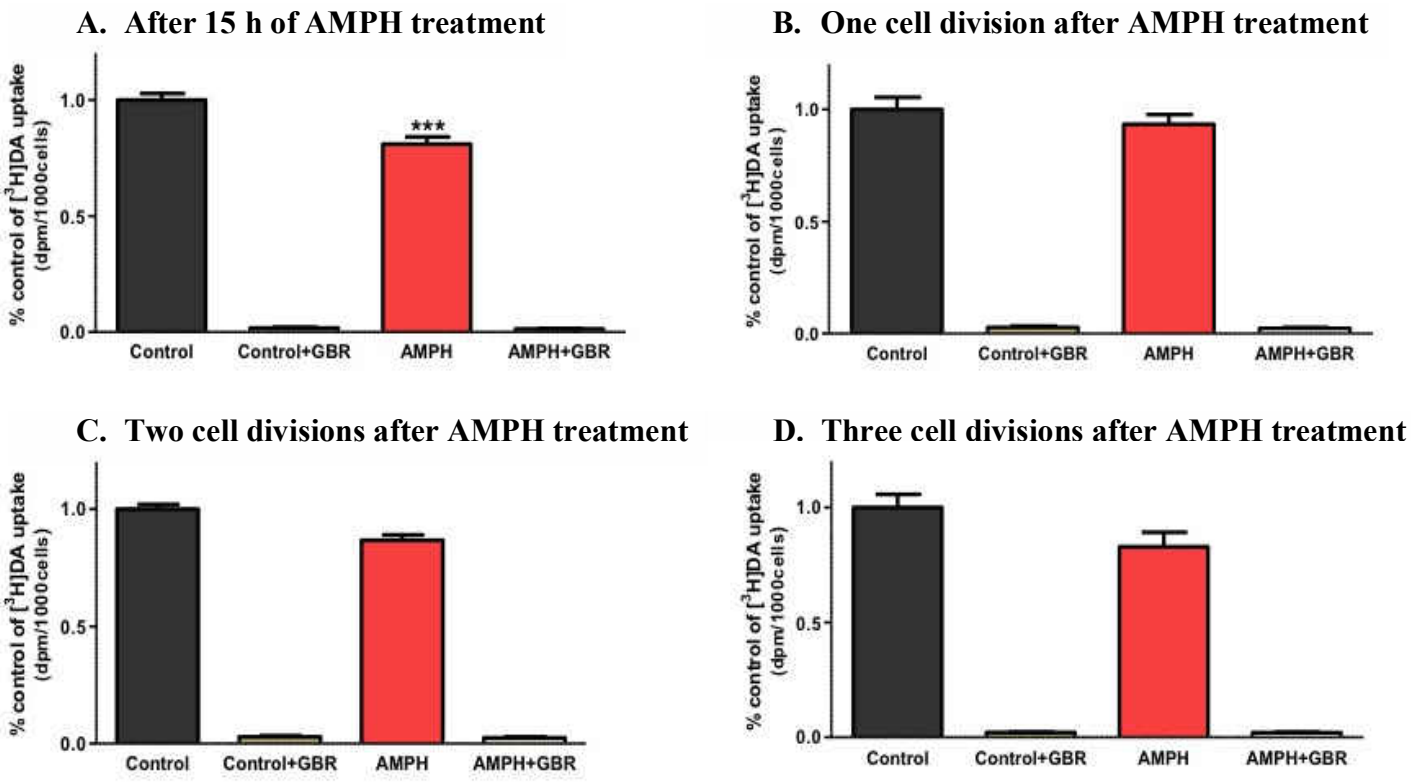


Figure 7: AMPH concentration of 1μM causes reduced dopamine uptake in hDAT expressing LLC-PK_1 cells after (*A*) 15 hrs of treatment but the significant reduction was not maintained after (*B*) one cell division, (*C*) two cell divisions, and (*D*) three cell divisions. 10μM GBR12935 completely blocked dopamine uptake validating the uptake we observed was specific to DAT. Statistical analyses were done by one-way ANOVA and Bonferroni's Multiple Comparison test, $p<0.05$, using Graphpad Prism software (version 5). Histograms represent dopamine uptake expressed as mean± SE of controls set to 100%, where ***= $p<0.001$.

