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AMPHETAMINE EXPOSURE DURING EMBRYOGENESIS LEADS TO LONG-TERM
AND TRANSGENERATIONAL INCREASE IN BEHAVIORAL RESPONSE AND
DECREASE IN DOPAMINE UPTAKE

By

Talus J McCowan
Bachelors of Science, Kansas State University, 2013

A Thesis

Submitted to the Graduate Faculty

of the

University of North Dakota

In partial fulfillment of the requirement

for the degree of

Master of Science

Grand Forks, North Dakota

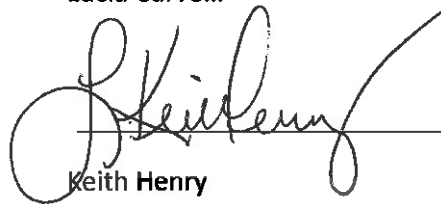
May

2017

This thesis, submitted by Talus J McCowan in partial fulfillment of the requirements for the Degree of Master of Science in Pharmacology, Physiology & Therapeutics 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.



Lucia Carvelli

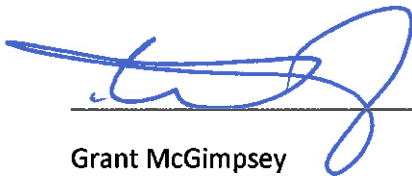


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This thesis is being submitted by the appointed advisory committee as having met all of the requirements of the School of Graduate Studies at the University of North Dakota and is hereby approved.



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Department Pharmacology, Physiology & Therapeutics

Degree Master of Science

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ABBREVIATIONS

- AADC Aromatic amino acid decarboxylase
- ADHD Attention deficient hyperactivity disorder
- AMPH Amphetamine
- BA Butyric acid
- BSA Bovine serum albumin
- *C. elegans* *Caenorhabditis elegans*
- DA Dopamine
- DAT Dopamine transporter
- DMEM Dulbecco's Modified Eagle's Medium
- FBS Fetal bovine serum
- KRH Krebs-Ringer HEPES
- L-15 Leibovitz's L-15 Medium
- L-DOPA L-3,4-dihydroxyphenylalanine
- HDAC Histone deacetylase
- NET Norepinephrine transporter
- PBS Phosphate buffer saline
- PDVF Polyvinylidene fluoride
- Pen Strep Penicillin and Streptomycin solution

- SDS PAGE Sodium dodecyl sulfate polyacrylamide gel electrophoresis
- SERT Serotonin Transporter
- SLC6 Solute carrier 6
- SWIP Swimming induced paralysis
- TH Tyrosine hydroxylase
- VMAT Vesicular monoamine transporter
- VPA Valproic acid
- VTA Ventral tegmental area

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Dedicated to my mom, dad, stepmom, and sisters

ABSTRACT

Amphetamine (AMPH) is widely prescribed for the treatment of ADHD and a highly abused substance in society, yet little is known about the long-term effects of the drug. Here, we used *Caenorhabditis elegans* (*C. elegans*) to establish a model for the long-term and transgenerational effects of AMPH exposure on behavior. Furthermore, experiments were conducted to explore the molecular mechanisms of AMPH that were altered by embryonic AMPH exposure.

C. elegans have a well characterized behavioral response to AMPH known as Swimming Induced Paralysis (SWIP). For the SWIP test, animals are placed in fluid, which normally induces a thrashing behavior. However, in the presence AMPH, the animals display a time- and dose-dependent paralysis. AMPH increases the levels of dopamine in the synapse by causing reverse transport through the protein known as the dopamine transporter (DAT), and the SWIP behavior has been shown to be dependent on dopaminergic transmission. We exposed embryos to either control solution alone (M9 solution) or 500 μ M AMPH dissolved in control solution for 15 hours. 4 days later the SWIP test was performed on young adult animals, revealing that animals previously exposed to AMPH as embryos displayed a higher response to AMPH. The progeny of both groups were tested for SWIP as well. Interestingly, the progeny of the animals exposed to AMPH as embryos showed a higher SWIP response with respect to the progeny of control animals, demonstrating that AMPH had both a long-term and transgenerational effect on the animals.

Because the SWIP behavior was previously shown to be dependent on dopaminergic transmission, we performed DA uptake assays using primary cell cultures made from F1 generation animals to investigate alterations in DATs ability to uptake dopamine. Results from the uptake assays showed that primary cultures made from the progeny of animals exposed to AMPH as embryos had reduced ability to uptake DA with respect to control cultures. To further investigate the reduced uptake ability following AMPH exposure, a human neuroblastoma cell line (SH-SY5Y) was exposed to 15 hour of AMPH, and 5 days later, a DA uptake assay using a concentration response of DA was carried out. Results showed that the cells had a reduced V_{max} with no change to K_m , suggesting a reduced amount of DAT in the cells.

We investigated changes in histone methylation as a mechanism for the long-term and transgenerational effect observed. Histones are proteins, which DNA wraps around to form the nucleosome, and methylation changes on histones can modify the binding of DNA to histones leading to a change in gene expression. Western blots of whole animal protein revealed a decreased level of histone 3 lysine 4 trimethylation (H3K4me3) in the F1 generation of AMPH exposed animals. Additionally, a reduction in the enzymes responsible for H3K4me2 methylation and H3K4me3 demethylation was observed in F1 progeny of AMPH exposed animals. Suggesting that AMPH exposure during embryogenesis alters methylation of specific histone markers.

Taken together, these experiments show that in *C. elegans*, AMPH exposure causes a long-term and transgenerational alteration in behavioral response to AMPH, which correlates to alterations

in DAT uptake ability.

INTRODUCTION

The Neuron and Neurotransmission

The primary cell types of the central nervous system are neurons, electrically excitable cells which communicate from one to another, and the supporting cells known as glia. There are several types of neurons, for instance, motor neurons responsible for communicating movement commands, sensory neurons responsible for signaling from sensory organs, and interneurons responsible for communicating between neurons. These cells typically consist of dendrites that are the primary area for receiving signals, a cell body also referred to as soma, and an axon, which sends electrical signals away from the soma for communication (Figure 1).

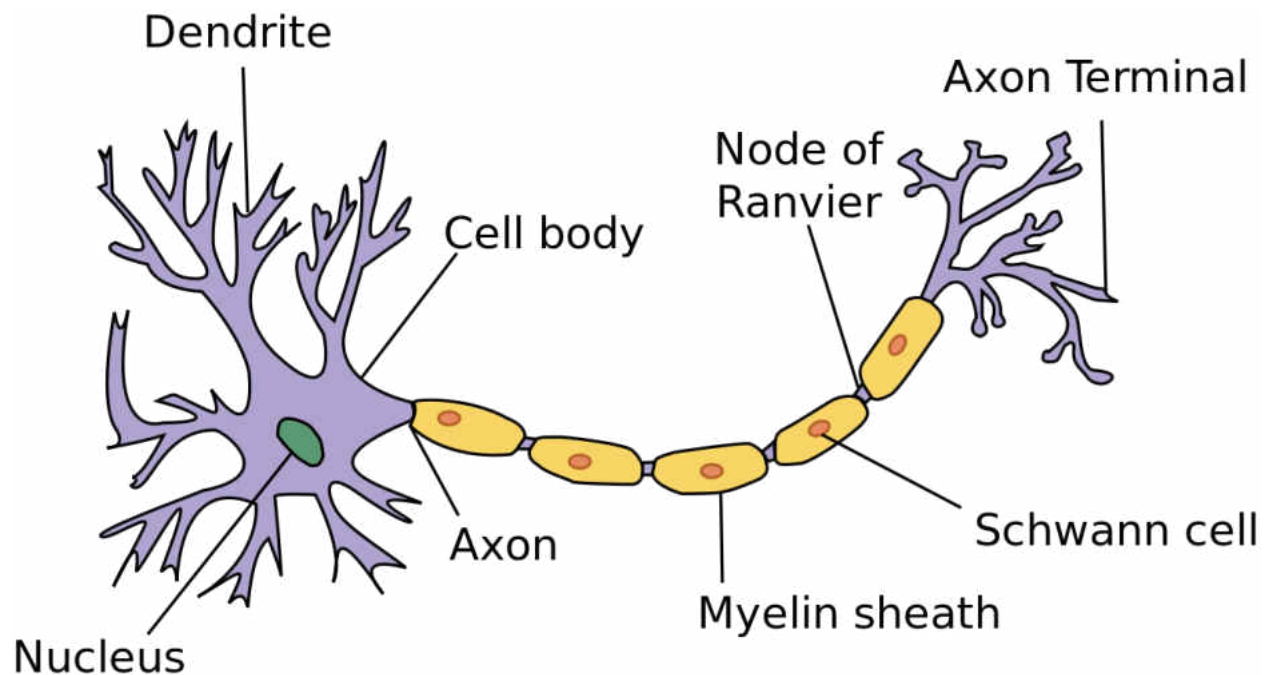


Image courtesy, US National Cancer Institute's Surveillance, Epidemiology and End Results (SEER) Program

Figure 1: Structure of the neuron

Neurons communicate with each other using electro-chemical signaling referred to as action potentials. In order for a neuron to send signals to another neuron they will form a synapse (Figure 2), a gap between the two cells that are very close but do not touch allowing for chemical transmission. These communications occur in a unidirectional manner through action potentials. Neurons at resting state are negatively charged typically around -70mV . This negative charge is established by the action of the sodium-potassium pump (also known as $\text{Na}^+/\text{K}^+-\text{ATPase}$). This protein is an antiporter, meaning it is a transporter, which moves 2 or more molecules in opposite directions. The sodium-potassium pump moves 3 sodium ions outside of the cells while moving 2 potassium ions into the cell, each against their concentration gradient. When the culmination of incoming signal depolarizes the neuron at the beginning of the axon known as the axon hillock, to the threshold potential (normally around -55mV) an action potential is triggered. Once the threshold potential is reached depolarization begins by voltage gated sodium channels located on the axon opening and allowing sodium to move with its concentration gradient into the cell, thus rapidly raising the membrane potential. Following a short period the voltage-gated sodium channels will begin to close, and go through a refractory period in which they cannot re-open which keeps the action potential from moving backwards. Additionally repolarization begins by voltage gated potassium channels opening, at this point the membrane potential is positive which causes the potassium ions to move out of the cell following their voltage and concentration gradients. These positive charged potassium ions moving out of the cells leads to the membrane potential lowering back toward resting potential.

As the action potential moves down the axon of the neurons it eventually reaches the end, known as the terminal button. When the depolarization reaches the button it triggers voltage dependent

calcium channels to open. This allows calcium to enter the cell. Within the button exist lipid vesicles known as synaptic vesicles. Synaptic vesicles have a protein known as vesicular monoamine transporter (VMAT), which transports neurotransmitters into the vesicle following the synthesis of the neurotransmitter. Neurotransmitters are chemicals responsible for transmitting the signal across the synapse. The entrance of calcium into the button begins the process of the synaptic vesicles fusing to the membrane, which releases their content into the synapse. The neurotransmitters released can then diffuse across the synapse to interact with receptors on the postsynaptic cell.

