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Aberrant Neurogenic and Epigenetic Processes Are Associated with
Depression Induced by Developmental Arsenic Exposure

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DISSERTATION

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DEDICATION

For Poppa,

I miss you every day.

**ABERRANT NEUROGENIC AND EPIGENETIC PROCESSES ARE
ASSOCIATED WITH DEPRESSION INDUCED BY
DEVELOPMENTAL ARSENIC EXPOSURE**

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ABSTRACT

Arsenic is a pervasive environmental contaminant derived from both natural and anthropogenic sources. Epidemiological evidence supports a correlation between arsenic exposure in drinking water and increased rates of psychiatric disorders, including depression, in exposed populations. Previous studies have established that perinatal exposure to arsenic (through all three trimesters of fetal/neonatal development, DAE) has long-lasting consequences into adulthood including deficits in learning and memory, molecular aberrations in the hypothalamic-adrenal-pituitary (HPA) axis, and depressive-like symptoms in mice. The studies described in this dissertation were designed to assess the impact of DAE on adult hippocampal neurogenesis (AHN) as a possible mechanism of action for arsenic-induced susceptibility to depression.

Three sets of experiments were developed to test the hypothesis that DAE induces aberrant epigenetic regulation of neuronal genes involved in AHN leading to depressive-like symptoms. First, arsenic-induced morphological and functional damage to the hippocampus was assessed: DAE reduced differentiation and survival of neural progenitor cells in the dentate gyrus (DG) of the hippocampus and induced depressive-like behaviors after exposure to a predator odor (2,4,5-trimethylthiazoline, TMT) in adult male offspring. DAE also blunted the HPA axis stress response, as measured by plasma corticosterone levels, after TMT exposure. Second, two treatment paradigms, environmental enrichment (EE) or chronic treatment with fluoxetine, were performed to determine the reversibility of damage and the causality of aberrant AHN as a mechanism of action. Both treatments ameliorated deficits in AHN, while fluoxetine prevented depressive-like behaviors and reversed arsenic-blunted stress responses in exposed animals. Third, morphological deficits in AHN were associated with abnormal gene expression in the DG of several factors in pathways associated with neurogenesis. Global histone modifications and next-generation sequencing following chromatin immunoprecipitation (ChIP-Seq) were performed to assess the regulation of gene expression in the DG. DAE increased levels of activating histone marks, including H3K4 trimethylation and H3K9 acetylation, specifically in the DG of adult males, with altered expression of their respective chromatin modifiers, Mll1 and GCN5/PCAF. ChIP-Seq analysis is currently underway to demonstrate the specific genes associated H3K4 trimethylation (indicative of increased gene expression) after DAE in the DG. The research presented in this dissertation demonstrates that aberrant AHN is a likely candidate for DAE-induced hippocampal damage leading to greater susceptibility to

stress-induced depression in adulthood via aberrant epigenetic regulation of gene expression. These studies provide a basis for investigating the effect of DAE on epigenetic programming during embryonic development leading to neurogenic deficits observed in adulthood.

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1.0 Epigenetic regulation of adult neurogenesis as a mechanism for arsenic-induced susceptibility to depression

1.1 Arsenic exposure and neurological function: an introduction

1.1.1 Arsenic in the environment

Arsenic is a common, pervasive environmental contaminant that affects almost every major organ system in the body including the brain. Exposure to arsenic mainly occurs through ingestion of contaminated food or water in which arsenic accumulates due to 1) natural sources such as the leaching of arsenic from geological features and 2) anthropogenic sources including industrial activities (i.e. burning of fossil fuels and smelting processes) and the use of products containing arsenic like herbicides, pesticides, wood preservatives, animal feeds, and semiconductors (EPA, 2014). Currently, the World Health Organization (WHO) considers a safe limit of 10 µg/L (parts-per-billion, ppb) arsenic in drinking water with a maximum permissible level of 50 µg/L (ppb). Despite these guidelines, arsenic exposure is a worldwide health concern as several millions of people are exposed to this metalloid at levels that well exceed the WHO standard; even still, epidemiological evidence suggests that low-level exposure to arsenic (in the ppb range) can be harmful to humans (Zierold *et al.*, 2004). Additionally, exposure can occur via several unregulated sources in which high concentrations of arsenic can be found: these include everyday products like wood varnish and fruit juice (Wilson *et al.*, 2012). Further, several countries including China, India, and Bangladesh do not have access to arsenic-free water and as such, the mass poisoning of these populations due to high amounts of arsenic in ground water is considered one of the most insidious worldwide health epidemics.

1.1.2 Arsenic metabolism

Inorganic arsenic, found in drinking water, exists in two oxidation states as As^{3+} (arsenite) and As^{5+} (arsenate), both of which readily accumulate in the body. Arsenite is more toxic than arsenate as it reacts with thiol groups in proteins and crosses cellular membranes, but both inorganic forms are considerably more toxic than their organic arsenical counterparts such as arsenobetaine, found in marine life (Marchiset-Ferlay *et al.*, 2012). While absorption of arsenic mainly occurs in the intestines via ingestion of arsenic-contaminated food or drinking water, some exposure can occur via absorption through the skin or inhalation via activities such as mining and ore smelting. Regardless of method of exposure, arsenic can accumulate in the heart, lungs, kidneys, liver, muscles, and brain (Singh *et al.*, 2011). Uptake of arsenic occurs adventitiously via transport systems that have developed for other metals such as selenium and phosphate or via transport proteins such as aquaporins and glucose transporters (Liu *et al.*, 2006; Rosen & Liu, 2009). Arsenic biotransformation after ingestion includes several metabolic REDOX reactions and associated methylation steps to form six arsenical species that are excreted from the body; these include the trivalent and pentavalent forms of inorganic arsenic and the mono- and dimethylated metabolites of each inorganic form. Several studies have demonstrated that the speciation of arsenic, not only the total exposure, impacts type and severity of molecular dysfunction (Dodmane *et al.*, 2013; Wang *et al.*, 2014). Additionally, the enzymes responsible for the metabolism of arsenic also play a role in the development of arsenic-related diseases; for example, a particular haplotype of arsenic methyltransferase *AS3MT* (for the monomethylation of the trivalent form, MMA(III)) is associated with more efficient metabolism of arsenic (Engstrom *et al.*, 2013).

Detoxification of arsenic after exposure actually produces more toxic species (conversion of arsenate to arsenite) using glutathione (GSH) and other thiol groups as reducing agents. The conversion to arsenite allows arsenic to pass through cellular membranes, as the ionization of As^{5+} at physiological pH levels prohibits this passage. Arsenite is then distributed throughout tissues into cytosol where it undergoes methylation, which is catalyzed by S-adenosyl methionine (SAM), the universal methyl donor cofactor. Arsenic is thought to deplete the levels of SAM in the body, particularly under chronic exposure conditions, leading to hypomethylation of DNA and other aberrant epigenetic processes including histone posttranslational modifications that require methyl groups (Ren *et al.*, 2011). However, the mechanisms by which arsenic impedes epigenetic processes are still under investigation.

1.1.3 Arsenic-induced neurotoxicity

Paradoxically, arsenic has been used as both poison and a therapeutic for centuries. Currently, arsenic trioxide (As_2O_3) is used to treat refractory or relapsed acute promyelocytic leukemia (APL), which is a variant of acute myeloid leukemia (AML). Arsenic readily crosses the blood brain barrier and thus leads to remission of symptoms for APL patients with central nervous system (CNS) involvement (Kiguchi *et al.*, 2010). Yet, arsenic's reputation is predominately nefarious: it acts as a co-carcinogen, and while it may be useful for APL patients, arsenic induces severe CNS damage resulting in both peripheral and central neuropathies. Chronic exposure to arsenic results in encephalopathy and toxic delirium with deficits in neurological functioning including learning, short-term memory, and concentration. Low doses often impair psychomotor

speed and attention, with particularly adverse effects on verbal processing and learning (Vahidnia *et al.*, 2007). Epidemiological studies on arsenic exposure and common neurological outcomes including deficits in learning and memory and the increased incidence of psychiatric disorders like depression will be discussed in detail in Chapter 2.

The mechanisms by which arsenic induces neurotoxicity are still under investigation: these include increased oxidative stress and interference with protein expression and function leading to disordered brain structure and increased neurodegeneration. Arsenic toxicity induces the release of reactive oxygen species and lipid peroxidases, which are concurrent with decreased levels of superoxide dismutase and glutathione (Singh *et al.*, 2011). Aberrant protein expression and function after arsenic exposure is associated with axonal deterioration and degeneration of dopaminergic neurons and increased apoptosis via activation of the MAPK pathway in cerebral neurons (Namgung & Xia, 2001; Vahidnia *et al.*, 2008). Additionally, the expression and metabolism of several neurotransmitters are impacted by arsenic exposure: these include acetylcholine, glutamate, adrenaline, noradrenaline, dopamine, and serotonin in specific brain regions including the frontal cortex and hippocampus, two regions associated with mental health disorders like depression (Tyler & Allan, 2014). The possible mechanisms of arsenic-induced neurotoxicity and the rodent and human studies that have been done to elucidate these mechanisms will be discussed in greater detail in Chapter 2.

1.1.4 Developmental arsenic exposure and depression

As the neuropathies associated with arsenic exposure have been well characterized, for the purposes of this dissertation, the connection between arsenic exposure and major depressive disorder (depression) will be the predominant focus. Research has established that depression is linked with the imbalance of amino acids, neurotransmitters, thyroid and adrenal hormones, nutrients and minerals, and fatty acids; however, the role of environmental factors in the etiology of depression, including toxins like arsenic is still under investigation. Exposure to lead, mercury, cadmium, manganese and arsenic have all been linked to the onset of depression, likely via altered neurobiological mechanisms in the CNS (Onishchenko *et al.*, 2008; Bowler *et al.*, 2011; Haider *et al.*, 2013; Scinicariello & Buser, 2014). As such, this dissertation will explore the mechanisms of arsenic-induced toxicity in the etiology of depression. It should be noted that the model of arsenic exposure used throughout these studies is that of developmental exposure to 50 ppb arsenic during the three trimester equivalents of pregnancy in the mouse model. The early pre- and postnatal environment has been demonstrated to impact behavior and susceptibility to psychiatric disorders in adulthood (Hochberg *et al.*, 2011). In several studies, negative environmental exposures (handling, poor maternal care, stress, toxins) induce alterations in the offspring's endocrine stress response and are likely associated with the behavioral endophenotypes of anxiety and depression (Hellemans *et al.*, 2008; Stiller *et al.*, 2011; Bolton *et al.*, 2013; Freund *et al.*, 2013). (The term, *endophenotype*, is used extensively in the field of psychiatry to denote particular intermediate phenotypes that are part of a heterogeneous complex illness based on genotype. Endophenotypes are measurable (molecular, behavioral, physiological) features that contribute to a disease,

and thus this word is the most appropriate for measures of animal behavior in describing characteristics of psychiatric disorders (Gottesman & Gould, 2003).) While the exposure to some environmental factors can be controlled, arsenic exposure, even in low doses, is unavoidable because of its ubiquitous presence in the environment. Research on arsenic exposure, hepatic function and the susceptibility to cancer have demonstrated that *in utero* arsenic can have long-lasting effects on the molecular biology of an organism well into adulthood. Therefore, we are interested in how low level exposure to arsenic during development can impact an offspring's susceptibility to depression. The following sections will introduce and discuss depression, its mechanisms of action, including adult hippocampal neurogenesis and epigenetic regulation of gene expression.

1.2 Depression: an overview

Major depressive disorder (MDD) is the most pervasive mental disorder in the United States and affects over 350 million people worldwide (Kessler *et al.*, 2005). The World Health Organization estimates the depression is *the* most expensive health care burden and accounts for in excess of 31 billion dollars per year in lost productivity time in the U.S. alone (Stewart *et al.*, 2003). Currently, mental health assessment utilizes guidelines established by the Diagnostic and Statistical Manual of Mental Disorders (DSM-V), published by the American Psychiatric Association. Patients may not have all symptoms, due to the heterogeneity of MDD; however, the type and severity of depression (rather than cause) can be distinguished. Two particular features in all depressive cases are 1) depressed mood with 2) loss of interest or pleasure. At least one of the following symptoms presents as well: significant alteration in weight along with reduced or

increased appetite; insomnia or hypersomnia; agitation or psychomotor retardation; fatigue or loss of energy; feelings of worthlessness or excessive guilt; reduced concentration; and recurrent thoughts of death or suicide. These symptoms present most of the day and nearly every day for at least two weeks causing significant distress or impairment of normal function. Other forms of depression include major depression with melancholia, a more acute form of major depression in which loss of reactivity is quite severe; psychotic depression; adjustment disorder with depressed mood; dysthymia; mixed anxiety and depression; post-natal (or postpartum) depression; and adolescent depression. Over 50% of patients with MDD suffer from current comorbid psychiatric disorders including anxiety, bipolar disorder, substance use disorder and personality disorder (Melartin *et al.*, 2002; Aina & Susman, 2006). While the type and severity of depressive states differ, current theories offer a few common mechanisms that may underlie the overall phenotype of “depressive-like” symptoms.

1.2.1 Animal models of depression

Modeling any type of human behavior in a rodent is difficult, but the complexity in modeling depression is compounded due to the variety of symptoms and the heterogeneity and unknown etiology of the disease. Recapitulating MDD in a mouse model requires the simplicity of measured phenotypes that can be reproduced and then evaluated in a rodent.

McKinney and Bunney established three sets of criterion in the 1960’s for establishing valid models of psychiatric diseases; these include face validity, construct validity, and

predictive validity (McKinney & Bunney, 1969). Each mouse model and subsequent behavioral assessments to identify depressive-like behaviors or other psychiatric disorders typically conform to one or more of these criteria. For face validity, the animal should exhibit symptoms that are similar to those observed in humans as measured using appropriate behavioral assessments (see below). A mouse model can have good face validity without appropriate construct (or etiologic) validity, in which the symptoms in both animals and humans are based on the same neurobiological mechanisms. This is the most difficult type of criteria for a mouse model of depression since there are several theories on the etiology of MDD (see sections below). If a model has both appropriate construct and face validity, then it should respond to pharmacological manipulation to have predictive validity, in that the animal will respond to treatment similar to humans. The more criteria that are met by an animal model, the more scientifically valid this model can be. Thus, for our purposes of the arsenic exposure paradigm, to determine that these animals have what we term “depression”, we must be able to detect depressive-like behaviors using appropriate behavioral assays (face validity), observe similar disease progression and neurobiological symptoms molecularly (construct validity), and observe similar response to treatments given to depressed patients (predictive validity).