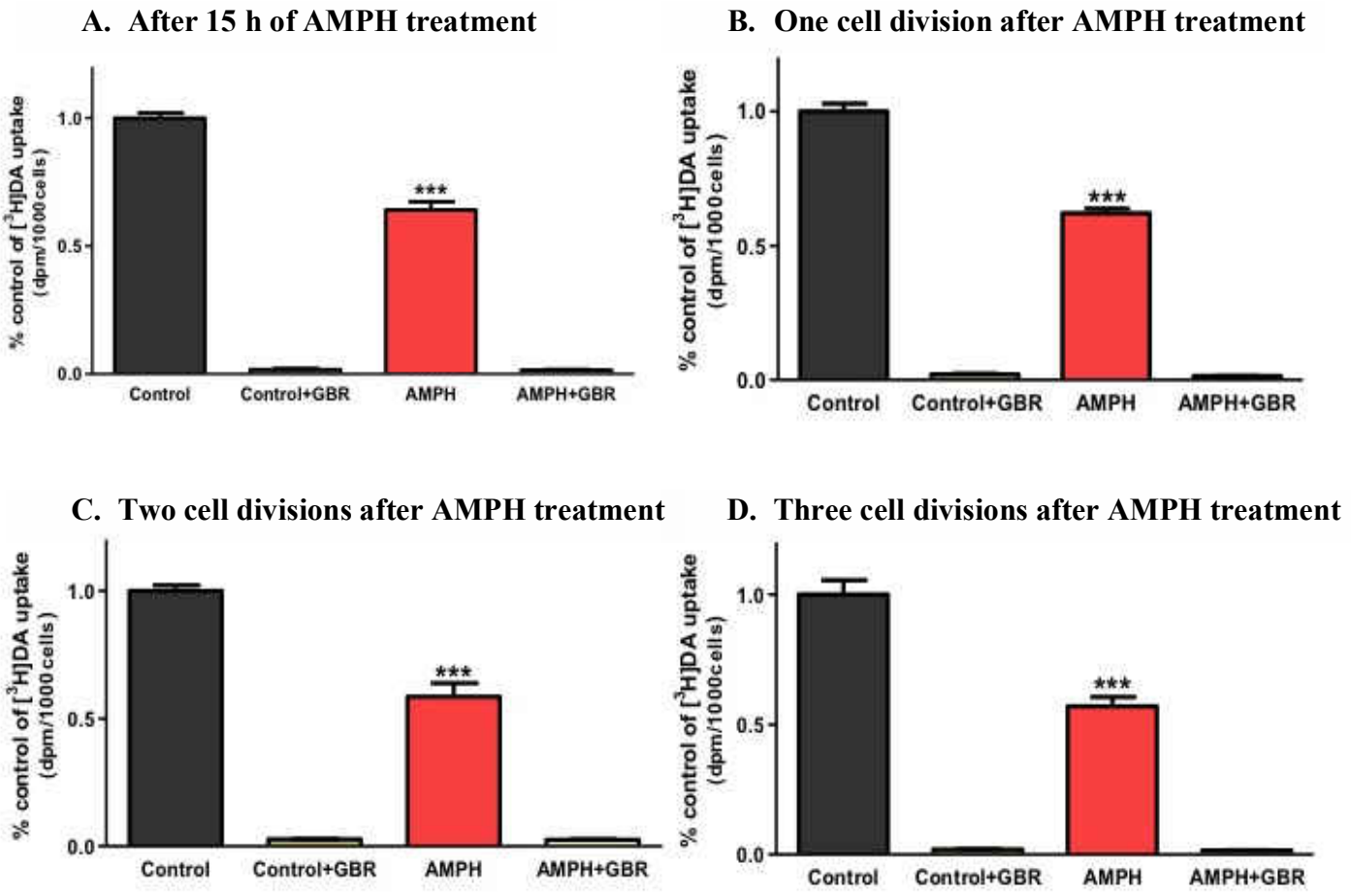


Figure 8: AMPH treatment of 50 μM causes reduced dopamine uptake in hDAT expressing LLC-PK_1 cells after (*A*) 15 hrs of treatment, (*B*) after one cell division, (*C*) after two cell divisions, and (*D*) three cell divisions. 10μM GBR12935 completely blocked dopamine uptake validating the uptake we observed was specific to DAT. Statistical analyses were done by one-way ANOVA and Bonferroni's Multiple Comparison test, $p<0.05$, using Graphpad Prism software (version 5). Histograms represent dopamine uptake expressed as mean± SE of controls set to 100%, where ***= $p<0.001$.

AMPH-induced reduction of dopamine uptake in SH-SY5Y cells is maintained in daughter cells

From our initial experiments in LLC-PK_1 cells, we found that AMPH treatment for 15hrs significantly reduces dopamine uptake in parent cells and this effect was transmitted and conserved up to at least three cell divisions where we saw similar percent reduction in dopamine uptake in daughter cells. These data led us to hypothesize that chronic AMPH treatment causes long term reduction in dopamine uptake by down-regulating the expression or activity of DAT.

To further test our hypothesis, we moved on from a heterologous system artificially overexpressing DAT to a cell line which brought us closer to the physiology of human dopaminergic neurons. In our next approach, we used the human neuroblastoma cell line- SH-SY5Y. SH-SY5Y cells have been used frequently, either in an undifferentiated state, or in a neuron-like differentiated state after induction with retinoic acid (RA). RA treatment has been shown to induce the expression of tyrosine hydroxylase (TH), suggesting a shift towards a DA neurotransmitter phenotype. Based on literatures the neuroblast-like morphology of these cells are not only positive for TH but also for dopamine-β-hydroxylase, the enzyme which is known to catalyze the conversion of dopamine to norepinephrine. Hence, SH-SY5Y has been used as a dopaminergic as well as a noradrenergic model system.

For our experiments, we took advantage of the neuronal characteristics of these cells and the endogenous expression of monoamine transporters (DAT, NET, SERT). We repeated the same experiments using SH-SY5Y to determine if the long term behavioral effects of AMPH we observed in *C.elegans* and in the initial experiments with LLC-PK_1 is reproducible. The experimental paradigm is similar where we pretreated SH-SY5Y cells with 1μM or 50 μM

AMPH or control solution for 15 hours. The cells were then washed three times to remove AMPH and allowed to undergo cell division(s). For one set experiments we performed uptake assays immediately after 15hours of AMPH treatment and for the rest of the experiments, after the cells crossed cell division(s) they were subjected to RA-induced differentiation for 5 days. After 5 days of differentiation [^{3}H] dopamine uptake assays were carried out to measure dopamine uptake in daughter cells.