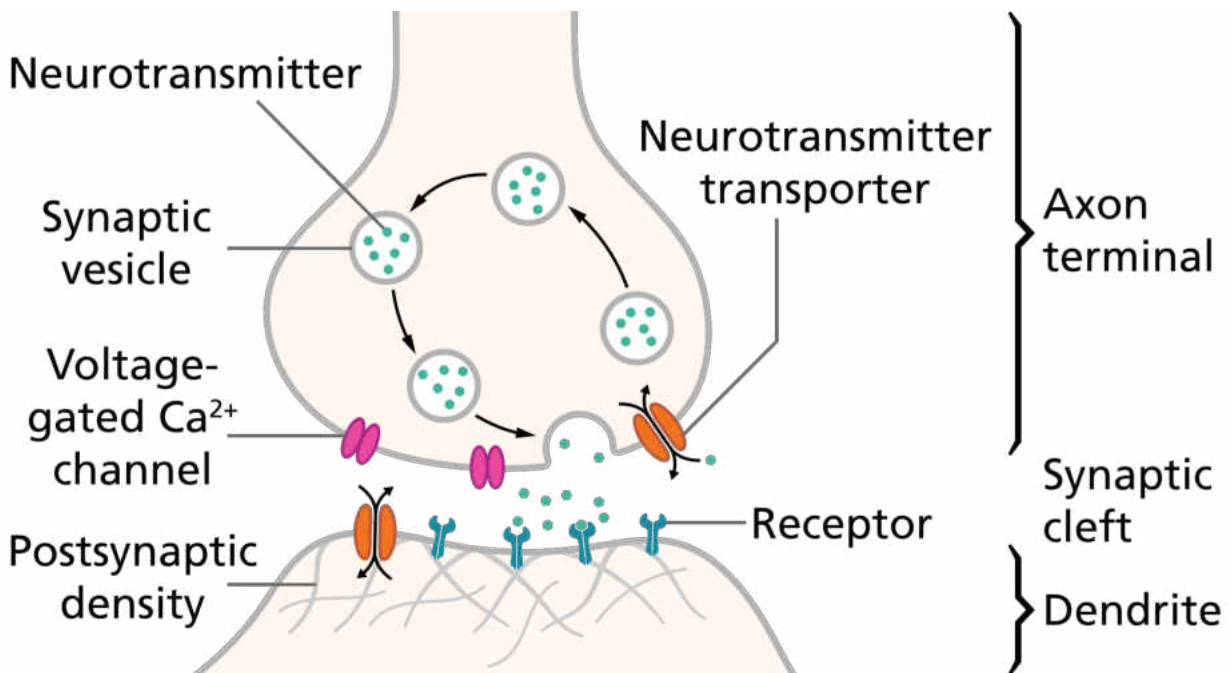


Image courtesy, Thomas Spletstoeser

Figure 2: The synapse

Dopaminergic Transmission

Dopaminergic transmission is significant in a number of physiological mechanisms including, reinforcement of reward, mood, cognition, and fine-tuning of movement. Additionally, alteration of dopaminergic transmission by addictive drugs has been shown to be the major process in the cause of addiction. There are 4 major dopaminergic pathways in the brain, the mesolimbic, mesocortical, nigrostriatal, and tuberoinfundibular.

The nigrostriatal pathway connects the substantia nigra to the caudate nucleus and putamen. This pathway has long been studied for its role in fine-tuning of movement, additionally Parkinson disease and other movement related disorders have been linked to disruption in this pathway [1]. The hypothalamus is connected to the pituitary gland via the tuberoinfundibular pathway. This pathway is important in regulating the release of prolactin [2]. The mesolimbic and mesocortical pathways have long been studied for their role in addiction. The mesolimbic pathway projects from the ventral tegmental area (VTA) to the nucleus accumbens, while the mesocortical pathway projects from the VTA to the prefrontal cortex [3].

Dopamine is synthesized from tyrosine through a two-step enzymatic process (Figure 3). First L-tyrosine is taken up by neurons where it is converted to L-3,4-dihydroxyphenylalanine also known as L-DOPA. The conversion from tyrosine to L-DOPA is carried out by the enzyme tyrosine hydroxylase (TH) and this reaction is the rate-limiting step in process of synthesizing dopamine. Next the enzyme aromatic amino acid decarboxylase (AADC) catalyzes the process of turning L-DOPA into dopamine by removal of a carboxyl group from L-DOPA. Both TH and AADC carry out their reactions to synthesize dopamine within the cytosol, following the

synthesis of dopamine it must then be transported into synaptic vesicles by the protein vesicular monoamine transporter (VMAT) [4].

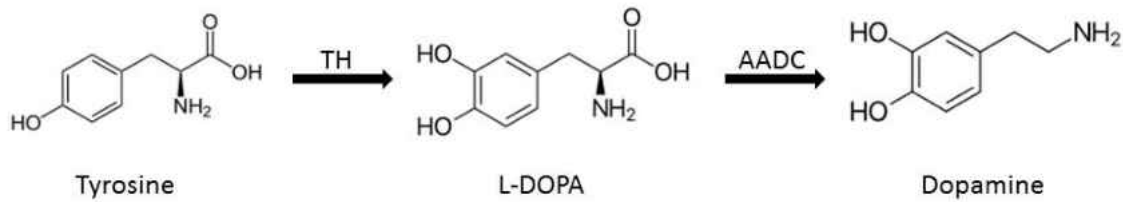


Figure 3: Synthesis of dopamine. L-Tyrosine is converted to L-3,4-dihydroxyphenylalanine (L-DOPA) by Tyrosine Hydroxylase (TH), followed by the conversion to dopamine by aromatic amino acid decarboxylase (AADC).

The dopamine transporter (DAT) is a major protein in the regulation of dopamine levels within the synapse because of its ability to uptake dopamine back into the presynaptic neuron. Once the transporter reuptakes dopamine, the neurotransmitter can be recycled by being repackaged into synaptic vesicles or broken down. DAT has 12 transmembrane domains and is from the SLC6A family of transporters, which also includes the serotonin (SERT) and norepinephrine transporters (NET). DAT acts as a symporter and uses the movement of Na^+ and Cl^- with their gradient to translocate substrate [5, 6]. This is thought to happen through an alternating access mechanism [7] in which the protein switches between inward facing and outward facing conformations, binding substrate in one conformation and releasing that substrate after switching. In addition DAT has been shown to have channel activity as well [8], which may also influence excitability in DA neurons.

Psychostimulant Drugs

Psychostimulant drugs such as AMPH, cocaine, or methylphenidate have a long history for their use as therapeutic agents but are also carry with them the ability to cause addiction making them an important subject of research. AMPH most common therapeutic use is to treat attention deficit hyperactivity disorder (ADHD), although it is also used in the treatment of narcolepsy and obesity. ADHD is characterized by a lack of concentration and shortened attention span, with hyperactive mood. ADHD is commonly diagnosed in children and teenagers with the average age of symptom onset being 7 [9], and typically treated with a single dose of AMPH every day. This method of treatment of ADHD leads to long-term exposure to AMPH, the effects of which have not been heavily studied.

AMPH also has a high potential for abuse especially when used recreationally. Additionally there is no current pharmacological treatment for AMPH addiction [10], psychotherapy currently being the only treatment.

AMPH and cocaine cause dependence by altering dopaminergic transmission, specifically causing an increase in dopamine within the synaptic cleft. DAT blockers such as cocaine bind the transporter in its outward facing conformation and stop the transporter from alternating to the inward-facing conformation [11]. AMPH also can bind DAT and block DA reuptake, however AMPH increases dopamine concentration within the synapse by causing reverse transport through the dopamine transporter [12]. During this reverse transport, AMPH is translocated inside the cell, where it can also cause reverse transport of dopamine through VMAT, emptying the contents of the vesicles into the cytosol [13]. This cytosolic dopamine then continues to gets reverse transported into the synapse by DAT. The dopamine can then act on the post-synaptic

cell and concentrations stay elevated because dopamine is not being re-uptaken by DAT, and levels of dopamine are only limited by diffusion.

Epigenetics Mechanisms of Amphetamine

Epigenetics refers to regulatory mechanisms that alter gene expression without altering DNA sequence. The term means “above the gene” and includes a number of mechanisms, from changes in chromatin structure such as histone modification, to direct methylation of DNA, and interactions with non-coding RNA. Changes in environment, or in the context of these studies exposure to drugs of abuse, can alter many of these mechanisms. The sum total of these epigenetic changes in the cell is known as the epigenome, and more recently studies have begun looking at the effects of epigenomic alterations following drug exposure on short and long-term gene expression changes. While the study of epigenetic modifications following drug exposure is a relatively new field, specific studies on AMPH have begun to show that both acute and chronic exposure leads to epigenetic changes, which affect gene expression (Figure 4) [14].

Histone modifications are one of the most studied epigenetic mechanisms for changes caused by AMPH exposure. Histones are positively charged proteins that form a histone octamer core composed of two copies of H2A, H2B, H3 and H4 proteins, which 147 base pairs of DNA wraps around [15], the DNA and histone proteins together form what is called the nucleosome.

Nucleosomes are condensed into a structure known as chromatin [15]. The N-terminal tails of histone proteins contain residues that can be modified in a number of ways such as methylation, acetylation, phosphorylation, and ubiquitination. These modifications alter the interaction of the histones with the DNA, which can lead to an open chromatin state known as euchromatin, that is

associated with up regulated genes, or a closed chromatin state known as heterochromatin, which is associated with down regulated genes.

The most commonly studied histone modification with relation to AMPH exposure has been acetylation, the loss of which has been related to gene repression. One of the reasons for studies in acetylation being more prevalent than other histone modifications is because of compounds such as butyric acid (BA) and valproic acid (VPA). These drugs are histone deacetylase (HDAC) inhibitors that readily cross the blood brain barrier, thus being valuable in investigating how acetylation changes alter behavior.

Although HDAC inhibitors have been useful in studying how acetylation changes on specific histones affects gene expression, there are discrepancies in the literature as to what effects histone acetylation has on behavior. One group has found that HDAC inhibitors potentiate the behavioral effects of AMPH [16, 17], while a number of studies have shown that HDAC inhibitors reduce or block behavioral changes induced by AMPH [18-22]. These discrepancies could be caused by a number of variables such as animal species, brain tissues investigated, or treatment paradigms.

Alterations in histone methylation have been shown following exposure to drugs of abuse. While less work has been done in this epigenetic mechanism with respect to AMPH, a previous paper has shown that chronic AMPH treatment leads to changes in histone methylation, specifically histone 3 lysine 9 dimethylation (H3K9me₂), at the c-fos gene promoter [23]. c-fos has been

studied highly with its relation to drugs of abuse; it is an immediate early gene that a number of drugs have been shown to increase, including AMPH [24].

c-fos itself has a very interesting interaction with drugs of abuse. c-fos is a member of the FOS family of transcription factors, which includes c-fos, FosB, and Δ FosB. Studies have shown that c-fos has a varied expression depending on if AMPH treatment is acute or chronic. Acute treatment of AMPH leads to an increase in c-fos expression [23, 25, 26], while chronic AMPH treatment leads to a decrease in c-fos expression [23, 25]. However, after chronic treatment with AMPH Δ FosB has been shown to increase [16, 23], and that increase persists even after withdrawal. This has led to speculation that Δ FosB may have a big role in the development of addiction to drugs [27-29]. The above-mentioned epigenetic mechanisms are believed to play a big factor in this molecular switching, as it has been shown that acute AMPH treatment increased H4 acetylation at the c-fos promoter, which fades after chronic AMPH treatments [23].

