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Intergenerational Effects Of Embryonic Cocaine Exposure In Zebrafish

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Intergenerational Effects of Embryonic Cocaine Exposure in Zebrafish

by

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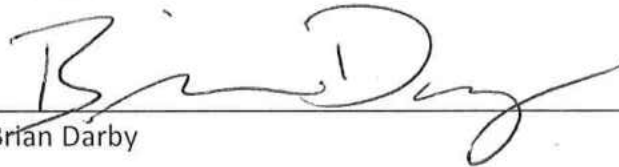Grand Forks, North Dakota

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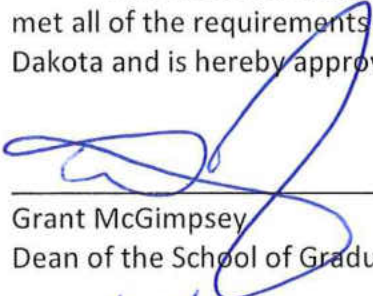
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ABSTRACT

Cocaine addiction has both genetic and environmentally driven components. Relatively recently several groups have suggested that epigenetic regulation of gene expression might be the mechanism linking environmental influence and inheritance in drug addiction. We have shown previously that embryonic cocaine exposure increases physiological and behavioral sensitivity to the drug in longitudinal adults. In this study, we provide evidence that the effects of embryonic pre-exposure to cocaine are intergenerational in zebrafish. In addition, we show how gene expression in the zebrafish telencephalon, which includes the teleost equivalent of the nucleus accumbens, changes during acute cocaine treatment during behavioral testing. These experiments outline gene expression pathways not extensively studied in the context of cocaine response. We show how embryonic exposure to cocaine affects these gene expression pathways when the longitudinal animals are acutely exposed. Finally, we show how chronic embryonic exposure affects these same pathways in 5-day old embryos.

INTRODUCTION

Dependency diseases as characterized by the Diagnostic and Statistical Manual of Mental Disorders-IV (DSM-IV) are a repeated maladaptive behaviors generating impairment or distress [1]. These disorders, including substance abuse disorders, have been characterized as diseases as they disrupt basic biological and behavioral function. Physical addiction refers to a biological phenomenon with both positive and negative symptoms. Positive symptoms include the rewarding psychological and physiological response associated with using. Tolerance is also associated with physical addiction, in which the body's response to a rewarding substance is attenuated following its first exposure. As a result, in substance abuse, recurrent use will require more of the substance to obtain the same physiological response as that experienced by a first time user. Additionally, physical addiction can include a hypersensitivity to a situation when there is a potential for substance exposure or addictive situation. This would involve increased impulsivity and drive to indulge in the substance. Physical addiction can be associated with physical withdrawal for substance and is often a cause for recurrent use and periods of relapse. Withdrawal produces many of the negative symptoms comorbid with physical addiction including depressive symptoms, lack of concentration, hypersensitivity to pain, and detrimental behaviors. Psychologically, addiction can also apply to a situation like gambling, but this disorder has been redefined by the DSM-IV as substance-independent dependency [1]. Diagnostically, dependency diseases have

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recently come to have a dimensional aspect with each component used to determine

severity and distinguish between dependency and abuse. There are three identified

components thought to contribute to the continuance of abuse and dependence and

they include 1) the substance and its physiologic action, 2) the individual that is using

the substance, and 3) the user's social environment including their relationships and

family history [2]. The listed components of the potential for abuse and dependence

imply addiction is a multifaceted disease including biological, psychological, and

sociological factors. Biological aspects of addiction stem from the release of

neurotransmitters and the stimulation of positive reward as a direct result of substance

use. Rewarding events or compounds stimulate the activity of the ventral tegmental

area, which projects dopaminergic neurons to areas like the nucleus accumbens and the

prefrontal cortex. Repeated substance use to has been thought to change synaptic

connections within this neuronal system, called the mesolimbic system. It has been

shown that this activation of the mesolimbic system occurs with rewarding chemical

substances, including drugs of abuse [3]. Stimulation of the dopaminergic neurons of

this pathway have been thought to not only reinforce drug use, but motivate behaviors

[4]. This positive reinforcement model has been widely accepted as a model for many

biological mechanisms of addiction.

Societal Impacts of Addiction

Addiction remains a large problem with a broad range of societal impacts despite

continued legislative bans worldwide. In a study conducted by the US Department of

Health and Human Services, as many as 10.2 percent of the US population over the age of 12 have recent illicit drug use [5]. In a similar survey, the Substance Abuse and Mental Health Services Administration concluded that 7.3 percent of females ages 12 and above reported to have used illicit drugs. Globally, around 7 percent of the population between the ages of 15 through 64 had recently used either cannabis, opioid, cocaine, or a type of amphetamine [6]. This accounts for roughly 324 million people. Furthermore, global deaths reported in 2012 due to substance abuse ranged from 95,000 to 225,000 people. Additional impacts of substance abuse include increase incidence of HIV infection and incarceration. WHO estimates place the global percentage of IV drug users currently living with HIV as high as 13.1 percent, with variations occurring demographically. In the United States, roughly 50 percent of the federal prison population stems from crime related to illicit drugs [7, 8]. An American study of the financial cost of substance abuse, opioid poisoning and overdose have accounted for an estimated 22.6 billion dollars that included emergency room visits, inpatient treatment, and overall losses in productivity [9]. Emergency room visits for opioid poisoning were often shown to coincide with alcohol or other illicit drug use. Drug statistics are often comorbid for other substances leading to a fluctuation in the numbers reported. Despite this limitation, the large impacts of drug use remain constant.

Reward and the Reward Circuitry

As previously mentioned, addiction is a multifaceted mental state that leads to its maladaptive behaviors. Of importance is its biological component. Generally

speaking, substance use generates a physiological response perceived as rewarding in

the mesolimbic system. Activation of this pathway causes the release of

neurotransmitters producing the physiological rewarding response. This perceived

reward alters behavior to increase the frequency of this reward. This reward serves as a

type of memory that is generated by changes in synaptic firing, increased

neurotransmitter release, and protein synthesis. These responses are governed by

genes in these brain regions. The repeated use or exposure to a substance has been

shown to create neuroplastic changes in gene expression as a means of learning and

potentiation of the behavior [10-12]. It is this plasticity of gene expression that produces

maladaptive behaviors and recurrent drug use. For the purpose of this thesis, I will focus

on the illicit drug, cocaine. Cocaine use generally includes feelings of pleasure, mania

and alertness, paranoia, flightiness, and anxiety [13]. The power of cocaine as it relates

to addiction and other drug-seeking behaviors stems from its activity in the nigrostriatal

and mesolimbic dopaminergic neurons and VTA. Cocaine crosses the blood brain barrier

via passive diffusion and has recently been shown to use the assistance of carrier

proteins on dopaminergic neurons for cellular uptake [14, 15]. After it has crossed this

barrier, cocaine has been shown to increase dopamine concentration and inhibit its

reuptake by dopamine transporter proteins upon binding to the dopamine transporter.

Increased dopamine concentration generates reward and is implicated in drug addiction

[16]. Within the VTA reside numerous GABAergic and dopaminergic neurons with both

neuronal groups sending projections to the nucleus accumbens and the prefrontal

cortex [17]. These regions are also home to the opioid receptors that mediate the sense

of pleasure as well as receptors responsible for the stimulatory effect of cocaine [18].

Moreover, dopamine released in these areas has been shown to occur over relatively

large areas and at axosomatic and axodendritic synapses [19]. This release of dopamine

is reported to also occur due to stimulation from GABAergic responsive neuronal

connections [20]. The reported research thus supports the idea that these neuronal

pathways including the VTA, the prefrontal cortex, and the nucleus accumbens, are

synaptically linked. It is through these pathways that cocaine exposure can stimulate a

physiological response generating physical and psychologic addition. Because of

cocaine's ability to inhibit the reuptake of dopamine, dopamine transporter knock-out

studies have been done. Knock-out of functional dopamine transport still produced mice

that self-administered cocaine [21].

Cocaine has been implicated to block monoamine transport in other neuron

transporters as well, indicating the possibility that redundancy pathways can still

develop reward following cocaine exposure [22]. Further, research has shown that

knock-in gene mutations of dopamine transporters insensitive to cocaine in an

otherwise functional dopaminergic system still allowed for rewarding behavior when

exposed to other rewarding compounds. [23, 24]. In this experiment, a dopamine

transporter was inserted into a transgenic mouse that was insensitive to cocaine. The

mutation attenuated cocaine reward while permitting normal food reward behaviors as

well as normal rewarding behaviors for other dopamine transporter agonists like

amphetamines, demonstrating the complexity of this reward system. In a study using a

cocaine relapse paradigm, it was shown that after sustained cocaine administration

dopamine concentrations were significantly lower in striatal and caudal brain regions following the treatment [25]. This corresponded to anhedonia and subsequent relapse. Research has suggested that some behavioral changes may be correlated to alterations in tissue migration within the reward pathways [26]. It is important to note that though these modifications in brain structure may occur, these changes seem to have no effect on overall brain volume suggesting that changes occurring in cocaine-exposed brains are related to the organization and the neurophysiology of neuronal projections. Cocaine has been shown to decrease arborization in the substantia nigra, the area of the brain responsible for reward and movement [27]. Those neuronal projections responsive to the inhibitory neurotransmitter GABA have been shown to be affected by cocaine exposure, resulting in changes in GABAergic neuronal patterning as they extend into the medial prefrontal cortex from the ventral prelimbic cortex [28]. As differentiation of patterning was observed, it should be noted that overall cell number was not found to be significantly changed. It appears that changes in brain biology are generating altered behaviors.

How do these biological reward pathways determine susceptibility to substance abuse and addiction? The answers lie in unique gene polymorphisms and changes to gene expression resulting from substance exposure. Recently, several gene polymorphisms have been implicated in increased addiction susceptibility [29, 30]. Polymorphisms in a mu opioid receptor and in the BDNF sequence have been highly investigated in their contribution to substance abuse disorders. Reward pathways and critical brain regions are highly plastic and readily changed. This means that these

regions function and synaptic connection as well as the expression of genes governing

these regions can be modified by substance use. Gene expression within these regions

has been thought to reinforce behavior and contribute to motivational cues for drug

use. Within the study of addiction there are several genes of interest that have been

highly studied.

Gene Expression Changes Regulate Reward

A gene shown to moderate the release of dopamine within the brain is brain-

derived neurotropic hormone or BDNF [31]. Neuronal projections found within the VTA

have been reported as substantial sources of the BDNF protein [32]. Consequently,

BDNF activation can stimulate multiple enzymatic pathways involved in dopamine

release and BDNF's importance in dopaminergic neuronal development has been

supported in the literature [33]. Bdnf binds to a tyrosine kinase linked receptor, TrkB,

and dimerizes allowing the complexed protein to phosphorylate downstream targets.

TrkB signaling has been indicated to provide anti-apoptotic activity in neurons of the

VTA and prevents loss of excitatory neurons in the forebrain [34]. These signaling

cascades generated through Bdnf and other neurotrophin signals have been implicated

in memory consolidation and neuronal plasticity [35]. Using these signaling pathways,

Bdnf has been shown to promote the recurrent use of cocaine and drugs of abuse in

animal models [36-38]. Studies of acute cocaine exposure indicated an increase in Bdnf

activity in the nucleus accumbens and a tendency to self-administer cocaine [36].

Animals treated with anti-BDNF antibodies to suppress BDNF activity in that brain

region, in contrast, showed a decrease in TrkB signaling, attenuated cocaine self-administration, and weakened tendency for relapse. In another study, exogenous supplementation of BDNF in the medial prefrontal cortex following self-administration training led to decreases in cocaine-seeking behavior [39]. These results taken together suggest Bdnf protein has differing effects within different regions of the brain. Dose-dependent experiments have shown differential Bdnf mRNA and protein concentrations within the medial prefrontal cortex and striatum of the mouse brain [40]. In an experiment conducted by Fumagalli et al. (2007) acute cocaine administration led to a robust increase in Bdnf mRNA expression in the prefrontal cortex. Researchers allowed for repeated cocaine administration and demonstrated an upregulation of a transcription factor that lead to increased Bdnf expression [40]. This upregulation generated neuroplastic changes in Bdnf expression. Studies using transgenic animals suggested nucleotide substitutions in the Bdnf sequence itself can affect substance use [30]. Human association data has addressed a link between compulsive alcohol consumption and the allelic alteration of a valine for a methionine at position 66 in the BDNF sequence leading to decreased activity of this protein [41]. Taking advantage of this knowledge, researchers generated transgenic mice expressing this allelic substitution and demonstrated increased alcohol consumption when compared against wildtype allelic mice [30]. BDNF is only one of several genes associated with addiction research. Another promising gene encoding a transcription factor is FBJ murine osteosarcoma viral oncogene homolog B or simply, FosB.

FosB is a member of the fos gene family and has been documented to play roles in motivation, reward, memory, and drug-induced neuroplasticity [42-44]. FosB possesses two isoforms and can be found as its shortened isoform, ΔFosB, within the nucleus accumbens and the striatum [45]. Both isoforms of FosB, FosB and ΔFosB, have been shown to be expressed there, but ΔFosB was found to be relatively more stable than its longer isoform [46]. Fos family genes have been indicated as a mechanism for natural rewarding processes including exercise, food in-take, and some sexual activities [42, 43, 45]. Drugs of abuse also stimulate this gene family. Acute exposure to rewarding drugs indicated increases in the ratio of ΔFosB to FosB. Interestingly however, this trend was shown not continue under chronic use conditions, suggesting the shorter isoform plays a more dominant role in this neuroplasticity of reward pathways. In a recent study with transgenic mice, mice were produced to allow for exogenous stimulation of the ΔFosB isoform in neurons of the nucleus accumbens [47]. Stimulation of these neurons led to significant augmentation of the rewarding effects of cocaine. Additional studies investigated the effects of overexpressing ΔFosB within striatal neurons [48]. In these transgenic animals, over-expression in striatal neurons led to rapid acquisition of cocaine self-administration at lower cocaine dosages. The results of this study imply that overexpression of this protein within the striatal neurons enhances the rewarding properties of cocaine in the animal and potentiates self-administration and suggested an explanation for relapse following a withdrawal period. Studying the effects of opioid abuse on ΔFosB displayed a similar result. Using a transgenic mouse model, researchers found inducible over-expression of ΔFosB lead to increased morphine reward and

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increased physical dependence [44]. Studies to understand a role of ΔFosB in alcohol

addiction are limited. A recent study investigating the use of electroacupuncture as a

treatment option shed some insight on this problem. Using electroacupuncture, there

was a decrease in alcohol consumption in rats when compared to sham-tested animals

[49]. The authors found a corresponding increase in the shorter ΔFosb isoform in the

core nucleus accumbens and dorsal striatal neurons when compared to untreated

animals. These results suggested that increased alcohol consumption occurred with an

increased ratio of ΔFosb/FosB [49]. The role of the genes BDNF and FosB provide sound

evidence of the neuroplasticity of our brains following drug use and abuse.

Factors Contributing to the Potential for Substance Abuse

An interesting caveat to the genetic vulnerability to addiction later in life relies

on one's personality. One well-documented endophenotype linked to substance abuse

is impulsivity. Impulsivity is an innate characteristic that is considered evolutionarily

beneficial when occurring in a small number of individuals in a population as it increases

potential for advantageous risk taking [50]. This increased risk taking has also been

demonstrated in individuals more likely to have a psychiatric or substance abuse

disorder [51, 52]. As human studies are often correlative and confounded with

numerous variables, animal models are a valuable resource. In an experiment using

adult rats, animals were behaviorally tested to select for a high-impulsivity phenotype

and this cohort was demonstrated more likely to persistently use cocaine regardless of

foot shock [53]. Studies using brain imaging techniques suggested reduction of

dopamine receptor 2/3 in rats' ventral striatum was predictive of substance abuse and impulsivity [54]. Imaging studies have suggested differential brain region volumes and increased impulsivity in human adolescents [55]. Impulsivity studies have been conducted regarding alcohol consumption as well. Mice with postnatal early and late alcohol exposure displayed poorer decision making skills when tested as adults [56]. These studies support a role for genetic makeup in mediating or contributing to behavioral consequences that underlie substance abuse disorders.

Environmental factors can also contribute to subsequent substance abuse disorder later in an individual's life. A well-studied environmental factor includes premature exposure to rewarding substances. In studies using a prolonged alcohol administration model system, alcohol led to changes in reward pathway neurochemistry in a dose-dependent manner [57]. Sustained alcohol exposure led to increases in the concentrations of the monoamines serotonin and dopamine. Research also indicated that prenatal exposure to alcohol led to altered grouping behaviors [58]. The increase in alcohol concentration during embryonic development decreased shoaling within treatment groups of adult zebrafish. Another environmental contributor to addiction is the exposure to stress. Research has demonstrated that early life stresses in male mice produced adult mice that self-administered methamphetamine significantly more than unstressed controls [59]. A similar result was obtained when pregnant dams experienced stressful stimulation, which led to increased self-administration of morphine in their pups during adult behavioral testing [60]. Social environment can also effect an organism's susceptibility to substance abuse. Cohorts of mice raised in

differing levels of arousal, as determine by opportunities for voluntary exercise, larger

living space, or toys, were shown to have differential conditioned preference to cocaine

[61]. This research concluded that increased environmental enrichment demonstrated a

protective quality against substance abuse.

Another prevalent environmental influence is nicotine, which acts to change the

amount of the neurotransmitter dopamine present within the reward pathways. Intake

of nicotine provides the brain with elevated concentrations of dopamine leading to the

reward circuitry being activated by the interaction of the tobacco alkaloid and nicotinic

acetylcholine receptors (nAChRs) on VTA dopamine neurons [62, 63]. Cigarette smoke

introduces numerous free radicals leading to an increase in fatty acid peroxidation, and

this increase is synergistic with simultaneous alcohol administration [64]. Lipid

peroxidation can lead to changes in mitochondrial membrane structure, action

potentiation, and synaptic cleft action. In adolescent rats tested using a nicotine

infusion paradigm, researchers adduced that increased mRNA expression of p53 was

shown in multiple brain regions as a direct result of cellular damage [65]. Embryonic

nicotine exposure has been indicated to have both physical and behavioral alterations in

animal models. Nicotine exposed dams produced offspring with gender specific

locomotion alterations, increases in nicotinic receptor densities, increases in anxious

behaviors, learning deficiencies, increased vulnerability to nicotine consumption, and

increased impulsivity [66-69]. These changes are thought to occur due to alterations in

gene expression in the cholinergic system. Gestational nicotine exposure was associated

with increased expression of various nAChRs in the forebrain of fetal rats [70].

Longitudinally, early exposure led to decreases in dopamine release from the nucleus accumbens in adolescent rats [71]. These results provide evidence that early exposure to exogenous stressors can have robust effects on the developing organism. The influence our environment has on our behaviors has been well documented, but how these behavioral changes come to be is still being investigated.

Early Drug Exposure Produces Longitudinal Effects

The result of pharmacologic insult to the developing individual during critical developmental periods has been associated with long term structural, behavioral, and physiological implications. The Substance Abuse and Mental Health Services Administration reported 5.4 percent of pregnant females ages 15-44 admit to drug use. Early cocaine exposure has been well-documented to induce a suite of age-related consequences. Infants exposed to early cocaine exposure (ECE) have shown difficulty in controlling arousal [72], have divided attention [73], and display alteration in discriminatory attention [74]. In toddlers, the effect of ECE has been shown to affect sustained attention, response times [75, 76], impulse control [77], communication skills [78], and planning and task organization [79]. Brain imaging on a group of exposed children, aged 8 to 10, displayed a decrease in overall grey matter and thalamic and putamen volumes [80]. Similar research has indicated reductions in corpus callosum regions, additional decreases in grey matter of the occipital and parietal lobes, and a decrease in circular cerebral blood flow [81, 82]. Dopaminergic areas, such as the left and right caudate nuclei, have shown to be diminished in children of addicted mothers

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[83]. Results of these studies are often confounded by multiple factors including malnutrition, multiple drug use, tobacco use, low socioeconomic status, route of drug administration, and gestational time and duration of use. Because of these confounding variables, it is often difficult to attribute causation to a single factor. Researchers have aimed to overcome this and other ethical hurdles with the use of animal models allowing a greater understanding of the effects of prenatal cocaine exposure.

Studies investigating the negative effects of ECE on early development in animal models have allowed for more controlled designs. These studies allow researchers to account for confounding variables such as multi-drug use and malnutrition. Numerous studies have indicated gestational cocaine has led to significant effects on mammalian birthweight [84-86]. ECE in primates demonstrated significant decreases in volume and in brain weights as well as alterations in the formation of cerebral cortical layers I-IV [86]. Here, radioactive labeling of thymidine to tract cell migration demonstrated improper movement of cortical neurons during brain development. Animal imaging studies suggested volumetric differences in reward-associated brain regions indicative of damage in axonal myelination as a result of cocaine exposure [26]. These observed differences in neuronal development however have been controversially reported. Further, gestational cocaine exposure has been shown to hinder myelogenesis in synaptic pathways of the brainstem of mice [87]. The physiological response of the brain has been shown to be affected by ECE. Metabolic responses as measured by glucose utilization were shown markedly reduced by ECE in the limbic and hypothalamic regions [88]. These results also indicated a sexually dimorphic response between male and

female rats with reductions in glucose utilization seen in more regions of the male brain than the female brain. Importantly, this study implied ECE resulted in decreased brain metabolic responses. Cocaine exposure has been demonstrated to alter brain physiology and structure. These differences have also been demonstrated to effect communication within reward pathways of these brains.

Cocaine's effect on neuronal communication and synaptic transmission occur due to their immediate changes in neurotransmitters that are released. This increased release of dopamine and other neurotransmitters is thought to govern the behavioral responses of exposed organisms. Moreover, the release of neurotransmitters is subject to sensitization following recurrent use and can result in changes of the mesolimbic pathway. The alterations seen in the mesolimbic pathway occur by modified expression of gene governing reward. Cocaine has been shown to act on gene expression and neuronal regulation early in development [89]. Research has demonstrated that the timing of ECE changes nodal-related protein expression and its related transcription factors in whole larval zebrafish samples. Modifications in expression that did occur in the transcription factors important for developmental genes were unique to the time that the exposure occurred. The authors quantified these changes using quantitative PCR and demonstrated that there was up- and down-regulation of these gene resulting from the different temporal exposure to cocaine at 24 hpf and 48 hpf [89]. This exposure to cocaine at 24 hpf and 48 hpf led to increased and decreased expression of tyrosine hydroxylase and the dopamine transporter, respectively. Further, alterations in the spatial arrangement of transcription factors that enable dopaminergic and

GABAergic neuronal development then, are thought to also control the structural

development of the reward pathways [90]. These changes may be what contributes to

changes in sensitivity to the drug in subsequent exposure events. Moreover, another

study noticed significant difference in axonal bundling in the anterior cingulate cortex of

ECE rabbits, as well as increases in the GABAergic neuronal projections [91]. Stanwood

et al. (2001) immunologically stained for tyrosine-hydroxylase, the rate-limiting enzyme

for catecholamine synthesis and found increased staining regions densely populated

within pyramidal neurons. This suggested the potential for increased excitatory

responses and was indicative of structural alterations within the limbic system and

prefrontal cortex pathways. ECE was shown to modify neuronal plasticity in animal

models with embryonic exposure. Patel et al.(2012) showed that prenatal exposure led

to changes in neural stem cell production in the subventricular zone. They used

immunohistochemistry to label for the protein nestin within type A and C progenitor

cells. The results indicated changes in distribution of this protein that differed between

the genders of the adult mouse brain. Taken together, these results indicate that

cocaine has long-term impact on the adult brains of animals exposed to ECE, leading to

changes in neuronal networks and signaling.

Behavioral analyses have indicated that ECE can contribute to abnormalities in

social behaviors [93]. In human research studies, ECE was correlated to earlier onset of

sexual activity [94]. The occurrence of early sexual behavior was strengthened by the

coincidence of early stress, namely exposure to violence. Third trimester ECE in children

of approximately 7 years of age displayed a positive correlation between this exposure

and increased impulsivity, inattention, and increased hyper-activity [95]. Longitudinal

studies have indicated an increased undertaking of risky behaviors in adolescent males

reported with ECE [96]. This increased risk-taking behavior was associated with increase

hostility and a lack of impulse control [97, 98]. Examination of ECE adolescents identified

potential alterations in oxytocin pathways leading to increases in aggressive tendencies

into adulthood [99]. These results support the claim that ECE provides lasting behavioral

consequences in individuals of ECE. Unfortunately, with human studies, there are

confounding variables such as multi-substance abuse, malnutrition, and parental

investment that complicate the interpretation of these results.