Uptake assays carried out after 15 hours of chronic 1μM AMPH treatment showed a significant reduction of 24% in dopamine uptake in AMPH treated cells with respect to cells without treatment (control) (Figure-9A) but the effect was not maintained after one, two, and three cell divisions (Figure- 9B, 9C, 9D).This result matched our initial experiments with LLC-PK$_1$ cells. Results from these experiments also revealed NET mediated dopamine uptake in these cells. When 100nM of GBR12935 was used to selectively block DAT, it failed to inhibit dopamine uptake. Since we obtained a robust dopamine uptake in these cells, we tested whether dopamine was reaccumulated via the other monoamine transporter NET, by adding a specific NET inhibitor-desipramine. We found that 100nM of desipramine completely blocked dopamine uptake (Figure 9 and 10) suggesting that the uptake is mostly mediated by NET in SH-SY5Y rather than DAT.

When the same experiments were repeated with 50 μM AMPH treatment for 15 hours, we saw a reduction of 45% in treated cells with respect to control (Figure- 10A). As the goal of these experiments were to determine if the effect of chronic AMPH treatment persists in daughter cells after many cell divisions, we performed uptake assays after one, two and three cell divisions. Our

results show a similar reduction of 45-50% in dopamine uptake in AMPH treated group vs control after cell division(s) (Figure- 10B, 10C, 10D). The complete inhibition of dopamine uptake by desipramine was seen repeatedly in all these experiments, validating that it is a NET mediated uptake and also suggesting that AMPH can induce its effect not only via DAT but also through other monoamine transporters like NET.

While performing the uptake assays, the cell count and cell viability were monitored in AMPH treated group and control to ensure AMPH treatment does not have a toxic effect on cell growth. In every experiment cells were counted prior to uptake assay and results show that there is no significant difference in cell number or viability between treated cells and control (Figure-11) validating that the AMPH induced decreased dopamine uptake we observed was not due to a decrease in cell viability in treated cells.

