Epigenetic Reprogramming Prompts Hereditable Behavioral and Genetic Changes in *C. elegans*

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Abstract

An animal's response to their external environment is regulated by highly conserved neural pathways. Environmental cues result in epigenetic alterations, or reprogramming, play a role in transgenerational inheritance. *C. elegans* sense external cues through chemosensation and communicates to conspecifics through ascarosides, a pheromone class. One ascaroside, osas#9, has been shown to trigger avoidance response alterations across multiple generations. Here in this paper I show that pre-exposure to osas#9 triggers neurologic responses activating the serotonergic and glutamatergic pathways in adult *C. elegans*. In addition, pre-exposure causes H3K4 demethylation, down regulating transcription activation and avoidance behaviors. Studying how the nervous system senses the environment and the downstream genetic machinery that triggers epigenetic reprogramming is an essential part in understanding what builds us outside of genetics.

Introduction

Environment can trigger heritable genetic changes

Many have debated the prominence of nature vs nurture. Do your genetics determine who you are and how you behave, or does your environment? Research has shown that the answer to this question is not simple; both genetics and environment are influential. Your genes will determine many of your characteristics, both physical and behavioral. Your opinion of foods or scents can be linked directly to your genetics. Taste is caused by molecules binding to receptors on sensory neurons. Whether you like what you are tasting may be determined by the type of receptors you have. For example, 8% of the population tastes soap when eating cilantro. This is caused by a single nucleotide polymorphism (SNP) (Eriksson et al, 2012). Those that do not have this SNP don't taste soap when eating cilantro. Since the genes for these receptors are passed down to you by your parents, taste preference is hereditary.

Environment also influences preferences or behaviors, since many behaviors are taught or learned. For example, bird songs, must be taught. Male zebra finches learn their songs from their father (Porotsky, 2019). Without a tutoring session from 'dad' during a bird's first few days, he wouldn't be able to create an original song in adulthood. Since songs are linked to the mating rituals, the bird would not be able to find a mate. In this sense, mating behaviors are directly linked to environment. A bird might have a healthy brain, capable of creating a song, but if never taught, the bird won't sing (Porotsky, 2019).

In recent years, the genetics vs environment debate has been complicated with the discovery of epigenetics, modifications to DNA outside of the base pair sequence. Changes are to the chromatin and associated proteins affecting expression patterns (Greer et al, 2014). Acetyl or methyl groups will be added to lysine residues on histone proteins, while methyl groups are added directly to the DNA. These molecular additions alter the way the DNA molecule interacts with histone proteins. DNA will either associate to histones more tightly, resulting in a downregulation of genetic expression, or more loosely, resulting in an upregulation (Greer et al, 2014). Epigenetic changes allow cells to differentiate in a developing organism. Every cell in a single organism has the same DNA, but the chemical modifications to the chromatin change which proteins are produced, allowing a heart cell to be structurally different from a liver cell (Francis, 2011).

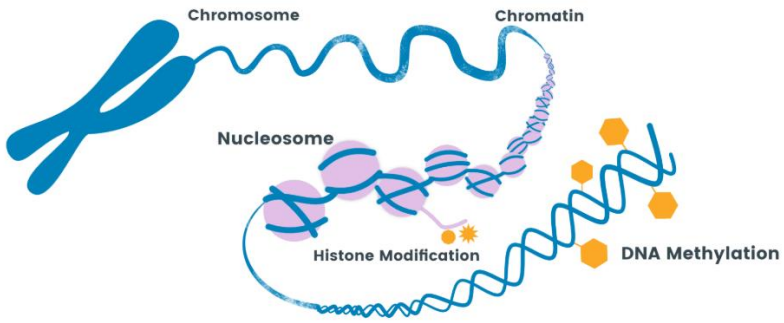

Figure 1: Epigenetic Modifications of Chromatin ("Touchpoint Epigenetics – NUGENIS", 2020)

The “epigenome” is the entirety of all modifications to histones and DNA. Throughout a lifetime, changes accumulate within the epigenome, causing different phenotypes to arise or be hidden. These changes are regulated by multiple site-specific methyltransferases and demethylases (Greer et al, 2014). Massive changes in the epigenome are called epigenetic reprogramming. Interestingly, epigenetic reprograming is triggered by environmental cues. One example of environmentally triggered epigenetic reprogramming occurred during World War II. In 1944, Germany controlled the northwestern region of the Netherlands. As the Allied troops advanced, the German forces destroyed all transportation infrastructure and flooded agricultural lands as they retreated (Francis, 2011). This resulted in food shortages, and the Dutch citizens were subjected to food rationing that only allowed 1,000 calories per day. Fetuses that experienced this famine while still in the womb were severely affected: the birth weights of “famine babies” were lower than babies born before or after the famine (Francis, 2011).

Starvation of the mother directly affected the child; children of the famine were prone to obesity, increased risk of schizophrenia, and depression, along with higher incidences of high blood pressure, heart disease, and adult-onset diabetes (Francis, 2011). The metabolomic changes observed in “famine babies” are believed to be caused by epigenetic reprogramming. This epigenetic

reprogramming was found to be hereditable because the *children* of "famine babies" were *also* prone to obesity despite the fact that children of the "famine babies" had zero-direct contact with the famine.

The Dutch famine epigenetic study is still ongoing- only a few generations have been observed since World War II. Because humans replicate slowly, only time will tell the full extent of the epigenetic reprogramming that occurred while in the womb. However, there is a drive to understand the effects of epigenetic reprogramming.

Nematodes as a model for Epigenetic studies

Due to ethical concerns and humans' slow reproductive rate, model organisms allow researchers to better study epigenetics. Human testing is difficult with a low number of study volunteers. Since epigenetics is a generational concept, it is also difficult to get multiple generations to volunteer and there are long wait times between generations in humans. To circumvent these issues, the nematode, *Caenorhabditis elegans*, can be used as a model organism. *C. elegans* have short life cycles of approximately two days (Brenner, 1974). After an egg hatches the worms will develop through a series of larvae stages -L1, L2, L3, and L4, before they reach adulthood (Figure 1). The roundworm was the first multicellular organisms to have a completely mapped and annotated genome, making genetic studies more feasible (The *C. elegans* Sequencing Consortium, 1998). Additionally, they are self-fertile hermaphrodites, allowing lineages to be easily followed and any changes in behavior traced back to a *single* parent. Although the majority of the population is hermaphroditic, a very small portion of males do exits- 0.1%. Males exist primarily for genetic diversity by mating with hermaphrodites (Narayan et al, 2016).

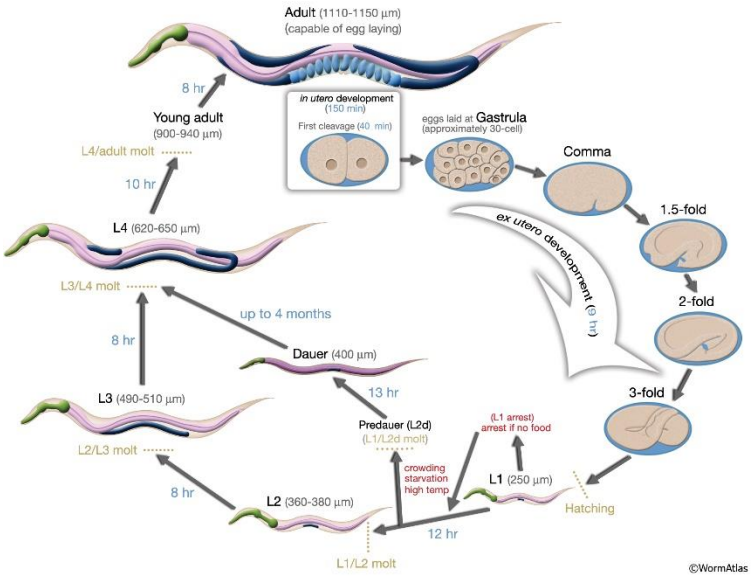

Figure 2: *C. elegans* life cycle. (Worm Atlas)

C. elegans are a prolific a model for the study of epigenetics. Although there is not methylation directly to the DNA, there is methylation to the histone proteins (Schaner & Kelly, 2006), making it a simplified version of the human epigenome. Methylation typically occurs between the 4th and 9th lysine residue on the Histone 3 (H3) tail, in a very similar fashion to human methylation. An addition of two or three methyl groups to the 4th lysine (K4) results in transcriptional activation, while addition of methyl groups to the 9th lysine residue results in transcriptional deactivation (Greer et al, 2014). *C. elegans* utilize SPR-5, a human ortholog of H3K4 demethylase, to remove methyl groups from the 4th lysine group, resulting in transcriptional deactivation. *C. elegans* also utilize the methylases SET-17, SET-30, and SET-26 to add methyl groups to either the 4th or 9th H3 lysine. Environmental cues will activate these enzymes causing changes to the epigenome, furthering behavioral and genetic changes.