Animal studies of behavior have substantiated some of the observed deficits in

human behavior as the result of ECE. In memory and learning analyses, mice with ECE

performed poorly when compared to untreated controls and were delayed in the

sensitization to pain in foot-shock experiments [100]. Mice pups with ECE have been

shown to wall-climb less than untreated controls indicative of abnormalities in

catecholamine levels in the striatum [101]. These mice also showed learning

abnormalities in matching maternal milk to their mother's odor when tested against

pair-wise controls. The behavioral aberrations as described are likely the result of

altered cognitive development as the result of ECE. Cocaine acts within dopaminergic

neurons leading early researchers to investigate how ECE played a role in dopaminergic

neurons. Mammalian studies indicated that ECE produced an uncoupling of D_1

dopamine receptors, which may lead to long-term behavioral dysfunction [102].

Neurotransmitters can act as morphogens as well as survival factors during

development and are critical to neuronal circuitry formation, so any disruption during this time period has lasting consequences on central nervous system function. Animal models exposed to ECE displayed abnormal grown in neuronal dendrites in brain areas associated with cognitive function and emotional regulation [103]. Moreover, numerous animal model studies have indicated alterations in signaling cascades of neuronal circuitry [104, 105]. A study was conducted using mice in an attempt to determine any cognitive deficits that resulted from ECE. Researchers assessed levels of Bdnf and its downstream effector, TrkB. Here, levels of the phosphorylated (thus, activated) TrkB as well as the levels of Bdnf mRNA were longitudinally increased in the frontal cortex [104]. This increased activation and increased expression may contribute to neuroplastic changes seen in organisms with ECE. A similar study addressing how the neuroplasticity comes about identified changes in AMPAR receptor function. Research by Baskshi et al. (2011) suggested ECE affected regulatory proteins that control the AMPAR receptor. Altered function of these receptors is thought to contribute to long-term potentiation and long-term depression of synaptic connections. Thus, ECE may contribute to the neuroplastic changes in reward circuitry that are thought to alter behavior. Cocaine exposure to the developing brain has lasting consequences, but the effects of ECE are not restricted to the nervous system and can negatively impact a broad range of physiologic responses with detrimental results for the individual. For example, ECE has been demonstrate to also have longitudinal effects on the cardiovascular system [106].

From the mentioned research, it is clear that cocaine produces numerous adverse outcomes for individuals with ECE. Placental mammals provide a sound model

system for mimicking the effects of ECE, but also include several limitations. Because of the internal development of placental animals, malnutrition can be a confounding variable for interpreting the results of addictive drug studies. Malnutrition as a result of ECE has been disputed within studies indicating food consumption and weight gain did not significantly differ, but results are controversial [85, 107]. Mammalian model systems include another unique confound. Because of their internal development the location of the developing embryo within the uterus may affect the amount of blood flow the individual receives. It has been experimentally determined that oxygen amount and correlatively the amount of blood the embryo receives may determine some cellular fates [108]. This differential blood flow may in turn, affect the concentration of drug the developing pup receives. This confound may affect the interpretation of longitudinal experiments with ECE. Additionally, due to this internal development, important organ systems including the brain, liver, and heart cannot be visualized without a cull. Zebrafish provide a large scale solution to these problems and have been recently used in addiction and behavioral studies [109-111].

Zebrafish as an Ideal Model Organism

Zebrafish (*Danio rerio*) have become a highly utilized vertebrate model organism. These small fish possess many advantages for researchers investigating mechanisms underlying complex disease. The easy manipulation of the small genome aids in introducing genetic mutations, leading to the generation of a myriad of mutant lines that are valuable to investigate developmental mechanisms and disease processes. The

external fertilization of zebrafish embryos allows researchers to control developmental

microenvironments and to reduce maternal variables like malnutrition that can impact

egg formation. Also, unlike the use of mammalian organisms, zebrafish development

can be viewed in a noninvasive manner. The small brain size of the adult zebrafish is

advantageous because it can be easily observed using intravital imaging equipment

[112]. Additional advantages when using zebrafish are the large clutch sizes and

relatively short generation time [113]. In addition, the zebrafish possess many

physiological features that are homologous to those of higher vertebrates. The

zebrafish brain has an amygdala-like structures and a reward circuitry that is

comparable to the mammalian brain [114]. As a result, zebrafish are an ideal model to

assess the neurophysiologic and behavioral consequences of ECE. In addition, there is a

high percentage of an amino acid structural homology between zebrafish and

mammalian dopaminergic receptors (D_2 and D_3), making zebrafish an ideal system for

studying the pharmacology of drugs of addiction [115].

Darland and colleagues have demonstrated the utility of the zebrafish model to

investigate the behavioral effect of cocaine and have linked these effects to genetic and

physiological responses. Previous studies from our lab provided evidence of a dose-

dependent, cocaine-induced conditioned place preference (CPP) in the zebrafish model

[116]. Interestingly, mutagenized family lines of zebrafish were insensitive to the effect

of cocaine within these behavioral studies, although specific genetic links were not

identified at that time. Using the percentage of individuals displaying impaired CPP, it

was suggested that this modified response was the result of a Mendelian inherited,

single gene mutation within gene networks of the dopaminergic pathway. This indicated zebrafish as a model for behaviors associated with reward.

Research has also suggested that zebrafish respond physiologically to cocaine in a dose-dependent manner [117]. Cocaine's effect on the cardiac system is mediated by both the central and peripheral nervous systems [118]. In the central nervous system, research suggests that cocaine results in increased sympathetic brainstem activation [119]. In the peripheral nervous system, the sympathetic inhibition of epinephrine reuptake or a probable increase in adrenal medullary stimulation leads to increases in heart rate and blood pressure [120]. Indeed, pharmacologic agents have been used to test adrenergic activity at sympathetic terminals in the peripheral nervous system [117]. In this study, it was observed that as cocaine dose was increased, the heart rate of adult zebrafish increased in a dose-dependent manner. The addition of a ß-adrenergic antagonist attenuated this increase in heart rate in the adult fish. Conversely, concurrent treatment with an α-adrenergic antagonist strengthened the effect of cocaine on heart rate increase. Further, 5-day old zebrafish treated with increased concentration of cocaine showed this dose-dependent increase in heart rate as well. This demonstrated the adrenergic systems control over heart rate was downstream of cocaine's effect as a monoamine. The results of these studies suggest zebrafish are a valuable model for the study of addictive drugs. As zebrafish are prone to lasting physiological consequence to ECE and share molecular regulatory pathways with mammals [106], this model is ideal for investigating how ECE generates lasting behavioral alterations governed by gene expression. A critical question in the field of

addiction is *how* are the gene expression and behavioral changes linked to ECE.

Emerging evidence suggests a role for epigenetics in these processes.

Epigenetics Mechanisms and Intergenerational Inheritance

Epigenetics is a means by which environmental factors generate persistent

changes in gene expression. Epigenetic modification occurs through a covalent

attachment of some functional group to a promoter sequence or DNA associated

proteins that can modify that segment of DNA's transcriptional potential. DNA

modification has been observed as an attachment of methyl groups directly to the DNA

sequence or as an attachment of these methyl groups to histone proteins. Several

mechanisms have been documented that led to differential DNA transcription.

Attachment of acetyl groups to histone proteins has been well documented as a means

altering transcription. Vassoler et al. (2013) and his team of researchers detected

increases in *Bdnf* expression as well as Bdnf protein in the reward circuitry of these male

offspring. Researcher observed an increased acetylation of histone 3 in the *Bdnf*

promoter regions in the sperm of self-administering paternal mice. Acetylation at these

histone proteins leads to the introduction of a positive charge, which changed the

accessibility of chromatin wrapped on its histone. In this open state, transcription of this

gene can occur at a greater rate. These results taken together suggest that the

substance abuse phenotype was passed on to offspring. This inheritance suggested an

epigenetic event was occurring and permitted the passing on of drug-related behaviors.

As mentioned, epigenetic alterations constitute changes in gene expression via mechanisms that do not alter the genetic code itself. Rather these changes in expression are the result of the thermodynamic probability for the RNA polymerases to interact with and access the template strand. Complex DNA as a double stranded molecule is wrapped around histone proteins forming a nucleosome. There are four histone proteins (H2A, H2B, H3, H4) that associate together and form this nucleosome octamer. Chromatin with 'open' regions are termed *euchromatin*, while regions tightly wrapped around these histone proteins are termed *heterochromatin* and are active or inactive for transcription, respectively. Chemical groups that can form an attachment to either DNA or the histone proteins change the accessibility of gene transcripts to the transcriptional machinery, thus changing access to template DNA. This accessibility is controlled by multiple molecular and enzymatic means, including modifications to the amino acid tails of the histone proteins, the addition of acetyl groups, methyl groups, phosphoryl groups, or methylation of the DNA sequence itself [121].

The effects of several epigenetic alterations have been reported in the literature. One model for chromatin remodeling that has been highly investigated is the addition of acetyl groups onto key histone amino acids by enzymes termed histone acetyltransferases. The addition of negatively charged acetyl groups typically to lysine or other positively charged amino acids neutralizes their charge, loosening the coiling of DNA to the histone proteins and promoting transcription[122]. Another highly studied modification occurring at histone tails is the addition of methyl groups by histone methyltransferases. Unlike the addition of acetyl groups, the addition of methyl groups

carries no charge and is thought to not affected that stability of the interaction between

DNA and chromatin. Methyl groups can be added to histone tails as single methyl, di-

methyl, or tri-methyl groups. These additions have varying effects on transcription at

histone tails depending on the amino acid targeted as well as the overall epigenetic

landscape [123]. In a study conducted by Maze et al. (2010), researchers reviewed the

role of a particular histone dimethyltransferase, G9a, which regulated the methylation

of the transcription factor ∇FosB [124]. In this study, experimental knockdown of the

G9a dimethyltransferase strengthened preference for cocaine in mouse behavioral

assays by augmenting dendritic projections in the nucleus accumbens of mice [124].

Another group of methyltransferases add methyl groups directly to the DNA strand,

ideally named DNA methyltransferases (Dnmts). Research has indicated five known

mammalian Dnmts: Dnmt1, Dnmt2, Dnmt3a, Dnmt3b, and DnmtL [125]. The zebrafish

genome has undergone a duplication event and thus possess a unique set of Dnmts

homologous to the mammalian enzymes. Mammalian Dnmt1 has been shown to

methylate CpG rich regions and act to sustain methylation patterns in cells undergoing

replication [126, 127]. Dnmt1 has been mentioned in methylation maintenance and is

highly expressed in cells undergoing mitotic processes [128]. Results of mutant lines

studies indicated that mouse embryonic stem cells with the Dnmt1$^{-/-}$ genotype showed

no significant decrease in de novo methylation [129]. These results suggested that de

novo methylation was largely catalyzed by Dnmt3a and Dnmt3b. In the zebrafish,

Dnmt3a is represented as dnmt6 and dnmt8 [130]. Mammalian Dnmt3b is represented

in the zebrafish as dnmt3, dnmt4, dnmt5, and dmnt7[131]. Indeed, another means of

epigenetic regulation used to modify gene expression includes a group of small

noncoding RNAs called, microRNA (miRNA). These are typically around 21 nucleotides in

length and act to modify gene expression patterns through complementary binding.

RNA strand complementarity to target sequence prevents the binding of transcriptional

machinery, consequently regulating the transcription of that particular mRNA. Research

has demonstrated this mechanism may play a role in some drug-seeking behaviors and

addictive neuropsychiatric disorders[132]. Research has suggested that alterations seen

in the expression of genes involved in memory and neuronal plasticity, such as bdnf

occurs through these small miRNAs.

Epigenetic mechanisms have been implicated in the responsiveness to

psychostimulants and drugs of abuse[121]. Research conducted by Baker-Andresen et

al. (2015) explored methylation patterns in the medial prefrontal cortex of mice [133].

Following the recurrent self-administration of cocaine, genome-wide sequencing

showed differential methylation patterns in 29 genomic regions of the mouse prefrontal

cortex when compared against untreated controls. Interestingly, differential

methylation patterns were observed following a period of cocaine abstinence,

suggesting cocaine induced lasting changes in brain regions associated with high

cognition, planning and sensory integration [133]. In another study, rats subjected to

chronic cocaine exposure showed decreased drug seeking behaviors when treated with

L-methionine, a methyl-donating compound [134]. Dr. Wright and her team observed

increases in the expression of DNA methylating enzymes, Dnmt3a and Dnmt3b, in the

nucleus accumbens. Additionally, the expression of a Fos family gene within the nucleus

accumbens and prefrontal cortex was decreased by L-Methionine treatment. These results indicated DNA methylation may play a role in addiction and in potentiating addictive behaviors within reward pathways by means of gene expression regulation [134]. Alterations in methylation patterns at certain CpG islands of gene promoters have been indicated to alter transcriptional potential, thus permanently changing gene transcription [135, 136]. If substance abuse in an adult organism can contribute to sustained DNA methylation changes, maintenance of these patterns could contribute to similar behavior in offspring, with the potential for particular vulnerability in those individuals exposed during brain formation. *The work presented here will test the hypothesis that ECE leads to persistent changes in gene expression as the result of an epigenetic mechanism and that these changes in expression govern reward-seeking behaviors.* We predict that epigenetic modification resulting from ECE can provide lasting gene expression changes and subsequently affect physiology and behavior in future generations.

METHODS AND MATERIALS

Fish husbandry

Zebrafish (*Danio rerio*) were raised at the University of North Dakota and

fostered according to recommended protocols (The Zebrafish Book, Animal Welfare

Assurance #A3917-01, protocol 1403-7) on a stand-alone water system from Aquatic

Habitats [137]. Water for fish underwent reverse osmosis with 0.3 g/L Instant Ocean

(Instant Ocean Spectrum Brands, Blacksburg, VA, USA) and 0.1 g/L bicarbonate and

conditioned on the system to our specifications, hereafter known as fish water. Fish

experience a 10-14 dark-light cycle. In the morning, fish are fed brine shrimp and in the

afternoon with pelleted food. All fish tested behaviorally are from an AB line obtained

from Harvard Biological Laboratories (Harvard University, Cambridge MA) and bred in

house for 10 years. Larval fish from a reporter line that use the α-tubulin promoter to

express green fluorescence protein (GFP) within the central nervous system were used

for embryonic tissue harvest [138]. GFP is expressed in the forebrain structure of adult

and larval zebrafish and was used to distinguish tissues for dissection (Figure 3).

Drag Exposure Model
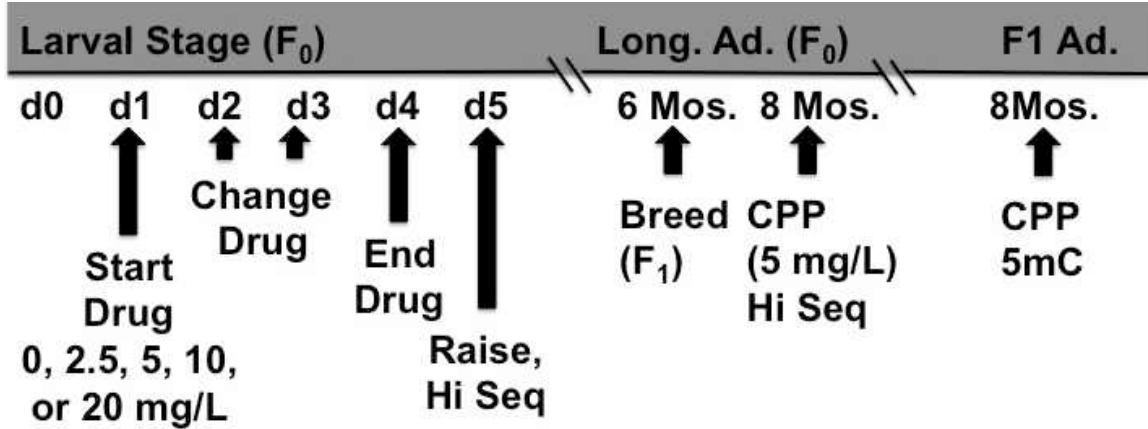
Overall Experimental Design
Figure 1: The treatment paradigm outlining the overall experimental design is shown. Fish embryos were collected and washed on d0 defined as fertilization. Initial treatment began on d1 in a 6-well plate with drug being replaced each morning (d2 and d3). Drug was removed on d4 and washed away (3X). Fish for longitudinal experiments including adult breeding, CPP and Hi Seq experiments were placed on the stand alone system on d5. Longitudinal experiments were conducted at 6-8 mos. F_1 fish were tested behaviorally or used for methylation experiments (5-mC) at 8 months.

The basic experimental paradigm is outlined in Figure 1. To model ECE, fish were collected the morning following a parental cross. Embryos were rinsed with fresh fish water and placed in a petri dish at d0. The following day fish were staged and screened for defects. Healthy specimens at a density of 25-40 fish/well were placed in a 6-well plate and treated with concentrations (0μM, 2.5 μM, 5 μM, 10 μM, and 20μM) of cocaine (Sigma-Aldrich, NIDA) as done previously [106, 117]. Previous data from our lab showed adult zebrafish display a dose-dependent behavioral response to cocaine in the ranges tested here. Parental generation (F_0) zebrafish were treated with cocaine hydrochloride dissolved in 5ml of fish water on d1-d3. We chose this developmental

28

window of 3-day treatment as it shows little neurotoxicity and is thought to have little

effect on dopaminergic architecture of the developing larval zebrafish [106]. On the d4,

fish were rinsed 3 times to remove cocaine and on d5 placed on the system. Parental

fish (F_0) from each treatment group were raised and crossed at 6 months to produce the

F_1 generation of zebrafish. This cross produced a clutch that did not experience a second

exposure to cocaine. Fish of both F_0 and F_1 were then tested behaviorally using a

conditioned place preference assay at 6-8 months of age.

Another group of F_0 fish were generated to experience ECE with a simultaneous

treatment of Chlorogenic Acid (CA). CA acts to inhibit the dnmt enzymes [139]. DNA

methylation occurs through the enzymatic action of DNA-methyltransferases at the 5'

position of cytosine residues. Methyl groups are donated from S –adenosyl- 1 –

methionine. CA is a dietary catechol thought to non-competitively inhibit methylation by

increasing concentrations of S-adenosyl- 1 –homocysteine in turn reducing

concentrations of S –adenosyl- 1 –methionine, the methyl group donor [140]. Dose-

response experiments were conducted to determine an optimal concentration that had

no effect on survival. Measurements of body length were conducted with live

anesthetized fish using a ruler with recordings collected in millimeters. Weights for

these anesthetized fish were recorded with the scale tared to 100mL of water.

Percentages of survival were calculated based on the number of viable specimens

relative to the initial clutch size. From this experiment, it was determined that 1 µM CA

was the threshold concentration that allowed for adequate sample size. Chlorogenic

acid must be dissolved in dimethyl sulfoxide (DMSO) at a stock concentration of 1 µM.

The groups for these experiments include an untreated group, a group treated with 1 µM DMSO, a group with 10 µM cocaine, a group treated with 1 µM CA, and a group treated with 1 µM and 10 µM cocaine hydrochloride. This treatment occurred as described above. Fish were raised into adulthood and tested at 8 months using Conditioned Place Preference (CPP).

CPP Behavioral Assay

CPP was adapted and recorded as previously described [116, 141]. Briefly, the testing apparatus is a 3-chambered rectangular tank depicted in Figure 2. Each chamber contained 1L of fish water and included 2 removable partitions. These removable partitions (or barriers) divide the lane into 3 compartments; the middle section contains twice the volume of the two smaller chambers of each lane (500 ml and 250 ml, respectively; 1 liter total). To allow fish to freely explore the testing apparatus, passable barriers were in place that had a 1" diameter hole in their centers. These were then replaced with a solid barrier for the isolations in the front or back chamber (Figure 2). Darland et al. (2012) measured water flow between the larger compartment and the smaller compartments using phenol red and determined water exchange was minimal (data not shown) [141]. Each lane remains separate from its adjacent lane and allows visualization of neighboring test subjects. Due to the anatomical features of zebrafish eyes, it is likely that the fish use additional extended visual cues. The rear portion of the tank has duct tape wrapped around. The proximal compartment is left open. Each 3-chamber testing tank is isolated from the adjacent testing apparatus by a white cardboard divider. The test tank allows for 3 fish to be tested together. The initial day of

testing included a 40-minute swim allowing for acclimation to the novel test

environment. Fish are placed in the center section of their lane and allowed to explore

the lane with open partitions in place. The 2nd and 3rd days included two isolation events

without cocaine treatment for 30-minutes each following an 8-minute baseline

measurement. The isolation occurred by removing the open partitions and replacing

with closed barrier. On day 2, the isolation occurred first in the front section for 30-

minutes and then again in the rear compartment for 30-minutes. Day 3, the isolation

events occur first in the rear for 30-minutes and then, the front section for 30-minutes.

Baseline measurements occurred before the isolation events on Day 2 and 3. On day 4,

preference for either the front or rear section is recorded during the baseline

measurement and determined during the isolation events. The first isolation occurs in

the chamber the fish preferred the most and the last isolation occurred the chamber

preferred the least.

It is on this day of CPP testing that conditioning occurs. Cocaine hydrochloride

was dissolved in fish water and added to the least preferred chamber during the second

isolation event. Day 5, the conditioning was conducted identical to day 4. Day 6

consisted of a baseline measure to quantify any potential change in preference as the

result of conditioning. Measurements were recorded two separate ways due to limited

technologic resource. In one lab, measurements were recorded using TopScan3.00

(TopScan 3.00 Clever System Inc. 2011). In the other lab, preference was recorded using

a ternary code, where 1 indicated presence in the front chamber, 2 in the central

chamber, and 3 in the rear compartment. Fish that did not move were scored as '0' to

reflect the characteristic freezing behavior associated with a stress response in zebrafish. Any fish exhibiting these stress-related behaviors, such as freezing, for greater than 50% of the reading were excluded from analysis. CPP experimental protocol was adjusted to account for behavioral results. Optimal assay concentrations were based on previously published work. Darland et al. (2001) showed that adult zebrafish display a dose-dependent response to cocaine using CPP, with the optimal range from 0 to 20 mg/mL [116]. In addition, we have shown that zebrafish respond physiologically to cocaine in a dose-dependent manner, with a characteristic shift in cardiac function [117]. For both behavioral and physiological assays, peak responsiveness to cocaine occurs at the concentration of 10 mg/mL (10 μM). Based on these studies, we treated our initial cohorts of zebrafish with a submaximal cocaine concentration of 5mg/mL to determine any change in longitudinal responsiveness.

The F_0 generation CPP included an untreated control, 2.5 μM/L cocaine, 5 μM/L cocaine, 10 μM/L cocaine, and 20 μM/L cocaine. These concentrations were shown to initiate a behavioral response in a dose dependent manner [116, 117, 141]. In preliminary studies with the F_1 generation, preliminary studies we observed some change in preference in untreated fish. To account for this phenomenon, we adjusted the CPP protocol. Experimental results indicated an innate preference to the front chamber, regardless of cocaine treatment. Changes in the location of isolations from the zebrafish preferred chamber to isolations occurring only in the rear chamber led to greater conditioned response. We added another control group to confirm that the observed behavior was a direct result of cocaine exposure. We treated a group of fish

with 2.5, 5, and 10 μM/L lidocaine, an anesthetic. Lidocaine acted as a negative control because it can imitate the local anesthetic action similar to cocaine, but does not stimulate reward by inhibiting monoamine transporters.

CPP Assay Tank
Figure 2: CPP-testing apparatus is shown. Arrows indicate isolation chambers. The red arrow indicates the back chamber and the blue arrow indicates the front chamber. Each isolation chamber has a removable divider with a hole in its center allowing free exploration. Conditioning occurs in the back chamber with an impassable barrier in place.

Tissue Collection and Brain Dissections

In order to determine the affect cocaine exposure had on gene expression, we collected telencephalic tissue from larval and adult zebrafish on the basis that this region is thought house regions that are structurally homologous to mammals and are responsible for the rewarding properties of cocaine, including the nucleus accumbens and dorsal striatum [142]. The critical regions are outlined in GFP expressing fish and reflect the areas dissected (Figure 3). We have previously demonstrated that cocaine exposure led to significant gene expression changes in the regions of adult brain [141].