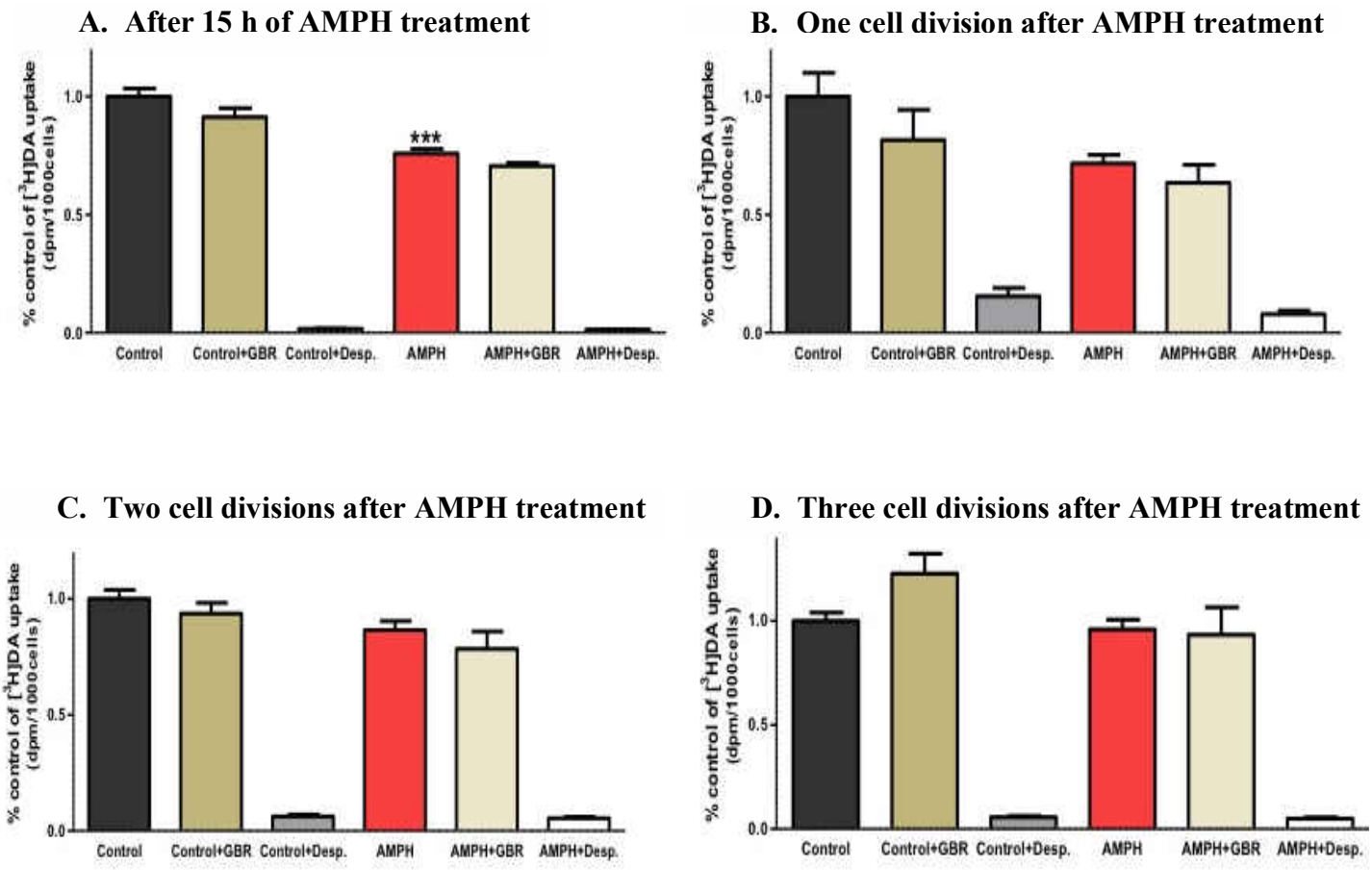


Figure 9: AMPH at a concentration of 1 μM causes reduced dopamine uptake in SH-SY5Y cells (*A*) 15 hrs after treatment but the effect was not maintained in RA differentiated SH-SY5Y cells- after (*B*) one cell division, (*C*) two cell divisions, and (*D*) after three cell divisions. 100nM GBR12935 failed to block dopamine uptake whereas the same concentration of desipramine (Desp.) inhibited the uptake, validating that the dopamine uptake we observed was moreso specific to NET than DAT. Statistical analyses were done by one-way ANOVA and Bonferroni's Multiple Comparison test, $p<0.05$, using Graphpad Prism software (version 5). Histograms represent dopamine uptake expressed as mean± SE of controls set to 100%, where ***= $p<0.001$.

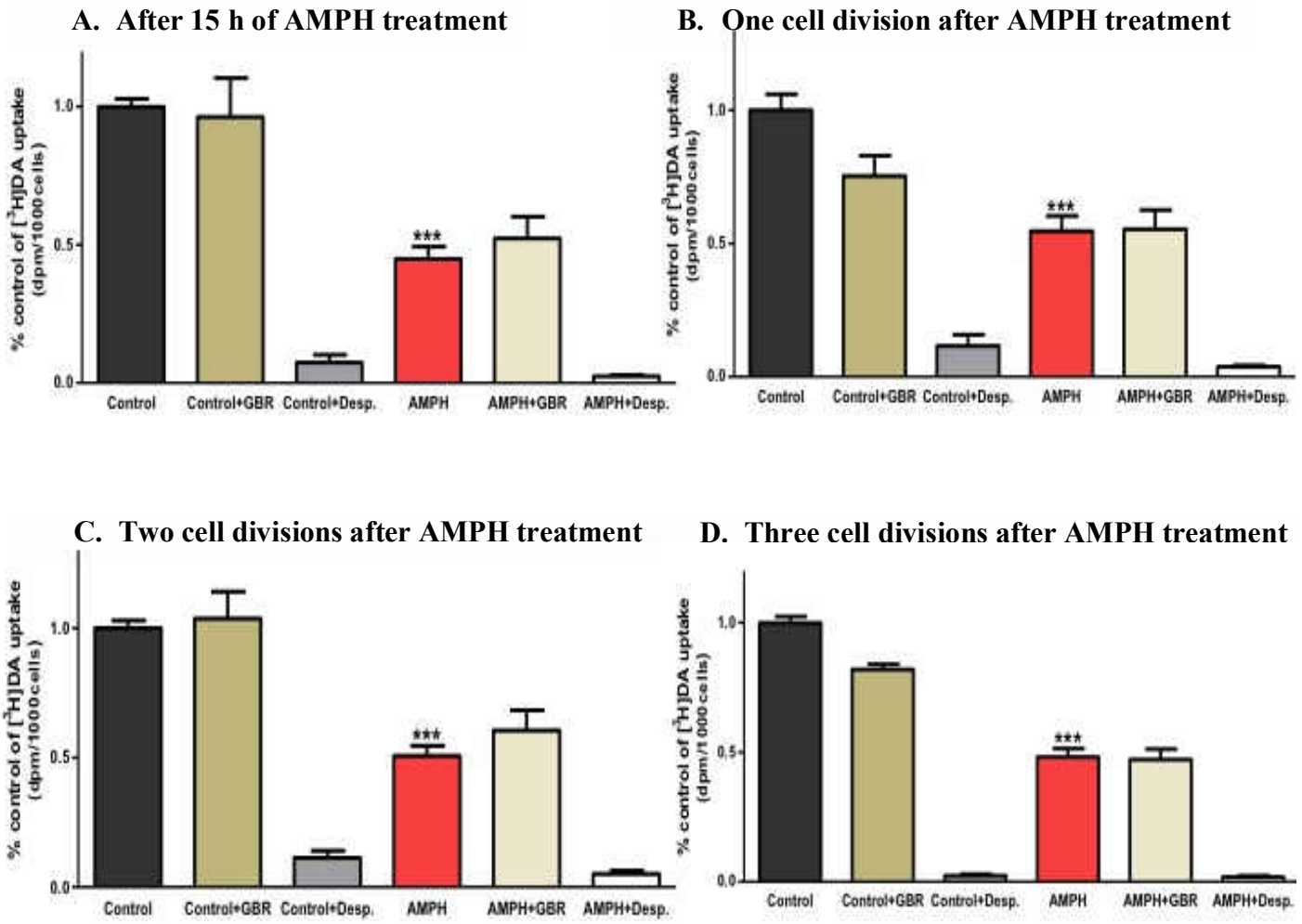
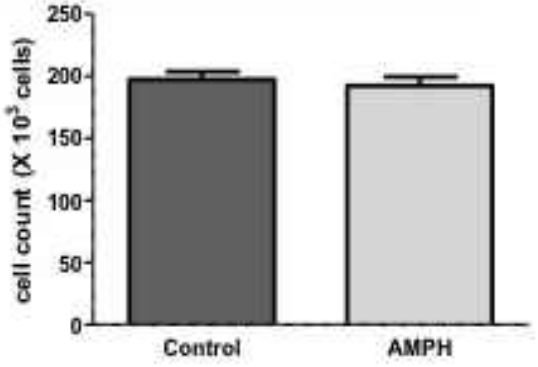
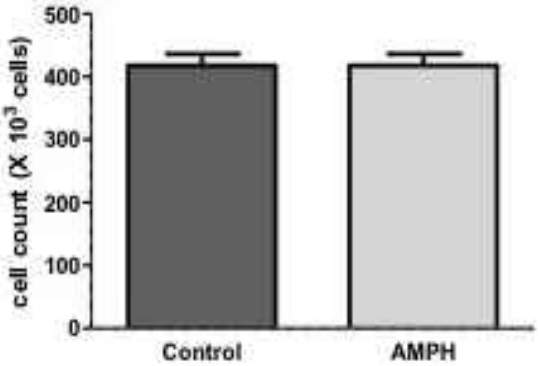