There are several models of depression used in research today: these include lesion models where animals are bulbectomized, pharmacological models that use glucocorticoids or reserpine to induce depressive-like symptoms, genetic models that use various molecular theories of the etiology of depression as a basis for knock-ins or knock-outs of gene function, and stress models in which different types of stress (chronic,

intermittent, restraint, etc.) are used to induce a depressive-like state. Discussing each of these models is beyond the scope of this introduction, as we are not attempting to create a model using arsenic, but rather assess the effect of arsenic on the risk of developing depression. In humans, chronic arsenic exposure has been correlated with an increased risk of depression, while adult rodent offspring exposed to arsenic during the perinatal period exhibit depressive-like symptoms in traditional behavioral assessments (see below) and demonstrate face validity. Thus, in order to establish that arsenic induces depression in a rodent model, we must demonstrate etiologic validity (i.e. molecular mechanisms of depression) and predictive validity (responses to antidepressants). These criteria have been assessed in Aim 1 of these dissertation studies.

1.2.2 Behavioral assessments of depression

To determine if a rodent model achieves these criteria set by McKinney and Bunney and demonstrates endophenotypes associated with depression appropriate behavioral assessments must be conducted. However, each behavioral assessment has its own limitations. As depression can be characterized (in humans) as having at least the overall criteria of depressed mood and loss of pleasure (anhedonia), behavioral assays have been developed to measure these symptoms in rodents. While animals cannot have a “depressed” mood, there are three particular paradigms in which despair-based behavior can be construed as “depression”; these include the forced swim task (FST), the tail suspension task (TST), and learned helplessness (LH) task. These three assessments are based on the hypothesis that animals will attempt to escape aversive stimuli, and lack of escape may be indicative of depressive-like behavior (Crawley, 2007).

When rodents are placed in water they all exhibit acute escape behaviors including climbing and swimming; after a period of time, these behaviors cease and the rodents display immobility. The latency to cease swimming and the total immobility can be considered a measure of depression compared to control counterparts. This task was developed in the 1970's and is referenced as the Porsolt forced swim task; experience in the FST can induce this "despair" based behavior in rodents, which will display increased immobility and decreased latency if they have undergone chronic uncontrollable stress (stress model of depression). Additionally, animals provided antidepressants typically exhibit active escape behavior longer than control counterparts given a vehicle drug. The tail suspension task is used to measure escape oriented behaviors as well; however, this task is not appropriate for rodent strains like the C57BL/6 mice used in our studies that naturally exhibit tail climbing behavior (Mayorga & Lucki, 2001). In both tasks, lack of active coping behavior (swimming or climbing) is interpreted as depressive-like behavior. However, it should be noted that there is some disagreement in the behavioral neuroscience field as to whether immobility observed in the FST is actually depression or simply a passive stress coping behavior. Additionally, some researchers purport that the FST assesses stress-induced anxiety rather than depression, as it is unlikely that a short period of inescapable stress induces depression in a wild type mouse. However, the FST in particular provides good predictive validity for antidepressant compounds and is thus used for high throughput screening of potential therapeutics (Dedic, 2011). Another form of inescapable stress that results in passive versus active coping responses is the learned helplessness task. Animals are exposed to uncontrollable and unpredictable aversive shocks for a sufficient period of time that will result in deficits in escape behaviors; after

a period of time, animals are provided with a means to escape the shock. The measure of 1) latency to escape the shock and 2) the attempts to escape are indicative of “learned helplessness”. The rodent learns that it has no control over the shock stimulus and is helpless to avoid the stressor; this acquired behavior is quite long-lasting in animals and is considered closer to a model of depression due to appropriate face validity (Buccafusco & Buccafusco, 2009). This theory of learned helplessness, developed by Martin E.P. Seligman, was based on the notion a depressed individual believes that the outcome of a situation is independent of his or her actions and thus feels powerless in a situation (Seligman, 1972). The learned helplessness task also provides appropriate predictive validity in response to antidepressants, and thus was used for behavioral despair assessments in the studies described in this dissertation (Chourbaji *et al.*, 2005).

1.2.3: Confounding factors in depression assessments

Since excessive anxiety can emerge as part of depression and many patients with some form of depression have comorbid anxiety symptoms, it is difficult to distinguish between the two in certain behavioral assessments (Berton & Nestler, 2006). For example, increased anxiety-like behavior can emerge as hyperlocomotion leading to increased active swimming behavior in the FST (and thus decreased immobility); this behavior could then be interpreted lack of depressive-like symptoms (Gardier & Bourin, 2001). Depression is considered a pathological condition, whereas anxiety may be construed as a normal cognitive response to certain situations. Anxiety behavior activates the stress response, which an animal can use to initiate mobility in response to a threat; this response may be considered protective on some level (Dedic, 2011). However, excessive

anxiety in response to stimuli not considered threatening can evolve into an anxiety disorder and may be part of a depressive phenotype. For our purposes here, we will only discuss anxiety measures that are related to depression.

Mice are naturally inquisitive and inclined to explore novel environments, although they do show aversion to well-lit environments. Thus, the time exploring an open well-lit area can be used to determine level of anxiety and locomotion. Arsenic differentially impacts locomotion, with low levels resulting in increased locomotion and high levels resulting in decreased locomotion (See Chapter 2); thus assessment of behavior in an open area can be used for analysis of alterations in locomotion that might play a role in the response to behavioral tasks aimed at determining depressive-like behavior. The open field test (OFT), or novel exploration of an unknown area, is typically used for determining state anxiety, while trait anxiety is determined by measuring exploration of an environment the animal has already been habituated to (or the second day of the OFT). This behavioral task takes advantage of conflicting tendencies: avoidance of a potentially dangerous area that is brightly lit and the inherent exploration of novel environments (Dedic, 2011). Additionally, the open field test has been used extensively for prediction of anxiolytic outcomes of various drugs; however, the use of the elevated plus maze and the light/dark box is more common (Griebel & Holmes, 2013).

Anhedonic behavior, indicative of loss of pleasure for normally rewarding activities including sexual experience and consumption, can be measured in animal models as indicative of depressive-like behavior. Chronically stressed animals that display

depressive-like symptoms in other behavioral tasks also lack normal responses to typically rewarding stimuli. The preference for highly palatable foods, including sugar, can be used to characterize hedonic behavior; this test is called the sucrose consumption test and assesses the preference for the sugar solution over water. However, as with other measures, there are confounds with this assessment, in that food and water deprivation preceding the test for 18-24 hours may affect the behavior and thus the interpretation of reduced sucrose preference (Mateus-Pinheiro *et al.*, 2014). Additionally, the metabolic and sensory influence of such a task cannot be ruled out either, and some literature suggests that sucrose preference lacks predictive validity (Forbes *et al.*, 1996). In general, mice display depressive-like symptoms manifested as decreased sucrose consumption, decreased ability to perceive associations between environment and reward (conditioned place preference), and decreased sensitivity to brain stimulation (self-stimulation) (Dedic, 2011).

1.2.4 Heritability versus environmental factors in the etiology of depression

The etiology of major depressive disorder, while extensively studied, is not well understood. As a result, of the 41 million people treated for MDD patients in the US, only 30% report receiving relief from depressive symptoms (NIMH, 2014). Many MDD patients never obtain full remission of symptoms likely due to biological variation of individuals. Indeed, the most recent genome wide association study (GWAS) on major depressive disorder reported that heritability only accounts for 40% of MDD cases and that no common genetic variant (specific loci reaching GWAS significance) is associated with MDD (Major Depressive Disorder Working Group of the Psychiatric *et al.*, 2013).

While single nucleotide polymorphisms (SNPs) have been identified in many of the MDD cases, including in genes associated with Wnt and serotonin signaling, thus far, none of the analyses have sufficient power and only account for a small proportion of heritability. Further, monozygotic twin studies of depression demonstrate high discordance of the disorder and individual variability in gene expression (Byrne *et al.*, 2013). The non-genetic risk of development of depression is poorly defined, but typically emerges as a manifestation from many common experiences including childhood trauma, emotional stress, physical illness, and even viral infections. As such, the role of environmental factors in the etiology of MDD is widely acknowledged but still requires further investigation.

Depression has mostly been studied in the context of stress-induced depression, using mouse models; yet maladaptation to stress leading to depression is highly variable, and very little is known about what makes some individuals more “resilient” to stress or adversity and others more vulnerable to it (Krishnan & Nestler, 2008). Over that past few decades, toxins like arsenic have been implicated in the pathophysiology of depression, as epidemiological studies have demonstrated significant correlations among individuals exposed to toxins and the incidence of depression. These studies are discussed in detail in Chapter 2. They have collectively provided convincing data that arsenic exposure may underlie some of the MDD cases around the world. However, the mechanism by which arsenic imparts this toxicity on the brain to induce a depressive-like endophenotype is still under investigation by our lab and others.

1.2.5 Antidepressant actions give rise to the monoaminergic hypothesis of depression

The current state of the literature on the pathology of depression is mostly derived from the pharmacology of the antidepressants used to treat MDD patients. As with many scientific discoveries, the antidepressant actions of compounds were serendipitously found as a side effect in the treatment of other illnesses. Iproniazid, originally developed for the treatment of tuberculosis, showed significant antidepressant action in TB patients, while imipramine (Tofranil) was developed as a treatment for schizophrenia (in which it monumentally failed, but was successful as an antidepressant) (Alamo & Lopez-Munoz, 2009). Iproniazid, an irreversible nonselective monoamine oxidase inhibitor (MAOI), prevents the breakdown of several monoamine neurotransmitters or biogenic amines including catecholamines (dopamine, norepinephrine, adrenaline) and indolamines (serotonin, melatonin, histamines). Imipramine, a tricyclic antidepressant (TCA), acts primarily on the serotonin transporter (SERT) and has affinity for the norepinephrine transporter (NET) and some affinity the dopamine transporter (DAT). The use of these drugs that modulate monoaminergic function, while initially developed for other treatments, improved depression in as many as 50% of treated patients (though some with severe side effects). This favorable response to these monoaminergic agents gave rise to the “monoamine hypothesis of depression”, suggesting that depression is caused by deficits in monoaminergic signaling particularly the noradrenergic and serotonergic pathways (Krishnan & Nestler, 2008).

The serotonergic system modulates several behavioral and physiological responses in the body, including the modulation of mood, emotion, sleep, and appetite. In the context of

depression, it is thought that synaptic serotonin levels are depleted; thus, many antidepressant drugs pharmacologically block the reuptake transporters in presynaptic neurons. This allows for increased extracellular concentration of serotonin in the synaptic cleft leading to greater interaction with serotonin receptors on the postsynaptic cell, ultimately alleviating depressive symptoms (See Figure 1.1). Further support for this hypothesis derives from a subset of patients who developed depression after treatment with an antihypertensive drug called reserpine, whose main mode of action is the depletion of monoamines (Krishnan & Nestler, 2008). Currently, the use of TCAs and MAOIs has declined as new drugs, particularly selective serotonin reuptake inhibitors (SSRIs) like fluoxetine and paroxetine, which have fewer side effects,

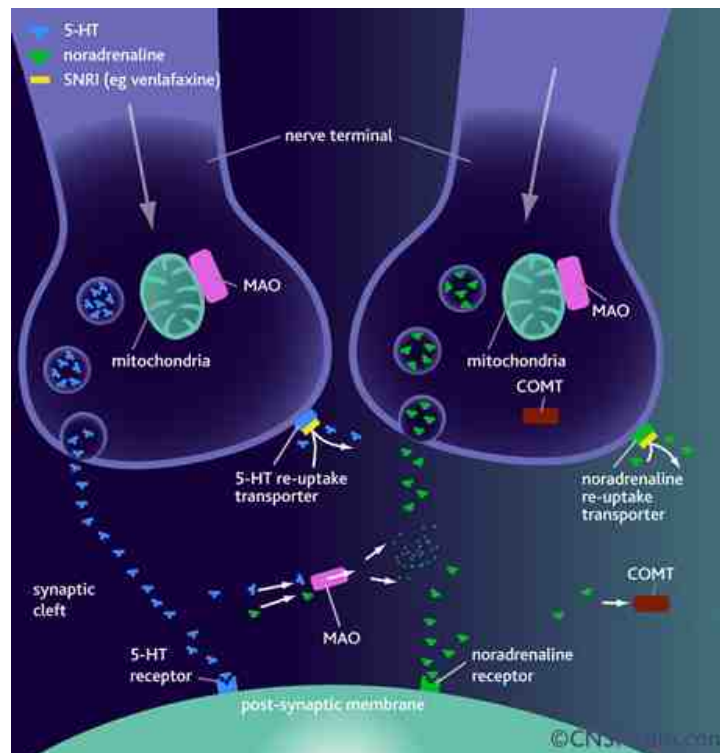


Figure 1.1. Selective serotonin reuptake inhibitors are commonly used as antidepressants. (CNS Forum)

have dominated the market for their efficacy and selectivity. While the pharmacological actions of these drugs are quite rapid, with plasma levels of serotonin increasing within thirty minutes of administration of the drug, the alleviation of depressive symptoms takes several weeks, suggesting that the pathophysiology of depression is more complicated than the monoamine hypothesis purports.

1.2.6 The neurogenic hypothesis of depression

Postmortem analysis of patients with depression has demonstrated alterations in the structure of the hippocampus, particularly neuronal atrophy and reduced granule cell numbers (with normal progenitor cell populations) in the dentate gyrus (Duman, 2002). MDD patients treated with SSRIs however do not exhibit hippocampal atrophy or reduced cell numbers compared to untreated patients (Boldrini *et al.*, 2013). Additionally, the dentate gyrus of the hippocampus preferentially responds to antidepressant treatment with a concurrent rise in the number of neural progenitor cells and subsequent number of granule neurons at the same time of alleviation of behavioral symptoms. As such, the neurogenic hypothesis of depression asserts that newborn neurons in the hippocampus are required for efficacy of antidepressants and regulation of mood (Eisch & Petrik, 2012).