To ensure no suffering occurred to the animals, they were first anesthetized in tricaine (methanesulfonate, Sigma-Aldrich) then sacrificed with dissections conducted essentially as described [141].The adult zebrafish head was removed at the 7th pharyngeal arch and a sharpened surgical probe was used to pierce the teleost skull medially and circumlaterally. The skull was then peeled away to expose the rostral portion of the diencephalon and the entire telencephalon. Using small surgical spring scissors, an incision was made to separate the caudal telencephalon from the rostral diencephalon. This cut enters through the diencephalic ventricle caudally to the dorsal telencephalic area. Then, rostral cuts were made to separate telencephalic tissue from the olfactory bulb. To separate the telencephalic tissue from optic tissue, the surgical scissor was inserted into the mediolateral space in the eye socket clipping off the optic nerve. The cartilage surrounding the socket was then cut away to expose the ventral telencephalon and optic chiasm. An incision at the optic chiasm freed the telencephalon. Which was then be removed from the 'cradle' of cartilage using forceps. Two telencephalons were used per sample. Care was taken to ensure that no tissue cross-contamination occurred during the dissections.

Embryonic dissections were done to harvest larval telencephalic tissue. Green fluorescent expressing larval zebrafish were anesthetized within a petri dish using tricaine and collected in a RNAlater solution. Larval fish were then centrifuged (≥10,000 rcf). Then, larvae were placed in a new petri dish with 600 μL RNA later and viewed under a fluorescent scope. In order to collect the corresponding telencephalic tissue in the larval zebrafish, fish were laid on a lateral side and the eyes was removed using a

tungsten needle. The developing telencephalon then lay dorsal to the olfactory bulb and could be excised. Because of the small tissue sample present within the larval telencephalon each treatment group would include >50 telencephalons.
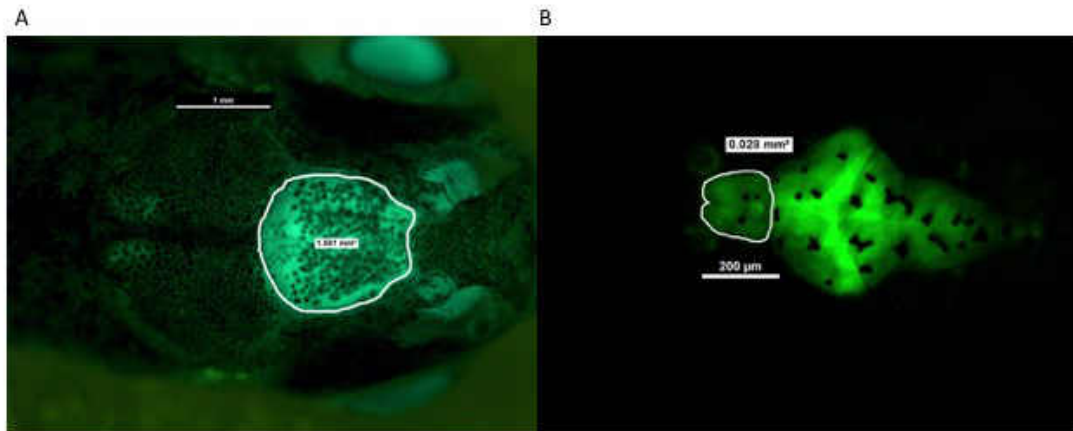
Zebrafish Reward Circuitry
Figure 3: Shown above are telencephalons of A) an adult and B) a larval zebrafish expressing Green Fluorescent Protein. A) is a magnified image of the adult zebrafish forebrain structure. B) is a magnified image of the larval zebrafish forebrain.

Genetic Material Preparation and Gene Expression Verification

Quantitative polymerase chain reaction experiments (qPCR) were used to take advantage of the linear nature at which DNA is amplified to determine the amount of a known sequence. Detection appears as PCR products accumulate during the exponential amplification steps of the PCR reactions. Here, the first substantial increase in the PCR products reflected increased detection of the fluorescent marker and the initial amount of our targeted sequences. We anesthetized and culled groups of ECE larvae at 5dpf and longitudinal adults at 6 months. Dissections were conducted to collect telencephalic tissue from adult and larval zebrafish. Adult samples were gender matched and two forebrains constituted one sample.

To begin, our tissue collections were chemically manipulated to produce total RNA. Collected tissues were stored in RNAlater solution or collected and immediately used to generate RNA. RNA was purified using an RNeasy kit (the Qiagen RNeasy® Mini Kit). Briefly, aliquots of a solution containing 10μL of β-mercaptoethanol in 1mL of buffer RTL were added to the tissue samples and homogenized for approximately 10 seconds. The samples were then centrifuged at full speed (15000 rcf) for 3 minutes and the supernatant transferred to a new centrifuge tube. Samples were then treated with one volume (700 μL) of 70% ethanol, prior to transfer to a spin column. The spin column was centrifuged at 10000 rcf for 1 minute, the supernatant was discarded and the remaining sample was added and centrifuged. In the spin column, 350μL of reagent RW1 was added and centrifuged at 10000 rcf for 30 seconds. The flow through was discarded and an aliquot of 10 μL DNase and 70 μL reagent RDD was added to each column and allow incubate at room temperature for 15' to remove residual genomic DNA contamination. The column was washed with 350 μL of reagent RW1 and centrifuged at 10000 rcf for 30 seconds. Supernatant was again discarded. The spin column was treated with 500 μL reagent RPE and spun at 10000 rcf for 30 seconds, with this step repeated, but was then centrifuged for 2 minutes at 10000 rcf. The top of the spin column was transferred to a dry collection tube and spun for 1 minute at >15000 rcf. The top of the spin column was removed and placed in another new, dry collection tube. Finally, 30 μL of RNase-free water was added to the tube and incubated for 1' at room temperature. This was followed by a 1' centrifuge at 8 rcf to elute the total bound

RNA. The samples were analyzed for purity using a Nano drop and then placed in a -80°C freezer for storage.

From our RNA samples, we generated complementary DNA (cDNA) using two separate reverse transcription kits. iScript contained all the necessary components for reverse transcription of first strand DNA synthesis except the RNA template. Briefly, a total sample of 20 µL cDNA was produced. Each sample required 4 µL of iScript reagent and the equivalent of 200ng of RNA, with the remaining volume filled with RNase-free water. The other kit was from Applied Biosystems, RNA PCR core kit that produced 40 µL of cDNA. Here, reverse transcription mixture need was generated prior to use and added to an equivalent of 200ng of RNA. Each sample required 8 µLMgCl$_2$, 4 µL of reaction buffer, 4 µL of the 5 dNTPs, 2 µL of RNase inhibitor, 2 µL of reverse transcriptase, 1 µL oligos, and 1 µL of random hexamers. These amounts were pipetted into one stock solution and each sample received 34 µL of this solution. The remaining volume was an equivalent of 200ng of RNA template and RNase-free water. Incubation of samples from both kits was done according to their respective protocols.

Real time qPCR was carried out in order to quantify the specific gene of interest expressed in our tissue samples. Further manipulation of our cDNA samples was required for this step. Master mixes for the respective gene were generated that contained 0.5 µL of the forward and reverse primers(nmol), 9.5 µL RNase-free water, and 12.5 µL SyberGreen (Bio-Rad Laboratories, Inc). This volume was pipetted into 96-well plated with 2 µL of cDNA from our ECE groups for RT-qPCR analysis. Real time qPCR reactions were carried out in the CFX86 Real-Time System C1000 Touch Thermal Cycler.

Reactions were conducted according to protocol. Briefly, Taqman protocol occurred in 3 phases. Phase 1 occurred at 95° C for 3 minutes. Phase 2 and 3 were repeated 39 times for amplification of the desired gene fragment. Phase 2 ran for 10 seconds at 95° C and phase 3 occurred at 60° C for 30 seconds. Each gene was plated in triplicate. Primers were produced so that the forward primer was complementary and had slight overlap with the reverse primer for each gene (Integrated DNA Technologies, Inc.). This overlap prevented the inclusion of genomic DNA in our samples. Primers used in these experiments are described in Table 1.

Next Generation Sequencing and Transcriptome-wide Analysis

In order to observe which genes govern the acute and longitudinal result of cocaine exposure, we utilized Next-gen RNA sequencing (RNA-seq) techniques. Generally, Hi-sequencing technologies use an Illumina approach to produce high-throughput sequencing data. Briefly, RNA-Seq works in a series of three steps: 1) sample preparation, 2) Sequence, and 3) analysis. RNA-seq is a type of whole transcriptomic shotgun sequencing that relies on the existence of a series of short reads that resulted in 30 million 50 bp single end reads for each gene. It is an ideal approach to observe the transcriptome during a disease or drug induced state. For our experiment, Hi-Seq RNA analysis was conducted by the University of North Dakota Epigenetic Core Facility. Library preparation and Illumina Sequencing was performed by the core facility at the University of Minnesota and differential expression analysis was performed by Adam Scheidegger at UND's core facility. Tissue samples were generated using the paradigm

shown in Figure 1. ECE embryos raised to adults were then group to determine gene

acutely sensitive to cocaine exposure. Fish from each ECE treatment group were treated

in a mock CPP experiment and sacrificed at 8 months (Figure 1). During this mock-CPP,

fish from each embryonic treatment group were isolated and exposed to 5 mg/L for 1

hour. Additionally, a group of fish from each embryonic cocaine concentration were left

untreated. For each of the ECE groups treated with mock-CPP and those left untreated,

three replicates were done for a total of 24 total samples. Fish from untreated and

treated groups were then sacrificed. Groups from the F_0 generation at each embryonic

treatment concentration were analyzed following a mock-CPP experiment. A cohort of

ECE fish, that were left untreated with the mock CPP experiment, from each embryonic

cocaine concentration were also analyzed. Then comparisons were conducted

comparing all mock-CPP exposed fish against all untreated ECE fish. This comparison

revealed a list of genes likely affected by cocaine exposure and these were termed

cocaine sensitive genes (CS genes). A file of CS genes was arranged according to $\log_2$

increase and significant p-value. From this, the top 150 CS genes were identified. Our

top 150 genes were then assessed for interactions among each other. Expression of

these genes was analyzed at each cocaine concentration. This allowed us to compare

the change in expression of these CS genes.

In order to determine any interactions within our list of genes, I used the

database STRING [143]. STRING generates a dense web of direct and indirect protein

interactions based on literary review, experimental data, and estimated functionality.

Our RNA-seq data gave an interesting array of protein interactions and clusters that I

investigated using qPCR. Genes appearing at focal points of these clusters were selected for further investigation.

Bisulfite Sequencing Analysis

To further test the hypothesis that methylation of DNA governing CS genes is altering the reward behavior of our F_1 longitudinal zebrafish, we collected telencephalic tissue for a 0, a 2.5, a 10, and a 20 mg/L ECE cohort for bisulfite sequencing. Bisulfite sequencing treats each DNA segment with bisulfite, which will convert unmethylated cytosine residues to uracil. Cytosines that possess a methyl group will be left untouched, and in this way, bisulfite sequencing gives individual nucleotide resolution of methylation patterns. Fish were first culled in a pair and each sample consisted of two male fish. If two males were not available for each sample, we matched gender at a 1:1 ratio. Because methylation often affects transcription at the promoter site we needed to generate genomic DNA (gDNA). A Quick-DNA kit (Zymo Research; Irvine, CA) was used for this purpose according to manufacturer's protocol. Briefly, dissected tissue samples (≤25mg) were prepared as previously described and added to a protease buffer (95μL nuclease free H_2O, 95μL Solid Tissue Buffer, and 10μL Proteinase K; per sample) and mixed thoroughly for incubation. The incubation occurs for approximately 3 hours at 55^oC. Following incubation samples were centrifuged at 12000 rcf for 1 minute to separate out any insoluble material. Samples were then transferred to new centrifuge tubes and 400μL Genomic Binding Solution was added. Solutions were mixed and added to a spin column for centrifugation at 12000 rcf for 1 minute. The flow through and

collection tubes were discarded. The spin column was transferred to a new collection

tube,400μL DNA Pre-wash Buffer was added, and spun for 1 minute at 12000 rcf. The

collection tube was emptied and 700μL gDNA Wash Buffer was added and centrifuged

for 1 minute at 12000 rcf. The collection tube was again emptied, 200μL gDNA Wash

Buffer was added, and the column was spun for 1 minute at 12000 rcf. The collection

tube was then treated with 50μL of Elution Buffer in a new collection tube, incubated at

room temperature (20-25°C) for 5 minutes, and finally centrifuged for 1 minute.

Genomic DNA of our samples was then assessed for purity using a nanodrop and sent

for bisulfite sequencing. Whole genome Bisulfite sequencing was performed by Zymo

Research. Differentially methylated genes were sorted by p-value and data was

complied with the top 2000 genes. Samples from 0mg/L and 2.5mg/L F_1 zebrafish were

also sent for hydroxymethylation studies, with DNA collected as previously described. A

complete list of significantly hydroxymethylated genes was produced based on

significant based on p-value.

Statistical analysis

All statistical analysis was conducted using GraphPad Prism 7.0g ® (GraphPad

Software, Inc., La Jolla, CA). Normality of groups was assessed using D'Agostino and

Pearson normality test. ECE groups used in CPP were analyzed for average cocaine-

induced behavioral changes using one-way ANOVA. Groups were compared against one

another and against control fish using Bonferroni's multiple comparisons test. Kruskal-

Wallis test was used to analyze experiments in which the assumptions of one-way

ANOVA could not be met. Groups tested included longitudinally raised zebrafish that were treated with previously determined concentrations of cocaine that include 0, 2.5, 5, 10, and 20mg/L [106, 116, 117, 141]. Individual behavioral experiments were analyzed using Repeated Measures ANOVA. Baseline one 1 (BL1) was excluded from this analysis due to a novelty effect. Any significant results were followed up with a Bonferroni's correction. Testing to compare untreated fish against all treated groups from the F_1 cohorts were transformed using the log transformation to account for deviations from normality. Untreated and treated fish were then compared using Mann-Whitney test as assumptions of the student t-test could not be met. Due to the large group variability, we analyzed group variances using the Brown-Forsythe test.

Expression data was normalized to the reference gene ef1α [106, 116]. Cocaine sensitivity of our gene selected for validation was done by comparing all ECE groups of fish left untreated to all ECE groups acutely treated using student's t-test. In order to compare expressional changes, one-way ANOVA was used to determine any differences among ECE groups. Multiple comparisons were done using Dunnett's multiple comparisons test to compare all groups against the 0 coc group. When determining difference among all groups, Tukey's multiple comparisons test was used. In order to determine any significant affect between ECE treatment concentration and longitudinal acute cocaine exposure on gene expression we used a two-way ANOVA. Multiple comparisons were done using Dunnett's multiple comparisons test to compare all groups against untreated, 0 mg/mL ECE controls.

RESULTS

CPP Results in the Parent Generation

Parental generation fish that were treated embryonically with the five ECE dosages were raised and tested with CPP to determine any differential behaviors attributed to early drug exposure (Figure 4). We hypothesized that ECE would generate increased sensitivity to acute cocaine exposure in the adult zebrafish. Previous studies indicated zebrafish responded in a dose-dependent manner with maximal behavioral responses occurring at 10 mg/mL [116, 141]. Given this bell-shaped curve, we wanted to test our hypothesis and, therefore, conducted our behavioral assays with a submaximal dose of 5 mg/mL. The column represented by Unt CPP included fish from all ECE groups that were untreated during CPP testing. These fish showed no significant differences in conditioned preference when tested without cocaine ($p<0.35$, F=1.24, df=24). There was significant CPP behavior when comparing treated fish from each ECE treatment group against untreated fish (for 0 mg/L $p<0.02$; for 2.5 mg/L $p<0.03$; for 5 mg/L $p<0.01$; for 10 mg/L $p<0.001$). The ECE treatment group exposed at the highest concentration of 20 mg/L did not display significant CPP behavior. Our results shown in Figure 4, demonstrated a bell-shaped curve indicating maximal response in ECE treatment groups exposed at 10 mg/L. CPP values recorded for the 10 mg/L ECE fish were also significantly higher than fish exposed at the highest concentration during their development, 20 mg/L ($p<0.02$).
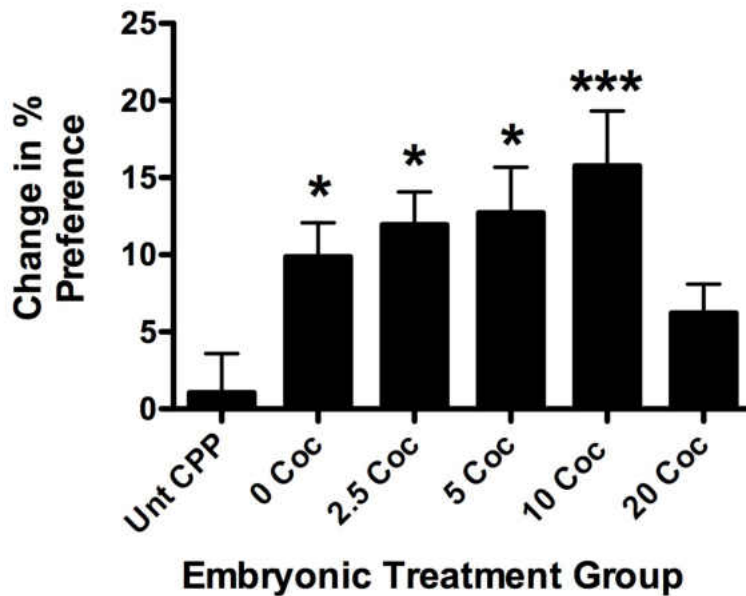
CPP Data in Parent Zebrafish Cohorts

Figure 4: Cocaine induced conditioned place preference change in embryonically treated adult zebrafish. Error bars represent ±95%, * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$ when comparing against untreated fish from all ECE groups using Dunnett's multiple comparisons test [106].

Illumina Sequencing of F_0 Zebrafish Cohorts

Groups of zebrafish with ECE were used for Hi-Seq transcriptome analysis. This was conducted to develop a profile of gene expression changes induced by acute cocaine exposure and to analyze how these changes were influenced by ECE. RNA-Seq analysis was conducted by the University of North Dakota Epigenetic Core Facility. Adult F_0 fish from each embryonic treatment concentration (0, 2.5 5, 10, and 20 mg/mL) were assigned to two groups for analysis. One group was left untreated. Another group was treated acutely with 5 mg/mL cocaine for one hour in a mock-CPP experiment. Fish from the four ECE groups that were left untreated were combined and compared to the four

ECE groups of the mock CPP exposed fish. Each of the four ECE groups was replicated

three times allowing comparison to be made between 12 groups. These comparisons

were conducted to give a list of CS genes with highly significant p-values. Following this

comparison, we returned to the untreated and mock-CPP exposed experiments for

comparison of each ECE group separately. This was done to interpret any effect that ECE

had on CS gene expression in response to acute cocaine exposure later in life. Analysis

of untreated and mock-CPP exposed fish gave a list of thousands of genes thought to be

cocaine sensitive. In the top 150 genes, as determined by adjusted significant p-values,

more than half of these CS genes showed cocaine-induced expression changes that was

affected by ECE. When comparing the mock-CPP treated and untreated groups of ECE

fish, a log change in expression was observed. The changes in the expression of our CS

genes revealed that there was a bell-shaped curve in their expression that reflected the

pattern seen in behavioral work, in that, expression was highest in the groups exposed

embryonically to 10 mg/mL in both untreated and acutely treated adults. This

expression tapered off at the maximal ECE concentration. The top 150 CS genes that

displayed this pattern of dose-dependent increased expression were entered into

STRING database to determine possible interactions among our genes [143]. The results

of our STRING diagram analysis using these genes indicated clustering of genes, some of

which have been implicated in addiction related research [30, 48]. The STRING-diagram

of clustered genes is shown with critical hubs circled (Figure 5). Several genes within

these hubs were subsequently analyzed for their expression using qRT-PCR, including

bdnf, Δfosb, inhbab, ddx5, eif4a1a, nr4a3, odc1, arf5, arg2, per2, and *mgarp*. We also

highlighted *nrxn3a* even though we did not try to validate it, because this gene was also

significantly hypermethylated and hydroxymethylated in the F_1 generation.
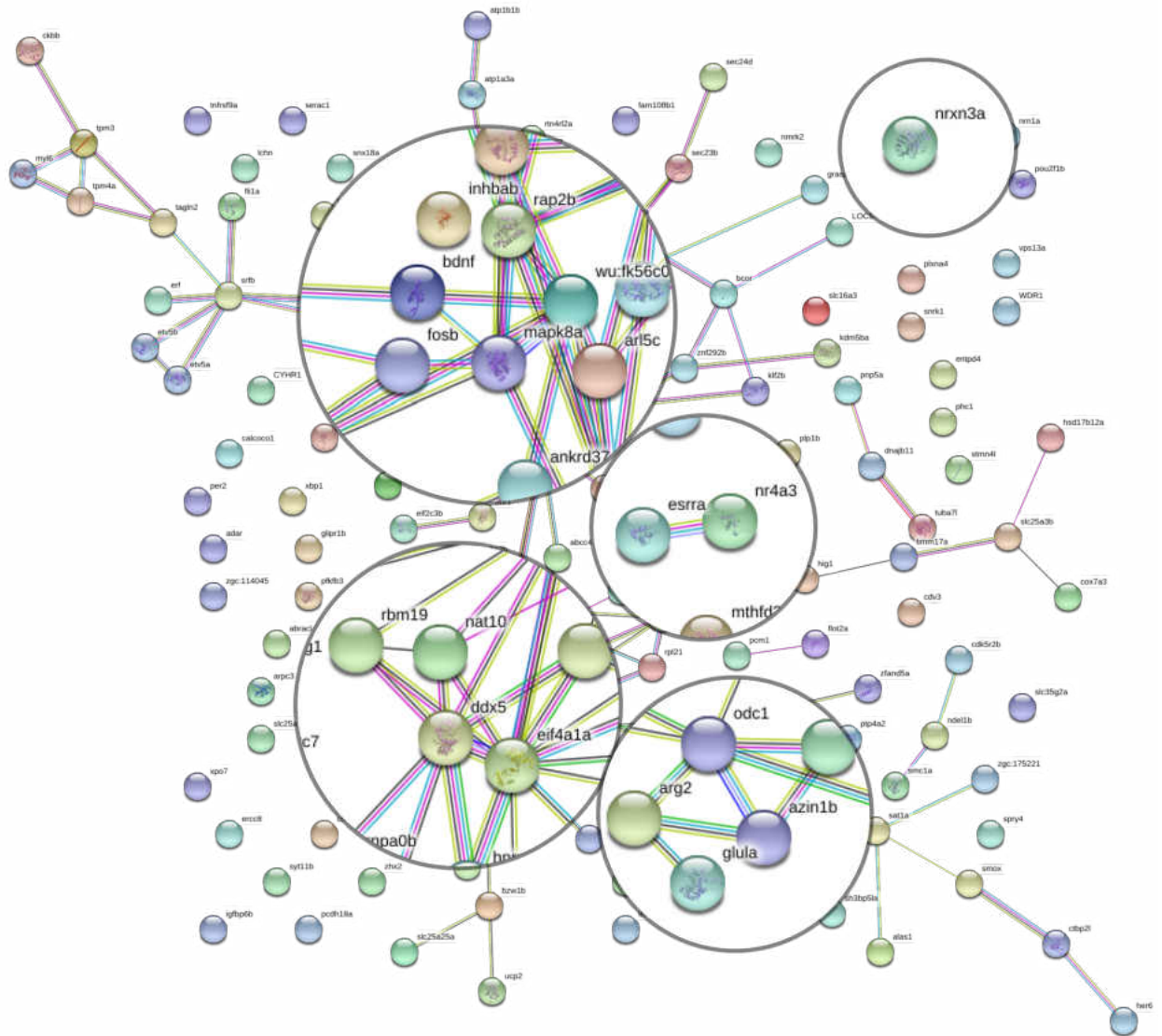
STRING Diagram of CS Genes
Figure 5: Example of STRING database image. Shown above are the top 150 most significantly affected genes according to HI-Seq. Circles indicate unique clusters of genes of which we selected CS genes for validation using qRT-PCR.

Verification of CS Sensitive Clusters
      The STRING database diagram shown in Figure 5 depicts the clustering of genes.