Figure 10: AMPH treatment of 50 μM causes 45-50% reduction in dopamine uptake in SH-SY5Y cells (*A*) 15 hrs after treatment. A similar percentage (45-50%) reduction was observed in RA differentiated SH-SY5Y cells after (*B*) one cell division, (*C*) two cell divisions, and after (*D*) three cell divisions. 100nM GBR12935 failed to block dopamine uptake whereas the same concentration of desipramine (Desp.) inhibited the uptake, validating that the dopamine uptake we observed was moreso specific to NET than DAT. Statistical analyses were done by one-way ANOVA and Bonferroni's Multiple Comparison test, $p<0.05$, using Graphpad Prism software (version 5). Histograms represent dopamine uptake expressed as mean± SE of controls set to 100%, where ***= $p<0.001$.

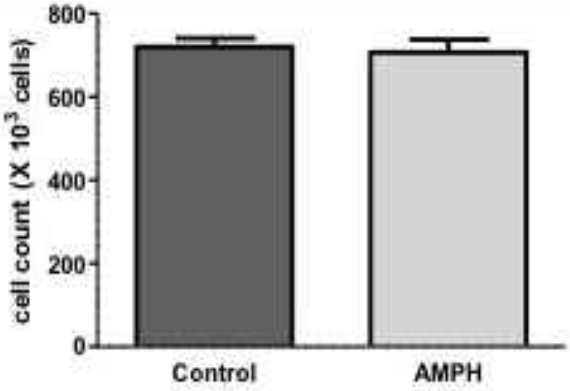
A. After 15 h of AMPH treatment



B. One cell division after AMPH treatment



C. Two cell divisions after AMPH treatment



D. Three cell divisions after AMPH treatment

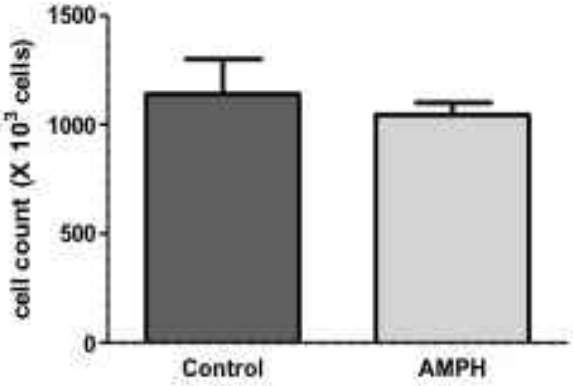


Figure 11: Cell count on the day of the experiments revealed no significant difference in cell number or viability between AMPH treated and control (A, B, C, D). Cell viability was between 99-100% in both groups. Statistical analyses were done by unpaired t-test, $p<0.05$, using Graphpad Prism software (version 5) and there was no significant difference between the two groups.

ELISA to detect AMPH revealed very low concentration of AMPH remaining in the cells prior to the uptake assay

The goal of our project was to establish that the effect of AMPH treatment on the catecholamine transporters is transmitted to daughter cells. In order to ensure that AMPH was completely washed out after 15 hours of treatment and by the time we performed our uptake assay, we used an ELISA kit to measure AMPH concentration in the cells prior to the uptake experiments.

Our results revealed very low levels of AMPH in treated cells which was close to the background concentration we measured in control cells (Table-1). These results validated that the decrease in dopamine uptake we measured in AMPH treated cells was not due to the presence of AMPH during the uptake assays.

Table 1: Very low-levels of AMPH was left over in cells prior to the uptake assays

	Conc.(ng/ml)	Conc. (fM)
Control	**3.03±0.78**	**6.47**
AMPH	**6.51±1.59**	**13.8**

ELISA results showing concentration of AMPH remaining in AMPH treated cells after cell division(s). Initially cells were treated with 50μM AMPH and then the drug was washed off after 15 hours of treatment. Cell lysates for ELISA were collected prior to uptake assays from both groups (treated and untreated). Very low concentration of AMPH was measured by ELISA which was close to the background concentration in untreated cells.

DAT protein expression in daughter cells following chronic AMPH treatment in progenitor cells

We hypothesized that AMPH induces long term reduction in dopamine uptake by down-regulating the activity or expression of DAT. Hence in parallel to the uptake assays done in SH-SY5Y cells, we also investigated whether chronic exposure to AMPH in progenitor cells alters the amount of DAT in daughter cells.

To look at DAT protein expression, we collected cell lysates from SH-SY5Y cells treated with 50 μM AMPH or vehicle for 15 hours before being differentiated with RA for 5 days. Cells were lysed in 100-400μl RIPA buffer and used for western blot analysis to determine DAT protein expression. We immunoblotted against the DAT protein using a DAT specific goat polyclonal antibody raised against amino acids 601 to602 of the C-terminus of hDAT. DAT levels were corrected for loading using Actin as a loading control. We blotted for β-Actin using a mouse monoclonal antibody raised against a recombinant protein corresponding to a region near the C-terminus of β-Actin of human origin. From our results we observed no significant difference in the total amount of DAT expressed in AMPH treated cells with respect to control cells (Figure 12).