A recent study investigated the heritable changes that arise from a hostile/parasitic environment. When worms are exposed to the pathogenic bacteria, *Pseudomonas aeruginosa*, for 24 hours, their adult progeny develop an avoidance response to the bacteria that is not displayed by their parents (Moore et al, 2019). This was an interesting result, as *C. elegans* are usually attracted to *P.*

aeruginosa, even though feeding on the bacteria leads to infection and later death. Although it was too late for the parental generation to survive by displaying avoidance behavior, they passed the information down to their naïve progeny allowing avoidance behaviors to arise. This behavior was regulated by changes in a variety of small (short RNA fragments between 18-30 nucleotides) which target specific genes (Moore et al, 2019). These small RNAs are believed to be involved in small-RNA-mediated gene silencing. This is evidence that environmental stresses, such as pathogenic bacteria, can epigenetically alter gene expression not only by direct modification of the histones, but also by increased expression of small RNA which will indirectly silence the genes.

Similar to the Dutch Famine, starvation results in epigenetic changes in *C. elegans.* Persistent gene expression and small RNA changes in *C. elegans* arose after severe starvation of a population (Rechavi et al, 2014). Starved L1 *C. elegans* enter a developmental arrest stage, called dauer (Figure 2). In this environmentally persistent dauer state, worms stunt their development and go into a period of "non-aging" to extend life. During this stage they are radially constricted, making them visibly smaller in size. They also close their oral orifice to conserve energy (Hu, 2007). During dauer stage there are alterations in the small RNA expressed. Surprisingly, these alterations have been shown to be heritably maintained for three generations (Rechavi et al, 2014).

C. elegans nervous system

As mentioned, *C. elegans* have a completely mapped genome. This includes ~20,000 protein-coding genes that are required for nervous system functions (Hobert, 2013). The nervous system is the most complex system of *C. elegans*. An adult hermaphrodite has 302 neurons which makes up just over 35% of somatic cells (Hobert, 2005). All connections between these neurons are mapped, creating a complete connectome for *C. elegans*. These neurons can be divided into three categories- sensory neurons, interneurons, and motor neurons (Hobert, 2005).

Most sensory neurons make up *C. elegans* chemosensory system (Bargmann, 2006). *C. elegans* utilize chemosensation for a variety of different behaviors, including communication between animals and searching for food. The chemosensory neurons are located at the head, or amphid, of the animal, with their cilia exposed to the environment through openings (Bargmann, 2006) (Figure 3). Some examples of sensory neurons include: the ASI neuron, which senses chemicals which trigger dauer formations, and the ASH neuron, which sense chemicals that trigger avoidance responses. At the tips of these cilia are numerous chemosensory receptors, which will sense chemicals from the surrounding environment. These chemoreceptors are typically G-protein coupled receptors (GPCRs), which trigger a signal transduction within the sensory neuron (Bargmann, 2006). Even though the receptors are almost exclusively localized to the cilia, signal transduction allows response to chemical stimulus to penetrate into the cell body. Once the signal reaches the cell body, an action potential may fire, and the signal will be passed on to interneurons.

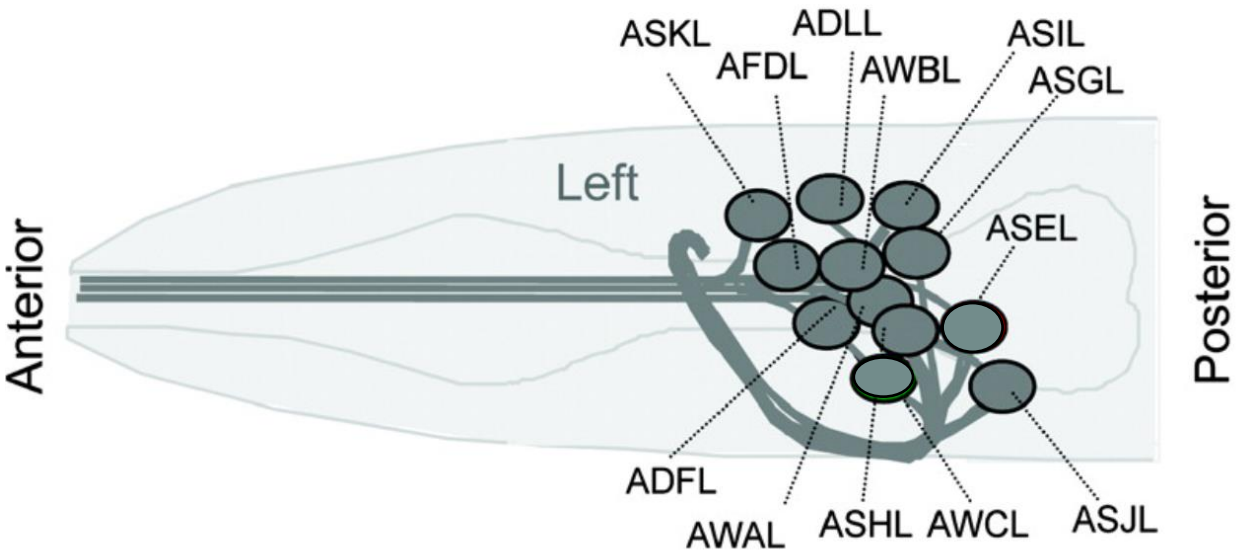

Figure 3: C. elegans chemosensory system. (Ortiz et al., 2006)

When a sensory neuron communicates with an interneuron, it must convert the electrical message that is carried by the action potential into a chemical message. This is usually done by releasing neurotransmitters into the synapse. *C. elegans* utilize tyramine, serotonin, dopamine, octopamine, and other neurotransmitters, similarly to humans. These neurotransmitters are synthesized within sensory neurons by a number of different catalytic enzymes (Hobert, 2013). For example, serotonergic neurons will convert the amino acid tryptophan into serotonin through a catalytic process that utilizes both TPH-1 and BAS-1. Serotonin will then be packaged into vesicles and released at the synapse (Figure 4).

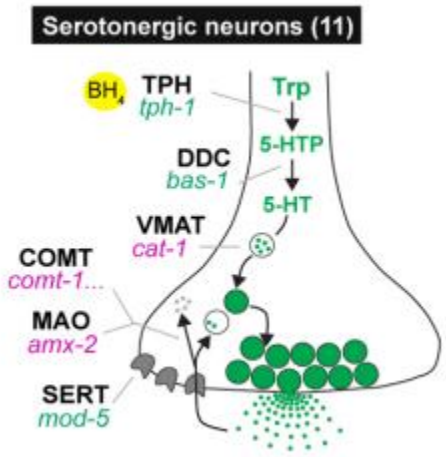

Figure 4: Serotonergic neurotransmission pathway (Hobert, 2013).

The neurotransmission pathways for dopamine, tyramine, and octopamine follow similar patterns as the transmission pathway for serotonin. They only differ by the enzymes used. For example, in the dopamine pathway, instead of TPH-1 catalyzing tryptophan, CAT-2 will convert catalyze it into a dopamine precursor molecule (Hobert, 2013). However, glutamate does not follow these synthesis patterns. *C. elegans* are unable to synthesize glutamate on their own. Instead, glutamate is ingested through food sources and then utilized by the nervous system for signal transduction (Hobert, 2013).

Each of these neurotransmitters modulate different responses within the animal. Dopamine modulates locomotive behaviors (Chase and Koelle, 2007). These behaviors allow a worm to stay in a

food source or search for new food sources. Dopamine also plays a role in altering/learning new behaviors based on previous experiences, both through mechanical sensation and olfactory sensation. For example, dopamine-mediated learning allows a worm to establish that repeated tapping or prolonged exposure of an odorant are not relevant, and they will eventually have no effect on the animal (Chase and Koelle, 2007).

Serotonin also modulates locomotive behaviors and egg laying behaviors, allowing *C. elegans* to respond to changes in the environment (Chase and Koelle, 2007). Serotonin is synthesized in neurosecretory motor neurons, which allows them to directly affect locomotion. Like dopamine, it plays a role in keeping an animal in a favorable food environment. There are numerous different receptors that bind serotonin, including GPCRs and a serotonin-gated chloride channel, MOD-1 (Chase and Koelle, 2007).

Glutamate has long been known to regulate learning and memory in invertebrates. Although the memory of worms cannot be accessed easily, it has been shown that glutamates facilitates habituation in worms (Brockie et al, 2006). Glutamate is also thought to contribute to foraging behaviors in *C. elegans.* This is done by altering the synaptic plasticity of neurons, strengthening or weakening connections. This is different than the type of learning mediated by dopamine.