Based on these clusters, genes thought to play central roles among the hubs were

selected for validation. These included *bdnf, Δfosb, inhbab, ddx5, eif4a1a, nr4a3, odc1, arf5, arg2, per2*, and *mgarp*. Quantitative PCR was used to quantify the expression in untreated fish from all ECE groups and compare it against acutely treated fish from each ECE groups for each gene to validate results of Hi-Seq analysis. Grouping fish left untreated and those treated from each ECE group did not lead to any clear indication of cocaine sensitivity among these genes. To determine any interaction between acute cocaine exposure in adult zebrafish with ECE and the untreated groups with ECE and its effect on gene expression, two-way ANOVA was conducted. According to two-way ANOVA, there was not a significant interaction between ECE treatment and acute cocaine exposure on gene expression in any of our selected genes. Due to the lack of interaction found from the two-way ANOVA, effects on gene expression as the result of ECE and acute cocaine exposure in the adult was analyzed using one-way ANOVA. This result would identify any effect on gene expression that was the direct result of ECE. All other genes were then normalized to *ef1a* as it has been demonstrated as a robust housekeeping gene in previous work [116]. One-way ANOVA reported no difference in gene expression of *ef1a* in either the treated or untreated groups. Early cocaine exposure had no significant effect on the expression of *bdnf, odc1, fosb, ddx5, nr4a3, arf5, arg2, eif4a1a,* and *per2* according to one-way ANOVA. Significant variation was observed in several genes. There were significant differences among the variances in *bdnf, ddx5,* and *nr4a3* ($p<0.038$, $F_{4,21}=3.081$; $p< 0.001$, $F_{4,21}=6.992$; $p< 0.012$, $F_{4,21}=4.222$, respectively). One-way ANOVA did indicate some significant expression differences between groups with ECE based on acute treatment, indicating an effect caused by ECE

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in subsequent exposure to cocaine. The gene expression of *inhibin a beta* (*inhbab*) was significantly modified by drug treatment ($p<0.014$, $F_{4,21}=3.979$). The Brown-Forsythe test indicated that there was significant variance among the groups ($p< 0.0001$, $F_{4,21}= 90.27$). Dunnett's multiple comparisons indicated the treated 2.5 mg/L ECE group was significantly elevated when compared to treated fish from the 0 mg/mL ECE cohort (Figure 6, $p< 0.05$, df=18). The student's t-test did not indicate any significant difference when comparing treated and untreated fish within an ECE treatment group. The mitochondria-localized glutamic acid-rich proteins (*mgarp*) was also significantly different among untreated and treated ECE groups ($p<0.028$, $F_{4,21,} =3.367$) with significant variance found among these groups ($p<0.0001$, $F_{4,21}= 34.37$). Dunnett's multiple comparisons test indicated significantly increased expression in the treated 2.5 mg/L group when compared to treated zebrafish with 0 mg/mL ECE (Figure 7, $p< 0.021$, df=18). The student's t-test did not indicate any significant difference when comparing treated and untreated groups within an ECE group.
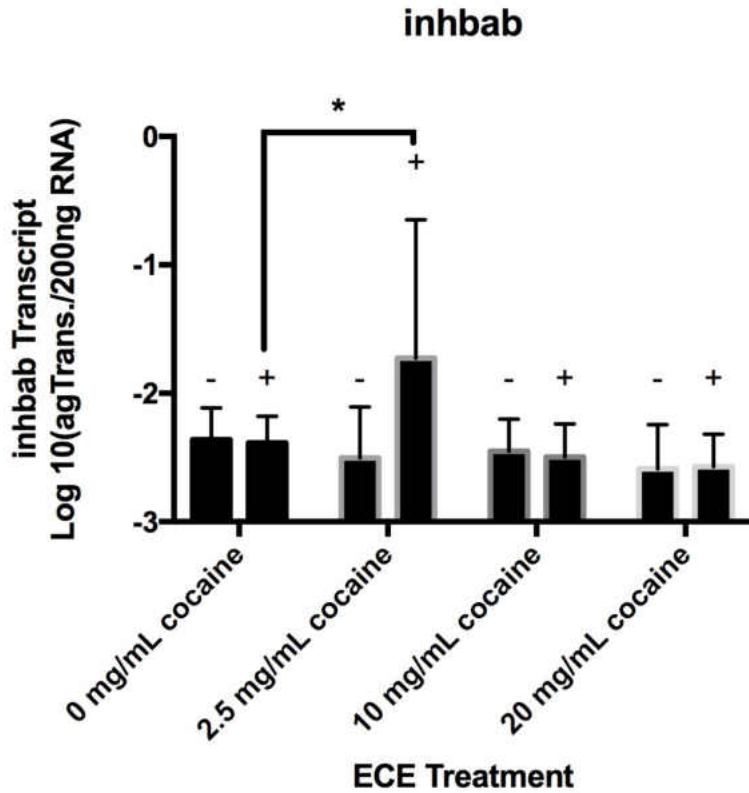
inhbab

Adult Zebrafish Expression of *inhibin A beta*

Figure 6: Expression changes of *inhbab* comparing untreated adult zebrafish with treated ECE groups. Error bars represent $\pm$95% CI, *inhbab* has been normalized to *ef1a*, * indicates *p* < 0.05 when compared against untreated controls using Dunnett's multiple comparisons test. (+) indicates zebrafish treated acutely with cocaine in adulthood. (-) indicates untreated zebrafish with ECE. The agTrans on the y-axis represents attograms of transcript.
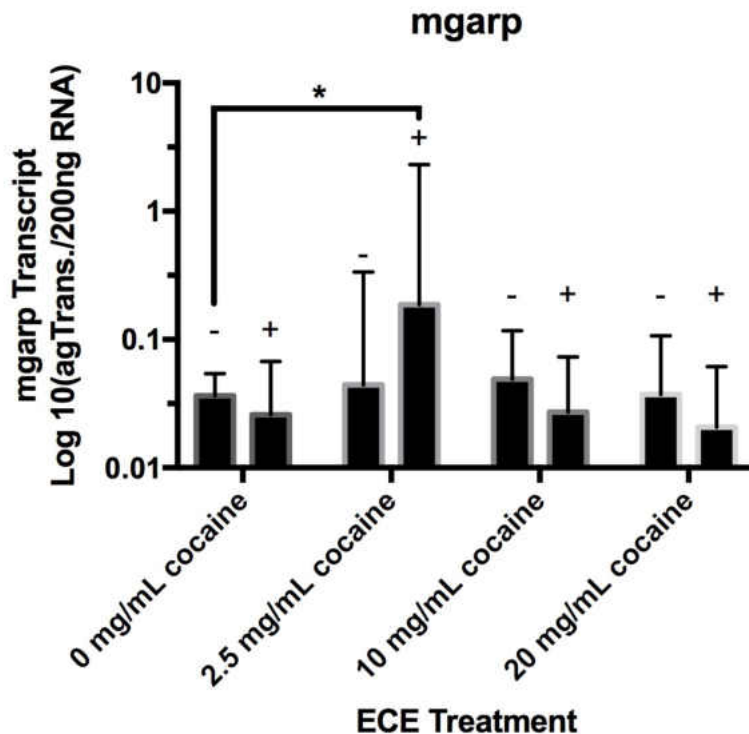
Adult Zebrafish Expression of *mitochondrial glutamic acid rich protein*
Figure 7: Expression changes of *mgarp* comparing untreated adult zebrafish with treated ECE groups. Error bars represent ±95% CI, *mgarp* has been normalized to *ef1a,* * indicates *p* < 0.05 when compared against untreated controls using Dunnett's multiple comparisons test. (+) indicates zebrafish treated acutely with cocaine in adulthood. (-) indicates untreated zebrafish with ECE. The agTrans on the y-axis represents attograms of transcript.

Quantification of gene expression was analyzed in larval zebrafish in order to demonstrate any expressional changes attributed to ECE. Again, expression data was normalized to the housekeeping gene *ef1a*, which was shown to be unaffected by ECE (p < 0.74, $F_{4,40}$ = 0.4989). The expression of *bdnf* was significantly affected according to one-way ANOVA by ECE (p < 0.05, $F_{3,32}$ = 3.846). Dunnett's multiple comparisons indicated a significant elevation in the expression of the *bdnf* gene as the ECE concentration increased from the untreated to 2.5mg/L group (Figure 8; p < 0.05, df = 32). There was no significant difference amongst other treatment groups when

compared to untreated larvae. *Delta fosB* was found significantly different among the

ECE treatment groups (p< 0.01, $F_{3,32}$= 6.06). When multiple groups were compared using

Dunnett's test, the 10 mg/mL treated larva displayed significantly decreased gene

expression when compared against the 0 mg/mL group (Figure 9; p< 0.05, df=32).

Expression of *ΔfosB* was significantly decreased in the 20 mg/mL treated larval zebrafish

(p<0.05, df=32). *Odc1* was not significantly altered due to ECE treatment (*p*<0.169). One-

way ANOVA indicated significant difference in expression in *nr4a3* due to ECE (Figure 10,

p<0.05, $F_{3,32}$=4.443). Dunnett's multiple comparisons test reported decreased

expression in all groups when compared against the control (*p*<0.05 for all ECE groups

compared to 0mg/mL, DF=32). One-way ANOVA indicated significant *eif4a1a* expression

changes attributed to ECE (Figure 11, *p*< 0.001, $F_{3,32}$=8.272). Dunnett's multiple

comparisons indicated all groups experienced a decrease in *eif4a1a* expression (Figure

11; *p*<0.01 for 2.5mg/L, *p*<0.001 for 10mg/L, and *p*< 0.001 for 20mg/L). Tukey's' multiple

comparison revealed there were no significant differences in *eif4a1a* when comparing

the treated groups amongst each other. The gene expression of *ddx5* was significantly

modified by ECE when tested using one-way ANOVA (*p*< 0.05, $F_{3,32}$=3.255). Dunnett's

multiple comparisons testing indicated 2.5 mg/mL treated larval zebrafish showed a

significant decrease in expression when compared against untreated controls (Figure 12,

p<0.05, DF=32). The *arf5* gene was differentially expressed due to ECE (*p*<0.05,

$F_{3,32}$=3.986). For *arf5,* according to Dunnett's multiple comparisons, there was

significant differences among all treated groups compared to controls (Figure 13, *p*<0.05

for all ECE groups compared to 0mg/mL, DF=32). Tukey's multiple comparisons test

identified no significant differences when comparing treated groups. The gene *mgarp*

also exhibited differential expression due to ECE in larval zebrafish ($p< 0.05$, $F_{3,32}=3.209$).

Dunnett's test indicated increased cocaine concentration during embryonic

development significantly lessened *mgarp* expression when compared against controls

(Figure 14; $p<0.05$ for 2.5 mg/L, $p<0.05$ for 10 mg/L; DF=32). The gene *inhbab* showed a

general trend of decreased gene expression, but one-way ANOVA revealed no

significant difference among groups ($p= 0.069$, $F_{4,40}=2.364$). Additionally, there was no

affect detected in the expression of *per2* and *arg2* among the ECE treated groups.
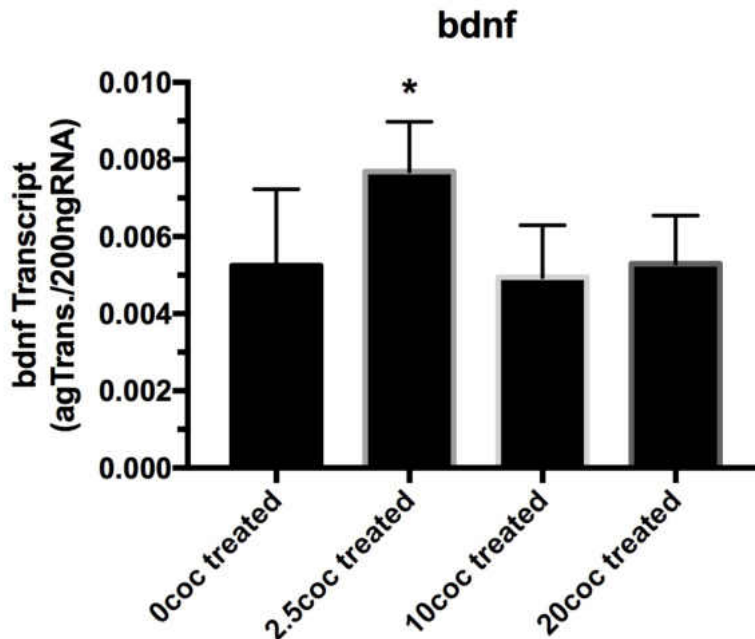
Larval Zebrafish Expression of *brain-derived neurotrophic factor*
Figure 8: Expression changes of the *bdnf* gene was seen in embryos treated with
cocaine. Error bars represent ± 95% CI, *bdnf* has been normalized to *ef1a,* * indicates $p <$
0.05 when compared to the untreated control group using Dunnett's multiple
comparison test. The agTrans on the y-axis represents attograms of transcript.
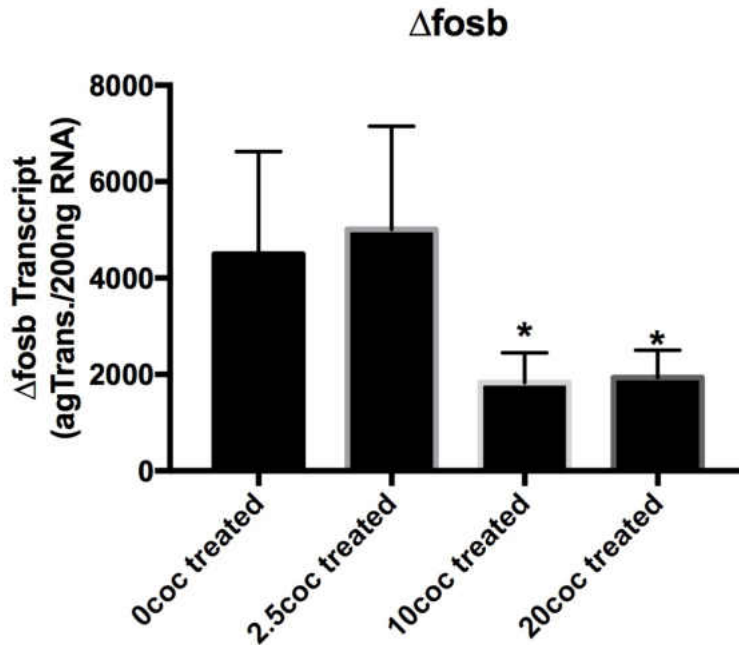
Larval Zebrafish Expression of *FBJ murine osteosarcoma factor B*
Figure 9: Expression changes of the *fosb* gene seen in embryos treated with cocaine.
Error bars represent ±95% CI, *fosb* has been normalized to *ef1a,* * indicates *p*< 0.05
when compared against all group means using Dunnett's multiple comparisons test. The
agTrans on the y-axis represents attograms of transcript.

Larval Zebrafish Expression of *nuclear receptor protein subfamily 4 group A member 3*
Figure 10: Expression changes of the *nr4a3* gene seen in embryos treated with cocaine.
nr4a3 has been normalized to *ef1a*. Error bars represent ±95% CI, * indicates *p*<
0.05when comparing against untreated ECE larva using Dunnett's multiple comparisons
test.

eif4a1a

Larval Zebrafish Expression of *eukaryotic translation initiation factor 4A1A*

Figure 11: Expression changes of the *eif4a1a* gene seen in embryos treated with cocaine. *eif4a1a* has been normalized to *ef1a*. Error bars represent $\pm$95% CI, * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$ when comparing against untreated larval using Dunnett's multiple comparisons test. The agTrans on the y-axis represents attograms of transcript.
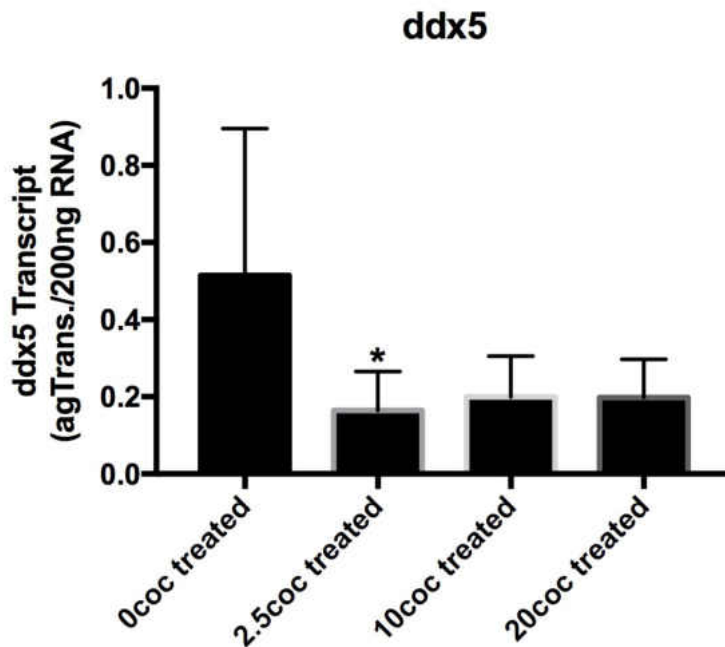
ddx5

Larval Zebrafish Expression of *DEAD box helicase 5*

Figure 12: Expression changes of the *ddx5* gene was seen in embryos treated with cocaine. Error bars represent $\pm$ 95% CI, *ddx5* has been normalized to *ef1a*, * indicates $p < 0.05$ and ** indicates $p < 0.01$ when compared to the untreated control

group using Dunnett's multiple comparison test. The agTrans on the y-axis represents attograms of transcript.
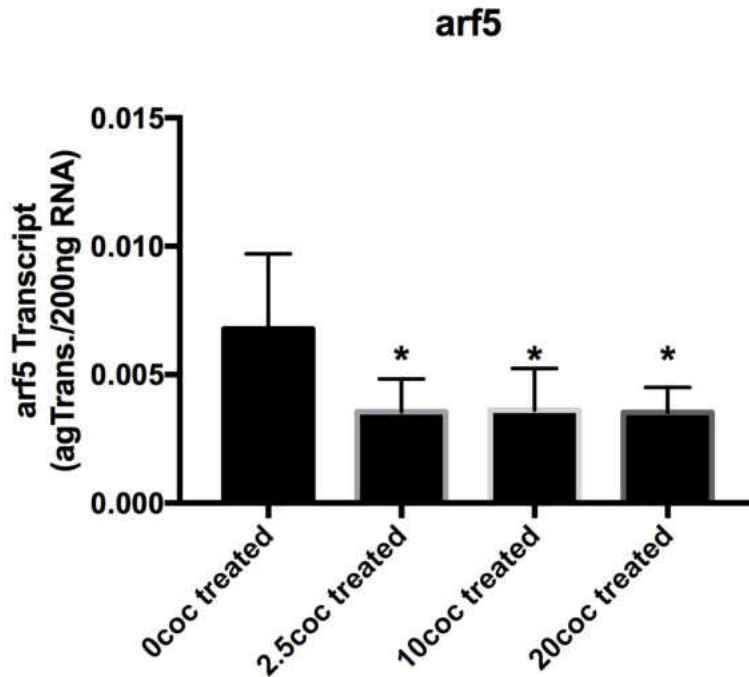
arf5
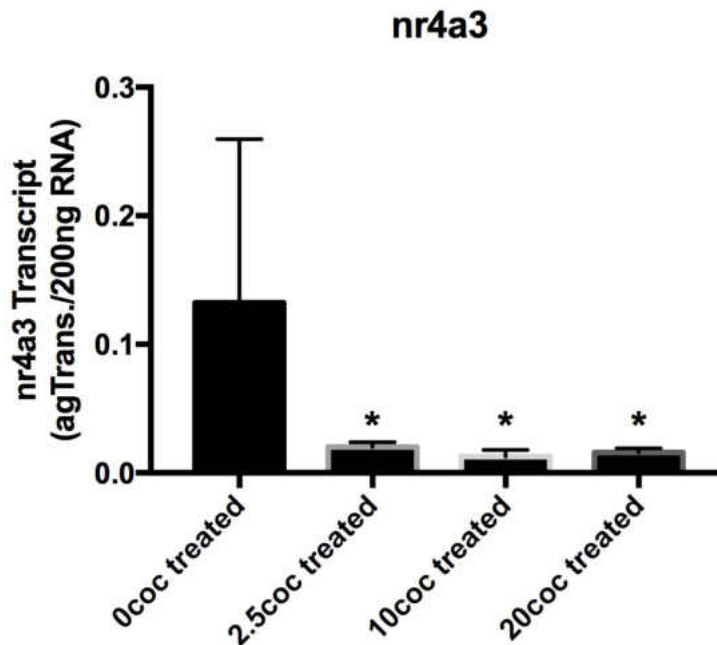

Larval Zebrafish Expression of *ADP-ribosylation factor 4*

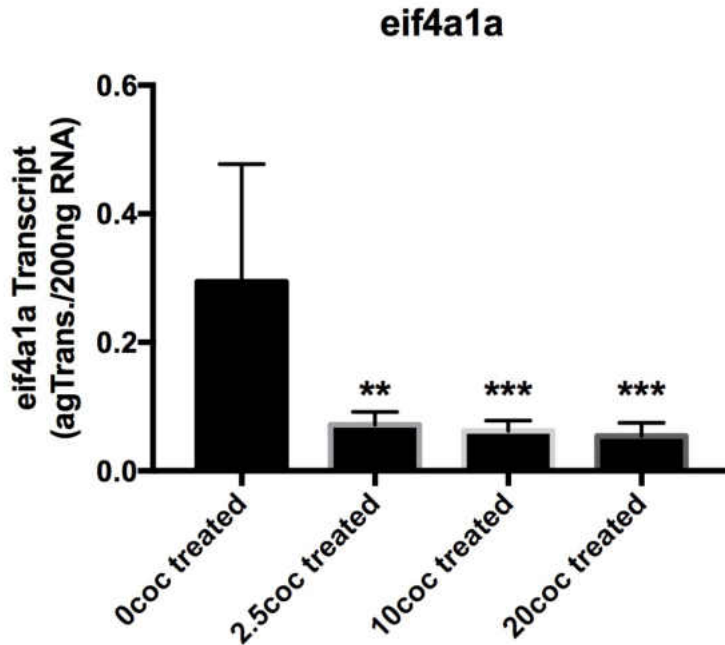Figure 13: Expression changes of the *arf5* gene seen in embryos treated with cocaine. Error bars represent ±95% CI, *arf5* has been normalized to *ef1a*, * indicates *p*< 0.05 when compared against all group means using Dunnett's multiple comparisons test. The agTrans on the y-axis represents attograms of transcript.
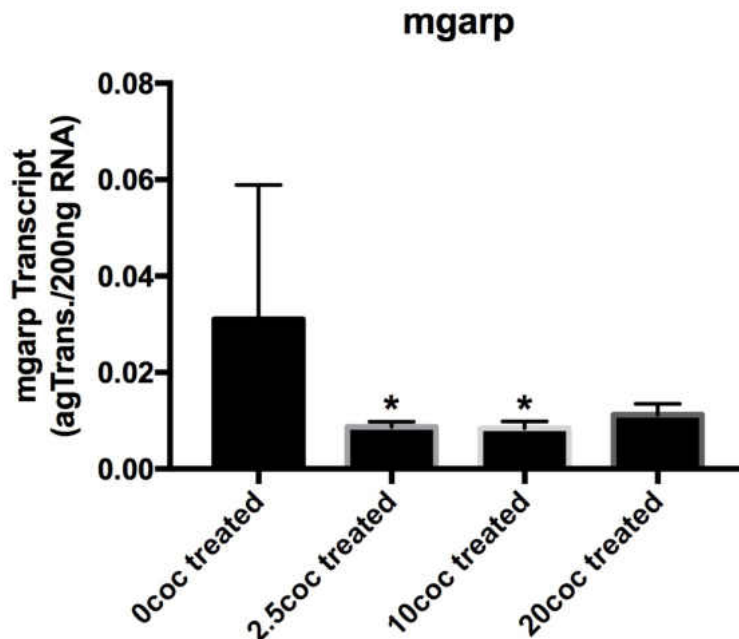
mgarp

Larval Zebrafish Expression of *mitochondrial glutamic acid rich protein*

Figure 14: Expression changes of the *mgarp* gene seen in embryos treated with cocaine. Error bars represent ±95% CI, *mgarp* has been normalized to *ef1a,* * indicates *p*< 0.05 when compared against untreated controls using Dunnett's multiple comparisons test. The agTrans on the y-axis represents attograms of transcript.

F_1 Generation CPP Assay Result

The parent generation fish exposed embryonically to cocaine (0, 2.5, 10, and 20 mg/mL) were crossed to produce the F_1 generation of fish. The F_1 generation was raised to determine any persisting behavioral effects that ECE may have on their offspring. Repeated measures ANOVA was conducted within individual CPP experiments to determine any differential CPP behavior that may be occurring between individual baseline measurements. In addition to acutely exposing our ECE cohorts to varying concentrations of cocaine, we also tested with three concentrations of lidocaine (2.5, 5, and 10 mg/mL) to serve as an additional control. Lidocaine has an anesthetic property similar to cocaine, but does not affect monoamine transport or reward and has been demonstrated to work on voltage-gated Na^+ channels in a similar manner [144, 145]. Lidocaine treatment did not generate differential CPP within any ECE group according to repeated-measures ANOVA. ECE appeared to affect how F_1 zebrafish responded to the testing apparatus as untreated fish gave generally higher CPP-responses. Repeated measures ANOVA indicated no significant differences among baseline recordings in any F_1 zebrafish in experiments testing with 1 mg/mL. F_1 zebrafish showed different conditioning responses in our behavioral assay based on cocaine treatment. Repeated measures ANOVA indicated the conditioning potential in the F_1 zebrafish in our experiment was dependent on the ECE of the parent generation.