DNA methylation is also an epigenetic mechanism, which occurs when cytosine or adenine is directly methylated on DNA. This methylation is associated with a decrease in gene expression. Very little research has been done investigating DNA methylation changes after AMPH treatment, although one study did find global DNA methylation increases in nucleus accumbens, orbital frontal cortex, and medial prefrontal cortex following drug withdrawal after 14 days of chronic AMPH treatment [30].

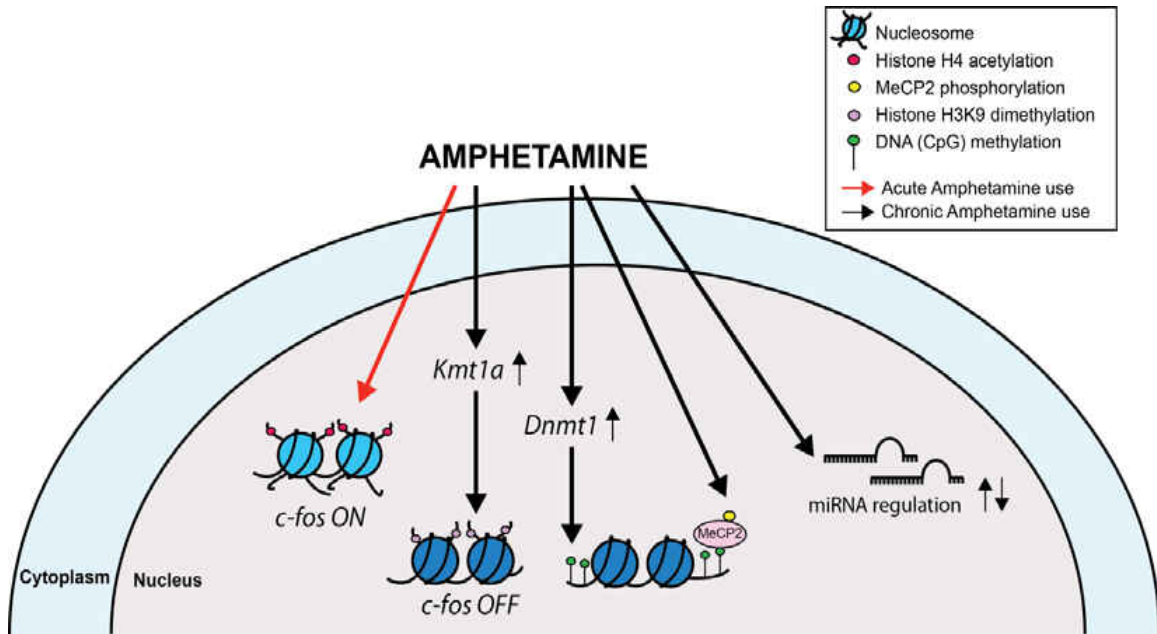


Figure 4: Epigenetic mechanisms altered by AMPH

Caenorhabditis elegans as a Model Organism

Caenorhabditis elegans (*C. elegans*) has long been used as a model organism to study a variety of topics as they provide a number of advantages over other model organisms. *C. elegans* is a hermaphroditic nematode normally found in soil, and feeding on bacteria. *C. elegans* have a relatively short life span, going from embryos to reproducing adults in a matter of 5 days. This makes them ideal in studying long-term and transgenerational effects as studies can be completed in a matter of a week or two, when similar studies in mammals would take months. Because *C. elegans* feed on bacteria and each animal can produce 200-300 embryos, these animals can be grown in large numbers easily on agar plates seeded with bacteria, leading to large sample sizes in studies.

The nervous system in *C. elegans* consists of 302 neurons, 8 of which are dopaminergic neurons. Additionally, the connectome, which maps neurons connected to each other, has been mapped in these animals, thus providing a great resource for studying their nervous system[31]. The endogenous function of dopamine within these animals is to control movement [32]. As the animal moves across a lawn of bacteria they get a release of dopamine, which has a downstream effect of slowing the animal, thus keeping them on the food source for longer.

Previous studies in *C. elegans* have proven them as a valuable resource in studying psychostimulant drugs that act on dopamine release [33-35]. Numerous studies have investigated a now well-characterized behavioral test known as Swimming Induced Paralysis (SWIP) [34] [33, 36-40]. For this behavioral test, the animals are submerged in fluid, where normally they display a thrashing or swimming behavior, but in the presence of a substance that causes dopamine release they paralyze. This paralysis happens in a dose and time dependent manner, and has been attributed to an increase in dopamine within the synaptic cleft [33, 34].

C. elegans are also an excellent model for studying epigenetic mechanisms. The histone proteins of *C. elegans* are highly homologous to histones of mammals [41-43] with a histone 3 and histone 4 being 97% and 98% identical respectively in amino acid sequence to mammalian histones. Due to the close conservation of these histone proteins they are likely to be modified in the same manner [44, 45]. However, *C. elegans* do not methylate DNA similar to mammals. In mammalian cells, the DNA is methylated on the fifth position of cytosine (5mC), which has not been seen in *C. elegans*. One recent study however, has shown evidence of adenine N6 methylation (6mA), which was shown to have transgenerational effects in animal phenotype

[46]. The finding of DNA methylation in *C. elegans* is still very recent and its role needs further research.

EXPERIMENTAL METHODS

Maintenance of *C. elegans*

The *C. elegans* strain N2 was used in all studies. Animals were kept at 20°C and were plated on agar plates seeded with *E. coli* (NA22 strain).

Amphetamine treatment of *C. elegans*

C. elegans embryos were treated overnight for 15 hours in AMPH. Plates full with adult animals containing embryos were washed with water and collected in 15mL tubes. Tubes were spun down at 12,000 rpm and washed several times to clear bacteria. Following the last wash, animals were lysed by adding 2mL of bleach and 0.5mL of 10 N NaOH to the animals, and tubes were filled to 10mL with water. Animals were rocked in this solution for 3 minutes. After the 3 minutes, the tube was filled with egg buffer to stop the reaction, and then washed 3 times with egg buffer. Embryos were separated from debris using a sucrose solution. To do this egg buffer was pulled off from the pellet and 5mL of 60% sucrose was added then filled with water up to 10mL in the tube. The pellet was disrupted and was shaken to mix well and then spun at 12,000 rpm for 6 minutes. Following centrifugation, eggs floated at the solution meniscus and a sterile pipette was used to transfer eggs to a new tube and washed 3 times with water. The pellet was then resuspended in 10mL of M9 solution. 4.975mL of this solution was separated into two new tubes. One tube was used for the M9 control; the other tube had 25µL of 500mM AMPH added for a final concentration of 500µM AMPH. Tubes were then rocked overnight for 15 hours at

room temperature. Following the overnight incubation animals were washed 4 times with water, and then plated on agar plates seeded with *E. coli* and allowed to grow until behavioral tests were performed.

SWIP behavioral test

For this test 10-15 animals are collected with a platinum wire and placed into 40 μ l of 200mOsm sucrose solution with or without 500 μ M AMPH for 10 minutes with the number (%) of paralyzed animals recorded at minute intervals.

Primary cell culture of *C. elegans* embryos

Embryos were treated for 15 hours in either M9 solution or AMPH solution using the same method as for SWIP testing. The embryos were washed and plated following the 15 hours and allowed to grow to adults containing embryos of the F1 generation. The F1 generation embryos were gathered using the same bleach/NaOH procedure as in AMPH treating process. Embryos were separated from debris and washed with water. After last wash, water was removed and embryos were moved to 2 mL tube with 1 mL chitinase solution (1 U per ml). Embryos were incubated in chitinase solution at room temperature on a tube rotator. Incubation was carried out for approximately 1 hour or until 80% of embryos have their eggshell digested. Tubes were centrifuged at 3800 rpm for 3 min. Solution was then aspirated off and 1 mL of L-15 media (Leibovitz's L-15 media with 10% fetal bovine serum, 10 U ml⁻¹ penicillin and 50 μ g ml⁻¹ streptomycin adjusted to 340 \pm 5 mOsm) was used to resuspend the pellet. Repetitive pipetting was used to dissociate the cells by pipetting against the side of the tube 60 times then centrifuged. Solution was again aspirated off and 1 mL of L-15 was added then pipetting was

repeated approximately 30 more times while periodically checking the progress of dissociation. Progress of dissociation was checked by eye and when the majority of embryos were dissociated the tubes were again centrifuged, solution aspirated, and 1 mL of L-15 was added. Next the solution was filtered to remove hatched larvae, clumped cells, or undissociated embryos. 1 mL of L-15 media was put through 5 μm filters into 15 mL tube, followed by .5 mL of cell suspension and 2 mL of L-15. Solution was pushed through filter and filter was washed twice with 2 mL of L-15. Process was repeated with a new filter to filter the rest of the cell suspension. 15 mL tubes with cell suspension were then centrifuged at 3800 rpm for 3 minutes. Following centrifugation solution was aspirated until 5 mL of solution remained and pellet was resuspended. Cell counting was done by taking an aliquot of cell suspension and making a 10x dilution. 10 μL of this dilution was then added to 10 μL of trypan blue and loaded into cell counting slide for Countess Automated Cell Counter (Invitrogen). Following cell counting, cells were plated onto peanut lectin treated coverslips at 2 million cells per plate. The next morning after cells settled an additional 1 mL of L-15 media was added to cells. Peanut lectin treated coverslips were created the day before the procedure. First coverslips were sterilized by placing in ethanol and flaming. Then 400 μL of peanut lectin (.5 mg mL^{-1}) was spread on the coverslip and allowed to incubate for 30 minutes. Peanut lectin was then aspirated off and plates were left under UV light overnight.

DA uptake assays in primary cell culture of *C. elegans* embryos

Uptake assays were carried out 4 days after primary cell preparation. For the uptake assay cells were washed with 1 mL bath solution (145 mM NaCl, 5mM KCl, 1 mM CaCl₂, 5 mM MgCl₂, 10 mM HEPES, 20 mM D-Glucose, pH 7.2, 350 mOsm) 3 times, followed by incubation with

5nM [³H] DA in bath TAP (bath solution with 100μM of each ascorbic acid, trolozone, and pargyline) for 5 minutes with or without 10μM Imipramine present. Cells were then washed 4 times with 1 mL ice cold bath TAP solution. Lastly 500 μL 1% Triton-X was added and allowed to incubate for 10 minutes to lyse cells. Supernatant was collected and 8 mL scintillation cocktail (Research Products International Corp., Econo-Safe Biodegradable Counting Cocktail) was added before counting in scintillation counter (Beckman Coulter LS 6500 multipurpose scintillation counter).