C. elegans metabolism is utilized for communication

C. elegans utilize the chemosensory nervous system to communicate with conspecifics. The sensory system detects signals through pheromones called ascarosides. Ascarosides are small molecules that incorporate glycosides of the dideoxysugar, ascarylose (Schroeder, 2013). Each ascarosides is a metabolite, or a byproduct of metabolomic prosses within the worm. These molecules are biosynthesized within the worms and then secreted in the exo-metabolome via the gut. The ascarosides secretions can then be sensed by other worms, allowing for a lexicon of chemical signals through which *C. elegans* communicate.

These ascarosides secretions play a role in "a deeply intertwined regulatory network" (Schroeder, 2015). This network connects a worm's metabolism to communication and behavior through ascaroside sections. Different modifications to the lipid chain will elicit different behaviors in surrounding wor s sense, the worms can communicate through their metabolic processes. For example, the addition of *p*-aminobenzoate (PABA) to the end of the fatty-acid side chain, resulting in ascaroside #8 (ascr#8), elicits an attractive mating behavior in male *C. elegans* (Pungaliya et al, 2009; Narayan et al, 2016). This same ascaroside elicits avoidance in hermaphrodites. Alternatively, icas#3, which features an indol addition to the 2-position of the ascarylose sugar, attracts hermaphrodites (Srinivasan et al, 2012). The basic structure of ascr#8 and icas#3 is very similar, but modifications can either elicit a negative or positive signal in hermaphroditic worms (Figure 5).

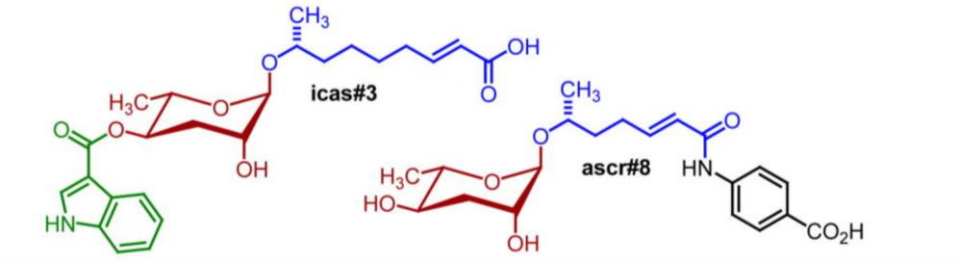

Figure 5: ascr#8 and icas#3 structure. Red) ascarylose sugar. Blue) fatty acid side chain. Green) indole group. Black) PABA group (Schroeder, 2015)

Starved, L1-arrest worms will secrete octopamine-succinylated ascaroside #9 (osas#9) (Figure 6) to communicate that food is not present. osas#9 is secreted into the exometabolome, sensed by nearby *C. elegans*, and elicits an avoidance response in starved worms, signaling to search elsewhere for food sources. This ascaroside is synthesized via the addition of octopamine to the ascarylose sugar of ascaroside #9 through succinyl linkage (Artyukhin et al, 2013). Octopamine itself is the synthetic successor of the neurotransmitter tyramine and its precursor, tyrosine. An enolase will add octopamine to the 2-position of the ascarylose sugar.

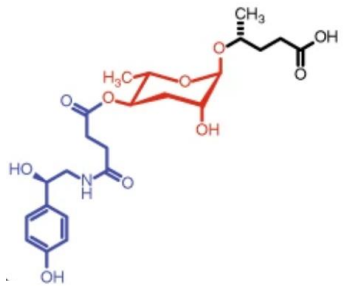

Figure 6: osas#9 structure. Red) ascarylose sugar. Black) fatty acid side chain. Blue) octopamine addition (Chute et al, 2019).

Metabolism and its connection to the epigenome

Metabolism and metabolites reveal what processes are occurring within an organism overall. In *C. elegans* metabolite excretion not only rids the worm of unnecessary molecules but transforms chemical metabolites into a lexicon used for communication with conspecifics. Some metabolites are used to modify nucleic acids and chromatin. Their availability is affected by activity of metabolic pathways. Thus, cellular metabolism constitutes a fundamental component of chromatin status and thereby of genome (Reid et al, 2017).

In humans, cellular respiration and exercise cause a decrease in the concentration of available ATP and an increase in available AMP (Cosentino and Mostoslavsky, 2013). This change in AMP/ATP ratio activates AMPK, a holoenzyme that will bind both AMP and ATP and encourage the digestion of lipids to satisfy energy demands. In recent studies, AMPK has been shown to crosstalk with a histone deacetylase (HDAC), resulting the hyperactivation of the HDAC (Cosentino and Mostoslavsky, 2013). This process connects the environmental condition, energy deprivation, to a metabolomic enzyme, AMPK, which activates an epigenome regulator. Although no studies have been conducted on the crosstalk of AMPK with HDACs in *C. elegans*, it is known that the AMPK pathway is conserved in the nematode worm (Ashrafi, 2007).

Recent studies in *C. elegans* suggest that carbohydrate metabolism is liked to regulation of the epigenome. Glycosyl groups are a byproduct of carbohydrate metabolism in *C. elegans.* Glycosylation of proteins is a common post translational modification in all eukaryotes. *O*-GlcNAcylation (O-GlcNAc) modifications are a byproduct hexosamine biosynthetic pathway (HBP) (Hanover et al, 2012). This pathway combines carbohydrate, amino acid, and fat metabolism into one pathway (Ashrafi, 2007). O-GlcNAc modifications are added to all any of the four core histones to regulate gene expression. Addition of O-GlcNAc modification typically occurs at a promoter, regulating the start of transcription. Typically, the addition and removal of O-GlcNAc modifications is highly dynamic. O-GlcNAc transferases and O-GlcNAcases interact with each other in a cyclic manner, consistently adding and removing O-GlcNAc modifications (Hanover et al, 2012). This cyclic process allows for fluctuations in genetic expression. One specific example of O-GlcNAc cycling is at the promoter of O-GlcNAc synthesis enzymes. The cycling maintains consistent levels of O-GlcNAc, activating transcription when O-GlcNAc levels are low and deactivating transcription when O-GlcNAc levels are high (Hanover et al, 2012). This pathway is an example of how metabolism can not only be affected by epigenetic regulation but can also impart alterations on the epigenome.

In addition to carbohydrate metabolism, epigenetic regulations have been connected to amino acid metabolism in *C. elegans* and other organisms. Proline, which has its own metabolic system, has the potential of being converted into α-KG, an epigenetic metabolite (Phang et al, 2013). Histone and DNA demethylases utilize α-KG as a substrate, using it to power their epigenetic modifications (Lu & Thompson, 2013). Enzymes responsible for the trimethylation of H3K4 have been shown to use mono-unsaturated fats, such as α-KG co-factors in *C. elegans* (Han et al, 2017). Thus, the metabolism of the amino acid proline can be coupled with epigenetic regulation, bridging epigenetics and metabolomic regulation together (Phang et al, 2013).

C. elegans metabolism has been connected to numerous biological functions (Figure 7). Metabolites offer a complex lexicon which can be utilized by *C. elegans* for communicative purposed. Metabolites also regulate protein function through post translational glycosylation modifications. When these glycosylations are additions to histones, or other chromatin structuring proteins, they have the ability to interact with epigenetic regulatory mechanisms. Additionally, amino acid metabolism creates byproducts that are used as ascarosides for communications, as precursors to neurotransmitters and as cofactors for chromatin restructuring enzymes. Overall, the metabolism of *C. elegans* is a complex system from which behaviors can be elicited and chromatin regulatory remodeling mechanisms can be influenced.