F_1 groups were acutely tested at different concentrations of cocaine including 1 mg/L, 2.5 mg/L, 5 mg/L and 10 mg/L. Comparisons were made to determine any differences that treatment had on F_1 fish when compared to untreated controls. Lidocaine did not induce CPP at any dose used and the CPP response was not significantly different when compared to one another. Lidocaine treated groups were then pooled for sake of comparison in Figure 15. One-way ANOVA was conducted comparing all untreated fish from each ECE group and indicated there was not a significant difference in CPP behavior (p=0.98). Because of this, fish from all cohorts of ECE treatment were combined. Comparisons were then made comparing pooled groups of all fish. Pooling of F_1 fish from each ECE group that were acutely treated, left untreated, and exposed to lidocaine were compared with one-way ANOVA and demonstrated significant different CPP behavior (p<0.01, $F_{2,304}$=6.107, data not shown). Tukey's post hoc experiment indicated no significant difference among untreated and treated fish (p=0.17). CPP behavior was significantly higher in treated fish when compared to fish exposed to acute lidocaine treatment (p<0.01, DF=304).

Analysis of longitudinal behavior in the F_1 generation based on ECE concentration revealed altered responsiveness based on acute cocaine treatment concentration (Figure 15). Results showed that only the 0 ECE group displayed significant CPP in the F_1 (p<0.001, $F_{5,67}$=4.787). According to Tukey's multiple comparisons testing, 0 F_1 fish responded significantly higher to 2.5 mg/mL cocaine when compared against our lowest treatment concentration ([1 mg/mL], p<0.05, DF=67). CPP data from the 2.5 F_1 cohort appeared to be unaffected by acute cocaine concentration,

as there were no significant behavioral differences observed (p=0.41). The 10 ECE F_1

group did not display differential behavioral preference resulting from acute cocaine

exposure (p=0.06). Graphic representation of CPP experiments conducted in the 10 F_1

group suggested heightened CPP behavior in a dose-dependent manner with maximal

responses occurring at 2.5 mg/mL, though not significantly different. The 20 F_1 cohort

did not present with differential CPP behavior based on acute cocaine treatment

(p=0.54). The results of CPP testing in the F_1 groups of zebrafish indicate a cocaine

insensitivity.
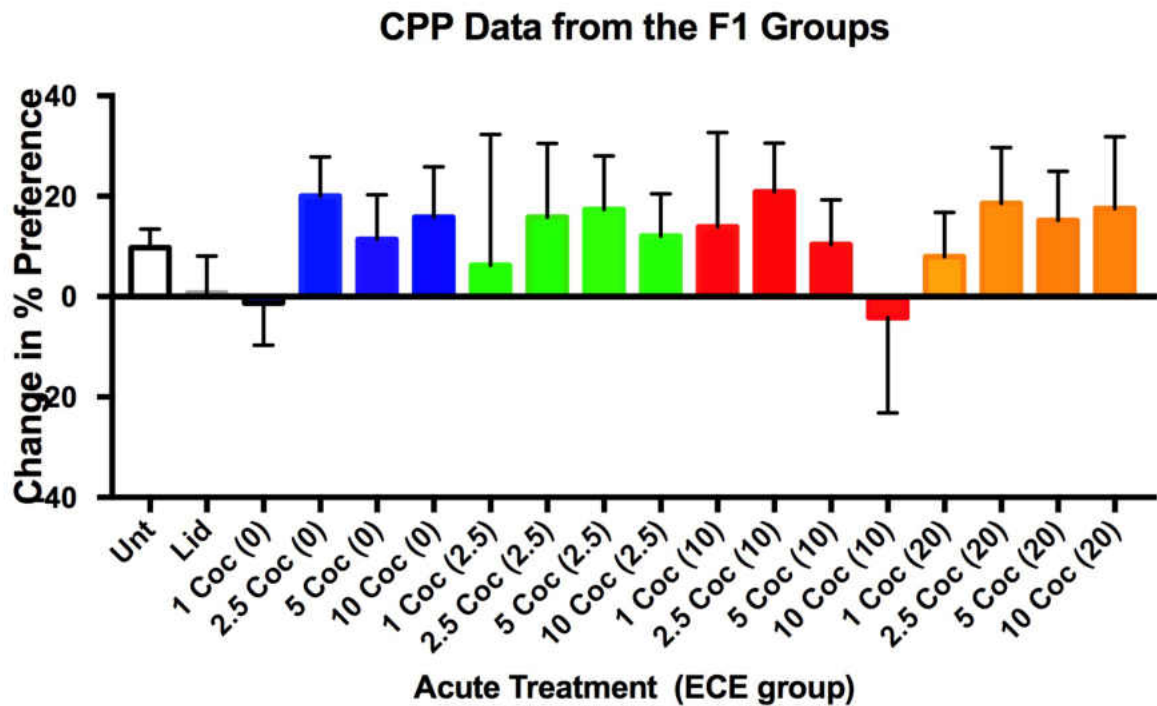
CPP Assay Data in F_1 Cohorts of Zebrafish
Figure 15: Cocaine induced conditioned place preference change in F_1 generation ECE
zebrafish. Data has been pooled from zebrafish from each ECE treatment and compared
based on acute cocaine treatment. Error bars represent $\pm 95\%$.

Analysis of 5-mC and 5-HmC Attachment Across the Genome

For this experiment, F_1 generation fish were culled and gDNA was produced for bisulfite sequencing. Methylation and hydroxymethylation data were collected and analyzed to determine if CS gene expression was modified by changes in the DNA epigenetic state to explain our decreased sensitivity in the F_1 generation. Whole genomic methylation patterns of hyper- and hypomethylation were observed across all 25 zebrafish chromosomes at increasing concentration of ECE cocaine exposure (2.5, 10, 20 mg/L) and compared against an untreated cohort. Differences seen among whole genome methylation patterns were analyzed by comparing individual genomic sequences across the various doses and by methylation status, either hyper- or hypomethylated. One-way ANOVA indicated that both significant hypermethylation and hypomethylation occurred on all zebrafish chromosomes. Overall, therefore, global DNA methylation is shown to be unaffected by ECE. An example can be seen on chromosome 3 in Figure 16. Hypermethylation was significantly affected on chromosome 3 (p=0.006, $F_{2,210}$=5.252). Multiple comparisons test with Bonferroni's correction indicated a significant decrease in hypermethylation between 2.5 mg/L and the 20 mg/L cohorts (p=0.006, df=210, mean diff.= -0.04). Chromosome 3 also displayed differences in its hypomethylation comparing increased cocaine exposure against 0 mg/L longitudinal fish (p< 0.0001, $F_{2,269}$=47.18). Hypomethylation was increased in both the 10 (mean diff.=0.08) and the 20 mg/L (mean diff.=0.10) F_1 groups when compared to the lowest dose, 2.5 mg/L (Figure 16, p<0.0001, df=269 for both comparisons). Global hydroxymethylation was also unaffected by ECE in the F_1 generation.

Differential methylation patterns were observed among numerous genes when

comparisons were made between ECE treatment and untreated groups (0 vs 2.5, 0 vs

10, 0 vs 20). Venn diagrams were constructed to determine overlap among the top 2000

genes that were significantly hypermethylated in each of the three comparisons. As

shown in Figure 17, there was minimal (<5%) overlap between the 890 genes that were

shown to be significantly hypermethylated as the result of increasing ECE in F_1 zebrafish.

Similarly, a Venn diagram was constructed comparing hypomethylation of the 979 genes

among the F_1 generation of ECE fish revealing ECE produced patterns of

hypomethylation that were relatively unique to each F_1 ECE group including little

overlap between the three ECE groups (<5%). Figure 18 shows the hypomethylation of

the 979 genes as ECE concentration increased in F_1 groups. The addition of data to these

Venn diagrams was done to determine any interaction between the methylation and

hydroxymethylation status. Grouping of the 890 genes thought to be differentially

hypermethylated with the 17,743 genes affected by hydroxymethylation revealed very

little overlap between modified genes. The results of this comparison indicated 39 genes

(0.2%) were found in samples with differential hypermethylation and

hydroxymethylation (Figure 19). The grouping of the 979 differentially hypomethylated

genes as the result of F_1 ECE and genes thought to be hydroxymethylated in 2.5 mg/mL

F_1 zebrafish revealed there was also minimal overlap (Figure 20). Globally, then it

appears, unique genes are affected by ECE primarily in the F_1 generation. Venn diagrams

with the inclusion of our top 150 CS genes generated little overlap as well. Comparisons

of CS genes with the three comparison groups of F_1 ECE hypomethylated genes revealed

there was no gene overlap. The analysis of CS genes compared to genes

hypermethylated in the three comparison groups revealed a single gene, neurexin 3a,

was found in all four (Figure 21). The list of CS genes was then assessed for overlap

within genes thought to be hydroxymethylated in 2.5 F_1 zebrafish. Here, 128 of the top

150 CS genes were shown to overlap within the 5-HmC gene list (data not shown).

Analysis showed there was differential methylation states occurring along the validated

CS genes. Figure 22 appears to show a pattern of increased hypermethylation of these

genes in F_1 of the 2.5 ECE group while the F_1 fish with higher ECE (10 and 20 mg/mL

cocaine) suggested hypomethylation. Hydroxymethylation of CS genes is shown in

Figure 23. The CS genes displayed general decreases in 5-HmC attachment. The results

of our methylation and hydroxymethylation studies indicate little global effect resulting

from ECE in the F_1 generation. There was little overlap observed between genes hyper-

and hypomethylated when compared to the CS genes revealed from Hi-Seq analysis.

There was also little overlap of hydroxymethylated genes by 2.5 mg/mL cocaine in the F_1

group when compared to our CS genes and genes hyper- and hypomethylated in F_1 ECE

groups. These experiments revealed nrxn3a as a commonly affected gene among these

studies.
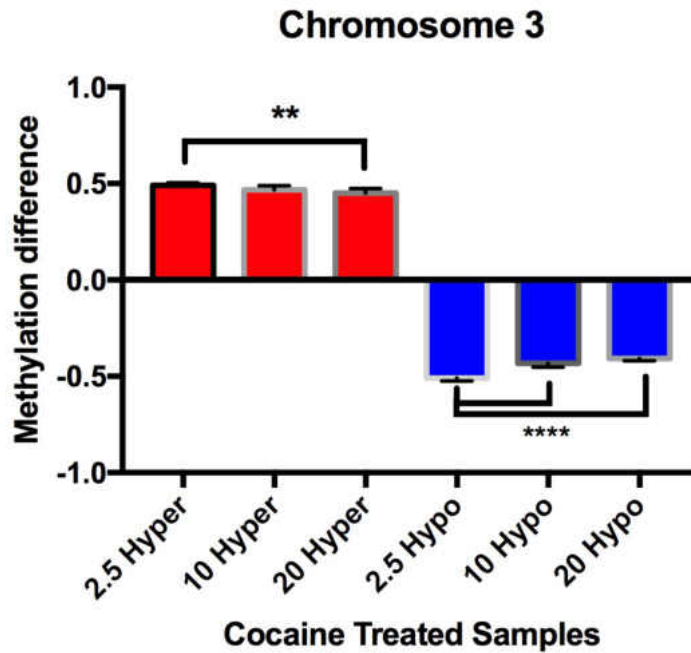
Chromosomal Methylation of Chromosome 3

Figure 16: Differences seen in hypermethylation and hypomethylation resulting from treatment with increasing cocaine concentration. Error bars represent the 95% confidence interval. ** indicates $p<0.01$ and $p<0.001$ according to Tukey's multiple comparisons tests. Each bar seen above represents difference in methylation patterns at that acute treatment concentration when compared to untreated samples.

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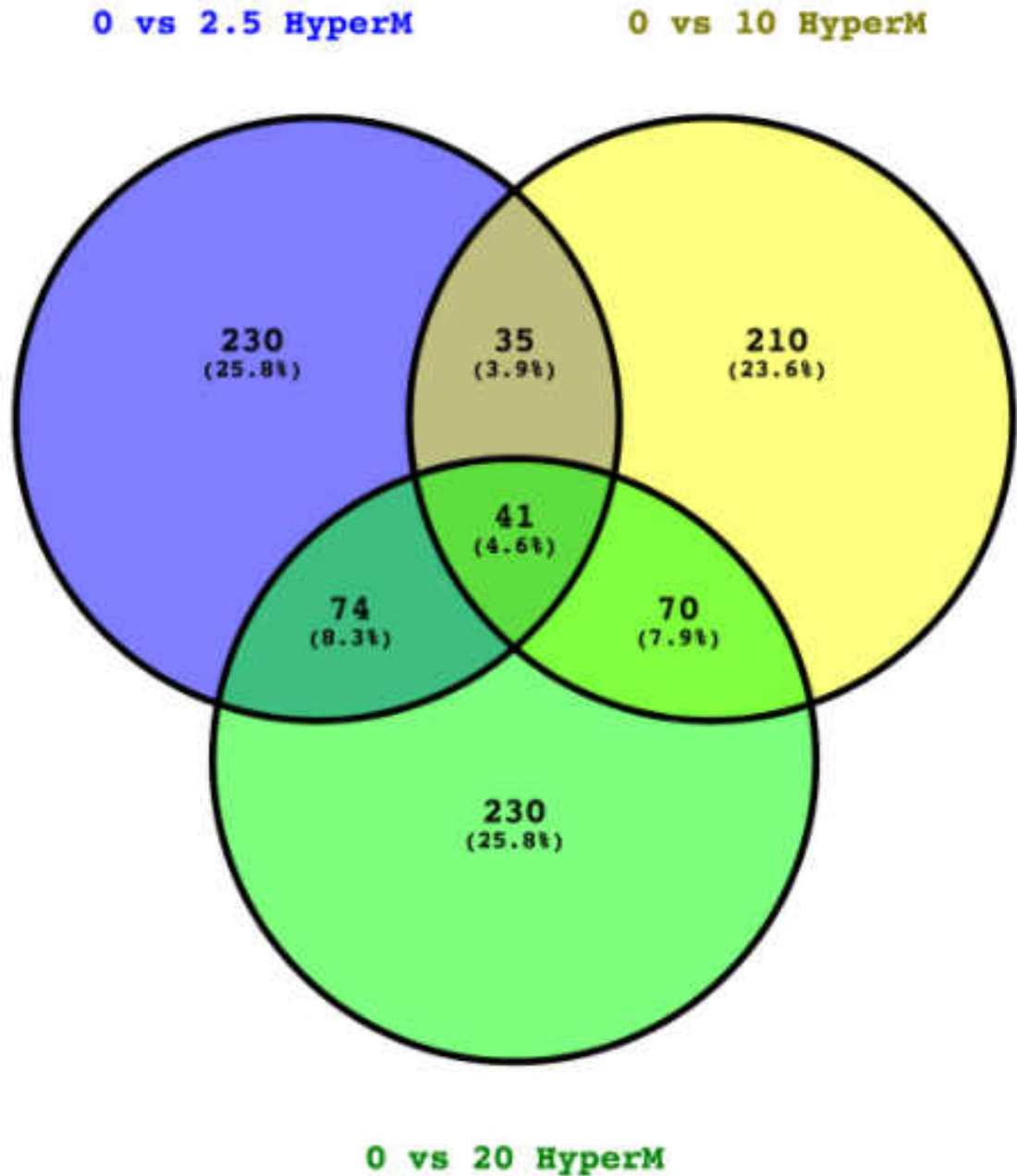
Venn Diagram of Hypermethylated Gene in F_1 Zebrafish
Figure 17: Venn diagram representing hypermethylated genes based on ECE treatment. 0 vs 2.5 represents 380 genes hypermethylated when comparing the 2.5 F_1 cohort against the 0 F_1 cohort. 0 vs 10 represents 356 gene hypermethylated when comparing 10 F_1 cohort against the 0 F_1 cohort. 0 vs 20 represents 415 genes hypermethylated when comparing 20 F_1 cohort against the 0 F_1 cohort.

Venn Diagram of Hypomethylated Gene in F_1 Zebrafish

Figure 18: Venn diagram representing hypomethylated genes based on ECE treatment. 0 vs 2.5 represents 392 genes hypomethylated when comparing the 2.5 F_1 cohort against the 0 F_1 cohort. 0 vs 10 represents 414 genes hypomethylated when comparing 10 F_1 cohort against the 0 F_1 cohort. 0 vs 20 represents 431 genes hypomethylated when comparing 20 F_1 cohort against the 0 F_1 cohort.
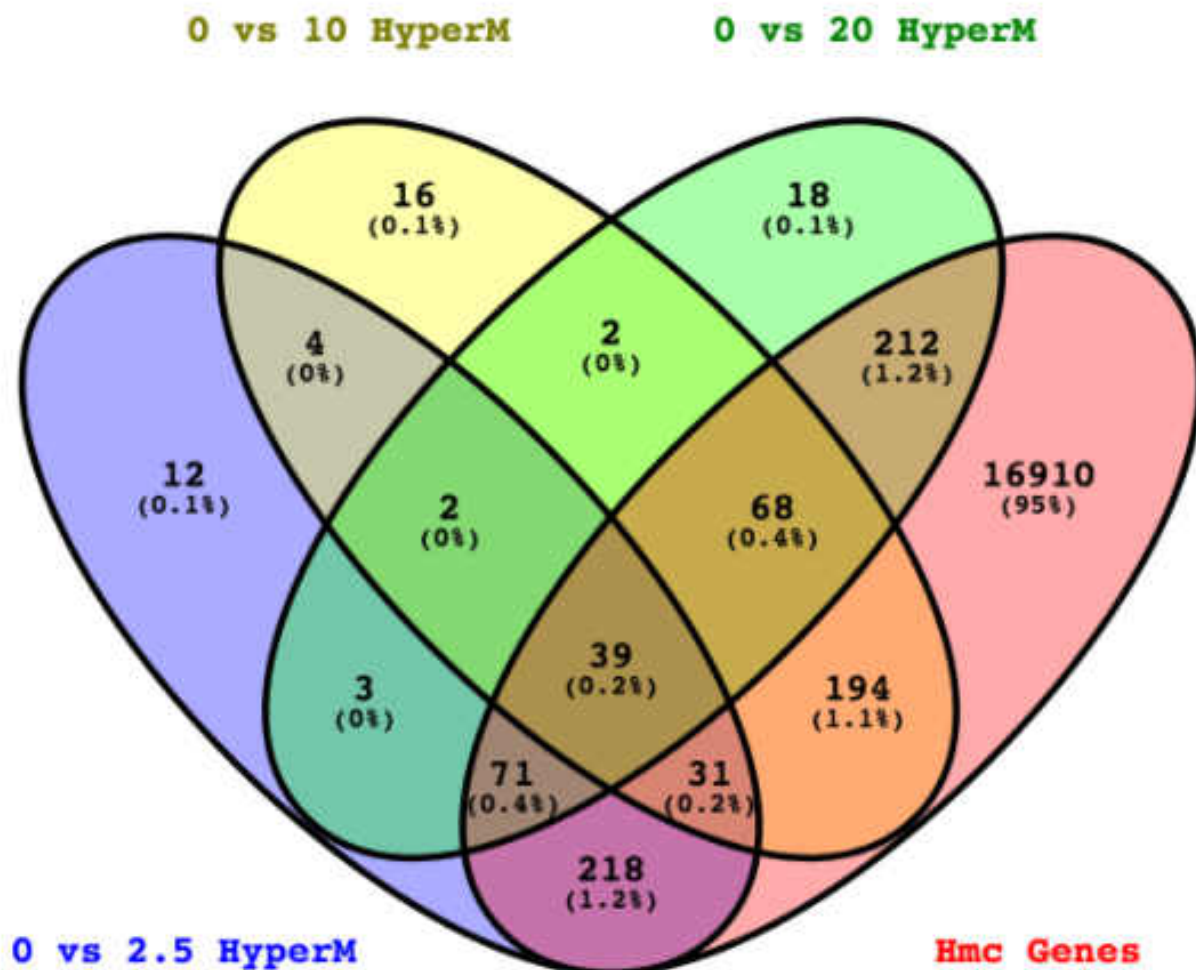
Venn Diagram of Hypermethylated & Hydroxymethylated Genes in the F_1 Zebrafish
Figure 19: Venn diagram representing hypermethylated genes based on ECE treatment when compared with 17,743 hydroxymethylated genes in the F_1. 0 vs 2.5 represents 380 genes hypermethylated when comparing the 2.5 F_1 cohort against the 0 F_1 cohort. 0 vs 10 represents 356 genes hypermethylated when comparing 10 F_1 cohort against the 0 F_1 cohort. 0 vs 20 represents 415 genes hypermethylated when comparing 20 F_1 cohort against the 0 F_1 cohort.
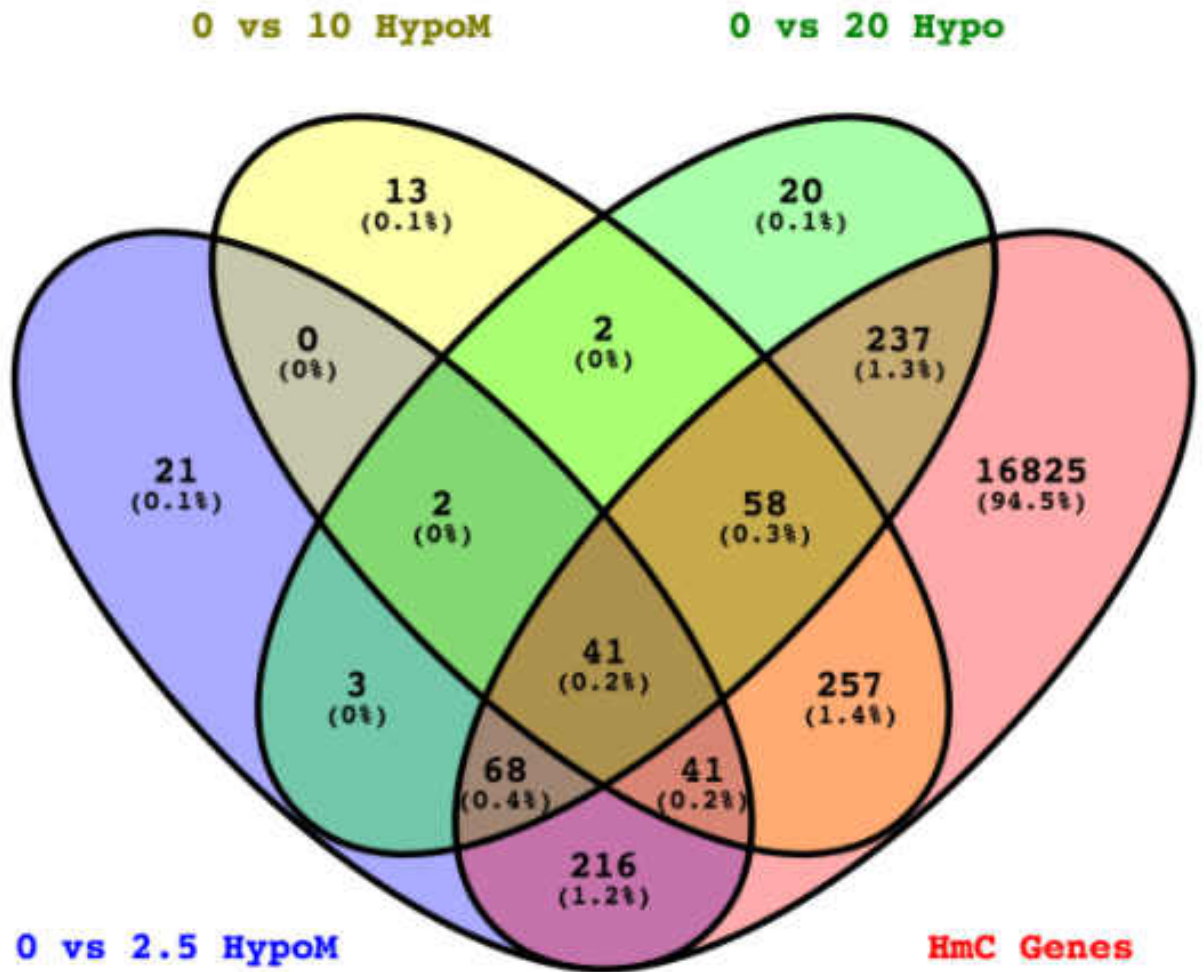
Venn Diagram of Hypomethylated & Hydroxymethylated Genes in the F_1 Zebrafish
Figure 20: Venn diagram representing hypomethylated genes based on ECE treatment compared against 17,743 hydroxymethylated genes in the F_1. 0 vs 2.5 represents 392 genes hypomethylated when comparing the 2.5 F_1 cohort against the 0 F_1 cohort. 0 vs 10 represents 414 genes hypomethylated when comparing 10 F_1 cohort against the 0 F_1 cohort. 0 vs 20 represents 431 genes hypomethylated when comparing 20 F_1 cohort against the 0 F_1 cohort.
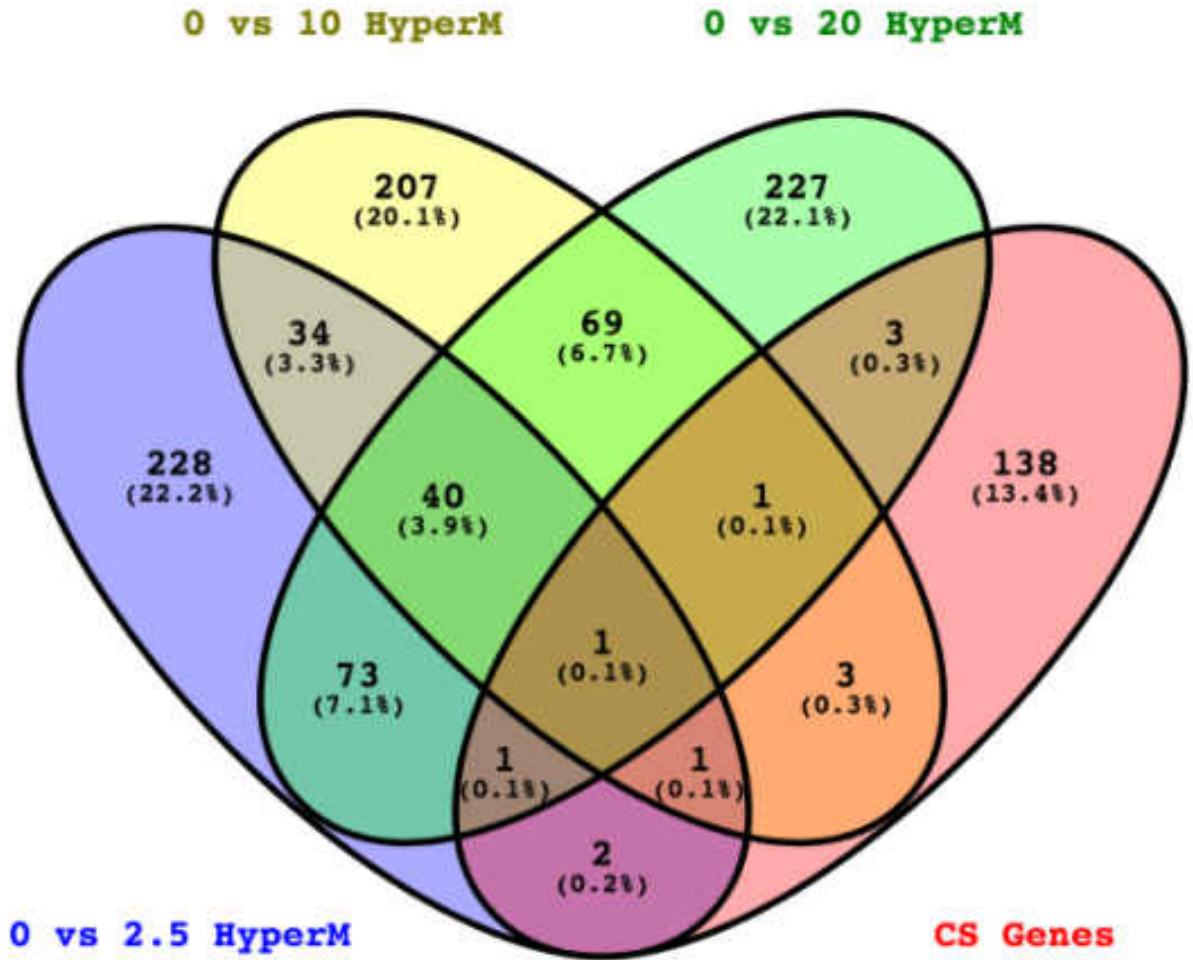
Venn Diagram of Hypermethylated Genes in F_1 Zebrafish and CS Genes
Figure 21: Venn diagram comparing 150 CS genes with genes demonstrated differential hypermethylated in the F_1 fish. 0 vs 2.5 represents 380 genes hypermethylated when comparing the 2.5 F_1 cohort against the 0 F_1 cohort. 0 vs 10 represents 356 genes hypermethylated when comparing 10 F_1 cohort against the 0 F_1 cohort. 0 vs 20 represents 415 genes hypermethylated when comparing 20 F_1 cohort against the 0 F_1 cohort. Neurexin 3a is revealed as a unique overlap among the four comparisons.
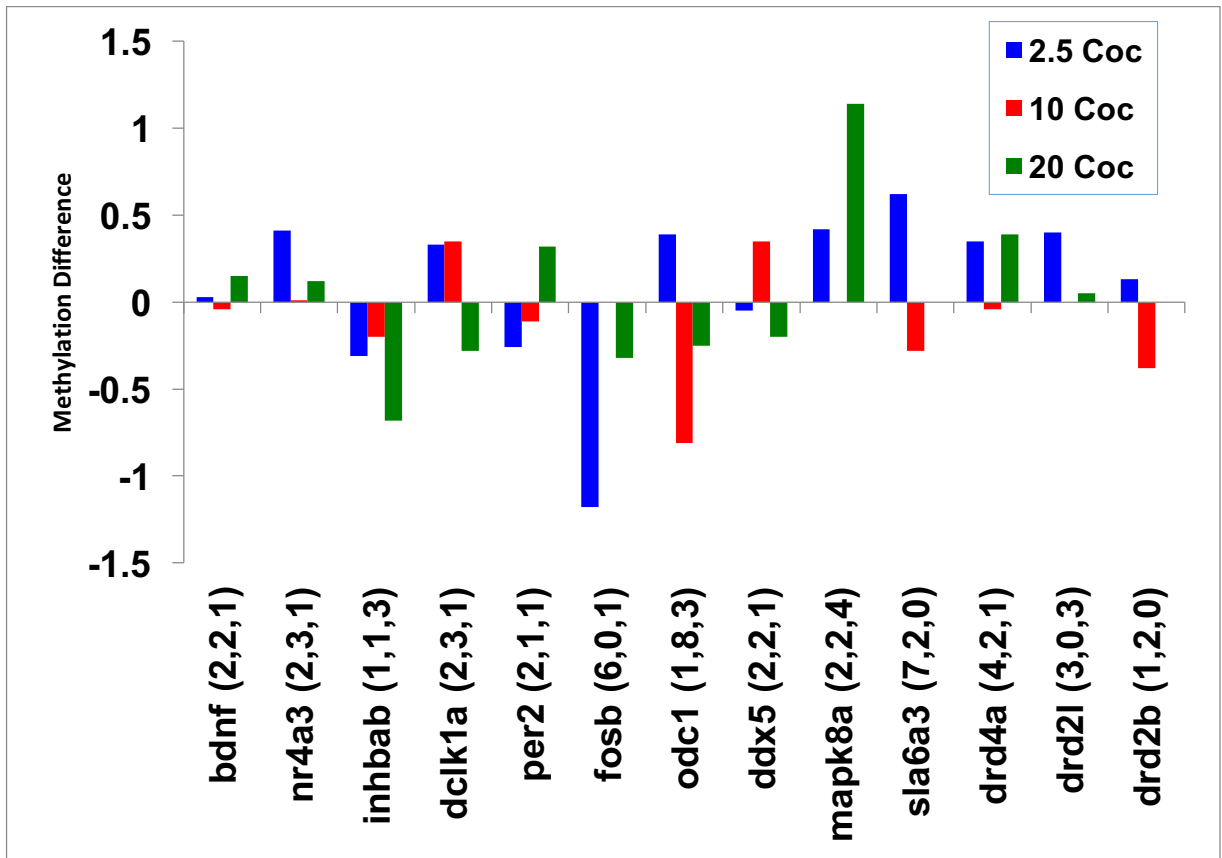
Methylation Ratio Among CS Genes