Seminal studies determined that intact neurogenesis was required for the efficacy of antidepressants, including fluoxetine, for amelioration of depressive-like symptoms in rodent models (Malberg *et al.*, 2000; Malberg & Duman, 2003; Santarelli *et al.*, 2003). Additionally, chronic fluoxetine treatment increases survival of neural progenitor cells in control animals (Wang *et al.*, 2008), and attenuates certain stress-induced depressive

behaviors via neurogenic mechanisms in adult rodents (David *et al.*, 2009). The delay between the initiation of antidepressant treatment, like fluoxetine, and the alleviation of depressive-like behavior in rodents and humans is comparable to the amount of time required for maturation of a newborn neuron, approximately 4-6 weeks (Santarelli *et al.*, 2003; Eisch *et al.*, 2008). Further, adult neurogenesis (as discussed below) is extremely sensitive to modulation of the stress axis (hypothalamic-pituitary-adrenal axis, HPA), with increased stress leading to concurrent decreases in neurogenesis along with increased depressive-like behaviors in rodents.

Indeed, as with the monoamine hypothesis, there are several caveats: in some studies, ablation of newborn neurons in the hippocampus did not result in depressive-like symptoms in rodents. Some antidepressants seem to have an effect on mood independent of adult neurogenesis though this varies based on the type of behavioral assessment used to measure depressive-like symptoms (David *et al.*, 2009). However, there are a number of studies demonstrating the functional importance of adult neurogenesis in the hippocampus in which cognition is greatly diminished after ablation and subsequent risk of psychiatric disorders increases. The strongest evidence of the role neurogenesis plays in depression is that 1) treatments that ameliorate depressive-like symptoms in rodent and in humans normalize aberrant neurogenesis, 2) blocking neurogenesis inhibits the molecular and behavioral responses to antidepressants in rodent models, and 3) the one common underlying external factor associated with depression, namely stress, has deleterious effects on neurogenesis correlated with increased incidence of depression (Hsieh & Eisch, 2010).

1.2.7 The contribution of stress in the etiology of depression

The contribution of environmental factors in the etiology of depression is quite high as the genetic risk for developing depression is only 40 percent. Environmental factors can include toxin exposure (discussed later), gender, adverse childhood experiences, trauma, personality traits, and stressful life events. Many epidemiological studies suggest a correlation between stressful events and an increased risk for developing depression. Everyone experiences stress differently such that the vulnerability to depression is quite difficult to assess; however, there are molecular indications of the association between stress and depression that are common among humans and rodent models.

1.2.7.1 The hypothalamus-adrenal-pituitary axis

A stressor is considered any (real or imaginary) disturbance in homeostasis for an organism. The neuroendocrine and autonomic responses to a stressor are coordinated through hypothalamic-pituitary-adrenal (HPA) axis (See Figure 1.2). Classically, the initiation of the stress response begins in the paraventricular nucleus (PVN) of the hypothalamus (though there is some evidence of frontal cortex initiation); PVN neurons excrete adrenocorticotrophin releasing hormone (CRH) and arginine vasopressin into the pituitary gland. These hormones induce the release of adrenocorticotrophic hormones (ACTH) from the anterior pituitary, which act on the adrenal cortex to stimulate synthesis and secretion of glucocorticoids (corticosterone in rodents and cortisol in humans). Glucocorticoid production and secretion then modulates the feedback inhibition of the stress response by interaction with glucocorticoid and mineralocorticoid receptors (GR and MR) in various tissues, including almost all structures in the brain in addition to the

components of the HPA axis itself (cortex, hippocampus, and pituitary gland). Activation of GR and MR in the frontal cortex and hippocampus, in particular, provides negative feedback on the HPA axis and reduces the tone of the stress response (Tsigos & Chrousos, 2002; Joels & Baram, 2009).

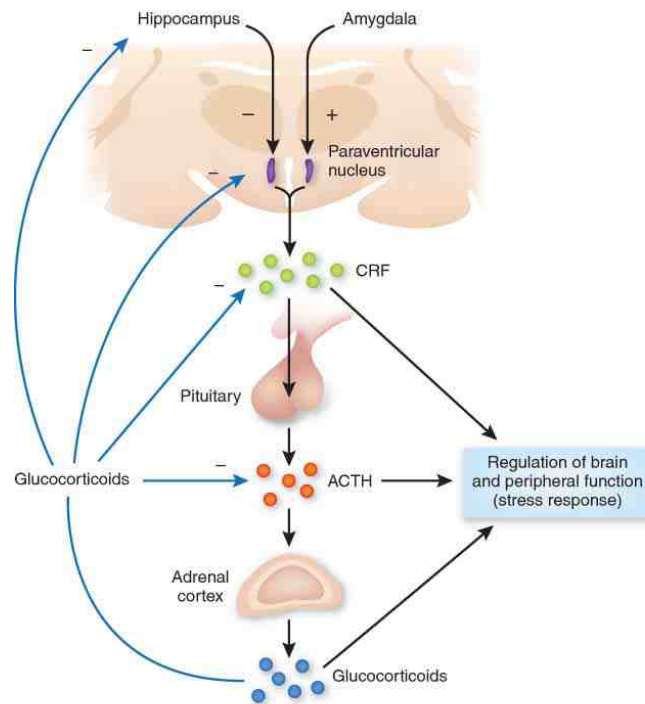


Figure 1.2. The hypothalamic-adrenal-pituitary axis regulates the stress response. (Adapted from Hyman, 2009)

A recent meta-analysis of 414 studies on the relationship between HPA dysfunction and depression suggests that depression is highly correlated with hyperactivity of the HPA axis, although the variability of this association is dependent on subtypes of depression (Stetler & Miller, 2011). The authors determined that in the studies assessed, a majority of depressed patients had elevated cortisol and ACTH compared to non-depressed controls; however, they did not find a correlation between altered CRH, although many studies have reported reduced levels of CRH in depressed patients. It should be noted that

among the studies assessed, different factors might affect the methodologies in measuring these hormones including tissue type, time of day, age, and subtype of depression. Yet, the prevailing theory is that hyperactivity of the HPA axis is due (in part) to reduced feedback by endogenous glucocorticoids: depressed patients given dexamethasone (DEX, a synthetic glucocorticoid) do not exhibit suppression of HPA activity, while healthy patients respond readily to even small doses of DEX, indicative of appropriate feedback inhibition as measured by reduced cortisol levels up to 24 hours later (Pariante & Lightman, 2008). Additionally, reduced GR function in various tissues, including the brain, has been measured in patients with successful antidepressant therapies typically associated with increased GR expression. There is sufficient evidence in the literature that excessive stress exposure, mediated through increased levels of cortisol and eventual HPA axis dysfunction, damages brain structures and their function, leading to impaired plasticity and a subsequent endophenotype of depression (Stepanichev *et al.*, 2014). Using the arsenic exposure paradigm described above, the Allan lab has found many perturbations in the stress axis that provide evidence suggesting that arsenic induces dysregulation of the HPA axis (Goggin *et al.*, 2012). Yet, how these perturbations eventually lead to depression is unknown and currently under investigation.

1.2.7.2 Stress inhibits neurogenesis

Dysregulation of the HPA axis in depressed patients has been reported in many studies, though the particular neurobiological mechanism likely varies as both hyper- and hypo-activity of the HPA have been reported (Anacker *et al.*, 2011a). One mechanism by which stress leads to increased depression is via perturbation of adult neurogenesis; that

is, the cascade of reduced negative feedback inhibition in response to stress likely contributes the neurogenic hypothesis of depression. Neurogenesis is highly sensitive to many factors (as discussed later), particularly stress. Several animal models of depression induced by acute or chronic stressors concurrently reduce adult neurogenesis (Cameron & Gould, 1994; Malberg & Duman, 2003; Yun *et al.*, 2010). Indeed, even in non-depressed animals, social isolation, chronic social defeat stress, or even sleep deprivation result in deficits in neurogenesis. One form of acute stress that will be used in these dissertation studies is brief exposure to a predator odor (trimethylthiazoline, TMT, a component of fox urine), which activates the HPA axis, produces anxiety-like behavior, and after chronic exposure, decreases many components of neurogenesis. There have been a few conflicting reports as to the effect of stress on neurogenesis, however, these are likely due to species variation and non-standardized methods (Danzer, 2012). The mechanism by which stress reduces neurogenesis is still under investigation; however, one common theory is that reduced granule cell number results in loss of GR and MR leading to impaired modulation of HPA axis feedback, particularly in response to the DEX test (Snyder *et al.*, 2011). The next section will discuss neurogenesis in greater detail as a candidate mechanism of toxin-induced depression.

1.3 Adult neurogenesis: a candidate mechanism of action

Adult neurogenesis can be broadly defined as the birth of new neurons from existing neural stem cells that functionally incorporate into the surrounding neural circuitry. This mechanism of neuronal birth was previously thought to occur only during the embryonic and perinatal stages of life in the brain, as the brain was perceived to be a stable, rigid

structure as perpetuated by Ramon y Cajal. Research from Altman and Das in 1965 challenged the prevailing theory of the rigidity of the brain, but it was not until the 1990's, due to the advent of a lineage tracer bromodeoxyuridine (BrdU), when several groups simultaneously published observations of newborn neurons in the brains of songbirds and adult mammals including humans (Eriksson *et al.*, 1998; Gould *et al.*, 1998; Cameron & McKay, 2001). Since then, an explosion in research focusing on the mechanisms and functions of adult neurogenesis has been underway.

1.3.1 Components of adult neurogenesis

Adult neurogenesis occurs in two neurogenic niches in the brain: the subventricular zone of the lateral ventricles where neuroblasts migrate along the rostral migratory stream to the olfactory bulb and differentiate into interneurons, and the subgranular zone of the dentate gyrus in the hippocampus, in which new dentate granule cells are produced (See Figure 1.3) For the purposes of this introduction, the discussion will focus on adult hippocampal neurogenesis (AHN).

The process of adult neurogenesis is multifaceted and can be divided into several components. Each neurogenic niche contains a pool of neural stem cells (NSC; type-1 progenitor cells) with radial-glia like properties that can either symmetrically divide to maintain the neurogenic niche or asymmetrically divide, undergo fate determination, and give rise to neural progenitor cells (NPCs). These NPCs, also referred to as transiently amplifying cells (TAPs or type-2 progenitor cells), migrate into the granule cell layer

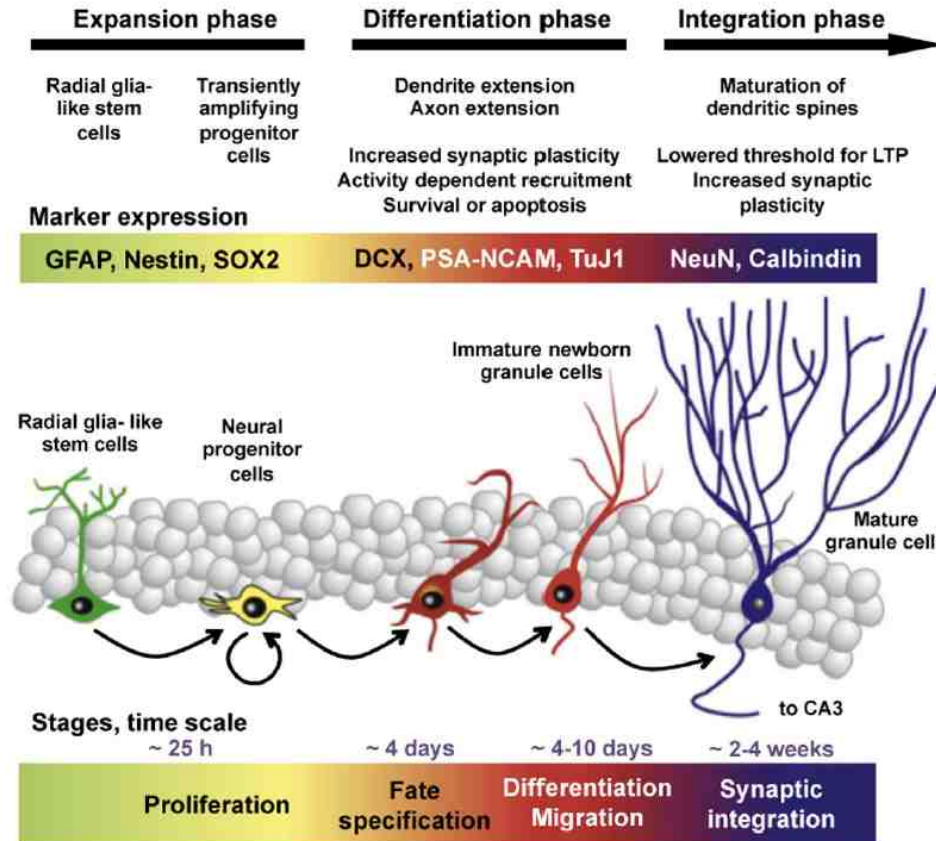


Figure 1.3. Adult hippocampal neurogenesis is comprised of many phases. (Adapted from Schouten et al. 2012)

(GCL) and undergo either division themselves to form more progenitors or several stages of maturation into excitatory glutamatergic granule neurons. Neuroblasts derived from NPCs exhibit the microtubule associated protein doublecortin (DCX) along with PSA-NCAM; eventually these newborn neurons elongate their axons and dendritic branches to establish synaptic connections with mature granule neurons for integration into the established circuitry. This integration time is approximately four to eight weeks, depending on the level of “maturation” assessed, and the histological markers NeuN, calretinin, and calbindin can be used to assess the existence of a mature newborn neurons (Ming & Song, 2005). Post-natal or adult neurogenesis reflects embryonic neurogenesis, except that these processes occur in a mature microenvironment and integration occurs

into pre-existing circuitry (Mateus-Pinheiro *et al.*, 2011). As such, each component of AHN is heavily regulated and depends on a complex choreography of gene expression. The various processes of AHN give rise to the heterogeneous cell types that comprise the dentate gyrus, and several markers can be used to identify each of these stages. During the mitotic phase of the cell cycle, NSC and NPCs can incorporate BrdU into their DNA during the replication process (S-phase); this mark is then persistently expressed in all daughter cells (though it fades as the number of mitotic cycles increases) and can be detected using immunohistochemistry. Additionally, mitotic activity can be followed through endogenous Ki67, which is expressed during all phases of mitosis. Combinations of markers and morphological assessment of labeled cells are used in concert to identify cell types in the dentate gyrus to determine three overall processes: proliferation of progenitors, differentiation of those progenitors into immature and mature neurons, and the survival of these cells over time.