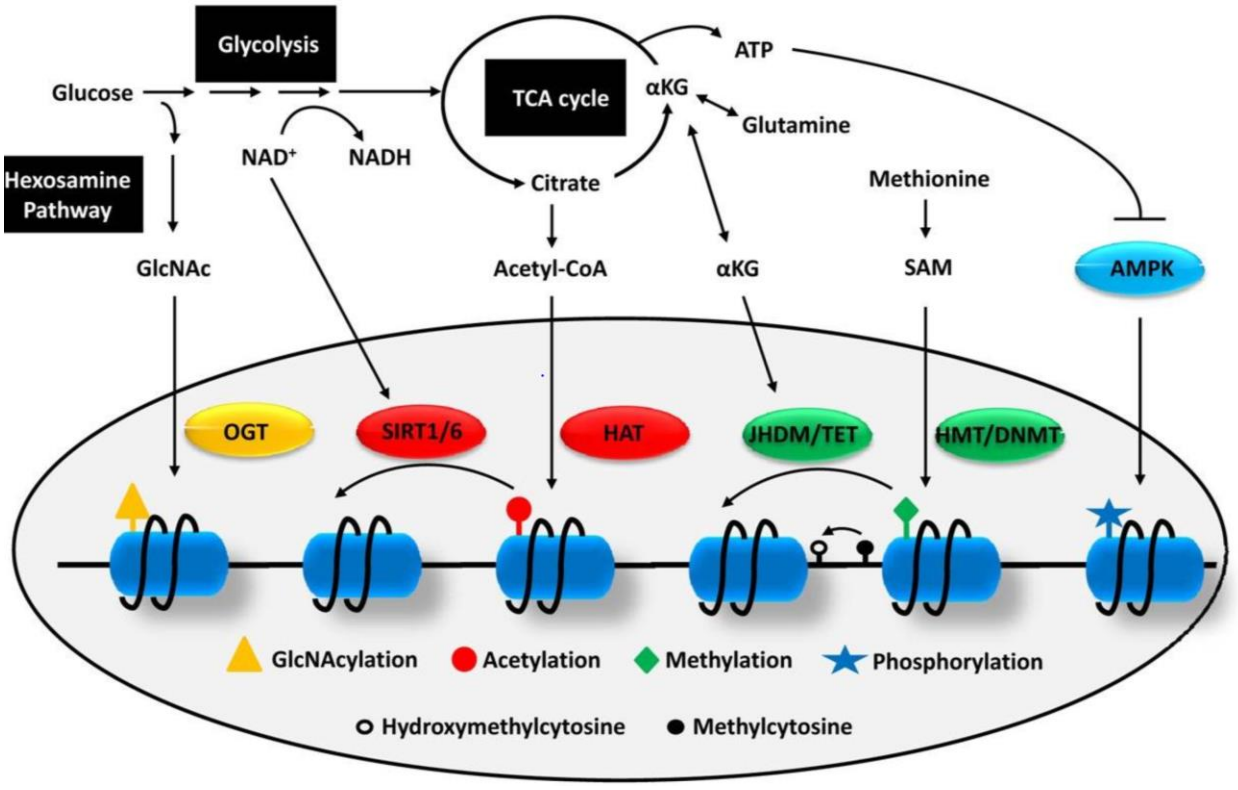

Figure 7: Metabolic Connections to Epigenetic Modifications (Lu & Thompson, 2013).

The use of a secondary metabolite to trigger epigenetic reprogram *C. elegans*

Previous studies developed a paradigm for testing epigenetic reprograming of osas#9 sensation (Garver & McGlame, 2018). It was found that when pre-exposed to osas#9-containing agar plates during embryo and L1 larvae stages, *C. elegans* exhibited decreased osas#9 behavioral responses in adulthood. The loss of behavioral response was passed from the parental generation to at least three subsequent generations (Garver & McGlame, 2018).

This thesis aims understand how this epigenetic event is regulated in *C.* elegans. The project extends previous data, establishing within how many generations the information is inherited. One major hypothesis of this project is *that removal of H3K4 demethylation process through an spr-5 knockout mutation will prevent behavioral alterations due to epigenetic reprogramming*. Additionally, this project worked to fully understand the involvement of neurotransmission pathways in epigenetic reprogramming, discovering the *utilization of serotonergic and glutamatergic pathways*. This project also investigated the impact of epigenetic reprogram on all biological processes through investigation of the metabolome.

Methods

Strain maintenance and acquisition

The N2 strain of *C. elegans*, obtained from the *Caenorhabditis* Genetics Center (CGC), was the original strain derived by Sydney Brenner. Brenner derived the N2 strain from a soil culture collected in Bristol, England by Ellsworth Dougherty in spring of 1964 (Riddle et al., 1988). This strain serves as the wildtype strain for most behavioral and genetic baseline research performed.

The strains MT15434 (*tph-1*(my280)II), CB1112 (*cat-2*(c1112)II), MT9668 (*mod-1*(ok103)V), and KP4 (*glr-1*(n2461)III) were all acquired from the CGC and used for neurotransmitter analysis. PS7953 (C23H4.2(sy1163)), a *cest-8* mutant was created in Frank Schroeder's lab at Cornell University. BR3417 (spr-5(by134)I) was also acquired from the CGC and used for histone methylation analysis.

All strains were maintained on 60 mm nematode growth media (NGM) plates seeded with OP50 *E. coli* to provide an adequate food source and were passed every few days to prevent starvation and overcrowding. Plates were stored in the 20 ˚C incubator throughout the course of the project.

osas#9 plates

1 uM osas#9 was added to NGM mix in 35 mm plates. The plates were stored at 4°C prior to use.

Epigenetic reprogramming by exposure to osas#9

1 µM osas#9 plates were seeded with OP50 *E. coli* strains. A young adult hermaphrodite was placed on the plates and allowed to lay eggs for 24-48 hours. Once eggs hatched and were in the L1 larvae stage the herms were removed. The L1 worms were washed with approximately 0.5 mL of M9 buffer into a 1.5 mL conical tube. (M9 buffer is created by combining 3g of KH_2PO_4, 6 g of Na_2HPO_4, and 5 g of NaCl into a liter of water.) The worms were allowed to settle by gravity for 10-15 minutes. After worms had settled, 10 µL of the buffer were pipetted from the bottom of the tube to ensure worms were present and transferred onto a seeded 6 cm NGM agar plate. The L1 worms were grown to adulthood, and either used for analyses or procreation of the next generation.

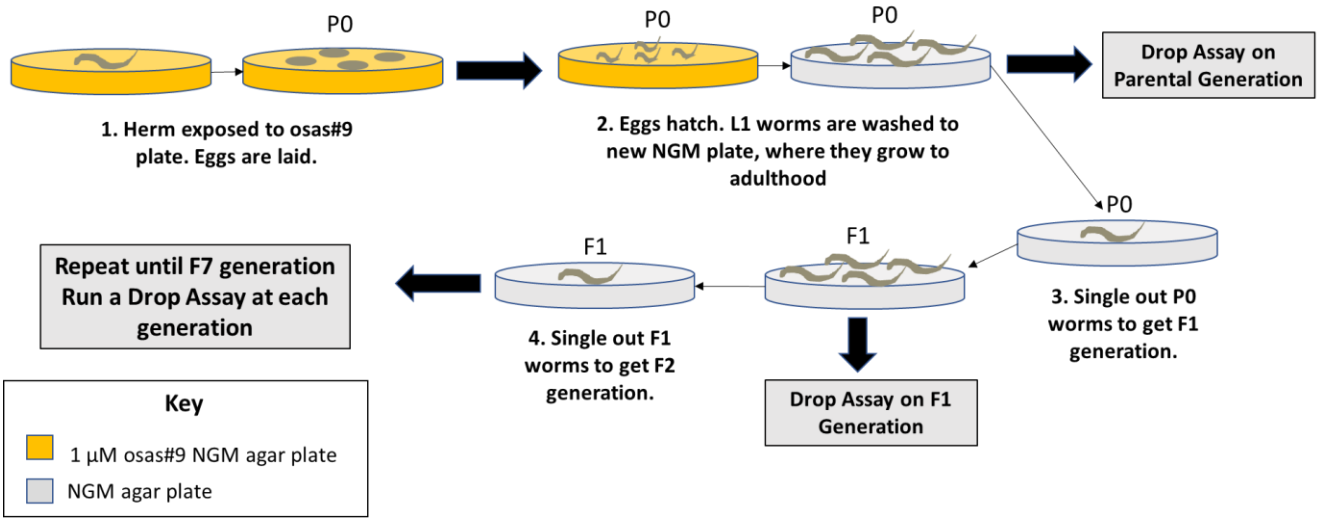

Figure 8: Experimental Paradigm

Generation maintenance

New generations were maintained by placing an adult hermaphrodite onto a seeded 35 mm plate and allowing it to lay eggs. The hermaphrodite was then removed, and the eggs were allowed to hatch and grow to adulthood on the same NGM plate. Adult worms would be used for either production of the next generation or analyses.

Avoidance assay

C. elegans were picked from 35 mm plates onto 60 mm NGM agar plates. Copper rings were used to prevent worms from leaving plate during an hour-long starvation period. The copper rings were dipped into 70% ethanol then flamed to ensure sterilization. The fire also heated the rings allowing them to sink into the agar and create a strong barrier.