Figure 22: Methylation of CS genes. The data was reported as methyl group attachment to total methylation site of a chromosomal segment ratio. The y-axis represents the difference in methylation ratio between each ECE concentration and untreated fish. Listed along the x-axis is the gene name and in parentheses are the read numbers found at each cocaine concentration. Bars represent the difference in average methyl reads along the gene segment.
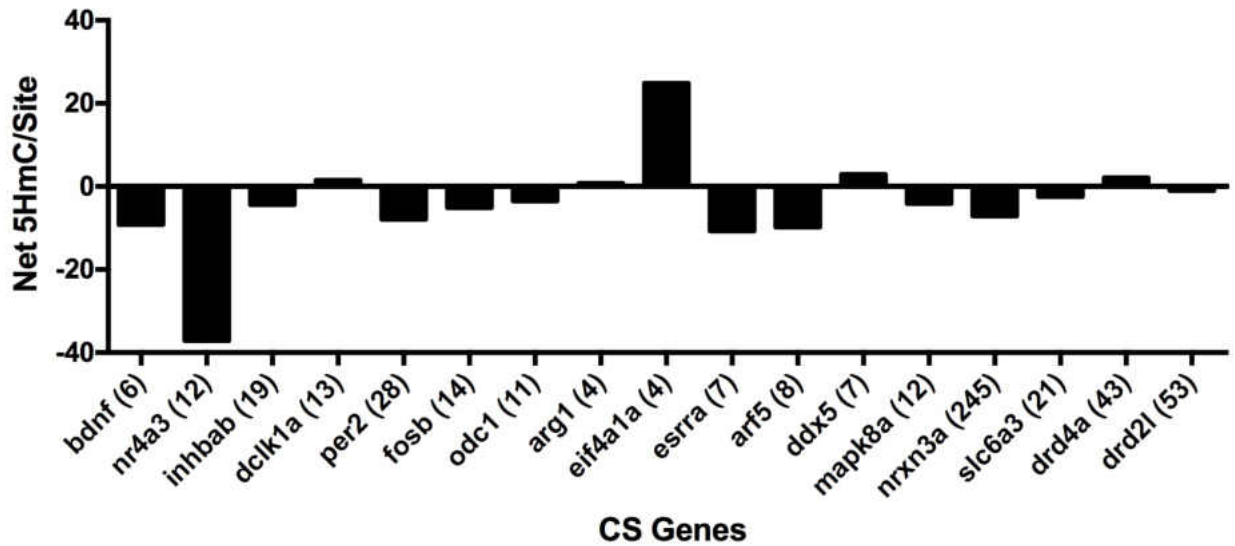
5-HmC Read Numbers on CS Genes

Figure 23: Hydroxymethylation patterns on several CS genes. The y-axis represents net numbers of HmC groups found at read sites of the CS genes. Listed along the x-axis is the gene name and in parentheses are the read numbers found in zebrafish from the 2.5 F_1 ECE when compared against untreated 0 F_1 zebrafish

CPP Assay in Cohorts of Zebrafish Exposed to CA

Testing the hypothesis that longitudinal behavioral changes are governed by DNA methylation, we generated longitudinal adult zebrafish treated with a potent DNA methyltransferase inhibitor, chlorogenic acid (CA). Groups included an untreated control, 1µM dimethyl sulfoxide (DMSO, as the vehicle control), 10 µM cocaine, 1µM DMSO+10 µM cocaine, 1 µM CA, and 1 µM CA+10 µM cocaine. Fish were raised to 8 months and tested sing the CPP assay. Initial behavioral results were analyzed across all testing days using repeated-measures ANOVA. It was observed that when treated as adults, fish exposed embryonically with 1 µM CA+10 µM cocaine showed significant changes in preference in CPP testing (Figure 24, $p<0.0081$, $F_{2.811, 22.49} = 5.183$). No other

embryonic treatment conditions led to significant differences in baseline

measurements.

One-way ANOVA reported differential behavioral outcomes based on

chlorogenic treatment groups (Figure 25, $p<0.005$, $F_{6,172}=3.183$). In these experiments,

post-hoc testing using Bonferroni's correction indicated a significant increase the CPP

response of fish treated embryonically with 1 μM CA+10 μM cocaine when compared

against fish left untreated embryonically ($p=0.0033$, df=172, mean diff=-15.3%). Fish

treated embryonically with 1 μM CA displayed significantly less CPP-like behavior when

compared to the 1 μM CA+10 μM cocaine treatment group (Figure 25, $p=0.032$, df=172,

mean diff= -15.%). Though not statistically significant, a pattern emerged that groups

with ECE displayed greater changes in CPP when compared to fish left untreated,

suggesting cocaine as a principle factor contributing to longitudinal behavioral

differences.

Subsequent to behavioral experimentation, fish were analyzed to determine any

effect that embryonic treatment may have had on survival (%), weight (g), length (mm)

and gender ratio (%). Among the groups survival was as follows: untreated control

41.2%, 1μM DMSO 96.6%, 10 μM cocaine 72.8%, 1μM DMSO+10 μM cocaine 52.5% , 1

μM CA 85% , and 1 μM CA+10 μM cocaine 55%. Among these treatment groups, one-

way ANOVA indicated significant differences among the weights within each treatment

group (Figure 26, $p<0.0001$, $F_{5,130}=23.57$). Follow up experiments were conducted to

determine any significant differences among treatment groups. According to

Bonferroni's multiple comparisons test, 1μM DMSO had a significant effect on weight

when compared against untreated controls (p=0.025, df=130, mean diff=0.100 g). The cohort treated with 10 1μM cocaine lead to a significant decrease in weight when compared to untreated control (p<0.0001, df=130, mean diff=0.10 g). This trend continued among the other treatment groups. The cohort treated with 1 μM DMSO+10 μM cocaine were shown to have a significant decrease in weight against untreated controls (p<0.0001, df=130, mean diff= 0.08 g). Treatment with 1 μM CA led to the largest significant decrease in weight among a cohort when compared against untreated controls (p<0.0001, df=130, mean diff=0.126 g). Lastly, treatment of the cohort of fish with 1 μM CA+10 μM cocaine weighed significant less when compared against untreated controls (p<0.0001, df=130, mean diff=0.079 g). Using Bonferroni's correction to compare all groups against one another, it was noted that differences in weight did occur. Fish from the chlorogenic acid cohort weighed significantly less when compared against the 1μM DMSO cohort (p<0.0001, df=130, mean diff= 0.084 g). Treatment with 1μM DMSO+10μM generated a cohort that was significantly heavier than those of the cohort treated with 1μM CA (p<0.001, df=130, mean diff=0.045 g). Treatment of a cohort of fish with 1 μM CA+10 μM cocaine lead to significant weight gain when compared against fish treated with 1 μM CA alone (p<0.0007, df=130, mean diff=-0.047 g). Length of zebrafish was also significantly differentiated among the treated groups with the chlorogenic acid experiment according to one-way ANOVA (Figure 27, p<0.0001, $F_{5,130}$=10.08). Treatment with 1μM DMSO had no significant effect on length of zebrafish when compared against the untreated cohort (p>0.999). The fish treated embryonically with 10 μM cocaine were significantly shorter when compared to

untreated controls ($p<0.0006$, df=130, mean diff=2.77 mm). Untreated controls

displayed significantly greater lengths when compared against 1μM DMSO+10μM

cocaine ($p<0.029$, df=130, mean diff=2.16 mm). The cohort treated with 1 μM CA was

significantly shorter in overall length when compared against untreated control

zebrafish (*$p<0.0001$,* df=130, mean diff=4.06 mm). The difference in length was

attenuated when a cohort was treated embryonically with 1 μM CA+10 μM cocaine

($p<0.014$, df=130, mean diff=2.30 mm). The cohort treated with 1 μM CA was

significantly shorter when compared with fish treated embryonically with 1μM DMSO

($p<0.0001$, df=130, mean diff= 2.74 mm). Comparisons of 1 μM DMSO+10 μM cocaine

against the cohort treated with 1 μM CA indicated a significant decrease in overall

length ($p<0.012$, df=130, mean diff= 1.88 mm). The comparison of cohorts treated with

1 μM CA and 1 μM CA+10μM cocaine suggested that the addition of 10 μM cocaine

attenuated the decrease in length seen in the 1 μM CA group ($p<0.024$, df=130, mean

diff=-1.75 mm).

It is important to consider the effect that population density may have had on

these recordings. Due to the differential survival of the untreated cohort, populations of

fish in the untreated housing tank were low. This would coincidently allow for greater

growth among these fish. In order for validity of these experiments to be considered,

the experimental measures would need to be repeated.

1uM CA+ 10uM coc Embryonic Exp
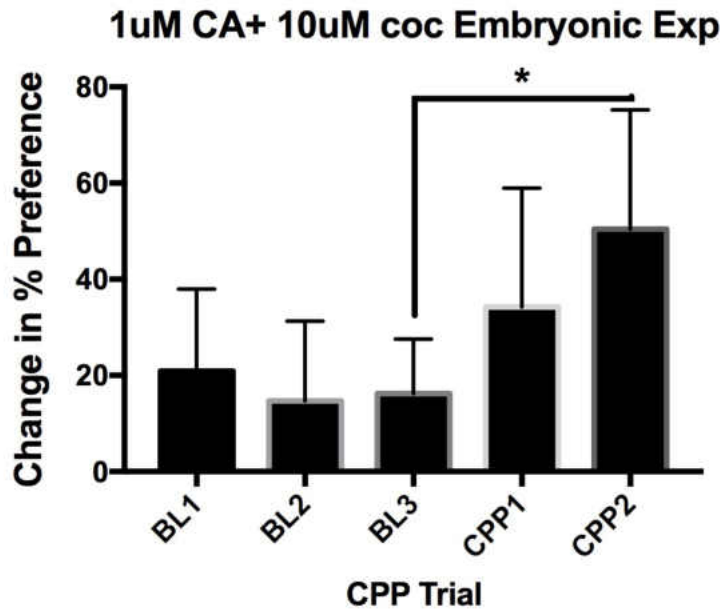
Repeated Measures ANOVA in Zebrafish Exposed to 1 μM CA+10 μM coc

Figure 24: Preference of zebrafish according to conditioned chamber. Initial drug administration begins following BL3 and is repeated following CPP1. CPP2 shows change in chamber preference. * denotes $p<0.05$ when comparing mean of each group with Bonferroni correction.

Overall Chlorogenic Acid Exp
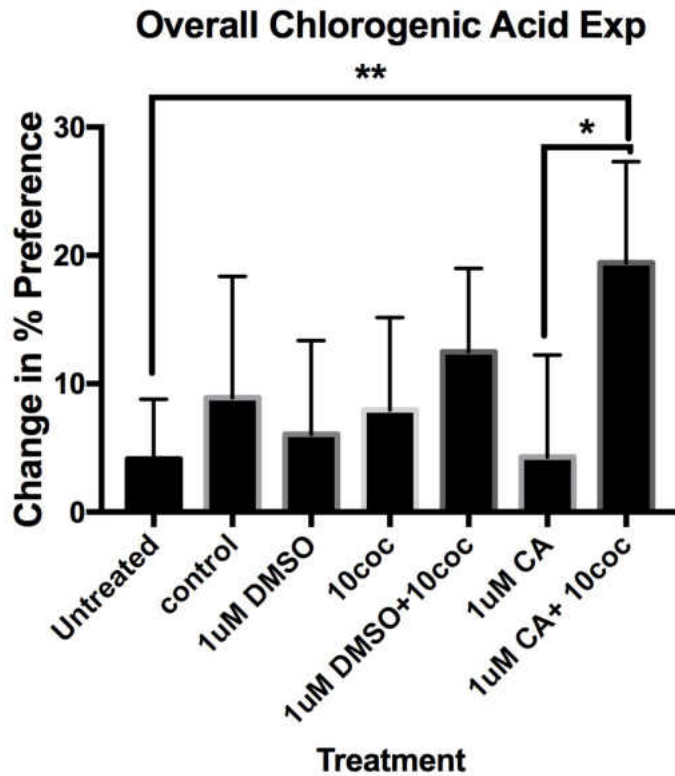
Overall CPP Data in Zebrafish Exposed to CA

Figure 25: Change in cocaine conditioned place preference to 5mg/L cocaine treated chamber in epigenetic drug exposed longitudinal zebrafish. ** denotes $p<0.01$ when compared against untreated controls with Bonferroni's correction. Error bars represent the $\pm$95% CI.

Chlorogenic Exp Weights (g)
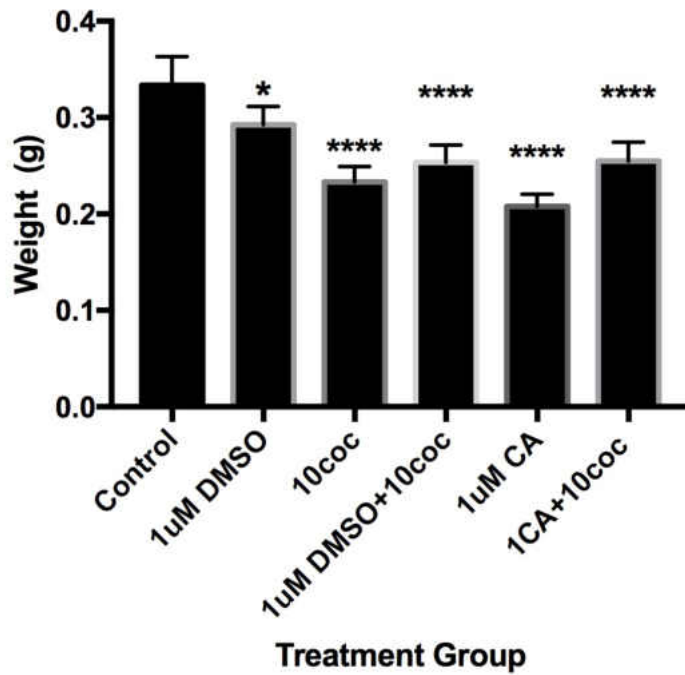
CA Experiment Zebrafish Weights

Figure 26: Weight associated with embryonic treatment zebrafish with dimethyl sulfoxide (DMSO), 10 μM cocaine (coc), 1 μM DMSO +10 μM coc, 1 μM chlorogenic acid (CA), and 1 μM CA+ 10 μM coc. *denote $p<0.05$ and **** denotes $p<000.1$ when compared against untreated controls using Bonferroni's multiple comparisons. Error bars represent the $\pm 95\%$CI.

Chlorogenic Exp Lengths (mm)

CA Experiment Zebrafish Lengths

Figure 27: Length associated with embryonic treatment zebrafish with dimethyl sulfoxide (DMSO), 10 µM coc, 1 µM DMSO +10 µM coc, 1 µM CA, and 1 µM CA+ 10 µM coc. *denote $p<0.05$, *** denotes $p<0.001$ **** denotes $p<0.0001$ when compared against untreated controls using Bonferroni's multiple comparisons. Error bars represent the ±95%CI.

DISCUSSION

The results of our study indicated ECE in the zebrafish generated longitudinal behavioral and gene expressional consequences. Zebrafish treated at increasing concentrations of cocaine during embryonic development showed a dose-dependent CPP response when exposed to cocaine again as adults. Our results were indicative of a longitudinal behavioral changes attributed to ECE. Analysis of gene expression in forebrain structure of larval and adult zebrafish demonstrated differential expression that was also dependent on ECE concentration. Larval zebrafish displayed a dose-dependent suppression in CS gene expression as cocaine concentration increased. RNA-Seq revealed a list of CS genes. Validation of the RNAi-Seq results of select CS gene expression in adult zebrafish forebrain was unsuccessful. Gene expression of several of the selected CS genes in adult zebrafish did not appear significantly affected by ECE. It may be that the limited number of samples used for these experiments did not allow for significant differences to be observed. As a possible explanation for changes in CPP behavior and gene expression, we investigated DNA methylation patterns, globally and along select CS genes. Bisulfite sequencing revealed little effect on global methylation, but we observed alterations in hyper- and hypomethylation along several CS genes. The CpG islands detected were observed in several promoter and coding regions of our selected genes. The addition of methyl groups to these regions may have longitudinal effects on gene expression, transcriptional potential, and gene isoform availability. The addition of 5-hydroxymethylcytosine (5-HmC) was also shown to be modified by the

exposure to 2.5 mg/mL cocaine along numerous genes. In addition, the pattern of 5-HmC addition on our CS genes indicated that exposure reduced the relative number of these epigenetic modifiers. It may be that these 5-HmC groups are a priming state of methylation, in that, the addition of 5-HmC acts as an intermediate between methyl group attachment and hypomethylation. Cohorts treated with chlorogenic acid demonstrated longitudinally altered behavior. Our results suggest insult exposure during critical developmental time periods has lasting consequences for behavior and gene expression.