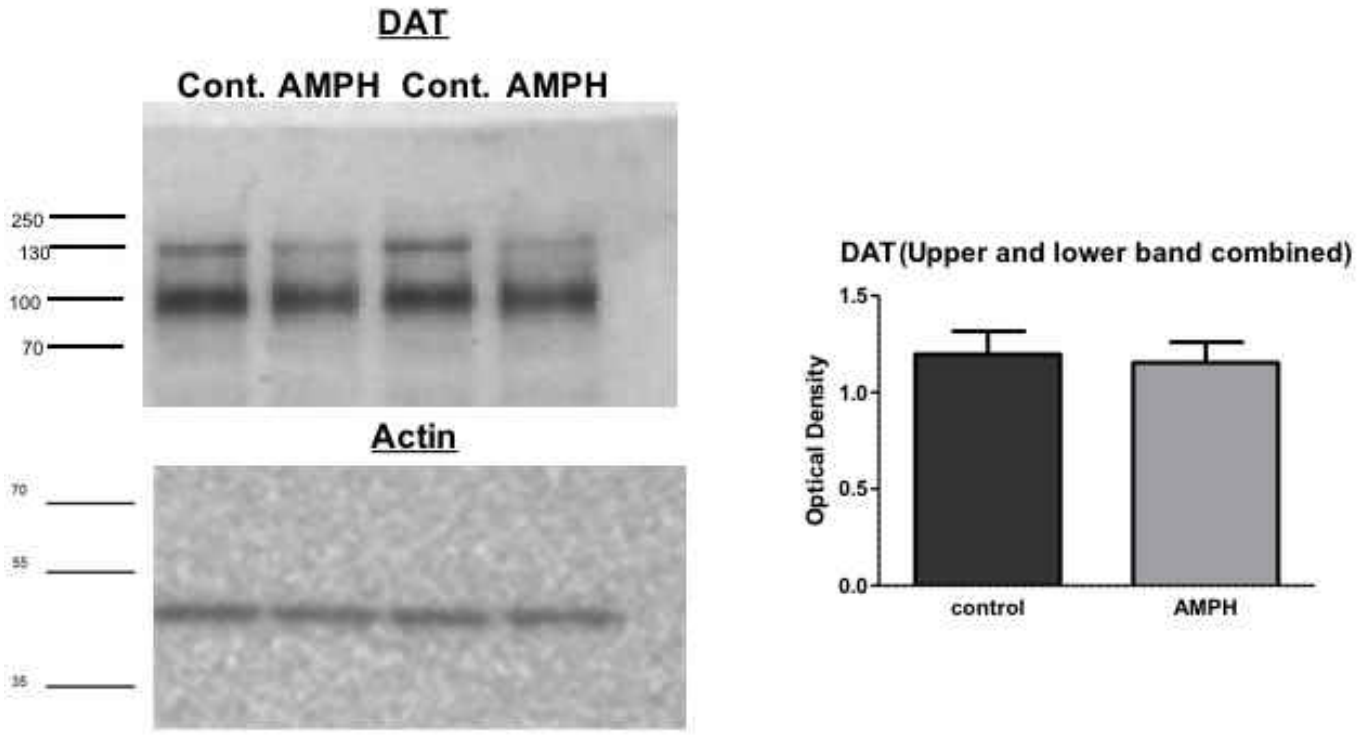


Figure 12: Western blot results showing no significant difference in DAT protein expression between AMPH treated cells and control, following chronic AMPH treatment in progenitor cells. Membranes were probed with DAT specific goat polyclonal antibody (1:1000 dilution in 3% BSA/PBST) and the bound antibodies were detected with anti-goat IgG secondary antibody linked to alkaline phosphatase (1:5000 dilution in 3% BSA/PBST). Statistical analyses were done by unpaired t-test, $p<0.05$, using graphpad prism software

DISCUSSION

The abuse of drugs can be split into categories which include illicit use of prescription drugs or use of illegal drugs that mostly rises from the inability to stop seeking drugs that ultimately leads to drug tolerance or physiological dependence. The possible pleasurable effects achieved from the abuse of psychostimulants are often the reason behind possible incremental increase in drug intake and gradual development of addiction to the substance.

AMPH is widely recognized for its role in treating ADHD, narcolepsy, chronic fatigue depression and several other disorders but despite its important medicinal role, AMPH and its analogues are highly abused illicit drugs all over the world. One of the primary molecular targets of AMPH, and its derivatives like methamphetamine, are the monoamine transporters. AMPH is known to induce excess extra-neuronal dopamine concentration by promoting dopamine efflux[27].

The use of AMPH in ADHD is a long term treatment and people taking AMPH for prolonged periods of time often develop addiction over months and sometimes for years. Popular prescription AMPHs like Adderall are often assumed to be safe as they are prescribed by doctors but abuse of Adderall can lead to an addiction that can be hard to break. Previous studies have illustrated the mechanism of how AMPH works on the monoamine transporters to induce dopamine efflux but very few studies have investigated the long term consequences of AMPH use.