Efflux assays in primary cell culture of *C. elegans* embryos

Efflux assays were carried out 4 days following primary cell preparation, using the same preparation method as for uptake assays. Medium was removed from the cells and washed 3 times using bath solution. 1mL of 5nM [³H]DA diluted in bath TAP solution was then added to cells and allowed to incubate at 30 minutes at room temperatures. To test [³H]DA loading, some dishes were then washed 3 times with bath TAP and lysed with 500μL 1% Triton-X for 10 minutes. The solution was then collected in scintillation vials and 8mL scintillation cocktail was added. All other dishes were washed 3 times with bath TAP solution. Then 1mL of 10μM AMPH diluted in bath TAP solution was added to the dish and allowed to incubate for varying times (1, 5, or 10 minutes). Solution was then collected in scintillation vials, following collection of solution dishes were washed with 1mL of bath TAP and 500μL of 1% Triton-X was added for 10 minutes. After 10 minutes lysed solution was collected in scintillation vials to measure the amount of DA still in cells. 8mL of scintillation cocktail was then added to all vials and counted in scintillation counter.

SH-SY5Y cell culture

SH-SY5Y cells are a human neuroblastoma cell line originally derived from bone marrow [47]. These cells have many characteristics of dopaminergic cells as they have been reported to express the dopamine transporter [48-50], tyrosine hydroxylase[50], and dopamine-beta-hydroxylase[49]. The cells were grown in T75 flasks at 37°C and 5% CO₂ in Dulbecco's Modified Eagle's Medium (DMEM), which was prepared by adding 50% Fetal bovine serum (FBS) and 1% Penicillin and Streptomycin solution in 500mL DMEM. Cells were passaged at 90% confluence. To passage cells they were first washed twice with sterile phosphate buffer saline (PBS). After PBS was aspirated following second wash 2mL of 1X trypsin was added to flask and incubated at 37°C for 5 minutes. Following incubated PBS was used to resuspend the cells and move them into 15mL tubes. Cells were then pelleted by centrifugation at 1500rpm for 5 minutes at 4°C. Two more washes using PBS were done using the same pelleting procedure. After the final wash cells were resuspended in DMEM and passaged into new T75 flasks containing 15mL of DMEM.

DA uptake using SH-SY5Y cell line

For uptake assays using SH-SY5Y cells, cells were detached using the same procedure as for passage and counted using Countess Automated Cell Counter. 150,000 cells were plated per well in 6 well plates with 2mL DMEM. 24 hours later AMPH was added to half the well at 50µM final concentration and given 15 hours to incubate. After AMPH exposure cells were washed twice with 2mL of PBS and then fresh DMEM was added. 5 days later uptake experiments were performed after insuring the cells had crossed a generation.

Uptake assay was carried out similar to the procedure in primary cell culture. Cells were washed with 1mL room temperature Krebs-Ringer HEPES (KRH) buffer 3 times. Then cells were incubated with [³H]DA in KRH TAP (KRH + tropolone, ascorbic acid, and pargyline). The DA was in a 1 part hot ([³H]DA) to 9 parts cold DA mixture, To obtain a final concentration of 1, 10, 50, 100, and 500nM; each concentration done in triplicate. Additionally in duplicates the experiment was performed in wells in which the KRH TAP solution DA was incubated in contain the inhibitor GBR 12935 at 10μM for control of non-specific binding and uptake. Following the 5-minute incubation with DA, wells were washed with 1mL ice-cold KRH TAP buffer 3 times, then lysed with 500μL of 1% Triton-X for 5 minutes. The solution was then collected into scintillation vials and 8mL of scintillation cocktail was added before taking to count in scintillation counter.

Quantitative reverse transcription PCR (RT –qPCR)

Embryos were treated with either M9 (control) or 500μM AMPH in M9 using the same procedure that was used for behavioral analysis. RNA extraction was done on L1 and L4 stage animals. First, animals were collected from plates and washed until no bacteria was present, followed by two washes in nuclease free water. After final wash, animals were pelleted and supernatant was removed, then 1mL of Trizol reagent was added and tube was shook for 90 seconds to lyse animals. Following 90 seconds on shaker tubes were vortexed and allowed to sit at room temperature for 10 minutes, then moved to 4°C for 10 more minutes. Next 200μL of chloroform was added and tubes were shook for 45 seconds followed by 3 minutes at room temperature. Tubes were then centrifuged for 12,000rpm for 15 minutes at 4°C. After centrifugation the aqueous phase was transferred to new 1.5mL tubes and 500μL of isopropanol

was added and mixed by inverting the tubes. Then tubes were incubated 10 minutes at room temperature and centrifuged at 12,000rpm for 10 minutes at 4°C. Solution was then discarded and pellet was allowed to dry. 1mL of 75% ethanol was added to resuspend the pellet and solution was moved to RNeasy column where manufacturer instructions were followed. Column was spun at 12,000rpm for 30 seconds at 18°C, and flow through was discarded. 700µL of RW1 buffer was used to wash column, followed by two washes with 500µL of RPE buffer. Column was then moved to a new collection tube and spun for 2 minutes to dry. Lastly the column was moved to its final collecting tube and 30µL of nuclease free water was added to center and allowed to incubate for 1 minute before final 90 second spin to elute RNA. RNA was run on 1.5% agarose gel after every collection to insure good quality. SYBR Safe DNA Gel Stain was used to image RNA, and NanoDrop 2000c was used to quantify RNA.

Reverse transcriptase reactions were carried out using iScript Reverse Transcription Supermix. Reactions were done by manufacturer's specifications, specifically 100ng of RNA was mixed with 4µL of iScript RT Supermix and nuclease free water was used to reach a final volume of 20µL. Reaction consisted of 3 cycles, priming for 5 minutes at 25°C, reverse transcription for 20 minutes at 46°C, and lastly RT inactivation for 1 minute at 95°C.

Following reverse transcription reaction a preamplification reaction was carried out. This reaction was necessary because without it some genes (specifically DAT) were expressing too low to be reliably quantified during quantitative real-time PCR. SsoAdvanced PreAmp Supermix was used to carry out the preamplification reaction. Manufacturer instruction were followed the reaction, 12.5µL of SsoAdvanced PreAmp Supermix was combined with 6.25µL of a primer

pool made from the same primers used later in the quantitative real-time PCR reaction. Lastly 1.25 μ L of nuclease free water and 5 μ L of cDNA product were added. The reaction was carried out as follows, 3 minutes at 95°C for polymerase activation, 15 seconds at 95°C for denaturation, 4 minutes at 58°C for annealing/extension. The denaturation and annealing/extension were repeated for 12 cycles. A 10X dilution of the cDNA was done after the reaction.

The next day the real-time quantitative PCR was performed. A master mix was made with 5 μ L of SsoAdvanced Universal SYBR Green Supermix, 1 μ L of respective primer, and 2 μ L of nuclease free water, per well. 8 μ L of master mix was added to each well, followed by 2 μ L of 10X diluted cDNA product from PreAmp reaction. Reaction procedure was as follows. 95°C for 3 minutes, followed by 41 cycles of 95°C for 10 seconds and 60°C for 30 seconds. Lastly 98°C for 10 seconds and 65°C for 31 seconds followed by a melt curve from 65°C to 95°C. Data was analyzed using the $2^{-\Delta\Delta C_t}$ method. For this method the average Ct value of actin (control) for each sample was calculated. Then each Ct value for gene of interest was subtracted by the actin Ct value average. The product of that subtraction was then subtracted by the average of the “calibrator” (F0 generation M9 sample). Lastly that value named target-calibrator was taken as 2^x where X represents the target-calibrator value. The final value represents fold change with respect to the “calibrator” in my experiments being the F0 generation M9 sample.

Western blots

Proteins were prepared from L4 stage animals following the same control vs. AMPH treatment protocol used in behavioral testing. Once animals were L4 stage they were collected into 15mL tubes and washed until cleaned of bacteria. Animals were then transferred to 1.5mL tubes and

washed twice with TNET buffer. Following washes with TNET animals were pelleted and put in TNET buffer with protease inhibitors, and then tubes were placed at -80°C for 10 minutes. Tubes were removed from -80°C and put on ice. Sonication of the samples was then carried out using a sonication wand in 10-second intervals 3 times. The wand was washed between each use and tubes were placed back on ice to prevent heating. After sonication was complete the tubes were centrifuged at 12,000rpm for 15 minutes at 4°C . Supernatant was collected after centrifugation into fresh 1.5mL tubes and placed at -80°C for storage.

Protein concentrations were determined by Bradford assay using NanoDrop 2000c with cuvettes. BSA in TNET was used to create a standard curve; each sample was then run to determine concentrations.

The SDS-PAGE procedure was run on gels with a resolving gel of 12% polyacrylamide and a 5% polyacrylamide stacking gel. $20\mu\text{g}$ of protein was run for each sample, and samples were mixed with standard loading buffer consisting of 5% 2-mercaptoethanol and boiled at 95°C for 5 minutes. Gels were run in 1X SDS page running gels at 140 volts for approximately 2 hours. The gels were then transferred to PVDF membranes, in 1X transfer buffer. Transfer was performed at 300mA for 1 hour.

Following transfer membranes were blocked using a 3% BSA in TBST solution for 1 hour. After blocking the membrane primary antibody was applied in a 1:1000 ratio and membrane was allowed to rock overnight at 4°C . The next morning primary antibody was removed and the membrane was moved into secondary antibody at 1:5000 ratio in TBST + 5% skim milk for 1

hour. 6 washes were then performed for 10 minutes each using TBST. Following the last wash membranes were treated with 1.5mL of Immun-Star AP Chemiluminescent Protein Detection Systems for 2 minutes. Membranes were then imaged on Omega Lum G Imaging System, and Photoshop was used for quantifying.

Materials used

Reagents

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% SDS); Phosphate Buffer Saline (1.37 M Sodium chloride, 2.7 mM Potassium chloride, 100mM disodium phosphate, 18mM potassium dihydrogen 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).

Glucose and AMPH were from Sigma Aldrich (St. Louis, MO); Sucrose, Dimethyl Sulfoxide (DMSO), HEPES, EDTA, Protease Inhibitor Tablets, Tween-20, Triton X- 100, BSA, Sodium Phosphate, Potassium Chloride, Sodium Chloride, Disodium Phosphate, Calcium Chloride, Potassium Dihydrogen Phosphate, SDS, Methanol, Glycine, β -Mercaptoethanol, Tris-HCl, and PVDF membranes were from Fisher Scientific (Waltham, MA); Trypsin was from Corning Cellgro (Manassas, VA); FBS was from Atlanta Biologicals (Atlanta, GA); DMEM and Penicillin/Streptomycin were from Thermo Fisher Scientific (Waltham, MA); Alkaline phosphatase substrate (ImmunStar) was from Bio-Rad (Hercules, CA).