ECE Led to Differential Responses to Cocaine

Cocaine exposure during embryonic development and how it sequentially affects behavior has been investigated in several model organisms. Our work has indicated that maximum behavioral response in adult zebrafish occurred with increasing cocaine concentration up to 10 mg/mL [116]. Within our study, F_0 zebrafish exposed at increasing doses of cocaine during an early developmental window showed significant increases in conditioned preference when tested at 8 months of age. These behavioral results were generated with acute treatment of a lower cocaine concentration of 5 mg/mL, indicating an increase in sensitivity to cocaine. This CPP behavior was again seen to be dose-dependent. ECE concentrations attenuated the CPP response in all F_1 fish treated with cocaine. These fish did not display a conditioned response, instead behaving similarly to untreated controls, appearing to be insensitive to cocaine. This result has been supported in the literature. In a study with ECE, male rats were taught to

self-administer cocaine and their offspring, specifically males, demonstrated a

subsequent lessening of cocaine self-administration and as well as decrease [146]. In

this study conducted by Vassoler et al. (2012), this inherited endophenotype was the

correlated with longitudinal acetylation at histone proteins associated with Bdnf

promoter sites. This result then, suggests that self-administration of cocaine in the

parental generation led to prolonged changes in behavioral responsiveness to cocaine.

Taken together, the results of our study may indicate that ECE in zebrafish led to a

longitudinal insensitivity to cocaine in CPP assays.

Analysis of CPP behavior in offspring of zebrafish exposed to ECE demonstrated

an intergenerational effect that can be attributed to cocaine. We used a range of acute

cocaine concentrations to assess possible altered sensitivity of F_1 zebrafish cohorts. CPP

testing was shown only significantly affected in the 0 F_1 cohort. The F_1 cohorts'

behavioral results suggested that ECE in the F_0 has an intergenerational effect. There did

appear to be an increasing response at the lowest acute dose (1 mg/mL), suggesting

that there actually was a positive response on cocaine sensitivity, but variability was too

high to be certain. This variability can imply that some F_1 fish with ECE did respond

highly to cocaine. It may be that these individuals are more susceptible to self-

administration and addiction as the result of intergenerational cocaine exposure. Here,

these fish gave a large CPP response to low acute cocaine exposure. In future studies, F_1

fish with large CPP responses to low acute exposure could be isolated following the CPP

assay. These 'high-responders' could then be crossed, raised, and genetically analyzed.

The results of this experiment may substantiate who succumbs to familial addiction and

how ECE produced lasting behavioral changes. Further investigation will be required to determine this result. In addition, F_1 had a higher background response than that seen in the F_0 and CA CPP experiments. This may have masked any positive CPP responses of F_0 ECE on F_1 fish. The response of the zebrafish to lidocaine and the low response to 1 mg/mL in the 0 mg/mL ECE fish provide further evidence that the baseline values in the F_1 fish were very high. Further studies will be need to explain the source of this high baseline activity.

A number of studies have investigated how early or prenatal cocaine exposure has affected behavior in individuals with ECE and their offspring. Prenatal cocaine exposure in mice was demonstrated to weaken the acquisition of cocaine induced CPP-like behavior [147]. The results described herein offer evidence that high levels of ECE led to cocaine insensitivity in adulthood. Malanga et al. (2007) reported behavioral testing in mice using CPP. Within their study, preference was significantly affected by physical properties of the testing chamber, specifically color. Similar to our result, it was reported that behavioral response showed an inverted U-shape, with maximal behavioral responses occurring at submaximal treatment concentrations. CPP-behavior returns to control following treatment with maximal treatment concentrations. From this result, the authors conclude that prenatal exposure to high concentrations of cocaine attenuated the conditioning effect of cocaine treatment in adulthood. ECE may also have affected how the mice responded to a novel environment. In a study with rats, chronic cocaine exposure resulted in a greater stress-response as measured by corticosterone levels in subjects undergoing the open-field assay [148]. This chronic

cocaine exposure then led to longitudinal alterations in stress-response signaling and stress behavior. The novelty of the new environment may have acted as a new stressor for the zebrafish of our experiment. Taken these results together, it may be that ECE in zebrafish altered the stress-response behavior and partially explains the high baseline recordings of untreated fish. The results of our study may further substantiate the result of the Malanga et al (2007) study. ECE in zebrafish led to increased CPP responsiveness in the F_0. Malanga et al. (2008) has further demonstrated that prenatal exposure to cocaine increases the rewarding nature of acute cocaine exposure later in adulthood of mice using brain imaging techniques [149]. The increased rewarding potential of cocaine shown in this study was sex dependent. In a self-administration experiment, gestationally exposed male mice were more likely to acquire the criteria for self-administration than untreated control mice [150]. In the work done by Rocha et al. (2002) the percent of mice that acquired cocaine self-administration displayed a bell-shape curve, similar to the result of our CCP data. These mice were also shown to self-administer cocaine more readily. Taken together then, it is clear that ECE generates longitudinal effects on behavior and on the reward response. Other researchers have shown that ECE produced deficits in conditioned learning [151], which could justify a poor learning and behavioral response. Spatial learning has also been shown to be stunted due to ECE [152]. Several studies conducted to assess working and spatial memory have indicated poor performance when compared against untreated controls [153]. Mice tested in Morris maze scored significantly worse than their untreated controls and demonstrated a delay in successful goal completion when tested using

Barnes maze test. This effect on working memory may contribute to the CPP data observed in the Malanga et al (2007) study. Similar to some mice model studies of prenatal cocaine exposure, our zebrafish did not display significant effects on locomotive behavior indicative of acute stress (data not shown). Cocaine has been correlated with hypersensitivity to acute stress in the mouse model [147]. Analysis of distance traveled in behavioral apparatus did not vary among the groups. Lastly, the F_1 cohort of zebrafish left untreated demonstrate a high basal CPP response without acute treatment. High basal response in this group may be the result of ECE in parental generations. The stress of the novel testing environment likely contributed to this result.

The intergenerational effects of cocaine exposure and associated changes are an area of active research. One possible explanation for the intergenerational change in sensitivity to rewarding substance is a shift in the epigenetic landscape that leads to changes in gene expression that effectively sensitize an individual [154, 155]. Vassoler et al. (2013) reported a robust acetylation change on histone proteins of *Bdnf* promoter in male rats as the result of male sires with self-administration of cocaine. This provided evidence of intergenerational consequence as the result of drug exposure. Interestingly, this epigenetic modification was not observed in female rats. Modifications in *Bdnf* mRNA were thought to be due, in part, to changes observed in the sperm of cocaine-experience paternal rats. Additional sexually dimorphic differences in responses have been observed with cocaine sensitivity. Intracranial studies of mice analyzing the threshold of brain stimulation following prenatal cocaine exposure demonstrated a lowering of threshold stimulation, indicating increased rewarding nature of cocaine

[149]. Measurements were recorded from cortical neuronal bundles thought to be

associated with reward. Rewarding stimulation would cause these neurons to fire, and it

was demonstrated that prenatal cocaine exposure lowered the threshold of excitability

for these neurons. This result was observed to occur only in male mice. The threshold of

excitability was not significantly lowered in female mice. This differential effect due to

gender may account for several differences observed in our study. Reports of

differential effects on cocaine sensitivity following ECE may reflect the large variance

observed in our CPP data.

Our CPP experiments did include some technical limitations that limit the types

of inferences we can make from our results. We were unable to separate male and

female zebrafish in our behavioral work due to limiting sample numbers. We had

previously demonstrated that cocaine had no significant effect on gender in zebrafish,

but within each cohort, numbers were not adequate to separate male and female fish

for CPP assays [106]. Due to reports of sexually dimorphic responses both behaviorally

and physiologically to cocaine in mammalian studies, future studies with zebrafish

should investigate the role that sex may have in the responsiveness to cocaine. In

addition, intergenerational analysis of ECE on F_1 fish would not truly include cocaine-

naïve cohorts. As their parent's germ line cells were exposed to cocaine as a result of

their ECE treatment, transgenerational effects on cocaine sensitivity will need to be

carried out through the F_2 generation. Following the results of CPP in the F_1 cohorts, it

became clear that fish appeared to become insensitive to cocaine concentration as

touched on previously. Further investigation using increasingly lower cocaine

concentrations would further have explained this change in sensitivity. Additional

studies will need to be conducted to elucidate the affect ECE has on transgenerational

behavior and physiological sensitivity.

ECE Produced Longitudinal Changes in Gene Expression:

In order to link changes in gene expression to the altered behavioral responses,

we analyzed transcriptome-wide changes in zebrafish forebrain tissue in response to

ECE and acute cocaine exposure. Running RNA-Seq analysis on cohorts of ECE fish

following acute adult exposure generated large lists of differentially expressed genes.

Narrowing this expanse using significance values gave lists of thousands genes modified

at the differing ECE concentrations. Here, we selected the top 150 genes with more than

half showing a dose-dependent increase in expression. Attempts to explain how the

changes in genes throughout the transcriptome interact with one another in circuit was

done using a protein association program known as the STRING database. We observed

groupings of these genes in association with genes highly linked to addiction research

[143]. The clustering of genes highly characterized in addiction research led us to

attempt verification of their expression in zebrafish (Figure 5). This was done using

quantitative PCR. One gene network determined following our analysis included *bdnf,*

fosb, mapk8, and *inhbab*. These genes have been loosely reported in connection with

one another based on literary review. Chronic drug use has been shown to modify

dopamine concentration, as well as tyrosine hydroxylase and *bdnf* expression [40, 156].

Increased *Bdnf* has been shown to have a negative effect on the rewarding properties of

morphine [157]. *Bdnf* supplementation lessened synaptic firing character in the ventral

tegmental area after mice were trained to self-administer, whereas reduction of *Bdnf*

expression led to increases self-administration of opioids in CPP testing. Transgenic

mouse lines expressing polymorphisms in the *Bdnf* gene displayed compounded

compulsive reward seeking behaviors in response to alcohol exposure [30]. Transgenic

lines possessing an amino acid substitution on the *Bdnf* gene showed elevated alcohol

consumption. This alcohol consumption was attenuated by virally supplemented wild-

type *Bdnf* protein [30]. *Bdnf* has also been investigated in association with the

rewarding actions of cocaine. Acute cocaine exposure promoted increased *Bdnf*

concentration through its TrkB-signaling [36]. The expression of *Bdnf* has been shown to

increase its expression in a dose-dependent manner and persistent pattern after chronic

cocaine exposure [40]. This increase in expression after chronic exposure is thought to

be the result of increased phosphorylation of its CREB activator. Studies of *Bdnf*

expression in mice with ECE indicated a significant decrease in *Bdnf* expression at

embryonic day 15 [104]. Longitudinally, however, *Bdnf* demonstrated increase signaling

activity in the dorsal midbrain through its TrkB receptor as well as displayed increased

mRNA expression. As chronic drug exposure changes upstream regulators, it can

contribute to lasting behavioral consequences downstream. One of its downstream

interactions was shown to occur with *fosb.*

The *fos* transcription factor shown in our STRING interaction diagram has been

reported to play a functional role in the reward associated with drugs of abuse.

Research has suggested the *fos* transcription factor as a means to produce a robust

change in brain [158]. *Delta FosB* has been shown to accumulate in several neuronal

projections following obsessive behaviors and produces lasting behavioral changes [42,

43]. Cocaine has been shown to induce increased expression of multiple isoforms of the

Fos transcription factors within the nucleus accumbens and its associated brain regions

of mice [159]. Chronic cocaine use is thought to prevent the buildup of *fos* family

transcription factors, and lead to adaptive and constant expression of *ΔFosB* [160].

Transgenic mice over-expressing *ΔFosB* within specific striatal neurons showed

significantly more lever presses for low-dose administrations of cocaine when compared

to their home cage controls [47, 48, 161]. It was also shown that mice that over-

expressed *ΔFosB* that were taught to self-administer, will continue to self-administer at

lower doses than initial training following behavioral extinction [48]. This suggested that

increased *ΔFosB* within these frontal lobe structures may increase sensitivity to cocaine

use as well as increase vulnerability to maladaptive use and relapse. Early nicotine

exposure in mice generated increased acquisition of cocaine self-administration

correlated with increased *c-fos* expression in the prefrontal cortex [162], suggesting

early developmental insult generates lasting expressional changes. In agreement with

this, in a study using Dutch Belted rabbits, ECE lessened *Fos* expression following acute

cocaine exposure in 20 day old rabbits in both the striatum and the cortex [163]. The *Fos*

transcription factors are associated with learning and memory as this transcription

factor produces robust changes in the brain and its increased mRNA expression, but not

translated protein, are associated with cocaine sensitivity [47]. This implies that ECE

may lead to decreased expression of these transcription factors following a subsequent acute exposure to cocaine in this rabbit model of addiction.

The last gene associated with this cluster was *inhbab. Inhbab* has been reported to play roles in numerous physiologic processes. As *inhbab* is homologous to activin A, sharing ß subunit, its roles in pluripotent stem cell growth and cellular differentiation have been reported [164]. This protein is a member of the TGF-ß superfamily. Its expression has been implicated in ovarian tissue as a regulator of FSH release. In addition, it has been shown to play roles in angiogenesis, inflammatory processes, and immunological responses [165-167]. Studies in the *Drosophila* suggest activin-like ligands are involved in neuronal cell development [168]. This result is in line with vertebrate studies implicating this gene in its role in early cell differentiation in several cell-type lineages [169]. Experimental evidence has suggested that *inhbab* gene expression, along with fos-family proteins and *bdnf* are induced following hypoxic brain injury [170, 171]. The expression of these genes in response to hypoxic injury seems to be spatiotemporally regulated by the injury itself. Human data has suggested the expression of *inhibin* protein in various region of the brain. Immunochemical experiments in post-mortem brain tissue has suggested biologically active forms of *inhibin* proteins in hypothalamic and forebrain tissue [172]. The interaction of these three genes may then, play a neuroprotective role following ECE.

Brief literary review of these genes may provide insight into an associated functional relevance. The clustering of *Δfosb, bdnf, mapk8*, and *inhbab* in our STRING diagram may play roles in a neuroprotective mechanism. Interaction between these

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genes has been reported in the literature in research involving neuronal protection and

neuronal formation. It has been suggested that early stress in postnatal rat pups lessens

the expression of *Bdnf* and the activation of a MAP kinase pathway [173]. Interactions

seen from STRING diagram clearly links *mapk8, bdnf, inhbab*, and *fosb*. *Bdnf* has been

shown to provide a neuroprotective mechanism through activation of secondary

protein, *inhbab* [174]. Here, *Bdnf* acts to limit neuron damage by preventing

excitotoxicity by repression of NMDA-receptor cell signaling. Increased induction of

Δfosb, inhbab, and *bdnf* have also been reported as the result of hypoxic injury [170]. A

common downstream protein of the MAPK pathway is a family of protein called jun

proto-oncogene (jun). This extracellular signaling has been associated with several

intracellular processes, including cell survival and cell death [175, 176]. Jun family

proteins are cited with fos family protein in association with apoptotic processes in

neurons of the substantia nigra [177]. *Fos* gene knockout studies have suggested that its

protein product plays a role in the prevention of neurodegeneration following

methamphetamine exposure [178]. Tinmarla et al. (1999) demonstrated that excitotoxic

damage to neurons of the substantia nigra were coincident with increased *fos*

expression. Due to the reports of protection against neuronal damage due to these

proteins, it is likely that this CS gene cluster is in some way responsible for neuronal

protection in our zebrafish following ECE. We did not quantify the expression of *mapk8*

within the work of this thesis, but it can be predicted that its expression would mirror

that of these select CS genes. Further, *mapk8* may lead to increased expression of *bdnf*,

and indirectly, increased expression of *inhbab* preventing excitotoxicity of neurons

present in the forebrain of zebrafish. Signaling through this MAPK pathway may also explain the elevated expression of fos family transcription factors. These results will need to be confirmed with future research.

Our STRING interaction diagram revealed other clusters of genes that may prove valuable in understanding the effects of ECE. One such cluster included several genes thought to affect modification of proteins, transcriptional and translational regulation, and protein transport [179, 180]. *DDX5* and its family are identified as RNA helicases associated with multiple cellular processes involving ADP-ribosylation and cellular survival [181]. *DDX5*, also known as *p68*, has been reported as a cofactor for RNA processing and cellular control [182, 183]. Experimental evidence has suggested that *Ddx5* acts like a molecular switch controlling downstream effector proteins responsible for apoptosis and developmental arrest resulting from DNA damage [184]. *Arf5* is from a family of GTPase proteins associated with cellular transport and cell adhesion molecules [185]. It has been suggested that *Ddx5* expression can be regulated by *Arf* proteins and limit the downstream proteins involved in the *p53* apoptotic pathway [186]. *Arf5* has further been implicated with Ca^{2+}-dependent activator proteins involved in dense core vesicular transport [187]. Ca^{2+}-dependent activator proteins recruits *Arf5* to the Golgi apparatus for proper exocytosis of vesicles. These proteins have been shown to be ubiquitously expressed in the mouse brain and involved in the transport of catecholamines as well as *Bdnf* [188]. Through these mechanisms of neurotrophin transport and regulation of apoptotic processes it can be implied that *Arf5* plays a significant role in neuronal survival. Our STRING interaction diagram indicated these two

proteins also interact with another protein, *eif4a1a*. The gene product of *Eif4a1a* is an elongation factor that is ubiquitously expressed in hippocampal neurons of the rat brain suggesting a role in memory plasticity [189]. Modifications in the translational potential in the hippocampus has been associated with spatial memory dysfunction and learning deficits [190]. As these pathways were believed to be affected by ECE, it may be that *arf5's* promotion of neuronal proliferation may allow for Hebbian synaptic formation of neurons within the forebrain structure. *Arf5* was shown to be differentially expressed as the result of ECE in larval zebrafish. This differential expression may reflect the functional state of these neurons. As *arf5* was shown to be a CS gene, this may indicate increased strengthening of neuronal synapses, thereby producing lasting behavioral consequences. In our work we did not confirm interaction with these proteins using immunochemistry experiments. Immunohistochemistry experiments may confirm decreased levels of *ddx5* and increased vesicular docking as the result of *arf5* recruitment to neuronal cell junctions. Further research is required for these questions. Additional pathways were also observed due to the clustering of CS genes. Namely, genes involved in the polyamine synthesis pathways.

Proteins associated with the polyamine pathway were shown to cluster from our analysis with STRING. Polyamines are important for many cellular processes, including cellular communication, cellular proliferation, and early development. These polyamines are generated from arginine, ornithine, and proline and are converted to polyamines by several biochemical pathways. Arginine is converted by arginase into ornithine which is acted on and decarboxylated by *Odc1*. Research has indicated *Odc1* can regulate

pluripotency of neuronal stem cells [191]. *Odc1* has been used as a biomarker for brain

cell development and it has been demonstrated that its expression is increased by

gestational cocaine exposure [192]. It was reported that this differential expression was

normalized in the prenatal period. Multiple reports support that the initial differential

expression of *odc1* occurs following ECE, but that levels of expression return to control

values in the postnatal period [193]. It is thought, however, that this suppression of

odc1 during early prenatal development will alter development of neuronal cells of the

exposed organism [194]. Cocaine exposure leads to vasoconstriction resulting from

norepinephrine reuptake inhibition and subsequent ischemia. This ischemia can lead to

hypoxic conditions in the brain and thus, produce damage to brain tissue. Research has

suggested that ischemia is a mechanism that can contribute to the suppression of *odc1*

expression [194]. Indeed, reductions in *odc1* have been shown to alter the synaptic

transmission of catecholamines [195]. Using pharmacologic means, repression of *odc1*

led to severe attenuation in synaptic development in catecholaminergic neurons in the

CNS and PNS. Quantitatively, the expression of *odc1* in our experiments produced data

similar to what has been reported in the literature. There was a trend of decreased

expression in larval zebrafish, though expression patterns were not significantly

different among ECE groups. Adult *odc1* expression was unchanged according to qPCR

as the result of zebrafish ECE. These results support the consequence of cocaine

exposure on *odc1* expression that has been reported in mammalian model systems. The

zebrafish model may demonstrate that there is not significant effect on the polyamine

pathway as the result of ECE.

Another unique set of interactions of the CS genes was detected using STRING. Here, it was revealed a clustering of nuclear receptor proteins occurred following ECE. These receptors belong to a class of nuclear receptors that include *nr4a1, nr4a2, nr4a3* that act as transcription factors and function independently of ligand binding [196]. This ligand independency labeled them as orphan receptors. These orphan receptors have been associated with lipophilic molecules as their increased expression has been associated with atherosclerosis in mice [197]. This family of receptors has also been established as neuroprotective proteins and associated with several pathologies. For example, *Nr4a2* has been shown as an obligatory factor for the development of dopaminergic neurons in mice [198]. This subfamily of proteins have high homology among its members and have been associated with maintenance and protection of midbrain dopaminergic neurons from excitotoxicity [199]. In addition, neuronal cell damage as the result of ischemia and glucose deprivation is lessened by the increased expression of *Nr4a* family proteins [200]. Taken together, there is a clear neuroprotective property of these proteins. It was shown to be among our CS genes following RNA-Seq analysis and displayed a bell-shaped pattern of expression. Verification of this cocaine sensitivity was conducted and revealed that *nr4a3* was differential expressed in larval and adult zebrafish tissue samples. Tissue collected from larval zebrafish demonstrated significant gene repression with increasing ECE concentrations. Quantification of *nr4a3* in the adult zebrafish suggested a bell-shaped expression pattern indicating an increase in expression as the result of ECE, though results were not significant. Studies of this gene family have suggested that activation of

this protein is an early biomarker for dopaminergic neuronal development [198]. In addition, these protein have been established to increase in their expression as the result of dopaminergic neuronal activity and could regulate the expression of dopamine producing enzymes and dopamine transporter proteins [201]. Real-time PCR did not successfully verify results of our RNA-Seq as there was no differential expression based on ECE, but this may be the result of large variation within the tissue samples. There was a general trend of increased expression in fish with 2.5 mg/mL ECE. It may be that low doses of cocaine induced increased expression of this gene, while increased concentrations reduced its expression or induced a secondary repressor. Further, *nr4a3* was shown to be associated with an estrogen-related receptor (*Essra*). Knockout experiments of the *Esrra* protein in the mouse animal model has suggested a functional role in several behaviors, including grooming, compulsivity and social functioning [202]. Knock-out or knock-down of *Esrra* in mice led to loss of operant conditioning with food as well as increased grooming, indicative of social abnormality. It is unclear from our work how direct the interaction between these two proteins is, but because both are affected by cocaine exposure, further analysis may generate interesting results.

The analysis of the RNA-seq data revealed unique interaction between genes found to be cocaine sensitive. Because of this, we attempted to verify gene expression changes as the result of ECE in both larval and adult zebrafish. Comparisons of acutely treated fish at each ECE concentration were made against fish from each ECE treatment group that were left untreated, giving us a list of CS genes that displayed a bell-shaped change expression. The results of our gene validation experiments using qRT-PCR did

not substantiate this result. Instead, there was little demonstration of a dose-dependent

change in expression among our genes. In most cases there was no significant difference

among the expression when compared to control fish. In nearly every tissue sample

there were large amounts of variation in expression of each CS genes. The amount of

noise in comparison to signal of expression may have limited the inference available

from our PCR experiments. Embryonic expression revealed unexpected results as well.

The gene *bdnf* showed a bell-shaped curve with a peak in expression when treated at

the lowest ECE concentration in larval expression. This expression pattern was similarly

observed in the adult samples. In larval samples, several genes displayed a significant

dose-dependent inhibition of gene expression. An example is clearly demonstrated in

Figure 10 of the results. Untreated larval zebrafish demonstrate significantly higher

levels of gene expression when compared to all treated groups. A critical difference in

the results seen in adult and larval PCR experiments may lie in the numbers of sample

used to quantify the expression in our experiments. The Hi-Seq experiments had 3

replicates of each of the 4 treatment groups giving 12 comparisons. Our PCR

experiments contain several tissue sample per amplified sample, but numbers of fish

from each treatment ECE cohort was limiting.

The difference between our results and those seen in the Hi-Seq may also be the result

of sample numbers. Moreover, the addition of methylation groups in promoter regions

of our selected gene may have resulted in the transcriptional differences observed.