Drops of either solvent control (SC, 1% ethanol) or 1 µM osas#9 in 1% ethanol were placed on the tails of forward moving worms. The drops wicked up the body of the worm through capillary action. The avoidance behavior was recorded for each worm. An "avoidance" was defined as a complete reversal of direction with two body bends or change of more than 90° from the direction the worm was traveling. The Avoidance Index (Equation 1) of a plate was calculated by dividing the number of worms that avoided by the total number of worms assayed.

$$Avoidance\ Index = \frac{Number\ of\ Drops\ Avoided}{Number\ of\ Drops\ Applied} \qquad (Equation\ 1)$$

This drop assay procedure was then completed again on the same 10 worms to generate an Avoidance Index. Up to 10 plates were analyzed per generation or condition.

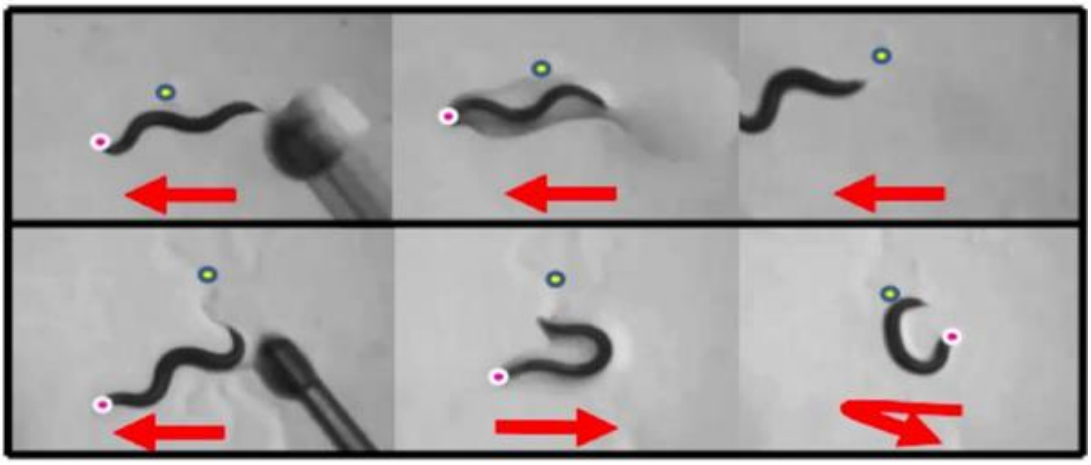

Figure 9: Representation of Avoidance Assay. In this assay, a drop of chemical is placed on the tail of a worm and allowed to wick up to the head of the worm generating contact with the sensory cilia. No response (top) is quantified when a worm continues in the same direction. Avoidance (bottom) is attributed when the worm either reverses direction or turns at least 90◦. The pink dot is a reference to the worm's head. The yellow dot is to allow a reference point to compare worm movement to.

Metabolomics

Performed at Worcester Polytechnic Institute:

Epigenetic reprogramming was induced in both N2 and PS7953 worms. Worm were frozen into pellets in the P_0 and F_3 generations. Pellets were generated by taking five 60 mm NGM plates and washing approximately 0.5 mL of M9 buffer from plate to plate and into a 1.5 mL Eppendorf tube. Worms were allowed to settle to the bottom and supernatant was transferred into a new Eppendorf tube. Another 0.5 mL of M9 buffer was added and once again the worms were allowed to settle. The supernatant was removed, and this time discarded. One more M9 buffer wash was performed to ensure all *E. coli* food strains had been removed. Supernatant was discarded and worms were centrifuged for 30 seconds at 6000 rpm to ensure pellet formation. Pellets and supernatant were frozen and stored at -80 ◦C.

Performed at Cornell University:

In order to extract metabolites frozen samples were lyophilized to dryness. Dried samples were extracted with 1 mL methanol. Dried worm pellets were then homogenized by being sonicated with a probe sonicator twice for 2 min, cycling 2 sec on and 3 sec off, with the sample sitting in a water bath. Extractions occurred on an orbital shaker set at 200 RPM 20 °C for 24 hours. Extractions were centrifuged at 12,000 G 10 min 4 °C, and supernatants were transferred to clean glass vials and dried on a SpeedVac (Thermo Fisher Scientific) vacuum concentrators. Dried material was resuspended in 35 µL of methanol, transferred to 1.7 mL Eppendorf tubes, centrifuged at 12,000 G 20 min 4 °C. Clarified concentrated extracts were transferred to HPLC vials and stored at -20°C until analysis.

High resolution LC–MS analysis was performed on a Dionex 3000 UPLC coupled with a Thermo Q Exactive HF hybrid quadrupole-orbitrap high-resolution mass spectrometer equipped with a HESI ion source. 1 uL of extract was injected and separated using a water-acetonitrile gradient on an Agilent Zorbax Eclipse XDB-C18 column (150 mm × 2.1 mm, particle size 1.8 µm) maintained at 40 °C. Solvents were all purchased from Fisher Scientific as HPLC grade. Solvent A: 0.1% formic acid in water; Solvent B: 0.1% formic acid in acetonitrile. A/B gradient started at 1% B for 3 min after injection and increased linearly to 100% B at 20 min, using a flow rate 0.5 mL/min. Mass spectrometer parameters: spray voltage 3.0 kV, capillary temperature 380 °C, prober heater temperature 300 °C; sheath, auxiliary, and spare gas 60, 20, and 2, respectively; S-lens RF level 50, resolution 240,000 at m/z 200, AGC target 3×106. The instrument was calibrated with positive and negative ion calibration solutions (Thermo-Fisher). Each sample was analyzed in positive and negative modes with m/z range 70 to 1000.

Comparative metabolomics was analyzed using the METABOseek platform. Metabolome data was compared between exposed and naïve worms of wildtype N2 F_3 generation in both targeted and untargeted ways, aiming to uncovering the metabolism changes of the F_3 generation worms. Same analysis was done to *cest-8* (PS7953) mutant to figure out the epigenetic difference between N2 and *cest-8* mutant.

Statistics

All transgenerational analysis was observed using a fold-change y-axis. Fold change avoidance indexes (Equation2) allow us to observe the avoidance behaviors that were elicited solely due to the osas#9 avoidance cue. Fold changes were created by dividing the osas#9 avoidance index by the solvent control avoidance index.

$$Fold\ Change\ Avoidance\ Index = \frac{osas\#9\ Avoidance\ Index}{SC\ Avoidance\ Index} \qquad (Equation\ 2)$$

Comparative statistics were performed using MatLab. ANOVAs were performed comparing each generation to the naïve, unexposed, worms.

Results

Epigenetic generational inheritance

In order to trigger and epigenetic reprogramming, wildtype, N2, adult hermaphrodites were placed onto seeded 35 mm NGM agar plates which contained 1 µM osas#9 within the agar. The hermaphrodites laid eggs, which grew up to be L1 worms. These L1 worms served as the parental generation (P_0) for this study as they were the first generation exposed to osas#9. Exposure at developmental state (egg to L1) allowed the ability for epigenetic reprogramming to occur.

The P_0 generation showed significantly decreased behavioral response to osas#9 with an avoidance index roughly around solvent control level (Figure 10). This decreased avoidance response was transgenerationally inherited in the F_1 generation, which had no previous exposure to osas#9, suggesting that an epigenetic reprogramming occurred, suppressing the avoidance response. This reprogramming was maintained through the F_5 generation. In the F_6 generation, the avoidance levels began to increase, returning to normal levels in the F_7 generation. This data shows that an epigenetic reprogramming can be triggered through the sensation of an environmental cue by chemosensory

system. It also shows that the reprogramming is robust and has the ability to be transgenerationally inherited for six generations (Figure 10).

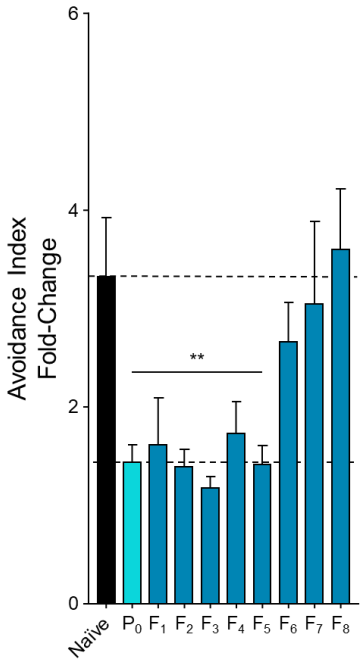

Figure 10: N2 Transgenerationally Inherited osas#9 Behavioral Response. Error bars denote SEM.