Equipment

Disintegration per minute (DPM) and counts per minute (CPM) of [3H] dopamine were determined using LS 6500 multi-purpose scintillation counter from Beckman. Weight measuring of chemical reagents was done on analytical balance from Ohaus. SH-SY5Y cells were maintained in a Nuair 2700-30 water-jacketed CO₂ incubator and splitting, plating and treatment of cells was carried out in Nuair class II type A/B3 class II biological safety cabinet laminar flow hood. Centrifugation was done using an Eppendorf micro centrifuge 5424R and Eppendorf 5810R centrifuge. SDS-PAGE and protein transfer to PVDF membranes during western blots was completed using Mini-Protean tetra electrophoresis apparatus and Mini trans-blot electrophoretic transfer cell from Bio-Rad. Power supply for SDS-PAGE and protein transfer was Bio-Rad powerpac 300. Power supply for RNA gels was Bio-RAD powerpac 3000. Omega LumTM G Imaging system was used for imaging PVDF membranes. Blots were quantified using Adobe Photoshop software. Graphpad Prism software was used for all statistical analyses.

RESULTS

Embryonic exposure to AMPH in *C. elegans* leads to increased behavioral response to AMPH in adult animals and their progeny

Whereas AMPH is a highly prescribed and abused drug, very little research has been done to investigate the long-term effects of the drug as well as any trans-generational effects. *C. elegans* have been previously used to investigate the molecular effects of AMPH on the dopaminergic system due to their unique behavioral response but no studies have investigated the long-term effect of the drug on the animals.

Here *C. elegans* were used to generate a model of both the long-term and trans-generational effects of embryonic AMPH exposure (Figure- 5). *C. elegans* embryos were gathered and were exposed to the M9 solution alone (control) or M9 + 500 μ M AMPH for 15 hours while rocking at room temperature. Following drug exposure all animals were washed and plated on agar plates seeded with *E. coli*, this generation was labeled F0. Three days later, when animals reached young adult stage the behavioral test known as swimming induced paralysis (SWIP) was conducted to investigate their response to AMPH. We found that the animals exposed to AMPH during embryogenesis had a greater response when challenged with 500 μ M of AMPH later as young adults (Figure- 6A).

Some plates of F0 animals were not used for behavioral testing and were allowed to grow to full adulthood when they contained embryos. The embryos of these animals were collected to create the F1 generation using the same procedure as when collecting for AMPH exposure, with the exception that no animals were treated in the F1 generation. All embryos were immediately plated and allowed to grow to the L4 stage. Remarkably even though animals in the F1 generation had no previous contact with AMPH, when challenged with AMPH at the L4 stage the progeny of animals exposed to AMPH as embryos showed higher response to the drug during the SWIP test (Figure- 6B). To our knowledge this is the first demonstration of a trans-generational effect of AMPH.

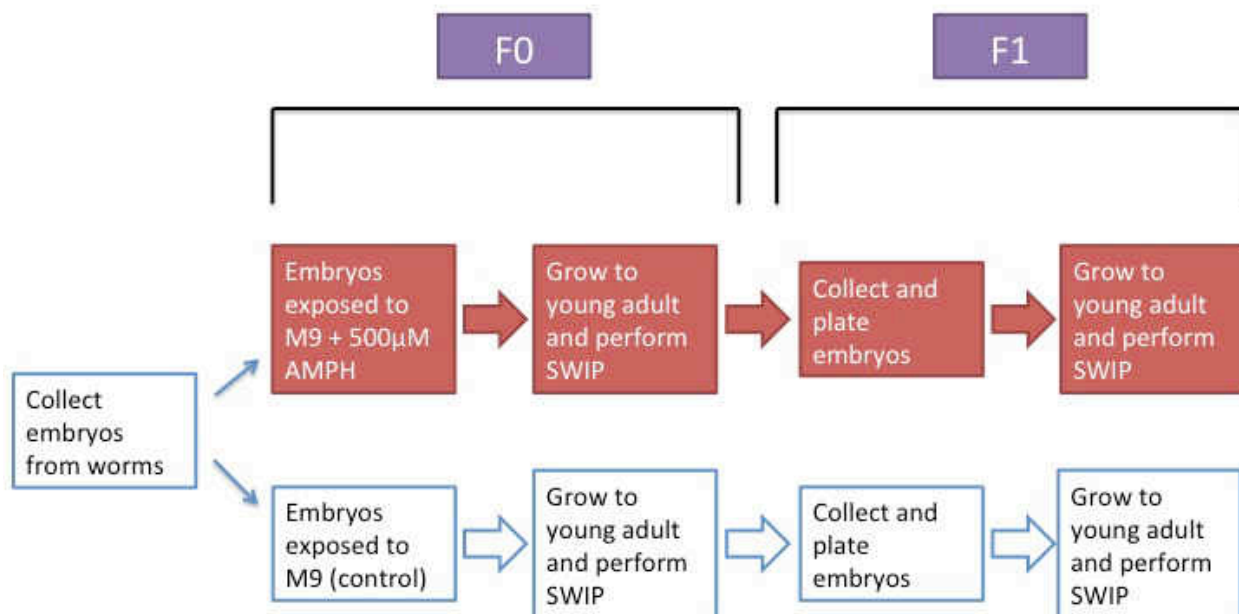


Figure 5: Experimental paradigm for AMPH exposure and SWIP behavioral test

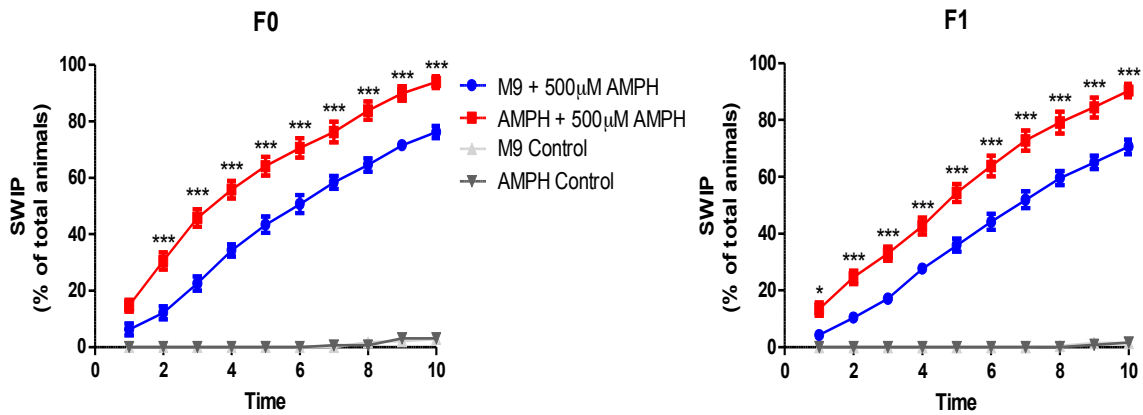


Figure 6: AMPH exposure during embryogenesis leads to increased AMPH induced SWIP both in the generation of exposure as well as their progeny. Animals exposed to AMPH as embryos (red squares) displayed higher SWIP response to AMPH with respect to animals treated with control solution (blue circles) (A). Additionally in the F1 generation (B) the progeny of animals exposed to AMPH (red squares) displayed higher SWIP response to AMPH, with respect to the progeny of animals treated with control solution (blue circles). No difference in SWIP was observed between M9 and AMPH exposed animals that were not challenged with AMPH (light grey triangles and dark grey inverted triangles, respectively). Statistical analysis was two-way ANOVA with Bonferroni's Multiple Comparison test, $P < 0.05$, using Graphpad Prism software version 5.04.

Embryonic AMPH exposure in F0 generation leads to reduced DA uptake in primary cell culture of F1 generation

Previous studies have characterized the mechanisms causing the SWIP behavior in *C. elegans*. These studies have found that an increase in DA within the synaptic cleft leads to paralysis [34], and a mutation that alters the dopamine transporter, D2-like dopamine receptors, or synthesis and packaging of DA, will alter AMPH induced paralysis in the animals [33].

Because previous studies have identified the dopamine transporter as a key player in the SWIP behavior [33, 34, 36], and reverse transporter through the dopamine transporter is how AMPH causes its effects, alterations in the ability of the dopamine transporter to uptake dopamine was explored as a potential mechanism for the altered behavioral response to AMPH. To accomplish this F0 animals were treated with either control (M9) or AMPH using the same procedure as in behavioral testing, except all animals were allowed to grow to adults containing embryos (F1 generation). Embryos were collected to generate a primary cell culture containing all the cells that would have continued on to create the F1 generation animals, including the dopaminergic neurons. After 4 days, when the DA neurons exhibit axon and dendrite processes, DA uptake assays were done using radiolabeled [³H]DA. [³H]DA was applied to the cell cultures for 5 minutes, after which cells were washed and lysed to measure [³H]DA content that was transported into the cells. This assay allows for the investigation of any variation in the F1 animals ability to uptake DA through the dopamine transporter, either through changes in transporter function or total number of transporter. Interestingly, the cell culture that was made from the progeny of F0 generation animals exposed to AMPH as embryos showed a decreased DA uptake of 45.7% with respect to control cultures (Figure 7). This assay indicates that the

previous AMPH exposure in the F0 lead to the F1 generation having a reduced ability to uptake DA. However, the assay does not indicate whether there are fewer transporters at the surface to uptake DA, or if the transporter that is present has a reduced ability to uptake DA. This does however give a potential mechanism by which the progeny of animals treated with AMPH as embryos show an increased SWIP response.

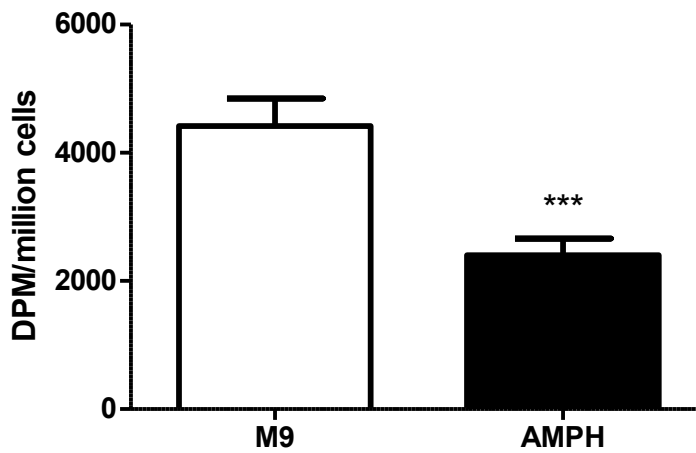


Figure 7: Embryonic AMPH exposure leads to reduced DA uptake in primary cell cultures of F1. DA uptake in primary cell cultures made from embryos of the F1 generation reveal a reduced DA uptake (45.7%) in the cultures made from the progeny of animals exposed to AMPH as embryos (AMPH) with respect to the progeny of animals exposed to control solution (M9). 10 μ M imipramine was used to calculate only specific uptake through DAT. Statistical analysis was students t-test, $p < 0.05$, using Graphpad prism software version 5.04.