Figure 1.3 depicts some histological markers that can be used to identify the various cell types within the dentate gyrus. For the studies described in this dissertation, identification of proliferation can be determined with BrdU, Ki67, PSA-NCAM, while immature and mature neurons are identifying using DCX and NeuN, respectively. Survival can be assessed by identifying the BrdU label many weeks after its incorporation in these cells. It should be noted that progenitor cells can also give rise to glia in a process known as gliogenesis; thus, fate specification is also a crucial step in the formation of these cells, as several external factors have been shown to alter the ratio of formation of glia and

neurons. For our purposes here, we will focus on mechanisms regulating the formation of neurons and the purpose of those neurons in the context of mental health.

1.3.2 The function of newborn neurons in the context of depression

The dentate gyrus is the first destination in the classic trisynaptic circuitry of the hippocampus. Glutamatergic granule cells of the dentate function to modulate the excitatory and inhibitory input from the entorhinal cortex, called the perforant pathway (Danzer, 2012). Mossy fibers, or the axons from the dentate granule cells, synapse on the CA3 pyramidal neurons, which provide inputs for the CA1 pyramidal neurons through their axons, termed the Schaffer collaterals. CA1 neurons then project back into the entorhinal cortex. Recent evidence suggests that the integration of new neurons in the dentate gyrus impacts the overall trisynaptic hippocampal circuit. How exactly these new neurons influence the circuitry, and ultimately, regulate emotion, is not well understood (Deng *et al.*, 2010); however, recent reports have provided evidence of the effect of new neurons on learning and memory.

Adult neurogenesis is a form of specialized synaptic plasticity. Newborn neurons are thought to play distinct roles in certain forms of learning and memory and in mood regulation (Shors *et al.*, 2002). While many studies have used different ablation strategies, rodent models, and behavioral tasks, the consensus in the literature seems to be that newborn neurons, particularly those aged one to three weeks, are important for several different types of hippocampal-dependent tasks including spatial learning and memory and pattern separation (Shors *et al.*, 2001; Snyder *et al.*, 2005; Cameron & Glover, 2014).

Pattern separation, shown to be important in both computational and experimental models (Deng *et al.*, 2010; Aasebo *et al.*, 2011; Sahay *et al.*, 2011), is of particular interest as newborn neurons likely aid in episodic memory. In many of the daily experiences we have, similar episodes or events need to be distinctive from one another in a spatiotemporal manner in order to remember these events in the appropriate context: this representation seems to be carried out by newborn neurons (Nakashiba *et al.*, 2012). Pattern separation may be critical in discerning between past and present experiences, as memory deficits and incorrect distinctions among events are common for patients with depression; indeed, a recent study showed a correlation between reduced ability to accurately complete pattern separation tasks and the severity of depression (Shelton & Kirwan, 2013). Further, depression is likely due, in part, to inaccurate processing of stressful events: a recent study by Cameron and Snyder demonstrated that newborn neurons were required to mediate the stress response and that reduced adult neurogenesis leads to concurrent inappropriate responses to dexamethasone and increased behavioral despair (Snyder *et al.*, 2011). The authors suggest that the negative control the hippocampus imparts on the HPA axis is via this particular subset of newborn neurons; this is fitting in that stress has been shown to negatively impact AHN and is associated with an increased risk of developing depression. While the particular role that newborn neurons play in the context of depression is still under investigation, one prevailing theory is that overgeneralization observed in several psychiatric disorders, including anxiety and depression, may derive from reduced AHN (Kheirbek *et al.*, 2012). Since the hippocampus integrates information from several brain regions, some of which are part of the limbic system as emotion is tightly associated with memory, newborn neurons likely

allow for lifelong adaptation to new experiences and remembering those experiences accurately (Kempermann *et al.*, 2008).

1.3.3 Epigenetic regulation of neurogenesis

The complex processes involved in neurogenesis are orchestrated by interactions among intrinsic and extrinsic elements, from internal transcription factors and epigenetic modifications to growth factors and neurotrophins from the surrounding milieu. The intracellular epigenetic program in particular is responsive to extrinsic physiological factors and environmental stimuli that allow it to “align” the progression of neurogenesis with the external demands of cognition (Mateus-Pinheiro *et al.*, 2011). Yet, what is known about the epigenetic regulation of neurogenesis is limited to select studies focusing on different epigenetic factors on particular components of neurogenesis; an overall picture of this regulation has yet to be formulated.

The current state of the literature suggests that the first phase of AHN, adult neural stem cell proliferation, is epigenetically regulated by the competing interactions of the trithorax (TrxG) and polycomb (PcG) chromatin modifying enzymes. The polycomb repressive complexes 1 and 2 (PRC1, PRC2) contain sets of proteins that are recruited to chromatin targets to catalyze the ubiquitination of histone 2A (H2AUb1) or the trimethylation of lysine 27 on histone 3 (H3K27me3) for suppression of gene expression, respectively. How the recruitment to particular targets occurs is not known, and the role that each protein plays has yet to be elucidated. However, one protein in PRC1 has been determined to be essential for neural stem cell maintenance: the zinc finger protein, B-

cell specific Moloney murine leukemia virus integration site 1, or Bmi1, which controls self-renewal of many different stem cell types in the body including NSCs (Lobo *et al.*, 2007). Deficits in Bmi1 result in loss of proliferation and renewal capacity via reduced repression (lack of H3K27me3 or H2AUb1) of genes coding for the cell cycle inhibitors p16, p19, and p21 (Molofsky *et al.*, 2003). Overexpression of Bmi1 leads to increased proliferation, at least in one study (Yadirgi *et al.*, 2011). Bmi1 requires EZH2, a protein in the PRC2, for repression of p16 and p19, suggesting collaboration between PRC1 and PRC2. EZH2 is expressed in embryonic neural stem cells and expression is lost as these cells differentiate into neurons; however, its role in adult neurogenesis has not been determined to date. Loss of either EZH2 or Bmi1 can lead to increased neuronal production (i.e. differentiation and maturation) but at the expense of a depleted progenitor pool. Evidence of the function of the PRC1 and PRC2 complexes and their respective proteins suggest that PcG proteins function to repress the expression of pro-differentiation genes to maintain the niche of neural stem cells (Ma *et al.*, 2010; Jobe *et al.*, 2012).

Conversely, TrxG complexes contain proteins associated with up-regulation of gene expression, including the SET domain factor Mll1, which contains H3K4 methyltransferase activity. Mll1 is required for neuronal differentiation in the subventricular zone of the lateral ventricles (the other location of adult neurogenesis in the brain) with increased H3K4me3 levels associated with activation of Dlx2 (Lim *et al.*, 2009). While Mll1 has been shown to bind to the Dlx2 promoter, MLL1-deficient mice do not have reduced H3K4me3 methylation of Dlx2. However, these mice do have

abnormally high bivalent chromatin domains containing both H3K4me3 and H3K27me3 modifications for poised transcription, particularly on *Dlx2*. This suggests that the TrxG complexes may recruit a demethylase for H3K27me3, though this protein has yet to be identified (Ma *et al.*, 2010). Demethylase proteins (as opposed to simply histone methyltransferases), likely play a role in adult neurogenesis, as *Jmjd3*, a histone demethylase for H3K27me3, has been shown to be required for fate specification and differentiation during embryonic development (Jepsen *et al.*, 2007). While few studies have been performed, there is evidence to suggest that histone methylation plays an important role in AHN, not only in the maintenance of the progenitor pool via repression mediated by PcG proteins, but also in neuronal maturation mediated by the TrxG proteins that allow for the transition from repressed to activate transcriptional states in postnatal neural progenitors.

Reports have provided evidence suggesting that histone acetylation and deacetylation are also required for various components of adult neurogenesis as rapid changes in acetylation can allow for better control of gene expression. But this regulation is still complex: while self-renewal of NSCs and NPCs have been shown to involve the histone acetyltransferase (HAT) *Querkopf* (Merson *et al.*, 2006), *Querkopf*-deficient mice have reduced neural differentiation, suggesting that this protein is required for both proliferation and differentiation in AHN. Indeed, increasing overall acetylation of histones via histone deacetylase inhibitors (HDACi) has been demonstrated to induce differentiation of NPCs in the hippocampus via up-regulation of transcription factors such as *NeuroD* and *Tlx1* (Ma *et al.*, 2010). However, deficits in HDACs can impair

differentiation as well, as knockout of HDAC2 reduces maturation NPCs in the adult brain (Jawerka *et al.*, 2010). As such, the precise roles for each histone acetyltransferase (HAT) and HDAC will no doubt need to be determined for AHN. HDAC2 in particular, while paramount for maturation of neurons during adult neurogenesis, inhibits pro-neural genes in postmitotic neurons when involved with the REST complex (see below). Studies using sodium butyrate (NaB) and suberoylanilide hydroxamic acid (SAHA), both HDACis, demonstrate that increasing acetylation reduces proliferation of NPCs while simultaneously altering the expression profiles of several transcription factors. Both HDACis decrease the expression of Sox2, Hes1, and Hes5, the transcription factors that maintain the stem cell niche, and increase expression of Neurod1 and Neurog1, pro-neural transcription factors important for differentiation (Zhou *et al.*, 2011a). Overall, it can be stated that HDAC activity seems to be important for maintenance of the proliferative pool of neural progenitors and that the use of HDACis can increase differentiation; however, more studies are required to elucidate the particular mechanisms for each group of HATs and HDACs. Additionally, it has been hypothesized that the rapid turnover of acetylation of histones, as compared with slower rates of demethylation, allows for more precise control of the timing of gene expression for cell cycle exit, fate specification, and differentiation programs in adult neurogenesis (Ma *et al.*, 2010).

There are several other epigenetic factors involved in adult neurogenesis, but our understanding of their roles is only as distinct subsets of proteins rather than a coherent picture of the overall regulation of neurogenesis. For example, methyl CpG binding protein 1, Mdb1 controls cell cycle exit via binding to fibroblast growth factor 1 (Fgf1),

to initiate differentiation (Li *et al.*, 2008). Mbd1 and methyl CpG binding protein 2 (MeCP2) associate with methylated DNA, known for repression of transcription, and are both highly expressed in the brain. Both of these proteins regulate NSC and neurogenesis via regulation of other epigenetic factors: for example, MeCP2 becomes phosphorylated during neuronal activity which affects its binding to brain-derived neurotrophic factor (BDNF), a neurotrophin that is critical for synaptic plasticity and maturation of neurons (Na & Monteggia, 2011). Removal of MeCP2 likely allows for recruitment of chromatin modifiers like HATs (and HDACs) to allow for increased transcription of BDNF for increasing AHN. Yet, MeCP2 allows for the transition from immature to mature neurons where it is highly expressed, and deficiency in this protein leads to reduced numbers of mature neurons (Smrt *et al.*, 2007). Additionally, Gadd45b, a DNA excision repair protein that also functions as an immediate early gene in the brain, initiates the rapid demethylation of several genes (including BDNF) involved in AHN after neuronal activity, thus allowing for the niche to respond to external stimuli (Ma *et al.*, 2009). To complicate matters, several microRNAs and long interspersed nuclear elements (LINE) have been shown to be important for regulation of AHN. For example, miR-137 represses the expression of EZH2 (in PRC2) leading to reduced H3K27me3 levels, allowing for the TrxG proteins to activate pro-neural programs (Smrt *et al.*, 2010); conversely, miR-184 is a target of Mbd1 for inhibition of maturation via Numb-like proteins, while miR-124 represses Sox transcription factors that are expressed for maintenance and renewal of NSC (Cheng *et al.*, 2009). Several new lines of query have suggested that the crosstalk among histone modifiers, DNA methylation, and microRNAs create the rich and intricate regulation of adult neurogenesis (Jobe *et al.*, 2012).

While many chromatin-modifying proteins have been separately identified to play a role in cell-fate specification and maturation of neurons, a collective underlying mechanism that coordinates the epigenetic regulation of gene expression in maturing neurons may be via the neuron restrictive silencing factor NRSF, also known as REST. This zinc finger protein binds to a conserved 21-23 bp motif (neuron restrictive silencing element, RE1) typically found in neuronal gene promoter regions. This sequence has been found in genes encoding ion channels, neurotransmitter receptors, and neurotrophins (Hsieh & Gage, 2005). REST complexes with mSin3A/B, Co-REST, and HDAC1/HDAC2. Co-REST recruits several chromatin-modifying enzymes for silencing genes including MeCP2, heterochromatin protein 1 (HP1), suppressor of variegation 39h1 (Suv39h1), and the histone deacetylases 1 and 2 (HDAC1/2) (Ballas *et al.*, 2005). Several genes important for proper AHN contain RE1 elements to which REST binds, including corticotrophin releasing factor (CRH), BDNF, and the serotonin 1A receptor (5HT_{A1}); these genes also play a role in the pathophysiology of depression and action of antidepressants (Otsuki *et al.*, 2010). However, REST may not only play a repressive role: double stranded RNA that matches the NRSE sequence that is co-expressed with NRSF in neural progenitor cells of the hippocampus has been shown to convert NRSF from a repressor to an activator by removal from neuron specific genes for the induction of neurogenesis (Kuwabara *et al.*, 2005). Additionally, NRSF may play a dual role for neural development: NRSF is released from neural genes as progenitors differentiate into cortical neurons and NRSE-containing genes are activated during neurogenesis (Ballas *et al.*, 2005). REST recruits its corepressors to NSC chromatin to reduce the expression of pro-neural genes for appropriately timed differentiation, as REST deficiency leads to

increased AHN at the expense of the renewal of NSCs and if left unchecked, reduces the progenitor pool ultimately leading to deficits in AHN (Gao *et al.*, 2011). REST also interacts with other transcription factors and epigenetic modifiers including cAMP response element binding protein, CREB, and miR-124 to control the switching from self-renewal to differentiation depending on the requirements of the niche, yet its role is still under investigation as REST seems to be required for proper AHN (Mateus-Pinheiro *et al.*, 2011).