The general interest of our lab is to look at changes happening from drug exposure, in the long term and across generations. Previous work done in the lab has already found that exposing *C.elegans* to AMPH during embryogenesis induces behavioral changes in adult worms. It has been found that in *C.elegans,* an excess of extracellular dopamine results in loss of motility in fluid- a behavioral phenotype termed 'swimming induced paralysis' (SWIP). Using SWIP assay it was shown that adult worms who were exposed to AMPH as embryos show increased SWIP when challenged with AMPH with respect to control groups (animals who never saw the drug). These data suggest that the animals have a memory of the drug exposure during their developmental stage. Even though the worms were only exposed to AMPH as embryos and never saw the the drug again, the exposure during development had a long term effect which resulted in increased SWIP behavior at a much later stage in the worm's life.

Since DAT is one of the major proteins which is targeted by AMPH to induce SWIP in *C.elegans*[28], we investigated the long term effect of AMPH in hDAT expressing cells, to determine if the effect of AMPH on *C.elegans* DAT is reproducible with hDAT. For our initial experiments, we worked with LLC-PK_1 cells which were stably transfected with hDAT. This helped us determine the effect of AMPH on dopamine uptake by the transporter over long term. We treated cells with two different concentrations of AMPH, 1μM and 50 μM, for 15 hours and performed [^{3}H] dopamine uptake assays after cell division(s). We know that for therapeutic purpose AMPH is prescribed in few μM range (1-10μM) but when abused the concentration is much higher. Hence, we chose to investigate AMPH induced effect on dopamine uptake once with a low concentration treatment (1μM) and then with a higher concentration of 50 μM[29]. Our results show a significant decrease in dopamine uptake in treated cells compared to

untreated cells when we performed uptake assay immediately after 15 hours of AMPH treatment. This significant reduction in dopamine uptake was transmitted and maintained in daughter cells after one, two and three cell divisions when 50 μM AMPH was used. These results demonstrated that the long-term effect we observed in *C.elegans* is reproducible in hDAT expressing cells. Since these experiments were done in a heterologous system, artificially over-expressing DAT, the results also helped us verify that AMPH induces a decrease in dopamine uptake by acting on the transporter either by down-regulating DAT expression on the cell surface or by impairing DAT activity to uptake dopamine.

To further test our hypothesis we repeated similar experiments in the human neuroblastoma cells, SH-SY5Y. These cells have an endogenous expression of monoamine transporters (DAT, NET, and SERT) and exhibit neuronal characteristics. The use of SH-SY5Y cells helped us mimic the experiments done in *C.elegans* where AMPH treatment during embryogenesis caused behavioral changes in adult worms. The results suggests that the presence of AMPH during the developmental stage has an effect on the neuronal precursor cells that later develop into dopaminergic neurons in *C.elegans* and we see a consequence of that at a much later stage in the worm's life. In the case of the SH-SY5Y cells, we treated the cells with AMPH before inducing differentiation with RA to determine if AMPH pre-treatment prior to differentiation, when neuronal precursor cells are present, induce a similar decrease in dopamine uptake in the long term.

Our experimental paradigm included treating cells with AMPH for 15 hours and differentiating the cells with RA once they have crossed cell division(s), followed by the uptake assay. Our data

revealed that dopamine uptake in differentiated SH-SY5Y cells were mostly mediated by NET instead of DAT which was significantly reduced in AMPH treated cells with respect to control cells up to three cell divisions. We ensured that the decrease in dopamine uptake in daughter cells was not due to the presence of AMPH during the uptake assays, by performing ELISA against AMPH which displayed very low concentration of AMPH left-over in cells that was close to the background concentration measured in untreated cells. Taken together, these data suggest that 'chronic AMPH treatment causes long term reduction in dopamine uptake via down-regulation of expression or activity of monoamine transporters like DAT and NET'.

With the SH-SY5Y cells we further investigated the expression of total DAT protein in daughter cells following AMPH treatment in progenitor cells and observed no significant difference in the total amount of DAT expressed in treated group with respect to control. This could imply several prospects like – (a)AMPH pre-treatment induces long term decrease in dopamine uptake not by altering the total transporter expression but via altering DAT expression only on the cell surface; (b) AMPH pre-treatment does not alter the number of DAT on the cell surface but down-regulates its capability to uptake dopamine from the synapse; (c) as we found out from the experiments with the SH-SY5Y cells that the dopamine uptake in these cells is dominated by NET, AMPH pre-treatment can decrease NET expression on the cell surface or impair its activity, both of which culminates in decreased dopamine uptake.

CONCLUSION

AMPH is a potent CNS stimulant and is widely known for its addictive properties. Several studies have already shown that chronic AMPH treatment promotes increased monoamine transmission and decreases their uptake into the presynaptic neuron but very few studies have investigated the possibility of this effect to persist in the long term. The unique aspect of our investigation is we were able to show how AMPH induced reduced dopamine uptake in parent cells is transmitted to daughter cells in the absence of the drug. The cells had a memory of the treatment and the effect was maintained up to three cell divisions. Since AMPH is one of the most effective drugs to treat children and adolescents with conditions like ADHD, evidences indicating a long term effect from the use of the drug should be deemed important when considering AMPH's therapeutic use and should be further investigated.