ECE Generated Changes in the Epigenetic Landscape

As our lab proposed that behavior was longitudinally affected by ECE and was governed by changes in gene expression, we analyzed DNA methylation as a means to produce intergenerational changes to the transcriptome and associated behaviors. It is widely accepted that DNA methylation is often associated with gene silencing [203]. The addition of methyl moieties to the 5' carbon on the nucleotide cytosine, prevents protein binding and alters the transcriptional potential of the DNA segment. Alterations in DNA methylation have been observed in other model organisms following prenatal exposure to various insults including heavy metals, nicotine, alcohol, and drugs of abuse [204-207]. Alcohol exposure during gastrulation has been demonstrated to decrease the presence of 5-methylcytosine (5-mC) groups along the neural tube and impede development [204]. Brain regions involved in memory and behavioral motivation, like the hippocampus have also been shown to be longitudinally affected by early drug insult. Research suggested that methamphetamine exposure during gestation led to changes in the epigenetic landscape of hippocampal DNA [207]. Mice with this differential methylation were also shown to have enhanced cocaine-induced CPP responses. The results of our study indicated whole chromosome methylation was likely unaffected by ECE in the F_1 generation of zebrafish. Along the 25 zebrafish chromosomes, there was significant differences seen in the hypermethylation state as well as significant differences seen the hypomethylation states. Because of significant hypermethylation as well as significant hypomethylation along nearly ever zebrafish

chromosome, it suggests no net change in global methylation. This did not mean that along these chromosomes there were not significantly changes in the methylation state of genes encoded by the chromosome. Analysis of genes with differentially methylated did indicate some different methylation patterns based on the ECE concentration. Low concentration ECE led to a generalized increase in methylation among our selected CS genes. In contrast, high concentration of ECE generally led to increased hypomethylation. It seems from our result that each concentration of cocaine led to altered methylation states of a unique set of genes. These concentrations then, appeared to act as a unique stressor to the transcriptome. Hydroxymethylation comparisons were made between the untreated and 2.5 ECE F_1 group. This concentration of ECE led to an overall decrease in 5-HmC moieties along the CS genes previously identified. How then does this relate to the expression observed within our CS genes? As mentioned, each concentration led to a unique set of genes with differential methylation. It may imply that methylation occurred uniquely to concentrations experienced as rewarding and unique to concentrations of cocaine found as harmful or stressful. We did not look at methylation and hydroxymethylation in the larval zebrafish, but if the result from the adult methylation data represents the methylation state in larval zebrafish it may explain the result. As the addition of 5-mC moieties was typically higher in the 2.5 F_1 ECE group, it may reflect the decrease in expression seen in the larval groups. The concentration may have been a threshold treatment, keeping expression of CS genes inhibited regardless of increased ECE treatment. The enzymes responsible for the addition of the methyl groups are Dnmts.

Because of the different functions of Dnmt enzymes, repression may occur at de novo methylation as well as repression in the maintenance of methylation [208]. The conversion of 5-mC to 5-HmC is controlled by ten-eleven translocase (TET) enzymes and is thought to have demethylation potential [209]. It is through the oxidation reaction of these TET enzymes that demethylation could occur and could represent a methylation intermediate between methylated and demethylated. Analysis of the gene *nr4a3* for example showed significant reduction in its expression in larval zebrafish as ECE dosages increased. There was no difference observed between the low concentration of 2.5 mg/mL and 20 mg/mL, suggesting the low dose was enough to substantially reduce its expression. The expression appeared to recover in the adult zebrafish following acute cocaine exposure. There was significant hypo- and hyper- methylation along chromosome 16, where *nr4a3* is found. This did not appear along the chromosome positions relative to the location of this gene. Here, there was substantial modification of 5-HmC groups found within the 3' regions upstream of exons 4, exon 6, and exon 9. This repression was seen in several of our genes, with a general increase and recovery of expression seen in the adult brain. Taken together these results support further investigation into 5-HmC addition and its implication into intergenerational drug sensitivity. Quantitative analysis to determine the functional activity of TET as well as the DNMTs in zebrafish would further clarify the DNA methylation state resulting from ECE.

The results of this study suggest the following:

- ECE led to increased sensitivity in the F_0, which lead to decreased sensitivity in the F_1

- Validation of Hi-Seq results revealed larval suppression of select CS genes by increasing ECE and adult expression appeared unaffected

- Clustering of CS genes affected by ECE appeared to be involved in neuroprotection

- F_1 Global methylation is unaffected by ECE in the F_0, but select CS genes showed differential methylation states based on ECE concentration

- Modified methylation landscapes as the result of CA exposure during development led to differential CPP behavior

Neurexin the Novel Addiction Gene

Neurexin (NRXN) is a unique, single-pass transmembrane protein found in the synaptic terminal of neurons that may be an ideal gene for further addiction research. Alterations in copy number and mutations in this protein have been associated with several neurodevelopmental and neuropsychiatric diseases. Neurexin has three genes in mammals: *Nrxn1*, *Nrxn2*, and *Nrxn3* and two isoforms of differing length, an α- and a ß-, generated by different promoters at differing splice sites [210-212]. These three neurexin genes are highly evolutionarily conserved. Further evolutionary analysis indicated that a specific duplication event in the zebrafish genome occurred leading to different paralogs at different linkage groups of this protein, so there are six *nrxn* genes in zebrafish [213]. Research has also indicated significant similarity among these gene

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sequences to neurexins found in humans [214]. Sequence analysis and cloning of *Nrxn3* indicated an internal base position difference present in the two *Nrxn3* genes. This difference was shown to be identical to the sequence of base pairs found within the longer *Nrxn3aα* gene and were seen in a splice region [211]. The differences in the bases found within the splice site led to a different translation result, with one protein becoming transmembrane and the other becoming a secreted protein as it no longer possessed the membrane-anchoring carboxyl tail [211, 215]. The transmembrane protein, due to its differing internal base, undergoes a 30 amino acid insertion-deletion mutation, whereas the secreted protein is produced as multiple in-frame stop codons are inserted in sequence. Further, inspection of glycosylation patterns indicated that *Nrxnß* undergo similar O-glycosylation of their C-terminus as the *Nrxnα*. This suggests they maintain a similar transmembrane orientation in the neuronal membrane. Knock-out studies of the three *Nrxn* genes lead to development consequence with varying severity. Triple knock-out of all three *Nrxnα* led to early mortality in mice, and varying knock-outs resulted in impaired respiratory function thought to contribute to the mortality [216]. *Nrxnß* triple knock-out is suggested to have little effect on survival, but did alter glutamatergic neuronal transmission and memory in mice [217]. For these proteins to carry out their function *trans*-synaptically, they must interact with other cell adhesion molecules. One such protein is neuroligin. Neuroligin proteins have been suggested to interact with neurexin proteins on post-synaptic neurons [218]. Neuroligins appear as dimers and contain an acetylcholinesterase-like domain that interacts with presynaptic neurexins in the extracellular spaces of synaptic junctions

[219]. Together this Ca^{+2}-dependent heterotetrameric complex is thought to be involved in synaptic maturation and synaptic formation during synaptogenesis [220-222]. In the zebrafish and humans, it has been shown early splicing events are highly controlled leading to the larger expression of *nrxnα,* whereas *nrxnß* increases in expression later in development, but are still minimally expressed [214]. It is thought that the different isoforms of neuroligin and neurexin undergo their own alternative splicing and this may govern the protein complexes that are found between the two proteins, though binding between affinities for the neurexin and neuroligin may be only slightly different between isoforms [223]. The neuroligins reside in the postsynaptic membrane and its varying isoforms have been associated with different inhibitory synapses [224, 225]. Recent evidence has supposed that these neuroligin and neurexin complex play substantial roles in social behaviors via excitation and inhibition control in the hippocampus [226].

Human studies have shown neurexin-3 (NRXN3) to be implicated in addiction and other mental disorders. In a study investigating NRXN3 single nucleotide polymorphisms, variations in the NRXN*3* gene were associated with smoking and addictive tendencies [227]. A similar study indicated variation in the NRXN3 gene was associated with alcohol dependence and was correlated with increased expression of the longer isoform, *NRXN3α* [228]. *Nxrn3α'*s physiologic function has been specified with animal knockout studies. Research by Aoto el al (2015) indicated that homozygous knockout of *nrxn-3 α/ß* had impact on synaptic function in cultured neurons. In their study, it was observed that conditional knock-out of *nrxn3* had differential impact based

on location in the brain, specifically the hippocampus or the olfactory bulb. Specifically, in cultured neurons, *nrxn3* was required for GABA release in olfactory neurons and is involved in AMPAR-mediated responses in hippocampal tissues [229]. Quantitative research has suggested that *Nrxn3ß* expression was altered in the globus pallidus in the mouse brain by acute cocaine exposure [230]. Differential expression of *Nrxn* isoforms in this and other regions of the basal ganglia support this genes role in the motivational cues associated with addiction. Our study identified substantial epigenetic modification of neurexin genes and, interestingly, it was the only gene found hypermethylated in all three of the ECE treatment groups when compared to CS genes in the F_1. Methylation data indicated a differential methylation state along the DNA encoding the neurexin gene. The shorter isoform, *nrxnß*, as stated previously, is expressed in smaller quantities when compared against the longer isoform [214]. The results of our bisulfite sequencing experiment did not reveal numerous 5-mC or 5-HmC attachments along the *nrxn3ß* sequence. The methylation patterns of *nrxn3α* indicated unique methylation patterns along the DNA sequence. Using the Ensemble sequences of this protein it was seen that an increase in methyl moieties were found at doses higher than 2.5mg/ml around exon 17. This increased methyl attachment was seen on the shorter isoforms of the *nrxn3* gene. Methylation ratio differences suggested low doses of cocaine led to hypomethylation whereas increasing cocaine to 10 mg/ml led to hypermethylation in the same regions along the segment. Hypomethylation as a result of low cocaine treatment continued along the DNA segment until around intronic region 18. A change in the methylation landscape of this gene was observed as the result of increasing

cocaine exposure. The increased concentration of cocaine exposure in the F_1 coincided with methyl attachment at positions matched to the longer isoforms. These differential methylation patterns may contribute to varying splicing capabilities, thus providing limited generation of the various isoforms. The study of this gene in our experiment also revealed differences in hydroxymethylation along the chromosome. The bisulfite sequencing method cannot, however, distinguish between 5mC and 5HmC groups [231]. Observation of our 5-mC and 5-HmC groups at various positions show no overlap along our *nrxn3*. These differential results may produce alterations in the entropically favorable neuroligin-neurexin complexes that present early in synaptogenesis. Due to the timing of synaptogenesis and our early cocaine treatment, it is likely that these cell adhesion molecules and their expression are modified. Due to reports of different complexes responsibility in excitatory and inhibitor neurons within the brain, it is likely that alterations here may longitudinally affect behavior. Research suggests the importance of these proteins in synaptic formation and protein folding [232, 233]. Here, reductions in neuronal cell formation and stability as the result of neurexin knock-down may produce augmented brain regions critical in higher cognitive processes, including motivation and reward. Knockout studies specific to the reward circuitry could identify specific roles to the *nrxn3* gene in the zebrafish as they relate to reward and motivation. As shown, differential methylation along the this *nrxn3* gene may generate sequences of varying length and as previously mentioned, alternate splicing along neurexins and neuroligins contributes to the complexes that form. How these cell adhesion molecules contribute to reward and longitudinal behavior remains to be investigated. Due to the

readily manipulated lines of zebrafish, knock-out studies of this kind can be undertaken. These results taken together with what has been reported in the literature of the human and murine *nrxn3* gene family imply the importance in understanding how these cell adhesion molecules contribute to altered reward and behavior in organism with early drug exposure.

CA Produces Differential CPP Behaviors

Understanding how drug exposure produces lasting consequence both behaviorally and genetically may provide insight into susceptibility toward drug abuse as well as provide potential treatment options. Persistent effects attributed to early drug exposure occur by several means including histone methylation and acetylation. For our experiment we looked specifically at DNA methylation. DNA methylation is an important process in regulating gene expression. Methylation arises as methyl groups are donated to CpG dinucleotides from the substrate S-adenosylmethionine (SAM). DNA methylation is associated with transcriptional activity that is important for processes like X-chromosome activation, early developmental processes, cell survival and differentiation, and genome integrity [125, 126, 234]. Mammalian Dnmts include three homologs [235]. It has been demonstrated that Dnmt1 is responsible for the maintenance of methylation patterns following DNA replication. This enzyme has been implicated in imprinting [236]. Dnmt3a and Dnmt3b are known for the *de novo* genome wide methylation of DNA sequences during development and cellular differentiation as well as in response to environmental stimuli [208, 234, 237]. There is homologous expression of Dnmts within

the zebrafish model also highly involved during development. The genome duplication

event of the zebrafish led to six paralogues for DNA methylating enzymes [130]. Dmnt3a

is represented by dnmt6 and dnmt8, whereas there are four DNA methyltransferases

for Dmnt3b, namely dnmt3, dnmt4, dnmt5, and dnmt7 [131]. Literary review

emphasized some discrepancies with classification scheme for these enzymes. Teleost

dnmt6 has been reported as dnmt3ab and dnmt3a2, while dnmt8 has been cited as

dnmt3aa [238]. Paralogues for Dnmt3b include dnmt3 (dnmt3b3), dnmt4 (dnmt3b1),

dnmt5 (dnmt3b4), and dnmt7 (dnmt3b,dnmt3b2) [131, 239]. Phylogenetic analysis has

suggested that dnmt3, dnmt5, and dnmt7 are unique to teleosts.

As epigenetic modification continues to grow in interest, research continues to

utilize different pharmacologic means to manipulate these modification processes.

Chlorogenic acid and other catechol-containing polyphenols have been demonstrated to

inhibit DNA methylation. As previously stated, DNA is methylated at cytosine residues

by the donation of a methyl group from SAM catalyzed by DNMTs. As SAM donates a

methyl group, SAM is demethylated to produce S-adenosylhomocysteine (SAH). SAH

acts in a negative feedback mechanism to inhibit Dnmt enzymes [240]. SAH produces

this inhibitory effect allosterically on the specific Dnmts through the use of different

moieties found within its overall structure [241]. CA contributes to this inhibition

through its reaction with Catechol-O-methyltransferase (COMT). Tea catechins like

chlorogenic acid have been well documented as efficient substrates for COMT [242,

243]. COMT catalyzes the addition of a methyl group to chlorogenic acid from the donor

SAM. Through this reaction, SAM is demethylated producing SAH. COMT-mediated

methylation of catechols will lower the endogenous SAM levels while simultaneously

increasing the concentration of SAH. This conversion of SAM and consequent

production of SAH furthers the inhibition of DNMT enzymes [240]. Another important

enzymatic function of COMT is the degradation of catecholamines. This enzyme is

ubiquitously expressed in nearly all tissues and in distinct brain regions in a small soluble

isoform as well as a large membrane bound isoform [244-246]. COMT drives the

exchange of a methyl group from SAM to one of the two hydroxyl groups present on the

catecholamine [247]. In the case of dopamine, it is methylated at either its 3'- or 4'

hydroxyl with preference to the 3'-hydroxyl. Methylation of dopamine produces the

metabolite 3-methoxytramine (3-MT). This 3-MT is then further metabolized by the

enzyme monoamine oxidase to form homovanillic acid [248]. This methylation of

dopamine has also been suggested to increase the release of dopamine from the

presynaptic terminal [249]. Inhibition of COMT occurs through reversible binding by

nitrocatechols. These compounds have the characteristic catechol ring. Some

nitrocatechols efficiently cross the blood brain barrier, including the drug tolcapone

[250, 251]. These compounds have varying incubation times altering kinetic parameters

and are thought to competitively inhibit COMT. COMT overexpression within a mouse

model was demonstrated to lead to increased capability for dopamine release within

the striatum [249]. Behavioral testing within this model system indicated stimulus

responsive learning was affected and further investigation indicated that this was not

the result of an effect on dopamine synthesis or transport, but possibly that increased

clearance generated increased release of dopamine presynaptically. Inhibition of COMT

is suggested to lessen dopamine degradation along the COMT-degradation pathway [252]. The degradation appears to continue, though along a different degradation pathway using monoamine oxidase enzymes.

Our results demonstrated that CA treatment during critical windows of dopaminergic development generated altered CPP behavior in adult zebrafish. Zebrafish with treatment of cocaine and CA experienced increased CPP behavior, while the treatment with CA alone appeared to attenuate this behavior. This may be explained in two ways 1) differential DNA methylation state or 2) a secondary effect on dopamine release within dopaminergic pathways that is regulated by COMT. It has been demonstrated that inhibiting Dnmt enzymes via pharmacologic means augmented preference for cocaine [253]. The results of our methylation data indicated that significantly differential methylation patterns over large chromosomal segments occurred in nearly every zebrafish chromosome. Additionally, 5-HmC moieties were shown to be placed along zebrafish DNA after exposure to a relatively low dose of cocaine. How chlorogenic acid treatment during embryonic development effects this process may be the result of a synergistic feedback mechanism (Figure 28).
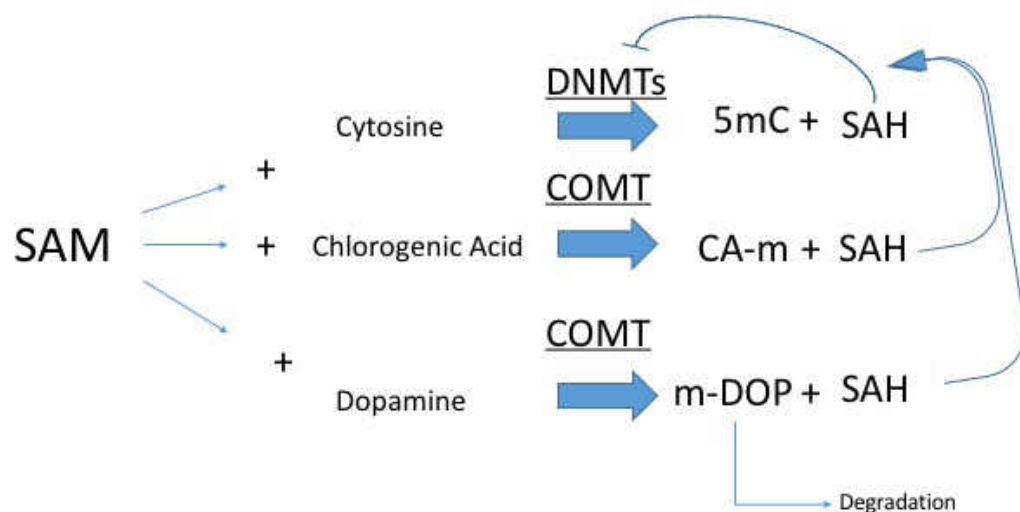
SAM Methyl Donating Biochemical Pathways

Figure 28: Schematic illustration displaying the transfer of methyl groups from S-adenosylmethionine (SAM) to the following substrates: Cytosine, Chlorogenic Acid, and Dopamine. In each reaction, SAM is used as the methyl donor and leads to the production of S-adenosylhomocysteine (SAH). It has been suggested that increased concentrations of SAH to demethylation of SAM, may produce a feedback inhibition of DNMT-mediated DNA methylation. Abbreviations: DNA methyltransferase enzymes, DNMTs; Catechol-O-methyltransferase, COMT; 5-mC, 5-methylcytosine; CA-m, methylated chlorogenic acid; m-DOP, methylated Dopamine.

The reactions of Figure 28 can be used to explain the findings from our study. In the case of zebrafish treated solely with CA, there was no significant change in cocaine induced preference. Embryonic treatment with CA may have lasting behavioral consequence in zebrafish. CA may lead to decreases in methylation of genes found in the reward circuitry of zebrafish and produce a longitudinal change in responsiveness to reward. Further, as zebrafish were not exposed embryonically to cocaine there should be no change in sensitivity to the drug as we had shown in the F_0 cohort. It would then

be implied that CA exposure led to differential responsiveness to cocaine longitudinally. Our results have suggested that ECE generated an increase in cocaine-induced CPP behaviors in adult F_0 zebrafish. Groups of zebrafish treated with both CA and cocaine exhibited a significant increase in CPP-like behaviors following acute cocaine exposure in adulthood. This could be indicative of a synergistic action of CA and cocaine. Because of the apparent hypersensitivity produced by ECE to subsequent cocaine exposure, additional treatment with CA may augment the behavioral response to cocaine in zebrafish. Because cocaine has been reported to inhibit the reuptake of dopamine at the synapse [254], acute exposure will lead to increased dopamine concentration at the synapse. It may be then that concurrent treatment with CA embryonically produced lowered endogenous levels of SAM that are not adequate to allow for the enzymatic methylation of dopamine by COMT. Because of this, dopamine will persist in the synapse for a longer duration. Concurrent decreased DNA methylation and ECE may enhance the cocaine reward seen in the adult. Cocaine may supersede the CA effect when treated embryonically. Exposure to CA during the development of the dopaminergic system may lower the concentration of endogenous SAM molecules. If COMT led to the significant methylation of chlorogenic acid, it would have simultaneously reduced the pool of available SAM molecules while producing SAH. This SAH will continue the reduction in DNA methylation, while lessening the methyl-donors available for catechol-containing compounds. As dopamine is less methylated is will remain in the synapse for a longer duration. In animals with ECE and the exposure to CA, dopamine will not be degraded as rapidly as well as its uptake in the synapse will be

inhibited by cocaine. These results taken together may explain why there was an increase in CPP-behavior in this cohort of zebrafish.

Chlorogenic acid treatment of zebrafish embryos appeared to have a significant longitudinal effect on both the weight and length of the zebrafish. This, however, may not be representative of overall health, as percentage of survival were different among treatment groups. Because of the low survival in our untreated cohort, low population density in the housing tank allowed for increased growth in this group. This experiment will be to be repeated for validation of the result. CA's effect on neuronal development remains under studied. Because of treatment during early developmental window, where this compound is generating its effect is misunderstood. Further research could elucidate the longitudinal physiologic result of this exposure. COMT's involvement in this altered behavioral phenotype suggests a need for further investigation. In the F_0 cohorts, it may be that COMT expression is inhibited in the adult zebrafish. This may govern an increase in dopamine release during acute exposure to rewarding compounds. In addition to cocaine's inhibition of catecholamine reuptake, decreased expression of catecholamine degradative enzymes may explain the heighted reward to ECE exposure individuals. How individual Dnmt enzymes modify the rewarding properties of cocaine following ECE remains unknown. Because of the easy manipulation of the zebrafish and the swift generation of mutant lines, dnmt knock-out experiments may reveal how these relate to cocaine-induced methylation.

APPENDIX

Table 1:

Primer sequences used for RT-PCR

Gene name	Gene ID	CG % (F/R)	Forward sense primers	Reverse primers	Size (bp) F/R
Brain-derived neurotrophic factor	bndf	50.0/45.5	cctgctgaatggtctc ctttac	cagctctcatggaact gaagta	22/22
FBJ murine osteosarcoma viral oncogene homolog B	fosb	52.6/55.0	acgagatgaaacgct gacc	cgtcggttcctacactt agc	19/20
Ornithine decarboxylase 1	odc1	55.6/50.0	atgccaagctggttct tc	tcgctccaaacttcac actc	18/20
Eukaryotic translation factor 1 alpha	ef1a	55.0/40.7	ctggaggccagctca aacat	atcaagaagagtagt accgctagcatt	20/27
Ribosomal protein L13	rpl13	50.0/50.0	tctggaggactgtaag aggtatgc	agacgcacaatcttga gagcag	24/22
ADP-ribosylation factor 5	arf5	37.5/47.6	cagaacacacagcgt ttaatcttt	agaaagctcctctgaa gattc	24/21
nuclear receptor subfamily, group A, member 3	nr4a3	47.4/50.0	ttcaagaggacggtg caaa	cacttctggaaacggc agta	19/20
Arginase 2	arg2	47.6/47.6	aacagaggagatcca caacac	gcaactatgtcaacgg ctaga	21/21
DEAD (Asp-Glu-Ala-Asp) box helicase 5	ddx5	50.0/47.4	caaattaggccggac agaca	tgaagagcaccaaca tgta	20/19
mitochondria-localized glutamic acid-rich protein	mgarp	50.0/47.6	aatgtggtcacaccag tctc	agatttaaccgggac gtcttc	20/21
Period circadian clock 2	per2	45.5/50.0	gtgggagacaagaaa gagatca	gcttggtcaaacccag tttc	22/20
inhibin, beta Ab	inhbab	55.6/47.6	ttcgcggagtcagga gat	ccttggctactttgag ggaaca	18/21
eukaryotic translation initiation factor 4A1A	eif4a1a	50.0/50.0	tgagaaggagtggaa gt	cggcgagtgttgatga agat	20/20

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