DA Efflux is not altered in primary culture following embryonic AMPH exposure

AMPH causes the increase in DA within the synaptic cleft by reverse transport of the dopamine transporter. Because of the reduced uptake ability of F1 cultures following AMPH exposure of the F0 generation, DA efflux from AMPH treatment was investigated. Here primary cultures were made using the same procedure as with the uptake assay, and efflux assays were carried out by incubating the cells with 5nM radiolabeled [³H]DA 30 minutes. To insure there was no difference in [³H] preloading between groups separate dishes were washed and lysed immediately after loading. All other samples were washed out to remove extracellular [³H]DA and 10μM AMPH was applied for 1, 5, or 10 minutes. Following AMPH incubation, extracellular solution was collected and measured for [³H]DA content. Cell dishes were then washed and lysed to check the amount of [³H]DA still within the cells.

The results of the efflux assay demonstrated that there was no difference in efflux caused by AMPH after 1, 5, or 10 minutes of AMPH treatment (Figures- 8A, 8B, 8C, respectively). Additionally after 30 minutes of preincubation with [³H]DA there was no difference in the amount of [³H]DA present in the cell before AMPH treatment (Figure- 8D). This was indicated both by having no difference in loading checked after the 30 minute preincubation (Figure- 8D).

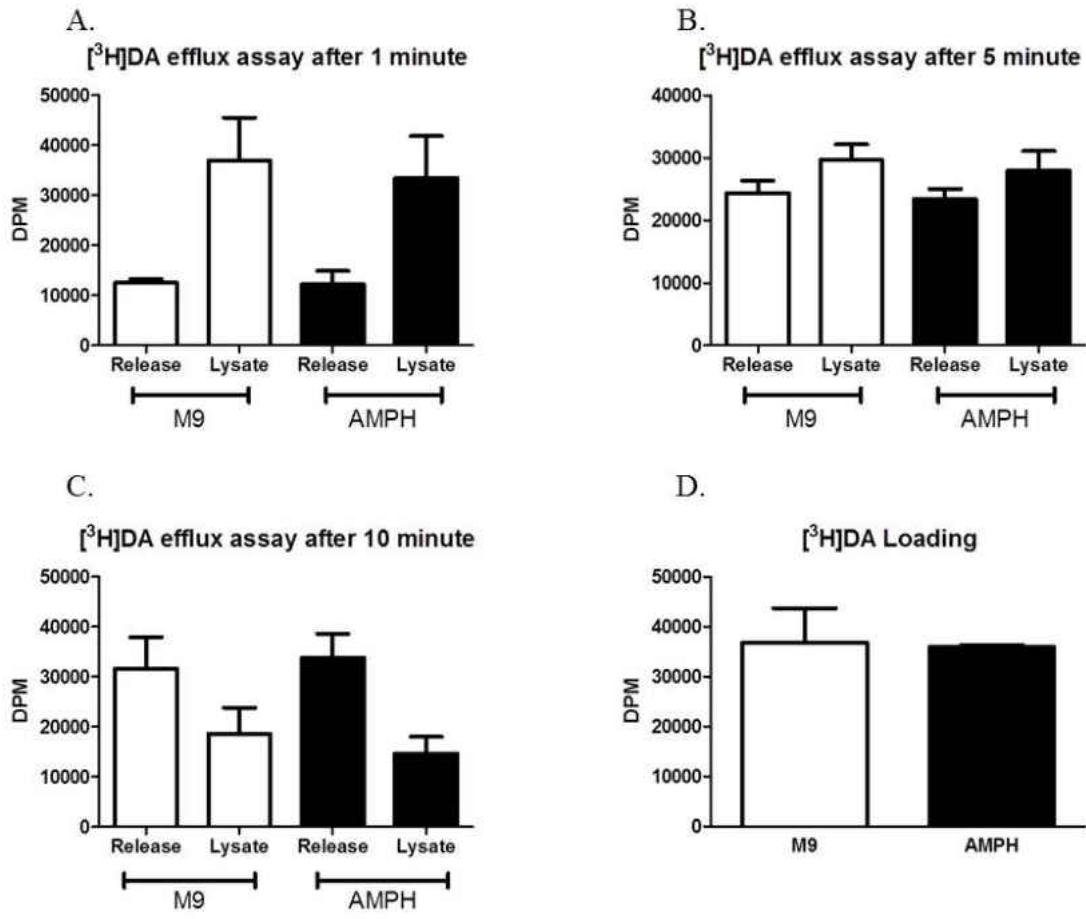


Figure 8: Embryonic AMPH exposure does not alter AMPH induced DA efflux. Efflux assays using primary cell cultures made from the F1 generation progeny of animals either exposed to AMPH (AMPH) as embryos, or control solution (M9) as embryos show there is no difference in efflux after (A) 1, (B) 5, or (C) 10 minutes of AMPH treatment. Additionally at none of the measured time points was there any change in lysate DA concentration, suggesting no difference in total DA loaded before AMPH treatment. To further test DA loaded, DA was also measured following the 30-minute pre-loading period (D). No difference was found between groups.

AMPH exposure reduces uptake in SH-SY5Y cells

Results of the uptake assay in primary cell culture showed that there was a reduced ability of F1 animals to uptake DA. This could be attributed to either a reduced function of the dopamine transporter or a reduced amount of the protein, or both. To further investigate these two possibilities, uptake assays were carried out using a concentration response curve. Unfortunately because *C. elegans* primary cell cultures required a large number of cells to produce a strong signal in [³H]DA uptake assays it has the restriction of providing a limited number of cell dishes to work with, which does not allow for a concentration response assay to be carried out. To circumvent this problem SH-SY5Y cells were used for the uptake. These cells are a human neuroblastoma cell line which has numerous characteristics found in dopaminergic cells. They have been reported to express tyrosine hydroxylase [50], dopamine-beta-hydroxylase [49], and most importantly the dopamine transporter [48-50].

150,000 SH-SY5Y cells were plated in 6 well plates and were exposed to 50μM AMPH for 15 hours, similar to that done in experiments with *C. elegans*. After AMPH exposure cells were washed and allowed to grow for 5 days. Uptake assays were then carried out using a concentration response curve of [³H]DA. Additionally, in some wells the assay was performed in the presence of 10μM of GBR 12935, a compound that blocks DA uptake through DAT. These wells were used to determine non-specific uptake, and was subtracted from total DA uptake.

The results from the concentration response curve showed that previous exposure to AMPH leads to a decreased ability to uptake DA (Figure 9) and is similar to the effect seen in primary cell culture (Figure 7). Michaelis-Menten kinetics were calculated from the concentration

response curve and indicated that previous exposure to AMPH leads to a decrease in V_{max} (0.446 pM/min/1000cells \pm 0.0328 for control, and 0.2596 pM/min/1000cells \pm 0.0280 for AMPH), with no change to K_m (10.23nM \pm 4.261 for control, and 14.37nM \pm 7.933 for AMPH). The Michaelis constant (K_m) is the concentration at which the reaction is at half V_{max} , and a change in this value would indicate an altered apparent affinity of the transporter, however no change was observed indicating that there was no alteration in transporter function. V_{max} represents the maximum rate at which the transporter can move substrate, which was decreased by AMPH exposure. These results suggest that in the SH-SY5Y cells, like in *C. elegans* embryonic cells, the previous AMPH exposure lead to a decreased number of DAT 5 days after AMPH treatment was terminated.

The reported K_m values for dopamine transport by DAT range from 460nM [51] to 2540nM [52]. The K_m values in our experiment for dopamine uptake in SH-SY5Y cells (10.23nM for control, 14.37nM) for AMPH is lower than what has been previously reported. However, the SH-SY5Y is a neuronally derived cell line and, therefore, is likely to express the appropriate DAT-interacting proteins and more closely mirror the regulatory events that occur in dopaminergic neurons. These components may be absent in other cell lines. SH-SY5Y cells have also been shown to have characteristics of noradrenergic cells [53], such as norepinephrine uptake [54], and our lab has previously shown SH-SY5Y cells transport dopamine through the norepinephrine transporter (NET) [55]. Importantly, the concentration of inhibitor used to determine specific uptake (GBR 12935 at 10 μ M) was sufficient to inhibit both the dopamine (K_i = 21.5 nM) and norepinephrine transporters (K_i = 225 nM) [56]. Therefore, the concentration of GBR 12935 used in our SH-SY5Y experiments do not allow for distinction between transport of dopamine by

DAT or NET, thus, our observed K_m value likely represents mixed contributions of dopamine uptake by both the DAT and NET transporters.

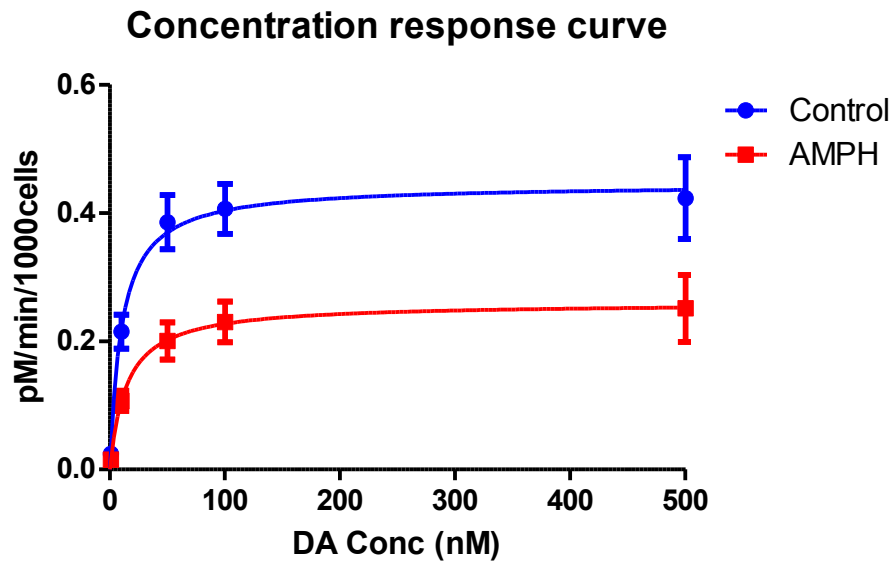


Figure 9: Previous AMPH exposure leads to a reduction in V_{max} in SH-SY5Y cells line. Uptake assay with concentration response curve of DA in SH-SY5Y cell line revealed that previous AMPH exposure leads to decreased DA uptake. Michaelis-Menten kinetics shows a reduced V_{max} ($0.446 \text{ pM/min/1000cells} \pm 0.0328$ for control, and $0.2596 \text{ pM/min/1000cells} \pm 0.0280$ for AMPH) following AMPH exposure, and no change in K_m ($10.23\text{nM} \pm 4.261$ for control, and $14.37\text{nM} \pm 7.933$ for AMPH). $10\mu\text{M}$ of GBR 12935 was used to calculate specific DA uptake through DAT. Michaelis-Menten kinetics were calculated using Graphpad Prism software version 5.04.