Overall, epigenetic programs in the dentate gyrus allow for the precise orchestration of transitions from one state of neurogenesis to the next. From proliferating progenitors to maturing neurons, these modifications allow the this special niche of the hippocampus to respond to external stimuli, such as when new information needs to be processed for pattern recognition and ultimately retrieved for spatial memory. Based on this dynamic response to external stimuli mediated through epigenetic factors, adult hippocampal neurogenesis seems to be particularly sensitive and highly responsive, thus making it a good candidate for the adverse actions of arsenic exposure. The next section explores these external stimuli that impact AHN.

1.3.4 The effects of environmental factors on neurogenesis

As we have previously discussed, adult neurogenesis is important for appropriate responses to stress, certain forms of learning and memory, and the efficacy of antidepressants. AHN is comprised of several processes including proliferation of NSCs, differentiation of NPCs, and survival and integration of newborn neurons into the

hippocampal circuitry. These processes are tightly controlled by epigenetic modifications for which current research is still elucidating the larger picture. Importantly, it is via these modifications that AHN can respond to the environment and to experiences to appropriately provide newborn neurons for hippocampal function.

There are many positive external stimuli that increase AHN. Physical exercise (running) stimulates proliferation of neural progenitor cells, and the mechanism for this enhanced component of AHN may be mediated through VEGF secretion, BDNF, and factors that promote vascularization of the brain (van Praag *et al.*, 1999b; During & Cao, 2006; Boehme *et al.*, 2011). Experience in novel and enriching environments promotes maturation and survival of newborn neurons (Kempermann *et al.*, 1997). Including a running wheel in an enriched environment synergistically enhances all aspects of AHN with a profound increase in the capacity for learning and memory (Fabel *et al.*, 2009; Bekinschtein *et al.*, 2011). Sexual experience increases cell proliferation and differentiation depending on timing and frequency, and even episodes of learning, where the task does not induce an overt stress response, can increase AHN. However, this potentiation of AHN via learning is dependent on the timing of maturation: newborn neurons primed with an activity to “learn from” will survive and integrate into the hippocampal circuitry (Dobrossy *et al.*, 2003). Of the average 5,000 neurons that are born each day in the adult mouse, almost half, if not most, die before differentiation and integration (Cameron & McKay, 2001). It is currently unknown why such a high rate of apoptosis occurs in the dentate gyrus; however, based on evidence suggesting that the first week after birth is critical, it is thought that learning or some “effortful” experience

will mediate the maturation and incorporation of these new cells into the circuit (Shors *et al.*, 2012). Indeed, newborn neurons that do survive and continue to develop are very stable and may permanently replace the developmental granule cells, though this idea is still under some debate (Dayer *et al.*, 2003). This “use it or lose it” phenomenon suggests that continued learning enhances AHN thereby allowing the brain to be primed for learning (Shors *et al.*, 2012). Learning itself can activate the HPA axis, and interestingly, most of the external factors that positively regulate AHN, including running, enrichment, and sexual experience, all require activation of the HPA axis for a stress response. Even the action of some antidepressants, particularly sertraline, an SSRI more commonly known as Zoloft, increases differentiation of neural progenitors via increasing expression of the glucocorticoid receptor (Anacker *et al.*, 2013). This finding is contrary to other reports suggesting that stress reduces AHN; however, it should be noted that a certain amount of stress is required for experience-dependent learning, and as such, the effect of stress on AHN is complex (Glasper *et al.*, 2012). One prevailing theory is that rewarding experiences (including learning) are likely to elicit a positive outcome on AHN, possibly mediated through reduced HPA tone via hippocampal GR or increased dopamine, oxytocin, and growth factors (Schoenfeld & Gould, 2012).

In general, stress is considered to be a negative regulator of AHN, with both acute and chronic stress impairing both proliferation and differentiation of NPCs. Different types of stressful events have been assessed for their impact on AHN and subsequent learning and memory; these include predator odor, intruder or dominant/subordination stress, electric shock, and physical restraint. The timing, duration, and type of stress play a role in how

severe these effects can be: acute stressors induce deficits in proliferation while chronic stressors reduce maturation and the number of differentiated NPCs (Schoenfeld & Gould, 2012). Even paradigms of stressful learning, including prolonged maze training, can impair proliferation, regardless of the learning outcome (Aztiria *et al.*, 2007). Other factors that reduce AHN include aging and neurodegeneration (Li *et al.*, 2009b; Sun *et al.*, 2009a), insulin deficits or resistance (Stranahan *et al.*, 2008), inflammation (Carpentier & Palmer, 2009), which reduces proliferation and fate commitment associated with increasingly reactive microglia (Monje *et al.*, 2003), and some forms of ischemia and seizure activity, though these particular events lead to increased proliferation at first followed by deficits in maturation, aberrant migration, and reduced synaptic connectivity (Jessberger *et al.*, 2007a; Jessberger *et al.*, 2007b; Ming & Song, 2011). Of particular interest is the negative effect of toxins on neurogenesis. There have been many studies demonstrating that alcohol intake impairs AHN, although these studies have conflicting results based on development, dose, and the extent and timing of exposure (Nixon, 2006; Valenzuela *et al.*, 2012; Gil-Mohapel *et al.*, 2014). In general, drugs of abuse, including nicotine, opiates, and stimulants, typically elicit reduced proliferation of NPCs (Hsieh & Eisch, 2010). Interestingly, the reduction in AHN induced by cocaine for example, may create greater vulnerability to the development of addiction itself (Yamaguchi *et al.*, 2004; Noonan *et al.*, 2010; Deschaux *et al.*, 2014). Exposure to heavy metals, particularly lead and mercury, reduces proliferation and differentiation of NPCs especially when exposure occurs during development (Falluel-Morel *et al.*, 2007; Rai *et al.*, 2010). Recent reports have demonstrated that even common contaminants such as bis-phenol A (BPA) reduce AHN and can lead to learning and memory deficits (Kim *et al.*, 2011b; Jang *et al.*,

2012). Chapter 2 will provide more detail on the effects of toxin exposure on hippocampal learning, memory, and neurogenesis, particularly in the context of arsenic exposure.

Environmental stimulus can have positive and negative effects on AHN, suggesting that the processes that comprise AHN are highly adaptive and yet sensitive. These processes provide a platform for the brain to respond to environmental cues likely mediated through epigenetic processes in order to maintain a responsive hippocampal circuitry for processing new information. To better understand how these epigenetic processes work, a brief introduction to epigenetics will be provided.

1.4 Epigenetics: the basics

In the 1940's, Conrad Waddington mused on the molecular underpinnings of cellular differentiation: how exactly do organisms develop from single cells into complex structures with multiple cellular phenotypes? To answer this conundrum, he proposed the notion of an “epigenetic landscape” in which fate determination, or a particular path in the landscape, could be “chosen” by a cell based on networks of gene interactions. This idea stemmed from his (and others) observations of what we refer to as differential gene expression, in which totipotent stem cells are able to differentiate into the various cell types of the body due to particular patterns of gene expression. Waddington coined the term epigenetics, inspired by Aristotle's “epigenesis”, to refer to the branch of biology that would study the “causal interactions between genes and their products which bring the phenotype into being” (Waddington, 1968). Based on our current understanding,

epigenetics, roughly translated as “above the genome”, may now be broadly defined as heritable, reversible changes in gene expression that are not the result of changes in underlying DNA sequences. For the purposes of neuroscience, epigenetics has been conceived as the bridge between nature and nurture: environmental factors including stress, toxins, and even experiences such as trauma or learning, influence gene expression patterns – which depending on cell type – may be heritable.

1.4.1 DNA methylation

There are several components of epigenetic gene regulation including DNA methylation (DNAm), histone posttranslational modifications (HPTM), and noncoding RNAs. DNA methylation is the most widely studied component of epigenetics. It occurs almost exclusively at the 5' carbon of pyrimidine ring of cytosine residues to generate 5-methyl cytosine at symmetric CpG dinucleotide sites in the genome, although there have been some recent reports suggesting that other nucleotides may also contain methylation (Rudenko & Tsai, 2014). Three highly conserved enzymes, the DNA methyltransferases (DNMT1, DNMT3A, DNMT3B), impart single methyl groups (CH₃) from the universal methyl donor S-adenosyl methionine (SAM). Mammals have less CpG sites than other eukaryotes likely due to progressive elimination from the genome as deamination of 5-methylcytosines to thymine residues (Antequera & Bird, 1993). Due to this gradual evolutionary loss, CpG dinucleotides only occur at 5-10% of the expected frequency (approximately 28 million CpGs in the human genome) and rough estimates suggest that only 60-80% of these single CpG sites are methylated (Smith & Meissner, 2013). These methylated CpG sites can occur in heterochromatin, or regions of the DNA not actively

transcribed. CpG “islands” are actually CG dense regions ranging from 1-4 kb and have the expected frequency of CpG content (G+C content of greater than 60% and ratio of CpG to GpC of at least 0.6) (Baylin *et al.*, 1998). These islands are typically present in 5’ regulatory regions of genes including transcriptional start sites and are conspicuously not methylated, with only 10% containing DNA methylation. Genes that contain CpG islands include most constitutively expressed genes (typically considered housekeeping genes) and genes important for developmental regulation. CpG dinucleotides within promoters without CpG islands are usually methylated based on the tissue specificity and are indicative of the transcriptional status of the gene, which has become the simplistic rule of this type of epigenetic mark. In essence, methylation of a CpG is indicative of repression of gene expression, while demethylation of a CpG can be indicative of active transcription. However, the bulk of DNA methylation is considered static across tissues throughout the lifespan of an organism, though the exceptions to these criteria occur in germline DNA and during early embryonic development when massive demethylation of the parental genomes occurs (Smith & Meissner, 2013). Indeed, since the most DNAm occurs in CpGs localized to heterochromatin and CpG islands are typically not methylated, the presence of a CpG dinucleotide within a promoter of gene of interest can be quite useful for studying gene expression. DNAm is critical to development of an organism, and in the context of developmental toxins, is the most widely studied epigenetic mechanism for toxicity. Arsenic exposure disrupts the balance of methyl donor groups (i.e., SAM), thereby resulting in aberrant DNA methylation. Discussion of arsenic’s effect on DNAm will be further discussed in Chapter 2. It should be noted a new line of query into epigenetic processes has emerged in recent years. The process of

active demethylation of DNA has not been elucidated to date, although demethylation of DNA likely occurs through TET-mediated mechanism including hydroxymethylation, which allows for activation of gene expression associated with behavior (Li *et al.*, 2014).

1.4.2 Histone modifications

Histone posttranslational modifications are another epigenetic mechanism that impact gene expression. Negatively charged DNA is counterbalanced by positively charged histone proteins that bind and package the DNA to form chromatin; this structure regulates the expression of DNA by allowing or inhibiting access to the genes (Bannister & Kouzarides, 2011). The core of the chromatin consists of the nucleosome, which is comprised of 147 bp of DNA wrapped ~1.65 turns around a histone octamer. The octamer is made of dimers of H2A and H2B and heterodimers of H3 and H4 with H1 acting as a linker. Variants of histone proteins, such as H2A.Z, are associated with different parts of the DNA like active promoter regions (Day & Sweatt, 2011), and recently, have been shown to be important for learning and memory (Zovkic *et al.*, 2014). The N-terminal domains of histone proteins or the “tails”, rich in basic amino acids, protrude from the nucleosome and can interact with adjacent nucleosomes or the DNA itself. These tails contain covalent modifications: these include acetylation, methylation, ubiquitination, and SUMOylation of lysine residues; methylation of arginine residues; phosphorylation of serine and threonine residues; and ADP-ribosylation of glutamate residues. These modifications or “marks” aid the diverse functions of nucleosomes including regulation of chromatin structure, recruitment of remodeling enzymes, influencing of transcription, and DNA repair, replication, and recombination. Histone

modifications are particularly dynamic and easily influenced by external factors, which for the brain, includes everything from emotional experiences to increased circulating neurotrophins. Yet, these HPTMs can be consistent in some cell types; for example, HPTM, along with DNA methylation, help maintain the “stemness” of neural stem cells (Smith & Meissner, 2013). Particular combinations of histone marks and chromatin remodeling enzymes confer structural variation to the chromatin: heterochromatin contains histone marks that recruit repressor proteins to inhibit transcription, while euchromatin contains “active” marks that are permissive for transcription. Remodeling of the chromatin allows for groups of nucleosomes to become more or less accessible to modify the availability of promoters to transcription factors and associated machinery, thereby regulating gene expression.

1.4.3 Chromatin modifying proteins

Remodeling of chromatin for gene expression is widespread in the brain and allows for dynamic regulation in both the developing and adult nervous system. There are several classes of enzymes that are responsible this remodeling, which is collectively considered the addition and removal of epigenetic marks and the recruitment of remodeling factors to alter gene expression. In general, methylation of a residue results in a neutral charge on the histone, and depending on the location of the lysine or arginine residue, can be associated with active or inactive transcription. Acetylation and phosphorylation of basic amino acids both result in a negative charge to the histone, which decreases the packing efficiency of nucleosome; thus these modifications are typically associated with active

transcription. For our purposes, we will limit the discussion of chromatin remodeling factors to those responsible for the acetylation or methylation of histone proteins.