REFERENCES

1. Liu, Z., et al., *Synaptic neurotransmission depression in ventral tegmental dopamine neurons and cannabinoid-associated addictive learning.* PLoS One, 2010. **5**(12): p. e15634.
2. Carr, F., *Neurotransmission: Sticking the brakes on.* Nat Rev Neurosci, 2015. **16**(9): p. 508-9.
3. Cauley, E., et al., *Neurotransmission to parasympathetic cardiac vagal neurons in the brain stem is altered with left ventricular hypertrophy-induced heart failure.* Am J Physiol Heart Circ Physiol, 2015. **309**(8): p. H1281-7.
4. Wakita, M., et al., *Nitrous oxide directly inhibits action potential-dependent neurotransmission from single presynaptic boutons adhering to rat hippocampal CA3 neurons.* Brain Res Bull, 2015. **118**: p. 34-45.
5. Stahl, S.M., *Neurotransmission of cognition, part 2. Selective NRIs are smart drugs: exploiting regionally selective actions on both dopamine and norepinephrine to enhance cognition.* J Clin Psychiatry, 2003. **64**(2): p. 110-1.
6. Cardot, J., *[The monoamines in molluscs. II. Dopamine and neurotransmission. Cardiac dopaminergic innervation in Helix pomatia (author's transl)].* J Physiol (Paris), 1979. **75**(7): p. 715-28.
7. Dajas-Bailador, F.A., et al., *Dopaminergic pharmacology and antioxidant properties of pukateine, a natural product lead for the design of agents increasing dopamine neurotransmission.* Gen Pharmacol, 1999. **32**(3): p. 373-9.

8. Drago, F., et al., *Dopamine neurotransmission in the nucleus accumbens may be involved in oxytocin-enhanced grooming behavior of the rat.* Pharmacol Biochem Behav, 1986. **24**(5): p. 1185-8.

9. Badgaiyan, R.D., *Imaging dopamine neurotransmission in live human brain.* Prog Brain Res, 2014. **211**: p. 165-82.

10. Chaumartin, N., M. Monville, and B. Lachaux, *[Dysphagia or dysphagias during neuroleptic medication?].* Encephale, 2012. **38**(4): p. 351-5.

11. Aggarwal, M., B.I. Hyland, and J.R. Wickens, *Neural control of dopamine neurotransmission: implications for reinforcement learning.* Eur J Neurosci, 2012. **35**(7): p. 1115-23.

12. Geiger, B.M., et al., *Deficits of mesolimbic dopamine neurotransmission in rat dietary obesity.* Neuroscience, 2009. **159**(4): p. 1193-9.

13. Stahl, S.M., *Neurotransmission of cognition, part 1, Dopamine is a hitchhiker in frontal cortex: norepinephrine transporters regulate dopamine.* J Clin Psychiatry, 2003. **64**(1): p. 4-5.

14. Seiden, L.S., *Brain monoamines and behavior.* Psychopharmacol Bull, 1975. **11**(2): p. 60-1.

15. Moron, J.A., et al., *Dopamine uptake through the norepinephrine transporter in brain regions with low levels of the dopamine transporter: evidence from knock-out mouse lines.* J Neurosci, 2002. **22**(2): p. 389-95.

16. Zhu, J. and M.E. Reith, *Role of the dopamine transporter in the action of psychostimulants, nicotine, and other drugs of abuse.* CNS Neurol Disord Drug Targets, 2008. **7**(5): p. 393-409.

17. Moritz, A.E., et al., *Phosphorylation of dopamine transporter serine 7 modulates cocaine analog binding.* J Biol Chem, 2013. **288**(1): p. 20-32.

18. Moritz, A.E., et al., *Reciprocal Phosphorylation and Palmitoylation Control Dopamine Transporter Kinetics.* J Biol Chem, 2015. **290**(48): p. 29095-105.

19. Kollins, S.H. and R.A. Adcock, *ADHD, altered dopamine neurotransmission, and disrupted reinforcement processes: implications for smoking and nicotine dependence.* Prog Neuropsychopharmacol Biol Psychiatry, 2014. **52**: p. 70-8.

20. Zhou, J., *Norepinephrine transporter inhibitors and their therapeutic potential.* Drugs Future, 2004. **29**(12): p. 1235-1244.

21. Hohmann, S., et al., *Association of norepinephrine transporter (NET, SLC6A2) genotype with ADHD-related phenotypes: findings of a longitudinal study from birth to adolescence.* Psychiatry Res, 2015. **226**(2-3): p. 425-33.

22. Pierce, R.C. and V. Kumaresan, *The mesolimbic dopamine system: the final common pathway for the reinforcing effect of drugs of abuse?* Neurosci Biobehav Rev, 2006. **30**(2): p. 215-38.

23. Sulzer, D., S.J. Cragg, and M.E. Rice, *Striatal dopamine neurotransmission: regulation of release and uptake.* Basal Ganglia, 2016. **6**(3): p. 123-148.

24. Sulzer, D., *How addictive drugs disrupt presynaptic dopamine neurotransmission.* Neuron, 2011. **69**(4): p. 628-49.

25. Robertson, S.D., H.J. Matthies, and A. Galli, *A closer look at amphetamine-induced reverse transport and trafficking of the dopamine and norepinephrine transporters.* Mol Neurobiol, 2009. **39**(2): p. 73-80.

26. Korecka, J.A., et al., *Phenotypic characterization of retinoic acid differentiated SH-SY5Y cells by transcriptional profiling.* PLoS One, 2013. **8**(5): p. e63862.

27. Haile, C.N., T.R. Kosten, and T.A. Kosten, *Pharmacogenetic treatments for drug addiction: cocaine, amphetamine and methamphetamine.* Am J Drug Alcohol Abuse, 2009. **35**(3): p. 161-77.

28. Carvelli, L., D.S. Matthies, and A. Galli, *Molecular mechanisms of amphetamine actions in Caenorhabditis elegans.* Mol Pharmacol, 2010. **78**(1): p. 151-6.

29. Berman, S.M., et al., *Potential adverse effects of amphetamine treatment on brain and behavior: a review.* Mol Psychiatry, 2009. **14**(2): p. 123-42.