Embryonic AMPH exposure leads to decrease DAT RNA in F1 generation

Results from the uptake assays pointed to the hypothesis that chronic AMPH exposure was leading to a decreased expression of DAT. There are no commercial antibodies currently available that target *C. elegans* DAT, thus measuring the protein itself would be very difficult. However measuring the RNA amount of DAT is very practical using quantitative real-time PCR, and could give us a greater insight into alterations in dat expression, with the caveat that RNA expression changes do not directly correlate to changes in protein levels. *C. elegans* embryos were exposed to AMPH from F0 and F1 generations using the same protocol for the SWIP behavioral test. However, when animals reached L4 stage, RNA was collected via Trizol reagent and processed using RNeasy Mini Kit. iScript reverse transcriptase was used to convert RNA to cDNA. After several attempts of RT-qPCR it was discovered that the DAT gene was expressing at too low of a level to be considered reliable when measuring. We hypothesized that the low DAT readings were due to the fact that total RNA from whole animal was collected and DAT is expressed only in 8-dopaminergic neurons out of 1031 total cells. To overcome this problem we performed a PCR reaction after the reverse transcriptase reaction using SsoAdvanced PreAmp Supermix. With this reaction the same primers were used as in the quantitative PCR, thus the genes of interest were specifically amplified to allow a greater signal during the quantitative PCR, allowing for more reliable Ct values. Quantitative PCR was carried out after the preamplification reaction and results were calculated using the $2^{-\Delta\Delta Ct}$ method, with the F0 M9 sample being used as the control sample.

The quantitative PCR demonstrated a decrease in dat-1 expression (32.6%) in the progeny of animals that were exposed to AMPH during embryogenesis (F1 AMPH) with respect to the

progeny of control animals (F1 M9) (Figure- 10). Surprisingly no difference was seen in the expression of *dat-1* in the F0 generation.

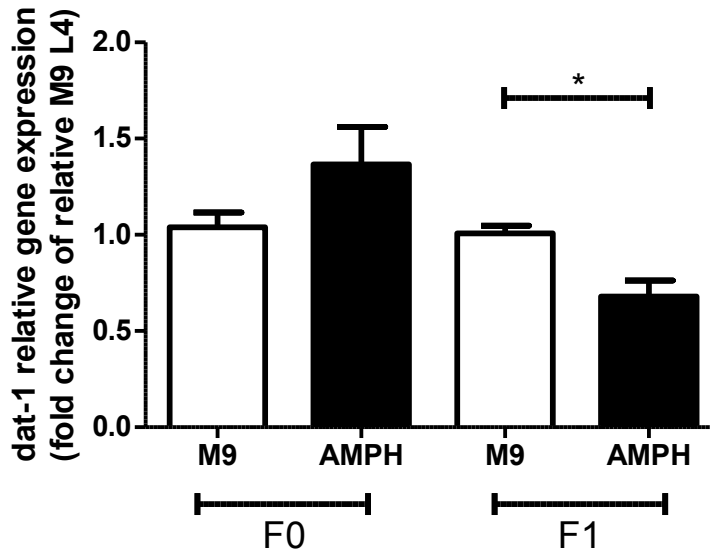


Figure 10: A reduction in *dat-1* RNA is seen in the F1 generation following F0 AMPH exposure. *dat-1* expression measured via quantitative reverse transcription PCR (RT-qPCR). F1 generation had a significant reduction in *dat-1* expression in AMPH group with respect to M9 group. No difference was measured between M9 and AMPH groups within the F0 generation. One-way ANOVA was used for statistical analysis with Bonferroni's Multiple Comparison as post-test, $p < 0.05$, using Graphpad Prism software version 5.04.

Western blots reveal a reduced level of histone 3 lysine 4 trimethylation in the F1 generation

The behavioral experiments along with uptake experiments demonstrate that AMPH exposure has a long-term and transgenerational effect. This means the animals are retaining a cellular memory of the previous exposure and this is in some way being transmitted to the next generation of animals. Epigenetic mechanisms are thought to be the way through which cells can hold a long-term cellular memory of previous exposure to drugs [23, 57, 58]. Moreover, it is believed that epigenetics is responsible for drug exposure having an effect across multiple generations [59, 60]. A number of epigenetic mechanisms could account for this. Histone modifications were chosen for investigating in these experiments, specifically histone methylation. Histone methylation was investigated because of previous implications in both transgenerational inheritance [61] and AMPH effects [23].

Animals were exposed to AMPH using the same procedure as for behavioral experiments. Following AMPH exposure animals were allowed to grow to the L4 stage, the same stage that behavioral testing was carried out, and that RNA samples were collected. At L4 stage whole animals were lysed and protein was collected. Initially, histone modification from whole animal protein lysates were analyzed by western blot for a number of histone markers, which were previously shown to be changed following treatment, and/or to be involved in transgenerational inheritance [61].

Western blot experiments revealed a significant decrease in histone 3 lysine 4 trimethylation (H3K4me3) (6.80%) (Figure- 11A) and histone 3 lysine 9 dimethylation (H3K9me2) (18.74%) (Figure 11B) in the AMPH group of F1 generation with respect to M9 control. No change was

seen in H3K4me3 or H3K9me2 in the F0 generation. Additionally the histone methylation markers H3K9me3 (Figure- 11C), H3K27me3 (Figure 11D), and H3K36me2 (Figure 11E) showed no difference in either F0 or F1 generation.

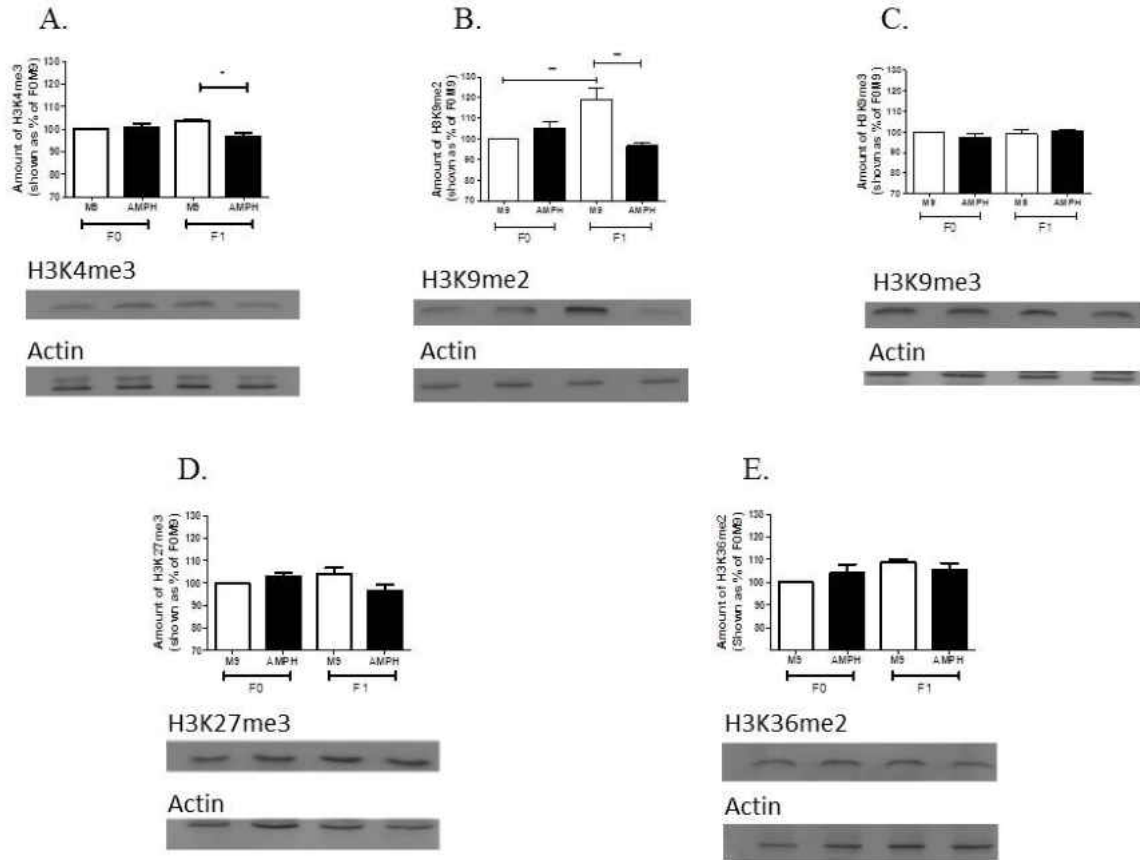


Figure 11: Western blots reveal a reduction in H3K4me3 and H3K9me2 in the F1 generation. Western blots of whole animal protein from L4 animals following AMPH exposure in the F0 generation and their progeny (F1 generation). H3K4me3 was reduced in F1 AMPH with respect to F1 M9 (A), additionally H3K9me2 was reduced in F1 AMPH with respect to F1 M9 and increase in F1 M9 with respect to F0 M9 (B). No change was observed in H3K9me3 (C), H3K27me3 (D), or H3K36me2 (E). Statistical analysis used was one-way ANOVA and Bonferroni's Multiple Comparison test, $p < 0.05$, using Graphpad Prism software version 5.04.

AMPH exposure alters the levels of enzymes responsible for histone methylation

There are a number of enzymes that are responsible for either methylating or demethylating histones. If the levels of these modifying enzymes are altered then the histone methylation status will be changed, thus leading to either increased or decreased gene expression. In addition changes in gene expression, which were correlated with alterations in histone methylation, have been observed even after withdrawal of drugs of abuse [57]. Because a change in H3K4me3 was observed (Figure- 11A) quantitative reverse transcription PCR (RT –qPCR) was used to investigate the levels of enzymes responsible for methylation (set-16) of H3K4me2 and demethylation (rbr-2) of H3K4me3. AMPH exposure was done in the same manner as all other experiments, and RNA was extracted at L4 stage, same as in behavioral experiments. RNA was then converted to cDNA using reverse transcriptase reaction, followed by quantitative PCR to measure expression levels of the two enzymes of interest.

Quantitative PCR showed that both set-16 (Figure- 12A) and rbr-2 enzymes (Figure 12B) had reduced expression in the progeny of AMPH exposed animals (F1 AMPH) (54.1% and 56.9% respectively) with respect to the progeny of control animals (F1 M9). Neither set-16 nor rbr-2 showed any expression changes in the F0 generation.