Acetylation of N-terminal tails of histone proteins is conferred by histone acetyltransferases (HAT), which provide an acetyl group from acetyl-CoA for lysine residues. Acetylation relaxes the nucleosome configuration by reducing the positive charge of lysine to reduce the interaction between the histone tail and the negatively charged DNA phosphate backbone. Currently, there are two classes of HATS (type A and B), each of which is further categorized into families based on sequence and functional homology. Type A HATs include proteins belonging to the GNAT, MYST, and CBP/p300 families. The p300 HAT and its homolog CREB binding protein (CBP) both induce activation of gene expression of several transcription factors that are paramount for learning and memory (Rudenko & Tsai, 2014). More is known about the action of histone deacetylase proteins (HDAC) in the brain: HDACs catalyze the inversion of the acetylation reaction to a deacetylated state, which packages the DNA into more condensed chromatin. There are 11 HDAC proteins (so far) separated in three classes: Class 1 HDACs (1, 2, 3, 8) and class 2 HDACs (4, 5, 6, 9, 10), both of which are cell type restricted. A third class of HDACs is the NAD-dependent sirtuin deacetylase proteins (SIRT). SIRT1 has been shown to play a protective role in neurodegeneration models of AD, such that activation of SIRT1 reduces synapse loss and improves learning and memory (Graff *et al.*, 2013). Additionally, inhibition of HDAC activity via HDAC inhibitors like valproate or sodium butyrate has been shown to increase learning and memory (Graff & Tsai, 2013); however, it should be noted that the presence of HDACs

in the brain is required for development. Research on the particular function of each individual HDAC is underway, but the overall consensus is that increased acetylation likely allows for more rapid gene expression of proteins necessary for learning (Rudenko & Tsai, 2014).

Histone methylation is the best characterized histone modification to date (Kouzarides, 2007). While acetylation has been exclusively associated with transcriptional activation of gene expression, the location and number of methyl groups on residues of histone proteins ultimately determines their effect on gene expression. It is unclear how methylation confers activation or repression, as methyl groups do not alter the charges of histone proteins. However, it is thought that the methyl groups provide binding sites for other proteins that may alter the nucleosome configuration (Arrowsmith *et al.*, 2012); as such, these “reader” proteins that respond to methylation of histones are likely responsible for gene expression changes rather than the methyl group itself. Methylation typically occurs on lysine residues, which can be mono-, di-, or tri-methylated and on arginine residues, which can be mono-, symmetrically or asymmetrically di-methylated. Arginine methylation on H3 and H4 occurs via the protein arginine methyltransferase (PRMT) family in coordination with cofactor associated arginine methyltransferase (CARM1). PRMT1 and CARM1 jointly catalyze methylation of mono and asymmetric dimethylation of arginine for gene activation, while PRMT2 induces mono and symmetric dimethylation for gene repression (Wang & Zhu, 2008). Both CARM1 and PRMT1 have multiple targets, but the factors and associated outcomes of arginine methylation are still relatively unexplored. Other histone methyltransferases (HMT),

unlike HATs, are more specific and usually have one or two substrate specificities. Additionally, at least for lysine residues, there are several HMTs that can methylate the same residue with the same number of modifications. HMT proteins that contain SET domain (suppressor of variegation enhancer of zeste trithorax) are responsible for catalyzing methylation of lysine residues. Only one lysine HMT, DOT1, which methylates H3 lysine 79, does not contain a SET domain; K79 lies within the histone globular domain and not the N-terminal tail. SET HMTs transfer a methyl group from the universal methyl donor S-adenosylmethionine. The SET HMT proteins of the mixed lineage leukemia (MLL) family have been demonstrated to be important for brain functioning. In particular, MLL1, which trimethylates H3K4, is important for neurogenesis and allows for expression of Hox genes during development and during proliferation in adult neural stem cells (Lim *et al.*, 2009). H3K4 trimethylation has been associated with gene activation almost exclusively, suggesting that the extent of methylation of certain lysine residues determines whether transcriptional activation or repression will occur. In addition to H3K4, methylation of H3K36 and H3K79 has been correlated to gene activation, while H3K9, H3K27, and H4K20 methylation are associated with gene repression. Several different proteins can impart each of these histone marks; for example, methylation of H3K9 can occur via SUV39H1, SUV39H2, G9A, or SETDB1 proteins (Wang & Zhu, 2008). Conversely, histone demethylase proteins come in two categories: those that require either 2-oxyglutarate (Jumonji family) or flavin (LSD or KDM family). LSD1 has been shown to be important for demethylation of H3K4 trimethylation and functions in the REST complex to deactivate gene expression

programs (Shi *et al.*, 2005). There are several other lysine demethylase proteins in each of the families, but their role in brain functioning is still uncertain.

1.4.4 Epigenetic mechanisms in psychiatric disorders

Every gene has underlying epigenetic components that mechanistically contribute to its regulation. In the past few years, it has become evident that DNA methylation and histone modifications interact with one another to confer epigenetic regulation of gene expression. Histone modifications, for their part, are important for several developmental and biological processes including learning and memory, adult neurogenesis, and recently, their dysregulation has been implicated as part of the pathophysiology of psychiatric disorders (Tsankova *et al.*, 2007). Additionally, several lines of evidence suggest that dysregulation of epigenetic processes may underlie some of depression's etiology.

One insight into the role epigenetic dysregulation plays in the etiology of depression is that antidepressant treatments have been demonstrated to impact histone modifications and chromatin modifying proteins. Electroconvulsive seizure therapy (ECS) is one of the most effective treatments for depression, although it is rarely used except for severe cases (Berton & Nestler, 2006). In rodent models of stress-induced depression, seizure therapy increases gene expression of both BDNF and CREB via increased H3 acetylation at both promoter regions in the hippocampus concurrent with amelioration of depressive-like symptoms (Tsankova *et al.*, 2004). CREB regulates the expression of BDNF, and BDNF, a neurotrophin, is critical for antidepressant efficacy and plays a role itself in the pathophysiology of depression, as described by the neurotrophin hypothesis of depression.

Additionally, several antidepressant medications function as inhibitors of chromatin modifying proteins: these include valproate, an anticonvulsant used as a mood stabilizer which also functions as an HDACi, and monoamine oxidase inhibitors, which in addition to inhibiting the breakdown of monoamines, also inhibit the histone demethylase LSD1, the enzyme responsible for trimethylation of H3K4, an activating HPTM (Sun *et al.*, 2013). Moreover, drugs originally intended as HDAC inhibitors have been demonstrated to have antidepressant effects (Schroeder *et al.*, 2007). Indeed, the use of chromatin modifying protein inhibitors in the treatment of depression has gained traction over the past few years with more studies demonstrating their efficacy.

The paradigms used to elicit depressive-like symptoms also induce altered chromatin modifications, suggesting a role of epigenetics in psychiatric disorders. In one study, chronic stress decreased histone acetylation, particularly in areas of the hippocampus, along with increased HDAC expression of all three classes (Ferland & Schrader, 2011). Interestingly, overexpression HDAC5 blocks the antidepressant actions of imipramine and is increased in animals exposed to chronic social defeat stress (Tsankova *et al.*, 2006). In humans, patients with major depressive disorder (unmedicated) have increased expression of HDAC2, HDAC5, HDAC6, and HDAC8, as measured in peripheral blood (Hobara *et al.*, 2010). Conversely, in one study, NAD-dependent SIRT HDACs, particularly SIRT1, 2, and 6, were decreased in depressed patients but not in patients in remission (Abe *et al.*, 2011). As such, the complete picture for the role of HDACs in depression is still not well characterized: it is currently thought that histone acetylation in particular allows for the adaptation to stress and maladaptation to stress may include the

increased expression of HDACs leading to greater susceptibility of depression (Sun *et al.*, 2013).

Some studies have demonstrated specific changes in other histone modifications that reverse upon antidepressant administration. In one study, chronic defeat stress increased H3K27 methylation at the P3 and P4 promoters of BDNF, leading to decreased expression in the hippocampus; treatment with imipramine, a tricyclic antidepressant, induced loss of K27 methylation, and increased H3K4 methylation and H3 acetylation at the same promoter regions, leading to up-regulation of BDNF (Tsankova *et al.*, 2006). As compared with HAT and HDACs, less is known about the histone methyltransferase proteins (HMT): one study demonstrated that in the nucleus accumbens, chronic social defeat stress reduced expression of G9a, SUV39H1, and CoREST, while simultaneously decreasing H3K9 methylation. Additionally, in another study, MLL and LSD1 were also decreased in the nucleus accumbens of mice that are considered more susceptible to developing depressive-like symptoms (Covington *et al.*, 2011). In assessment of the effect of stress for post-traumatic stress disorder, acute stress has been shown to increase H3K9 trimethylation in the dentate gyrus and CA1 region of the hippocampus, while chronic stress results in the opposite effect in both regions (Hunter *et al.*, 2009). The same research group subsequently performed ChIP-Seq analysis of H3K9 trimethylation in the hippocampus of PTSD-stressed rats, which revealed global increases in H3K9me3 and increased occupancy of retrotransposable loci along with increased expression of *Suv39h2* mRNA expression (Hunter *et al.*, 2012). Whether these effects are present in animals with stress-induced depression is still not known.

Overall, both histone acetylation and methylation along with their chromatin modifying proteins likely play a role in the etiology of psychiatric disorders, including depression. The brief discussion of the above studies demonstrates that the extent of aberrant histone modifications is dependent on the type of modification (acetylation or methylation), the location in the brain (nucleus accumbens, areas of the hippocampus, and other regions implicated in depression), and the type of stress (acute or chronic) that is used to induce a depressive-like phenotype. As such, more research on the epigenetic mechanisms of psychiatric disorders and the use of epigenetic therapies is underway.

2.0 The effects of arsenic exposure on neurological and cognitive dysfunction in human and rodent studies: a review

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Abstract

Arsenic toxicity is a worldwide health concern as several millions of people are exposed to this toxicant via drinking water, and exposure affects almost every organ system in the body including the brain. Recent studies have shown that even low concentrations of arsenic impair neurological function, particularly in children. This review will focus on the current epidemiological evidence of arsenic neurotoxicity in children and adults, with emphasis on cognitive dysfunction, including learning and memory deficits and mood disorders. We provide a cohesive synthesis of the animal studies that have focused on neural mechanisms of dysfunction after arsenic exposure including altered epigenetics; hippocampal function; glucocorticoid and hypothalamus-pituitary-adrenal axis (HPA) pathway signaling; glutamatergic, cholinergic and monoaminergic signaling; adult neurogenesis; and increased Alzheimer's-associated pathologies. Finally, we briefly discuss new studies focusing on therapeutic strategies to combat arsenic toxicity including the use of selenium and zinc.

2.1 Introduction

Arsenic is ranked first among toxicants posing a significant potential threat to human health based on known or suspected toxicity (Hughes *et al.*, 2011). This naturally occurring metalloid is a known poison, a co-carcinogen, and in lower concentrations has been shown to increase susceptibility to cognitive dysfunction (Naujokas *et al.*, 2013). Currently, the permitted concentration of arsenic in water is 10 µg/L (10 ppb). Yet, an estimated 100 million people worldwide are exposed to excessive amounts of arsenic via drinking water (in the ppm range). Many of these individuals obtain drinking water from

unregulated sources (wells) or live in regions where arsenic levels are high, as in Bangladesh. As arsenic leaches from rock formations into water sources as the water table recedes, exposure to high amounts of arsenic will continue to persist as the demand for clean water increases. This phenomenon particularly affects the Western region of the United States, where it is estimated that certain areas contain up to 3100 µg/L arsenic (31 ppm) in drinking water, on par with levels reported in Taiwan and China (Naujokas *et al.*, 2013). While the World Health Organization (WHO) and the Environmental Protection Agency (EPA) regulate water sources of arsenic, lack of strict regulations on food, beverages, and air quality can lead to increased arsenic exposure (Wilson *et al.*, 2012). Ingestion of arsenic activates metabolic pathways for excretion, resulting in a number of metabolites, some of which are more potent and toxic than the originally ingested inorganic form of arsenic (iAs), including mono- and dimethylated arsenicals (Kligerman *et al.*, 2003; Gosse *et al.*, 2013; Watanabe & Hirano, 2013), (Kruger *et al.*, 2009; Ventura-Lima *et al.*, 2011).

All forms of arsenic, including inorganic and methylated arsenicals, accumulate in many parts of the brain, with the highest accumulation in the pituitary (Sanchez-Pena *et al.*, 2010). Additionally, arsenic is a well-established teratogen that crosses the placenta during development, and in high concentrations, induces growth delays and neural tube defects (He *et al.*, 2007; Vahter, 2009; Ahmed *et al.*, 2011). Epidemiological studies reviewed in this paper have investigated the neurological and cognitive effects of arsenic on children and adults. Evidence presented suggests that chronic ingestion of arsenic alters a number of intelligence measures and induces learning deficits and mood disorders

like depression. Information on dose, concentration, extent, and method of exposure of arsenic will be given for each study. We provide a detailed review of the mechanisms involved in arsenic-induced toxicity in the brain. These include altered glucocorticoid signaling, cholinergic and monoaminergic signaling, adult neurogenesis and other forms of synaptic plasticity, and behavioral deficits including learning, memory, and locomotion. The last section of this review discusses new studies on therapeutics used in rodent models that may eventually be useful for remedying arsenic-induced neurological deficits in human populations.

2.2 Epidemiological studies on arsenic exposure: Cognitive assessment of children

Over the past two decades, various epidemiological reports have shown that arsenic exposure may alter cognitive function, particularly learning and memory during childhood. A recently published meta-analysis focused on the impact of arsenic exposure on intelligence measured by IQ tests (Rodriguez-Barranco *et al.*, 2013). Researchers concluded that arsenic exposure was associated with a 0.4 decrease in IQ in exposed children (Rodriguez-Barranco *et al.*, 2013). This appears to be a minor decrease in IQ, but these changes could have cumulative effects later on in life. For our purposes, we will provide a synopsis of epidemiological studies focusing on other neurobehavioral measures in children.