$n \geq 10$. One-Way ANOVA, Naïve P0 vs... ($p < 0.05$)* $p < 0.05$, ** $p < 0.01$

Since osas#9 exposure is able to trigger the epigenetic reprogramming, we believed that repeated exposure to minimal concentrations was responsible for transgenerational inheritance. Worms will typically begin to synthesize osas#9 during periods of starvation. However, like any chemical process, osas#9 is synthesized in minute amounts even without starvation. We hypothesize that this minimal exposure acts as a feedback mechanism that maintains the suppressed avoidance behavior in subsequent generations to the exposed. In order to test this theory, an enolase mutant was used (*cest-8*). This mutant prevents osas#9 from being synthesized by preventing the addition of the octopamine group to ascroside#9. This mutant was functionally adequate. It fed, traveled, and laid eggs in a similar manner to the wildtype N2 stain. It also avoided osas#9 at similar levels to N2 worms (Figure 11). This

suggests that even though the worms do not have the enzymes necessary for the synthesis of osas#9, they have maintained the cellular machinery necessary for its sensation, allowing the worms to have a behavioral response.

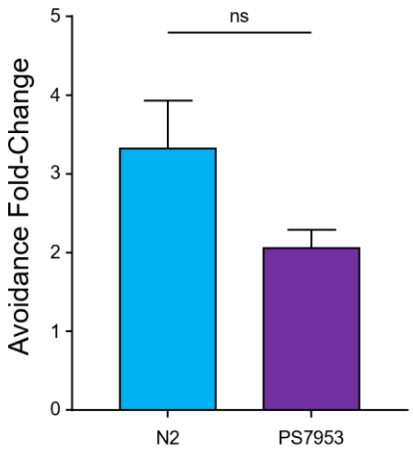

Figure 11: N2 vs *cest-8* osas#9 Avoidance Levels. Error bars denote SEM.

$n \geq 10$. One-Way ANOVA.

When the *cest-8* mutants were exposed to osas#9 in the same manner as the N2 worms a significant decrease in avoidance response was observed in the P_0 generation. This suppressed avoidance response was transgenerationally inherited in the F_1 generation, but not in subsequent generations (Figure 12). The avoidance levels returned to normal in the F_2 generation, making the epigenetic transgenerational inheritance less robust in this strain. Because there was no osas#9 synthesized in the enolase mutant, there was no maintenance of the epigenetic reprogramming, and the worms reverted back to their unexposed predispositions.

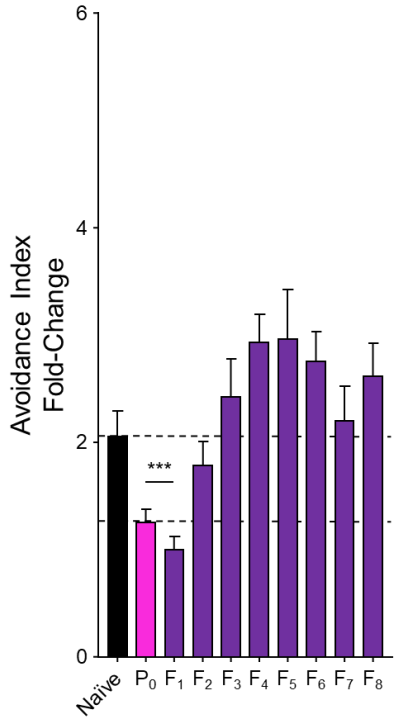

Figure 12: *cest-8* Transgenerationally Inherited osas#9 Behavioral Response. Error bars denote SEM

n ≥ 10. One-Way ANOVA, Naïve P0 vs... ($p < 0.05$)* $p < 0.05$, ** $p < 0.01$ *** $p < 0.001$

DNA demethylation mutant

The altered behavioral responses in both N2 and *cest-8* mutants suggest that osas#9 exposure is able to epigenetically reprogram at least one gene in the *C. elegans* genome. Because we saw suppressed avoidance/behavioral responses, we believed that the gene being reprogramed was being suppressed. To test this theory, we used an *spr-5* mutant, a mutant unable to make certain epigenetic changes. SPR-5 is a human ortholog of H3K4 demethylase. This enzyme removes methyl groups from the 4th lysine group of Histone 3, resulting in transcriptional deactivation. When *spr-5* mutants were pre-exposed to osas#9 there was no decreased avoidance response in either the P_0 or F_1 generations (Figure 13). This suggests that when the ability to unmethylated the 4th lysine is removed, preventing transcriptional deactivation, the epigenetic reprogramming observed in the N2 strain is unable to occur.

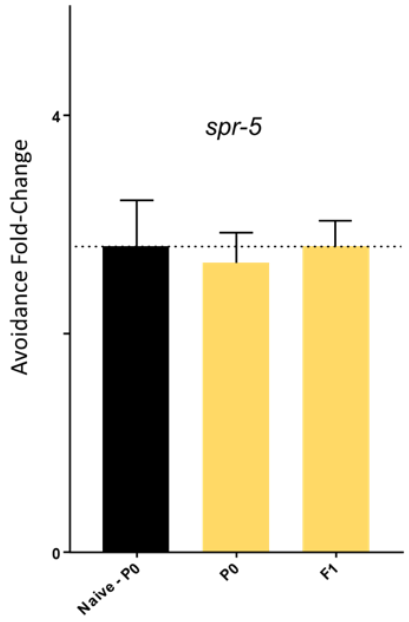

Figure 13: *spr-5* Transgenerationally Inherited osas#9 Behavioral Response. Error bars denote SEM. $n \geq 10$. One-Way ANOVA.

Neurotransmitter Mutants

The epigenetic reprogramming observed in the N2 strain occurred through the sensation of osas#9 through the sensory receptor, TYRA-2, is located on the ASH neuron. In the presence of osas#9, TYRA-2 triggers a signal to be transduced throughout the worm activating the chemosensory system. The complete connectomes for each neurotransmitter involved in osas#9 signaling through the chemosensory system has not been identified. We decided to investigate which of the neurotransmitters are involved with transduction of the osas#9 signal to initiate epigenetic reprogramming. This could help identify neurons which may be involved by using the connectomes. Mutants defective in dopamine synthesis, serotonin synthesis and sensation, and glutamate sensation were used as a starting point for this investigation.

Dopamine is synthesized from the amino acid tyrosine. Tyrosine is converted into a dopamine precursor molecule by the enzyme CAT-2. *cat-2* mutants were used to prevent the synthesis of dopamine in the worms. When the *cat-2* mutants were pre-exposed to osas#9, there was decreased

avoidance response in both the P_0 or F_1 generations (Figure 14). This suggests that dopamine is not involved in the signaling of the epigenetic reprogramming, since it was able to occur unaltered from the wildtype strain.

A similar approach was performed for serotonin. The enzyme TPH-1 converts tryptophan into the serotonin precursor molecule L-DOPA. When *tph-1* mutants were pre-exposed to osas#9, no decreased avoidance response was observed in either the P_0 or F_1 generations (Figure 14). Because the avoidance behavior was not suppressed, this suggests that serotonin plays a role in triggering the epigenetic reprogramming. To investigate this further a mutant for a serotonin receptor, MOD-1 was also tested. When *mod-1* mutants were pre-exposed to osas#9, the results were similar to *tph-1*. There was no suppressed avoidance behavior (Figure 14). Epigenetic reprogramming could not occur when either serotonin synthesis or serotonin sensation was prevented.

Because epigenetics is a form of cellular or genetic memory, we decided to also test the neurotransmitter glutamate for its involvement in epigenetic reprogramming. Glutamate is known to have strong ties to synaptic plasticity, learning, and memory in numerous different animals. *C. elegans* cannot synthesize glutamate. It is absorbed through their diet, packaged by glutamatergic neurons, and then used to transduce signals across synapses. Because there is no enzyme for glutamate synthesis, only a glutamate receptor was tested, GLR-1. GLR-1 is an ionotropic glutamate receptor which is responsible for allow calcium influxes into neurons. When a *glr-1* mutant was pre-exposed to osas#9, there was a decrease in avoidance response in the P_0 generation (Figure 14). However, this suppressed behavioral response was not transgenerationally inherited by the F_1 generation. This suggest that glutamate is involved with epigenetic reprogramming of the somatic cells, most likely localized in the neuron responsible for osas#9 sensation. However, glutamate is not involved with epigenetic reprogramming of the germline, so the offspring are unable to inherit the learned behavioral response.