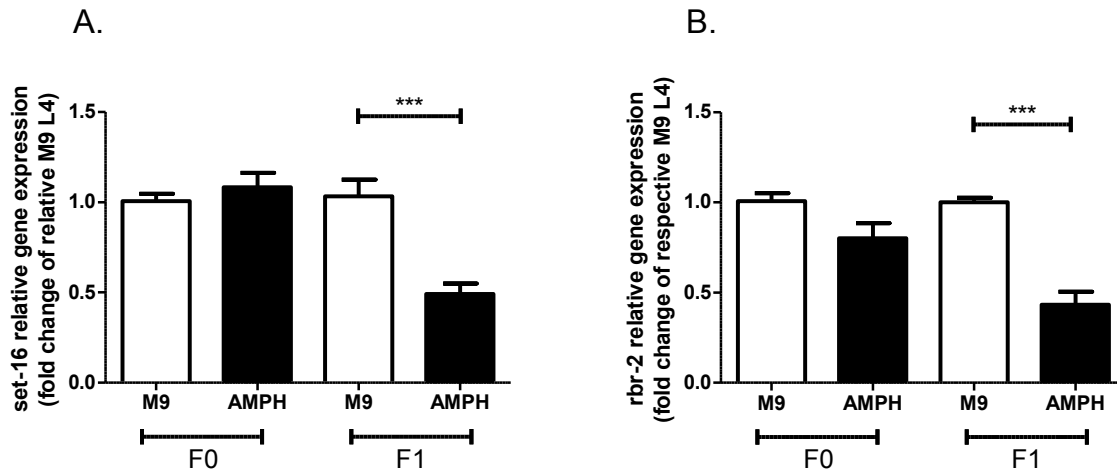


Figure 12: A reduction in RNA levels is observed in F1 of both the methyltransferase set-16 and demethylase rbr-2 enzymes. RNA concentrations of histone modifying enzymes following control or 500 μ M AMPH exposure in animals from F0 generation. In F1 generation set-16 was decreased in expression in F1 AMPH with respect to F1 M9 (A). Additionally rbr-2 expression was decreased in F1 AMPH with respect to F1 M9 (B). Statistical analysis was one-way ANOVA with Bonferroni's Multiple Comparison test, $p < 0.05$, using Graphpad Prism software version 5.04.

DISCUSSION

While numerous studies have focused on the molecular mechanisms of AMPH, there is a large gap in our knowledge of the long-term effects of the drug. Moreover even less research has been done to investigate any transgenerational effects of AMPH exposure. This is alarming as AMPH is widely prescribed for the treatment of ADHD, narcolepsy, and obesity. Moreover, the treatment regimen of AMPH often requires patients to take the drug for prolonged periods of time.

While AMPH can be successfully used to treat many diseases, it also carries with it the potential for abuse. This abuse can stem from either prolonged use to treat disease or from illicit recreational use. There are a number of reasons why people use AMPH recreationally. These include as an appetite suppressant to help in losing weight, for the stimulant properties of AMPH to combat fatigue, or as an aid in studying or tasks requiring an increase focus. The last of these reasons is a major contributor to teenagers or young adults abusing AMPH.

The initial focus of these studies was to investigate if lasting effects of AMPH could be observed and modeled in *C. elegans* behavior. Further, we asked whether any behavioral alterations would be transmitted to future generations. We did in fact find that if animals were exposed to 500 μ M of AMPH for 15 hours during embryogenesis, then washed of drug and allowed to grow to the L4 stage, they displayed a increased sensitivity to AMPH at this later stage. Specifically, the increased sensitivity was in the SWIP test. This behavioral test, in which animals are placed in fluid and paralysis is measured, has been well characterized, and the paralysis displayed can be attributed to increased DA levels within the synapse. For these experiments animals were

challenged with 500 μ M of AMPH in the SWIP test. Animals with previous exposure to AMPH had increased paralysis, thus these animals were either 1) releasing DA at a much faster rate, 2) were unable to clear DA from the synapse as fast, or 3) had an alteration in D2-like receptors. Using additional test cohorts that were not tested in the SWIP test we collected animals for the next generation (F1), and no exposure was conducted with these animals. They were simply plated and allowed to grow to the L4 stage. Once they reached L4 stage, the F1 generation was also challenged with 500 μ M of AMPH in the SWIP test. Surprisingly, the progeny of animals that were exposed to AMPH as embryos also displayed a higher SWIP response when challenged with AMPH, with respect to the progeny of control animals. This result suggests that the F1 generation progeny of AMPH exposed animals were having a larger buildup of DA in the synapse with respect to control animals, or some alteration to the D2-like receptors. These experiments are the first to model an altered behavioral response in a transgenerational manner following AMPH exposure.

Because of previous characterization of the SWIP response [33, 34], we can hypothesize that dopaminergic transmission is altered by the AMPH exposure in F0 animals. It is well known that AMPH exerts its actions through the DAT, thus to begin investigating potential mechanisms for the behavioral alteration we did uptake assays to explore if there was any change in the uptake ability of DAT. To examine this, F0 animals were exposed to 500 μ M of AMPH for 15 hours in exactly the same way as for the behavioral assay. The F1 generation animals however were isolated as embryos and used to create a primary cell culture. The assay revealed that cultures prepared from the progeny of animals exposed to AMPH had reduced [3 H]DA uptake with respect to control cultures. This result provides a potential mechanism by which the increase in

SWIP paralysis is observed. As mentioned above, AMPH exposure leads to increased levels of DA in the synapse, and if the neurons are not as capable of clearing the DA out of the synapse through DAT, then the animals will display increased paralysis. The uptake assay however does not reveal exactly why there is reduced uptake of DA, as this could be attributed to either a reduced amount of DAT total protein, or the number of DAT proteins present at the surface of neurons, or it could be caused by unaltered amount of DAT protein, but with reduced function. Further experiments were needed to reveal why the AMPH exposure lead to reduced uptake.

AMPH is well known to cause release of DA into the synapse by utilizing reverse transport of DA through DAT. Because of this, we hypothesized that AMPH exposure could alter the efflux rate of DA into the synapse in the presence of AMPH, which would then change paralysis rates. The same F0 treatment paradigm and F1 primary cell culture protocol was followed as in the uptake assays. Therefore efflux assays were performed, but they revealed no difference in DA efflux between the 2 groups (control and AMPH exposed animals). Lysates of the cells following release assays were also collected and measured for [³H]DA content. No difference was seen in lysate [³H]DA concentration leading us to believe that there was no difference in the preloading of [³H]DA after 30 minutes. Any difference in preloading could have an effect on the efflux assay but our method suggested that this was not the case. This was surprising as after 5 minutes of the uptake assays, we see a reduced uptake, but after 30 minutes of preloading of [³H]DA we observe no difference. Our current hypothesis is that with the extended time cells have during preloading, an equilibrium is reached, and thus both groups end up with similar intracellular concentrations of [³H]DA.

The uptake assay in primary cell culture showed that AMPH exposure in embryos of F0 generation was having an effect on DA uptake in F1 generation. However, as mentioned it does not indicate if this was due to reduced DAT expression or reduced DAT function. One way to further investigate the alterations in uptake is to perform concentration response experiments using different concentrations of DA. Thus, we used the SH-SY5Y human neuroblastoma cell line, which endogenously expresses DAT [48-50]. The cells were exposed to 50 μ M AMPH for 15 hours and then washed. Cells were allowed to grow and cross a generation, and 5 days later, uptake assays were performed using concentrations of [³H]DA between 1 and 500 nM. The inhibitor GBR 12935 (10 μ M) was used in some wells to inhibit uptake through DAT, allowing for the subtraction of nonspecific background. Similar to the results shown in experiments with *C. elegans*, a decrease in uptake was observed following AMPH exposure. Additionally, when Michaelis-Menten kinetics were calculated from the concentration response curve, a decrease in Vmax was observed in cells previously exposed to AMPH, with no change to Km. The results of this would suggest a decrease in surface DAT but no change in the function of DAT.

To further investigate if the level of DAT was changing following AMPH exposure, quantitative real-time PCR was carried out on animals at the same stage that behavior was tested (L4). The PCR revealed that RNA levels for DAT were decreased in the progeny of animals exposed to AMPH, with respect to control animals. Surprisingly no change was observed in samples from F0 generation. These results agree with results seen in uptake assays in both the *C. elegans* primary cell culture as well as the human SH-SY5Y line, and could explain a mechanism by which we see the increased sensitivity in behavioral testing in the F1 generation. That mechanism being that AMPH exposure decreases the dopaminergic neurons ability to reuptake

DA, thus when AMPH causes increased DA within the synapse the animals are not able to clear the DA as efficiently. This increased synaptic DA in turn explains the increased paralysis. The lack of change in RNA in the F0 generation was unexpected, as the increased behavioral sensitivity is seen in both F0 and F1. To further investigate this uptake ability of the F0 generation would need to be explored. Additionally the RNA levels of DAT could remain unchanged and still have varied levels of DAT at the surface of neurons as the protein is trafficked between the surface and intracellular storage and AMPH has been previously shown to effect this cycle [62, 63].

These experiments have shown that embryonic exposure to AMPH is leading to a transgenerational inheritance in increased behavioral response to AMPH, which uptake assays and qPCR experiments suggest is due to reduced DAT expression in F1 generation. Our hypothesis was that epigenetic changes could be responsible for the inherited changes, and we therefore began by looking at histone methylation as a potential mechanism. Five histone methylation markers were investigated using western blots. The 5 chosen histone modifications, H3K4me3, H3K9me2 and me3, H3K27me3, and H3K36me2 were based on previous research in epigenetics of drug abuse and previous research in transgenerational inheritance [57, 61].

Western blots revealed a significant decrease in histone 3 lysine 4 trimethylation (H3K4me3) in the progeny of AMPH- exposed animals with respect to the progeny of control animals. A significant decrease was also observed in histone 3 lysine 9 dimethylation (H3K9me2) also in F1 AMPH animals with respect to F1 M9 animals, and additionally a significant increase was seen in F1 M9 animals with respect to F0 M9 animals. These results show that even when looking at

whole animal protein lysates, the levels of epigenetic modifications were altered by AMPH exposure.

Because changes in H3K4me3 levels were observed, quantitative real-time PCR was used to investigate if RNA levels of the enzymes responsible for methylating and demethylating H3K4me3 were varied. Our qPCR experiments revealed that both set-16, the enzyme responsible for trimethylation of H3K4, and rbr-2, the enzyme responsible for removing the trimethylation from H3K4, had reduced expression with in the progeny of AMPH exposed animals (F1 AMPH) with respect to the progeny of control animals (F1 M9). These results suggest that remodeling is occurring at the epigenetic level. However we cannot tell from these experiments in which cell types the remodeling is occurring. Additionally, to identify where in the genome histone methylation is altered chromatin immunoprecipitation experiments need to be performed.

Conclusion

AMPH is a psychostimulant that is both highly prescribed and highly abused, while the long-term effect of this is not well understood. Additionally studies in other drugs of abuse are revealing that these substances may have effects that last across generations. Here we developed a model to study the long-term effects of AMPH and we found that chronic AMPH exposure during embryogenesis causes behavioral and functional change in adult animals and progeny. Experiments to reveal the mechanism by which AMPH was causing altered behavioral response to AMPH discovered that AMPH exposure during embryogenesis leads to reduced DA uptake ability within primary cell cultures created from the next generation. Further exploration revealed that reduced DAT expression was responsible for the reduced DA uptake, unveiling a

mechanism by which AMPH was having the transgenerational effect on behavior. Furthermore AMPH exposure was shown to alter histone methylation by acting through specific enzymes. These experiments highlight the importance of continued research on AMPH and its effects on dopaminergic transmission. Additionally consideration of these effects should be taken into account when prescribing AMPH, as this drug is currently the first line of treatment for ADHD.

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