Several studies have shown a relationship between low arsenic exposure (in the parts-per-billion range) and children's intellectual performance on a battery of cognitive tests that cover a number of skills and processes. For example, a 2007 study found a significant

association between urinary arsenic concentrations greater than 50 µg/L and poor scores on tests measuring visual-spatial reasoning, language and vocabulary, memory, intelligence, and math skills in 6-8 year old (y.o.) children from Mexico (Rosado *et al.*, 2007). It should be noted that researchers found a sex-specific effect of arsenic: males performed more poorly on all assessments compared to females. However, males had higher arsenic concentrations (mean 11.85 µg/L) in their urine, which could account for these differences. In another cohort of children in Mexico (6-7 y.o. with 55 µg/L urinary arsenic), researchers reported poorer scores in arsenic-exposed children on measures of language and vocabulary and a modest association with hyperactive behavior using the attention deficit hyperactive disorder (ADHD) index (Roy *et al.*, 2011). In India, a cross sectional study on 351 children (5-15 y.o.) exposed to an average of 147 µg/L arsenic in water throughout development and childhood found an association between arsenic exposure and poor performance in several measures focusing on vocabulary, math skills, memory, and overall cognition; however, confidence intervals and the age range of participants in this study were broad (von Ehrenstein *et al.*, 2007). When accounting for age range and other covariates (education, levels of arsenic, socioeconomic status), there was a dose-dependent relationship between arsenic levels in water and poor performance scores on intelligence measures (Wasserman *et al.*, 2004): children exposed to >50 µg/L arsenic had poorer intellectual performance than those exposed to <5.5 µg/L arsenic (Wasserman *et al.*, 2004). While low exposures to arsenic (< 2 µg/L) resulted in no measurable effects on IQ, moderate exposures (142 µg/L and 190 µg/L) resulted in decreases of 5 and 10 IQ points, respectively (Wang *et al.*, 2007). These findings, along

with other studies (See Table 2.1) support the association between moderate arsenic exposure and deficits in cognitive skills in children exposed during development.

Table 2.1. The impact of arsenic exposure on the cognitive abilities of children and adults

Cognitive assessment	Exposure	Age	Finding	Reference
Intelligence (IQ)	Low and High	Children	↓verbal IQ ↓total IQ	Caldern et al. 2001 [42]
	Medium	Children	↓total IQ	Rosado et al. 2007 [14]
	Low and High	Children (5-15 y.o.)	↓total IQ	Von Ehrenstein et al. 2007 [16]
	Low	Children (9-10 y.o.)	↓total IQ	Wasserman et al. 2004 [17]
	Low	Children (6 y.o. and 10 y.o.)	↓total IQ	Wasserman et al. 2007 [30]
	Low and Medium	Children (5 y.o.)	↓total IQ (females) ↓verbal IQ (females)	Hamandani et al. 2011 [26*]
Cognitive skills (reading, writing, vocabulary, math)	Medium	Children (6-7 y.o.)	↓capacity in figure design, vocabulary, letter sequencing	Rosado et al. 2007 [14]
	Medium	Children (6-7 y.o.)	↓capacity in vocabulary	Roy et al. 2011 [15]
	Low and High	Children (5-15 y.o.)	↓capacity in vocabulary, language	Von Ehrenstein et al. 2007 [16]
	Low	Adults	↓capacity in executive function, mental acuity, verbal skills	O'Bryant et al. 2011 [43**]
Visual perception	Medium	Children (6-7 y.o.)	↓capacity in visual search	Rosado et al. 2007 [14]
	Low and High	Children (5-15 y.o.)	↓capacity in picture completion, object assembly	Von Ehrenstein et al. 2007 [16]
Mental health	Medium	Children	↑risk for ADHD	Roy et al. 2011 [15]
	Low	Adults	↑incidence of depression	Zierold et al. 2004 [53]
	Medium	Adults	↑symptoms of anxiety	Dang et al. 2008 [50], Dang et al. 2009
	Low → High	Adults	↓quality of life and mental health	Syed et al. 2012 [47]
	High	Adults	↑symptoms of altered mental health	Fujino et al. 2004 [51]
	Low → High	Adults	↑insomnia ↓general health	Guo et al. 2007 [49]
	Low → High	Adults	↑risk of psychiatric disorder, depression, anxiety	Sen et al. 2012 [52]

Exposure

Low: less than 50 µg/L (ppb) urinary arsenic or water arsenic

Medium: between 50 µg/L (ppb) and 100 µg/L (ppb) urinary or water arsenic

High: more than 100 µg/L (ppb) urinary arsenic or water arsenic

As mentioned above, a common finding in these studies seems to be the differential effect of arsenic among young males and females; while this has not received much attention (Llop *et al.*, 2013), some research suggests that arsenic impedes growth and development in young females more than males, which in turn could impact cognitive function (Gardner *et al.*, 2013). In studies where urinary arsenic levels between males and females are comparable, females aged 5-15 y.o. had a larger negative response to arsenic

(Kang *et al.*, 2007). Overall, in addition to poor cognitive assessments, children exposed to arsenic in well water also have poor development scores (i.e. height), which correlates with lower intellectual function; this effect may be more pronounced in females than males (Wasserman *et al.*, 2008).

2.2.1 *In utero* and cumulative arsenic exposure

Most epidemiological studies focusing on the effects of arsenic exposure in children, with exposures occurring across several years, have reported impaired cognition regardless of the concentration of arsenic. Conversely, studies evaluating the impact of arsenic exposure only *in utero* fail to show consistent cognitive deficits. Analysis of arsenic in cord blood one day after birth (1.33 $\mu\text{g/L}$) and subsequent behavioral assessment (Brazelton Scale) showed an inverse correlation between arsenic levels and neurodevelopment in newborns in Nepal (Parajuli *et al.*, 2013). Yet, an early-life longitudinal study of pre- and postnatal arsenic exposure reported no significant correlations between maternal or child arsenic levels and psychomotor and mental development index assessments, behavior, or the maternal report of language acquisition in 18 month old children (Hamadani *et al.*, 2010). Additionally, infants born to mothers with urinary arsenic levels of 80 $\mu\text{g/L}$ during pregnancy showed no cognitive impairment when accounting for age, sex, and socioeconomic status (Tofail *et al.*, 2009). Measurable impaired cognition becomes evident later in life: a Bangladesh study from the same group demonstrated a correlation between the mother's and child's urinary arsenic content (80 and 51 $\mu\text{g/L}$ respectively) and verbal IQ and full scale IQ but only in females at 5 years of age (Hamadani *et al.*, 2011). Research focused on the adolescent time period

of development provides more insight, suggesting that long-term cumulative exposure is more harmful for cognitive function including alterations in pattern memory, attention set shifting (Tsai *et al.*, 2003), full scale IQ, verbal IQ, and functional memory (Wright *et al.*, 2006). The levels of arsenic among these different cohorts of teens were highly variable, ranging from 17.8 µg/L as assessed in hair (Wright *et al.*, 2006) to 132 and 185 µg/L arsenic in well water (Tsai *et al.*, 2003); thus, conclusions determined from comparing these studies are limited. Additionally, data from toxin assessment in hair has been deemed somewhat insufficient to predict health outcomes as information of toxin content varies according to gender, ethnicity, and pharmacokinetics (Harkins & Susten, 2003). However, all groups in these studies had developmental exposure to arsenic. As with other teratogens, the timing, dose, and duration of arsenic exposure seem to determine the extent of neurological insults on children, and these insults may occur during development to produce cognitive dysfunction in adolescence or adulthood.

Chronic moderate exposure to arsenic, more so than high acute exposure, may induce greater harm on intellectual function in children. Several investigations by Wasserman and colleagues showed that the relationship between arsenic exposure and cognition in children might be age dependent. In a study of arsenic-exposed children aged 6 y.o., researchers found a negative association between arsenic and performance on the Wechsler Preschool and Primary Scale of Intelligence (WPPSI-III) (Wasserman *et al.*, 2007); yet this association was not as substantive as the inverse association observed in 10 y.o. children, who had longer arsenic exposure (Wasserman *et al.*, 2004). Both cohorts of primary school children showed reduced intellectual function and performance,

reduced processing speed, and lower full-scale raw scores with arsenic exposures between 5-50 µg/L during development. Yet, the chronic nature of the exposure combined with the concentration of arsenic had the most adverse effect on cognitive ability and neurological functioning in children.

2.2.2 Factors comorbid with arsenic exposure

One difficulty in drawing conclusions of the effect of arsenic on cognitive ability is that exposure is commonly associated with other factors that could affect the outcomes of these studies (see Box 1). These include exposures to a mixture of metals, low socioeconomic status, and poor nutrition.

2.2.2.1 Metal Mixtures

The presence of arsenic in water or the environment does not preclude the presence of other elements; in fact, it is probable that a number of metals (Pb, Mn, Cd, Hg) are comorbid in individuals with arsenic exposure in the studies provided here. Reports on developmental exposures to heavy metal mixtures suggest that combined exposure is associated with greater risk for cognitive dysfunction, including behavior and impaired neurological (CNS) development (Rai *et al.*, 2010). The incidence of mental retardation is highly correlated with the presence of soil metals in rural areas (As, Cu, Pb, Mn, Hg), and the probability of intellectual disability in children increases as the concentration of arsenic and lead in the soil increases (Aelion *et al.*, 2008), (McDermott *et al.*, 2011). Yet, determining the effect of one metal in isolation is quite difficult. In several studies, the concentration of lead in blood, urine, or water (while low), still positively correlates with

arsenic levels (Parajuli *et al.*, 2013). Lead in particular is a known toxicant imparting severe neurotoxic effects on the brain especially in children. Of the studies described here, only a few discuss comorbid exposures and provide information about other metal concentrations. Poor scores on cognitive assessments have been reported in children exposed to both arsenic (slightly above and below the 10 µg/L EPA standard) and manganese (500 µg/L) in well water, though the arsenic and manganese interaction was not significant (Wasserman *et al.*, 2011). Arsenic, in particular, was associated with poor working memory and lower verbal comprehension scores as previously reported in a smaller prospective study ((Wasserman *et al.*, 2011)), although a significant manganese arsenic interaction was found in this study (Wright *et al.*, 2006). As such, further investigation on developmental neurotoxicology of arsenic in combination with other substances is warranted. The possibility that observations in epidemiological studies may be induced by comorbid factors in addition to arsenic cannot be ignored.

2.2.2.2 Socioeconomic status

Overall, arsenic exposure is correlated with lower socioeconomic status (SES) (Rosado *et al.*, 2007), and SES correlates with lower measures on tests assessing infant development and cognition (Tofail *et al.*, 2009). The effects of poverty and parental stress due to low SES are significant risk factors in neurodevelopment disorders (Drews-Botsch *et al.*, 2011), and parsing out these effects from arsenic exposure is quite difficult. Additionally, control cases used for epidemiological work are typically from more affluent areas without arsenic exposure. In China, cross-sectional analyses have shown that children who live in rural areas have lower IQ scores than children who live in urban areas (Kang

et al., 2007; Wang *et al.*, 2007; Dong & Su, 2009). Children residing in poor rural areas are more often exposed to arsenic, which may exaggerate the inverse associations found between arsenic in drinking water and IQ scores (Rocha-Amador *et al.*, 2007). Low SES is often associated with less education, especially in developing countries; one study reported that cognitive ability in children exposed to arsenic increased as maternal education increased from primary to secondary school (von Ehrenstein *et al.*, 2007). However, many epidemiological studies that controlled for education as a covariate still reported significant neurodevelopmental deficits imparted after arsenic exposure (Parajuli *et al.*, 2013).

2.2.2.3 Nutrition

Malnutrition can have a substantial negative impact on cognitive development and performance even in the absence of arsenic or metal exposure. Children lacking proper nutrition during development, such as iron deficiency, have deficits in neurological function, including cognition (Beard, 2003). Arsenic exposure is thought to increase the risk for anemia, which is a potential risk factor in blunted growth and development (Heck *et al.*, 2008). Current research has been focused on the possible connection between the lack of proper nutrients in diet during development (methionine, cytosine, protein, folic acid, vitamin B-12, choline, and betaine) and developmental delay (Heck *et al.*, 2009; Dominguez-Salas *et al.*, 2013). These nutrients are important as they boost the body's methyl donor availability, which is critical in the metabolism of arsenic. Increased consumption of methionine, cysteine, and protein aids in the methylation of arsenic, thus enhancing excretion in individuals exposed to arsenic (Heck *et al.*, 2009). The ability of

the developing body to metabolize arsenic and to properly execute epigenetic modifications could be impaired by the accumulation of arsenic. As such, understanding the nutritional status of the children in these studies may be critical to interpreting the effects of arsenic on cognition.

2.2.3 Summary of epidemiologic studies in children

Overall, we can ascertain certain patterns from the epidemiological literature on the association between arsenic exposure and cognitive performance in children. A number of studies have shown that arsenic induces cognitive deficits in children, even at low concentrations. Arsenic water levels or urinary arsenic levels correlate with poorer performance and scores on intelligence measures, and verbal IQ seems to be the most affected cognitive skill. These effects persist into adolescence, such that cumulative arsenic intake may be a greater factor than acute intake in cognitive dysfunction. Higher concentrations of arsenic exposure can alter growth and development in children, leading to neurological deficits, and females seem to be at greater risk than males, although few studies have systematically evaluated sex differences. Potentially confounding factors in these studies include the comorbidity of other metals with arsenic exposure, the correlation between high arsenic exposure and poor socioeconomic status, and poor nutrition in children exposed to arsenic (Calderon *et al.*, 2001). Studies on cohorts from around the world, including China, the U.S., Taiwan, Bangladesh, and Mexico, have all provided significant research supporting the negative effects of arsenic on childhood development and cognitive ability.