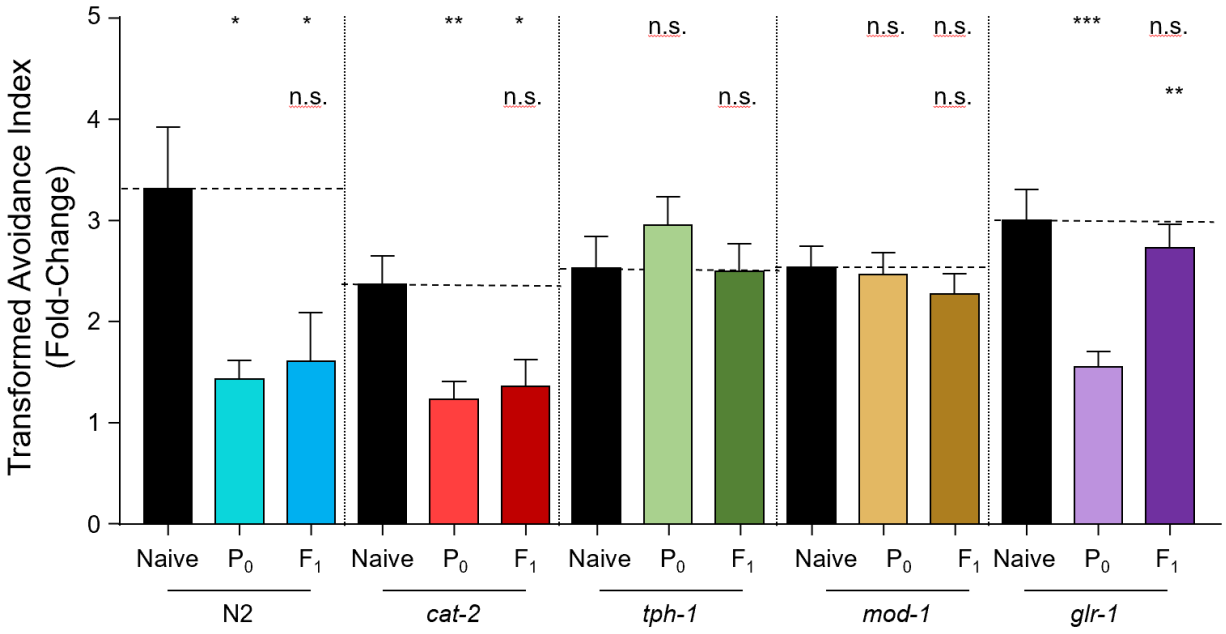

Figure 14: Neurotransmitter Mutants Transgenerationally Inherited osas#9 Behavioral Response. Error bars denote SEM. $n \geq 10$. One-Way ANOVA. Top-Row Stats: "vs. Naïve." Bottom Row Stats: P_0 vs. F_1

Metabolomics

Because it has been shown in past research experiments that the metabolome is connected to epigenome regulation, this project investigated the affect of pre-exposure to osas#9 on the metabolome in *C. elegans*. This was done to sixteen different worm samples, testing the parental generation and F_3 generations in both pre-exposed and naïve, unexposed conditions (Table 1). This was done for both the N2 strain which represented wild-type worms and the PS7953 worms which is the *cest-8* enolase mutant which cannot synthesize osas#9. The purpose of testing this mutant was to see how the osas#9 synthesis process affected possible changes to the epigenome. All sixteen samples were analyzed by mass spectrometry.

Table 1: Samples Tested by Mass Spectrometry			
Pellet#	**Strain**	**Generation**	**Treatment**
P1	N2	P_O	Exposed
P2	N2	P_O	Exposed
P3	N2	P_O	Naïve
P4	N2	P_O	Naïve
P5	PS7953	P_O	Exposed
P6	PS7953	P_O	Exposed
P7	PS7953	P_O	Naïve
P8	PS7953	P_O	Naïve
P9	N2	F_3	Exposed
P10	N2	F_3	Exposed
P11	N2	F_3	Naïve
P12	N2	F_3	Naïve
P13	PS7953	F_3	Exposed
P14	PS7953	F_3	Exposed
P15	PS7953	F_3	Naïve
P16	PS7953	F_3	Naïve

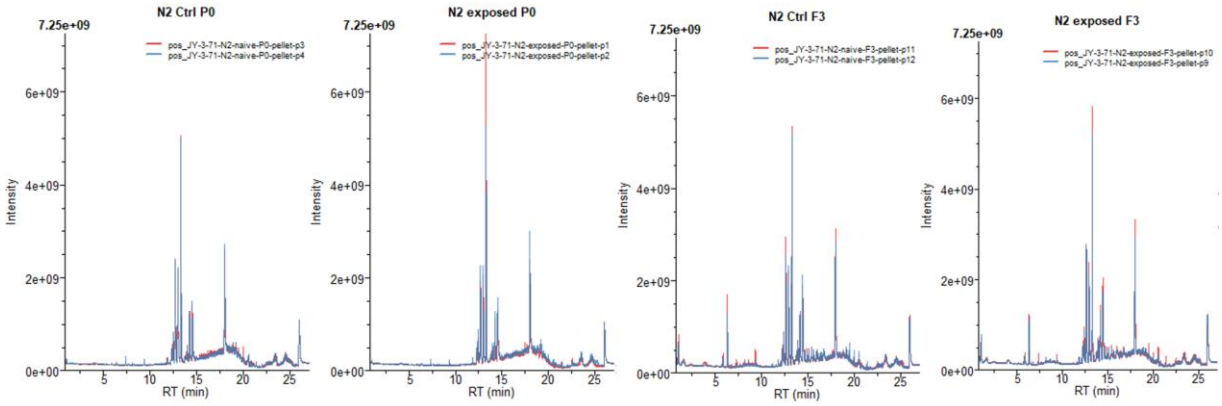

Figure 15: Positive Ionization Mass Spectrometry of N2 Worms

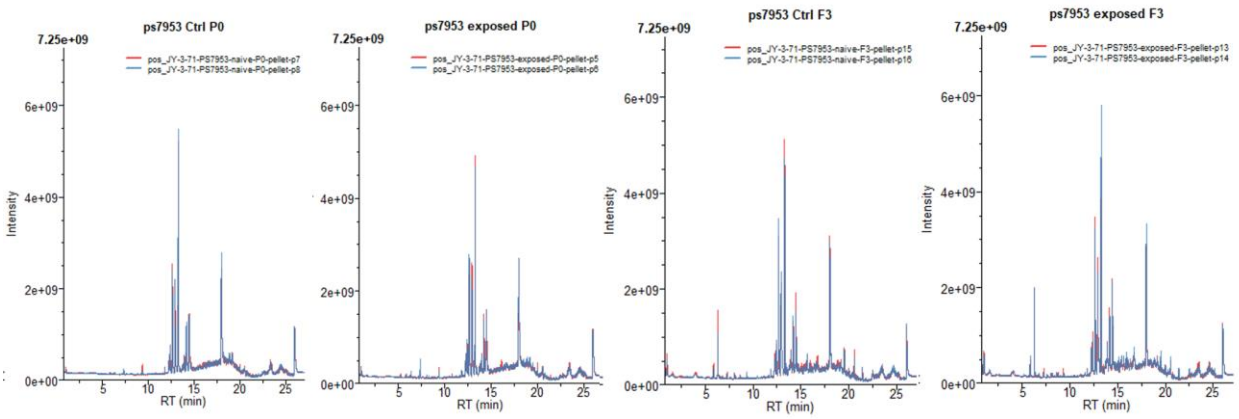

Figure 16: Positive Ionization Mass Spectrometry of PS7953 Worms

No major difference was observed between any of the N2 samples (Figure 15). There was no change in the peaks observed between pre-exposed and naïve N2 worms, meaning that there were no observable changes in their metabolism. This was unexpected as we hypothesized that the epigenetic reprogramming would create alterations to the metabolome, yet no pre-exposed generation displayed changed. This remained true for the P_0 generation of the PS7953 *cest-8* enolase mutant (Figure 16). The pre-exposed samples showed comparable metabolomic outputs as the control, unexposed, samples. However, there were observable changes with in the F_3 generation of the PS7953 samples. To investigate this further the PS7953 F_3 readings were broken down into different mass to charge ration (m/z). Significant peak differences ($p < 0.05$) were observed at m/z values of 234.03804, 455.18638, and 415.23794 (Figures 17, 18 & 19). In each of these cases there was a higher intensity reading in the control, unexposed worm samples than in the osas#9 pre-exposed samples. This means that there were more metabolites of the molecular weights examined within the naïve worms and a decrease in metabolism in the pre-exposed worms.

These results of the PS7953 F_3 generation are particularly interesting. This is the generation in which avoidance behaviors return to normal in the PS7953 strain (Figure 12). We hypothesized that pre-exposed F_3 generation would look similar to the unexposed generations, since the behaviors are the

same. However, this is not the case. The P_0 generations look similar to each other, while alterations in metabolites appear in the F_3 generation. Additionally, the N2 wild-type strain never displayed any alterations in the metabolome for any samples. Perhaps the metabolomic changes are a result of the cellular trigger to return the epigenome back to its unexposed disposition preceding the epigenetic event. This would explain why no alterations were observed in the N2 strain, as the F_7 generation would have to be investigated. It would also explain why changes in the metabolome appeared in the F_3 generation of the PS7953 strain.

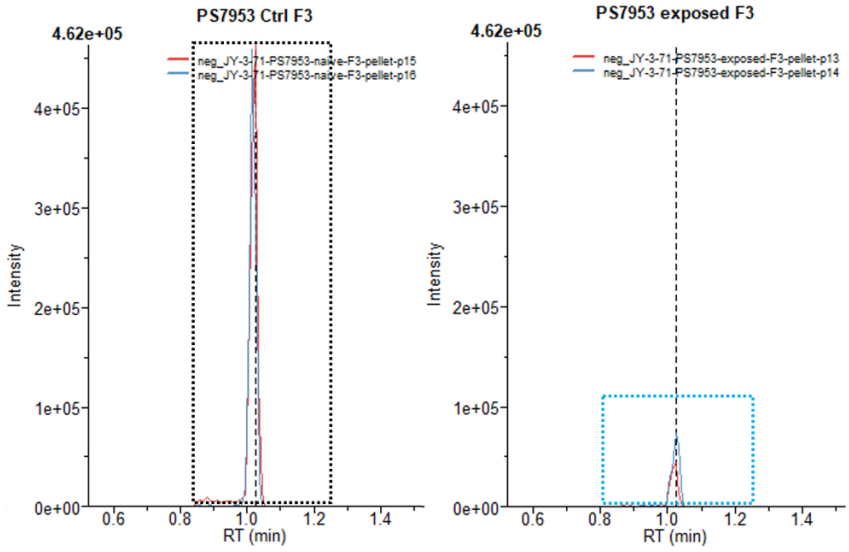

Figure 17: PS7953 F_3 Generation Mass Spectrometry Results at m/z of 234.03804.

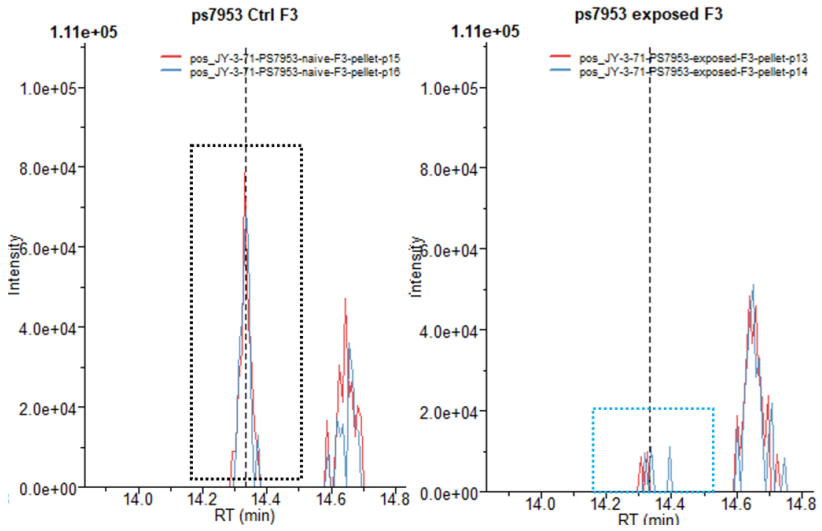

Figure 18: PS7953 F_3 Generation Mass Spectrometry Results at m/z of 455.18638.

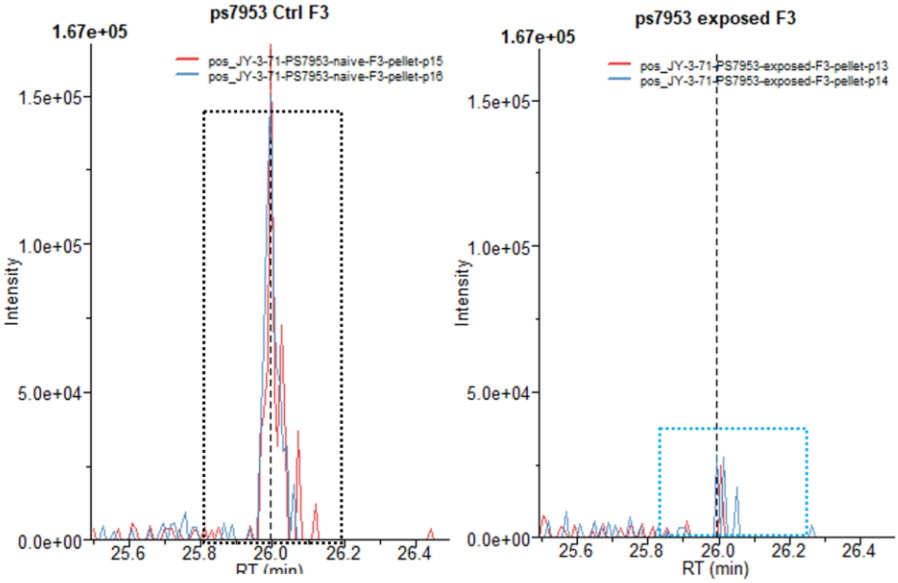

Figure 19: PS7953 F_3 Generation Mass Spectrometry Results at m/z of 415.23794.

Discussion

In this study I demonstrated that the chemosensory nervous system of *C. elegans* can be used to trigger epigenetic reprogramming. When worms were pre-exposed to osas#9 in their early developmental stages, they displayed decreased avoidance in adulthood. This decreased avoidance was

transgenerationally inherited through the F_5 generation, with the assistance of minimal osas#9 exposure in these finial generations. When osas#9 synthesis is removed as a biological process from *C. elegans*, the decreased avoidance behavior is only inherited through the F_2 generation.

This epigenetic event is possible through the demethylation of H3K4. Methylation of the 4th lysine on Histone 3 promotes transcription activation. Removal of these lysine groups promotes the deactivation of transcription and genetic suppression. Additional studies could be performed to determine if there are other epigenetic modifications regulating this reprogramming event. Perhaps another H3K9 is methylated in response to osas#9 pre-exposure, promoting transcription deactivation. Or perhaps other modifications such as acetylation or O-GlcNAc are involved. These could all be tested through the creation of mutants for each of these enzymes.

Additionally, the specific gene affected by the epigenetic reprogramming could be investigated. At the moment the hypothesis is that TYRA-2, the receptor responsible for osas#9 sensation, may play a role in the epigenetic downregulation of avoidance behaviors. Analysis of mRNA levels preceding and following pre-exposure would inform us about the number of transcripts for TYRA-2 being synthesized. If the number of mRNA transcripts is decreased in response to pre-exposure, then this would account for the decreased avoidance as less TYRA-2 receptor molecules would be present on the chemosensory neurons.

This study has also demonstrated the involvement of neurotransmitter is the triggering of epigenetic reprogramming. A reprogram of the epigenome typically occurs in response to an environmental change or trigger. Since, the nervous system is utilized by organisms to perceive the environment, it is logical that it would be involved in sensing when an epigenetic reprogramming event is necessary. In the epigenetic event shown in this paper, serotonin and glutamate display involvement

in triggering the reprogramming. While serotonin was involved in reprogramming the somatic system of the worm, glutamate was involved in reprogramming the germline.

The *glr-1* mutant used in this study yielded intriguing results. It is fascinating to imagine that glutamate signaling pathways were utilized for the triggering of epigenetic reprogramming of germline cells in developing *C. elegans*. It is possible that the specific mutant used in this study, which displays loss of function-gain of function aspects, is at fault for these interesting events. In order to ensure that these results are correct other *glr-1* mutants should be investigated.

Additionally, this study revealed a possible mechanism for the return of the epigenetic reprogramming back to its original modification through the use of the metabolome. Mass spectrometry revealed differences in the metabolites produce by the F_3 generation of the PS7953 strain. These *cest-8* mutants inherited decreased avoidance behaviors for only a generation, increasing in the second, and returning to normal in the third. It is possible that the metabolomic changes observed through mass spectrometry signal the epigenome remodifying itself to its unexposed disposition, while no alterations to the metabolome are triggered while the epigenetic reprogramming modifications are intact. In the future the F_7 generation of the N2 strain should be investigated to see if the same metabolomic alterations arise when this wild-type strain begins displaying avoidance behaviors again.

Overall, this study showed the complexity of epigenetic events. In analyzed how chemosensory signals could be utilized to trigger what would be interpreted as environmental changed in *C. elegans*. This study showed how the signal could be passed from the chemosensory system to the rest of the nervous system through serotonin and glutamate pathways. Once the signal is internalized by the worm, specific epigenome modifications can be altered to regulate a gene. In this specific event H3K4 demethylation was necessary for the suppression of avoidance behaviors. Additionally, a possible trigger

for loss of the reprogramming event and return to a normal epigenome has been identified within the metabolome.

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