2.3 Epidemiological studies on arsenic exposure: Cognitive assessment of adults

Most epidemiological research has focused on cognition in children, and reports on the effect of arsenic exposure on adult cognition are limited. However, a series of studies has recently revealed a significant correlation between arsenic exposure and altered adult cognition, particularly for symptoms associated with Alzheimer's disease. The FRONTIER project is an ongoing cohort study focused on a group of West Texas residents in an area that contains arsenic levels close to the EPA standard in groundwater (3-15 $\mu\text{g/L}$). Long-term and continuing chronic exposure to low levels of arsenic via drinking water significantly correlated with poorer scores in tests examining language, visual and spatial skills, and executive function, all of which indicate cognitive dysfunction (O'Bryant *et al.*, 2011). Poorer scores in global cognition, processing speed, and immediate memory were also found; these particular deficits have been associated with Alzheimer's disease (Gong *et al.*, 2011; O'Bryant *et al.*, 2011). Individuals exposed to 10.6 $\mu\text{g/L}$ arsenic scored significantly worse on global cognitive assessment compared to those exposed to 6.5 $\mu\text{g/L}$ arsenic, suggesting that 10 $\mu\text{g/L}$ EPA standard may not be sufficient to prevent arsenic-induced cognitive deficits (Gong *et al.*, 2011).

There are several caveats to the FRONTIER studies. The Mini-Mental State Exam (MMSE) used for cognitive assessment has been criticized for its bias against less educated participants (Rosselli *et al.*, 2006; Franco-Marina *et al.*, 2010); indeed, FRONTIER participants in the 10.6 $\mu\text{g/L}$ group did have less education than controls (Gong *et al.*, 2011). Additionally, most of these assessments rely on verbal responses, reading, and writing, while other tests require on proper auditory perception and visual

acuity. The average age of the 434 FRONTIER participants in these studies was 62 years old, thus if arsenic exposure affected sensory, perception, or communication, participants may perform poorly even if no cognitive impairment exists. Finally, the arsenic concentrations in both of these FRONTIER studies were determined using the ArcGIS program based on ground water measurements made by the Texas Water Development Board; direct measurement of arsenic was not performed. However, despite these limitations, the findings presented here are important in supporting that cumulative, long-term exposure to low levels of arsenic, levels considered safe by the EPA, can impart cognitive deficits in adults.

2.4 Epidemiological studies on arsenic exposure: Psychological assessment of adults

While the number of studies concerning cognitive and mental disorders after toxic exposures has increased over the past ten years, specific characterization concerning arsenic exposure and psychological health requires more epidemiological investigation. Of the studies presented here, several point to a strong correlation between arsenic toxicity and mental health status. These data are reported from all over the world including China, Bangladesh, India, and the United States. Altered cognition is typically concurrent with disturbances in mental health. As such, it is difficult to assess the validity of studies primarily focused on psychological health in arsenic-affected individuals, as the previous data we have presented shows a strong correlation between arsenic exposure and cognitive deficits. However, despite the overlap between psychological and cognitive disorders, there is substantial evidence to suggest that arsenic exposure increases the risk of impaired cognition and enhanced susceptibility for mood disorders.

2.4.1 Bangladesh

Bangladesh has one of the highest reported arsenic exposure rates in the world, and yet, few studies have explored the relationship between arsenic exposure and mental health. A recent cross-sectional study, published in 2012, found that patients with arsenic poisoning reported a lower quality of life and scored lower on a validated mental health index compared to individuals without arsenic exposure (Syed *et al.*, 2012). Arsenic poisoning has strong societal implications in Bangladesh, often resulting in individuals with arsenic exposure ostracized from the community (Syed *et al.*, 2012). This lack of social acceptance could contribute to the lower quality of life and mental health scores in these individuals; however, other studies on the effects of arsenic and mental health concur with those reported in this study.

2.4.2 China

China accounts for approximately half of the world's arsenic production and consequently its pollution (Garelick *et al.*, 2008), and there are many regions that contain naturally high amounts of arsenic in soil deposits. Arsenic toxicity studies from China typically focus on symptoms of neurotoxicity, including loss of hearing, loss of taste, blurred vision, and tingling/numbness in the limbs (Guo *et al.*, 2007). Altered mental health is comorbid with these ailments, including a prevalence of insomnia (Guo *et al.*, 2007), anxiety and depression (Dang *et al.*, 2008), and symptoms of distress (Fujino *et al.*, 2004a). Assessments of mental health were congruent with education levels of the participants; however, as described above, exposure to metal mixtures is common, and participants exposed to metals in one study showed significantly greater urine and hair

concentrations of arsenic, lead, and cadmium than control participants (Dang *et al.*, 2008). Additionally, water in these villages contained between 15-1860 µg/L arsenic, much higher than the Chinese Drinking Water Standard of 50 µg/L.

2.4.3 India

In India, over 1.5 million people have been exposed to high levels of arsenic with more than 200,000 cases of arsenicosis. Cross-sectional analysis of over 1169 arsenicosis patients between the ages of 18-65 y.o. revealed that 19% of patients developed some type of psychiatric disorder, compared an average 7% prevalence of mental disorders in India (Sen & Sarathi Biswas, 2012). Of the 19% of patients, most were categorized as having depression and/or anxiety; yet, the prevalence of depression in India is only 3.7%. The participants in this study were from seven different villages, 90% of which had arsenic levels ranging from 25-900 µg/L. Most patients were males with low SES and a 25% rate of unemployment. As noted in the studies from Bangladesh, it is difficult to determine if these patients exhibit depression due to the state of their overall health (arsenicosis) or if depression is a manifestation of the arsenic toxicity. However, in Indian society, arsenicosis patients are able to maintain their traditions, kinship, and cast relations to cope with stress of the disease (Sen & Sarathi Biswas, 2012). Similar to observations of the general population, anxiety and depression were the most common psychiatric disorders in arsenicosis patients; however, lower SES, the diseases associated with arsenic poisoning, body image, and low self-esteem are predisposing factors of psychological issues in this population.

2.4.4 United States

The US has more stringent standards of arsenic water quality than other countries (the EPA limit is 10 µg/L), yet this concentration may not be low enough as suggested by the literature on cognitive dysfunction. A 2004 cross-sectional study covered a wide range of locations in areas with low concentrations of arsenic and revealed a significant association between low arsenic exposure (2-10 µg/L) and poor mental health, particularly depression. Individuals with cumulative, long-term exposure to low concentrations of arsenic (2-10 µg/L) for more than twenty years were significantly more likely to exhibit depressive symptoms than those drinking less than 2 µg/L arsenic (Zierold *et al.*, 2004). Interestingly, participants consuming more than 10 µg/L arsenic were also more likely to have had cardiac bypass surgery, high blood pressure, and circulatory issues than those drinking less than 2 µg/L arsenic in water (Zierold *et al.*, 2004). Unlike studies from India, Bangladesh, and China where low levels of arsenic were not assessed, this study suggested that low exposure, under EPA limits of 10 µg/L, is associated with higher rates of adverse mental health. However, evaluating the validity of the conclusions from this study is difficult because of the lack of the critical methodological information, (e.g., participant health status, and the type and analysis of the assessment tools), which are all important in interpreting the findings.

2.4.5 Summary of epidemiologic studies in adults

There are several limitations to these studies, including some with small sample sizes, all with variable levels of arsenic toxicity, and arsenic-affected individuals typically living in rural areas and having lower SES. Additionally, assessment of dose-response

relationships and mental health has not been determined: it would be useful to have high and moderate arsenic exposures compared in the same study. While it is difficult to disentangle the contribution of arsenic and that of general health in many of the studies conducted in countries outside of the U.S., the findings presented thus far demonstrate a correlation between arsenic exposure, cognitive dysfunction, and mental health. There is clearly substantial evidence that arsenic exposure diminishes cognition and increases mood disorders in human populations. Animal and invertebrate studies have replicated many of these observations and aided in the search for the mechanisms for the adverse health impacts of arsenic.

2.5 Mechanisms of Action

It is difficult to use the epidemiological literature to identify mechanisms, as long-term exposures to arsenic are likely compounded with exposures to pollution, poor diet, and low SES. Basic science research is poised to control for these confounding factors, including extent and timing of exposure. This research has determined that arsenic imparts its toxicity on the body via a number of mechanisms. These include the depletion of methyl groups affecting epigenetic profiles, the uncoupling of oxidative phosphorylation and increased reactive oxygen species, the inhibition of thiol-containing enzymes and proteins (including the depletion of glutathione), altered signal transduction and cell proliferation, and reduced DNA repair inducing genotoxicity (Hughes *et al.*, 2011; Watanabe & Hirano, 2013). As these mechanisms have been discussed in detail in other reviews, we cover mechanisms particularly related to the brain. These include hippocampal dysfunction; glutamatergic, glucocorticoid, cholinergic, and monoaminergic

signaling; pathways associated with Alzheimer's disease; and synaptic plasticity, particularly neurogenesis. To begin, we briefly provide results from the few studies focusing on neural epigenetic patterns altered after arsenic exposure.

2.5.1 Epigenetic patterns

Arsenic accumulation and subsequent toxicity is likely mediated through multiple mechanisms of action. Of particular interest over the past few years is the impact of arsenic on the epigenome. While there have been several studies determining the impact of arsenic on epigenetic regulation in cancer cells, the liver, and other parts of the body, very few have focused the brain. Exposure to 3 and 36 ppm arsenic throughout gestation increased DNA methylation on two genes involved in neural plasticity in rat cortex and hippocampus (Martinez *et al.*, 2011). Hypomethylation of these genes in both regions was observed after four months of cumulative exposure to arsenic; these animals also displayed deficits in fear memory as well, although the link between hypomethylation and memory was correlational. Since arsenic metabolism requires methyl groups derived from S-adenosylmethionine (SAM) for excretion, it is plausible that arsenic depletes SAM leading to alterations in DNA methylation. *In vitro* studies corroborate this assertion: 25 μ M arsenic exposure for 24 hours depleted SAM concentrations, increased global DNA hypomethylation, and repressed DNMT1 and DNMT3a expression (Reichard *et al.*, 2007). Epidemiological work has shown that DNA methylation is affected by arsenic in human populations as well, including umbilical cord blood, but discussion of those studies is beyond the scope of this review (Reichard & Puga, 2010; Kile *et al.*, 2012; Bailey *et al.*, 2013; Koestler *et al.*, 2013). We can determine that

exposure to arsenic does induce epigenetic modifications to the DNA, which may result in aberrant gene expression even in the brain; however the link between DNA methylation on particular genes and cognitive deficits has yet to be elucidated.

Other epidemiological studies have determined the impact of arsenic ingestion on histone modifications. Arsenic compounds have been shown to alter gene expression and posttranslational modifications (PTM) of histones *in vivo*; interestingly, researchers found a differential effect of arsenic on global histone modifications among males and females (Chervona *et al.*, 2012c). *In vitro* assessment of low doses of arsenic on histone modifications has also been performed; however, detailed discussion of this research is beyond the scope of this review (Zhou *et al.*, 2008; Zhou *et al.*, 2009; Chervona *et al.*, 2012c). Rodent studies with prenatal exposure to 100 µg/L arsenic demonstrated reduced global acetylation on lysine 9 of histone 3 (H3K9ac) in the cortex and hippocampus of postnatal day (PND1) pups, which was correlated to altered learning in adulthood (Cronican *et al.*, 2013). Exposure to 3 and 36 ppm arsenic throughout development up to four months reduced myelination (for which methylation is required) and dimethylation of arginine residues on histones (Zarazua *et al.*, 2010). This could result from the altered expression and function of epigenetic modifiers or transcription factors, as arsenic impacts zinc-finger protein expression and function (Zhou *et al.*, 2011b).

These studies demonstrate that arsenic impacts DNA methylation and histone modifications and alters the enzymes responsible for regulating these modifications. The effects of arsenic on the epigenome are related to the dose and extent of arsenic exposure

as seen before, but also type of histone methylation mark, gene, and sex. As such, in determining mechanisms of arsenic toxicity it will be important to control for these variables both in epidemiological and molecular studies in the future. Additionally, cumulative low-level exposure to arsenic likely occurs over generations. The literature suggests that females are differentially more affected by arsenic than males. Since females are the key source of transgenerational effects (3rd generation ova are exposed to the *in utero* environment), arsenic could be impacting transgenerational epigenetics, including the imprinting of genes (from both males and females). This area of research could provide an insight into the effects of arsenic on the brain and body, yet there are no published reports on arsenic exposure and transgenerational epigenetic mechanisms to date.

2.5.2 The hippocampus

Studies on the mechanisms of arsenic-induced toxicity have established that arsenic alters learning and memory in behavioral assays and impacts multiple neurobiological processes including those of neurogenesis and cholinergic, glutamatergic, and monoaminergic signaling pathways. Recent work using animal models has revealed potent alterations in hippocampal function, morphology, and signaling leading to altered cognitive behavior after arsenic exposure. While the exposure paradigms and concentrations of arsenic have been highly varied, the overall conclusions have been congruent between studies (See Table 2.3).

Table 2.2. The impact of arsenic on behavioral tasks in rodent studies

Type of cognition	Task	Exposure	Finding	Reference
Learning and memory	Morris water maze (MWM)	Adult, chronic, high	↓ acquisition	Luo et al. 2009 [71]
		Adult, chronic, low	↓ acquisition	Sharma & Sharma, 2013 [66]
	Fear/cued conditioning	Adult, acute, low	↓ recall	Cronican et al. 2013 [63]
	8-way radial arm maze	Developmental, chronic, low	↓ acquisition	Martinez-Finley et al. 2009 [67]
	Y-maze; MWM	Adult, chronic, high	↓ acquisition	Jing et al. 2012 [68]
	Radial water maze	Adult, acute, low	↓ acquisition	Cronican et al. 2013 [63]
	Novel object exploration	Developmental, chronic, low	↓ performance	Martinez-Finley et al. 2009 [67]
Locomotion and motor	Startle/reflex response/ spontaneous alteration/ developmental battery tests	Adult, acute, low	↓ performance	Cronican et al. 2013 [63]
		Developmental, low → high	↓ coordination ↑ response time to completion no change in low group No change in either group	Luo et al. 2013 [86] Gandhi et al. 2012 [87]
	Rotarod	Developmental, low → high	↓ coordination ↑ ataxia	Yadav et al. 2009 [85]
	Spontaneous locomotor activity	Adult, subchronic, high	Dose-related	Bardullas et al. 2009 [83]
		Adults, chronic, low → high	↑ locomotion with low dose ↓ locomotion with high dose ↑ locomotion females	Rodriguez et al. 2010 [84]
	Total movement	Adults, chronic, low → high	no change in low group ↓ locomotion	Bardullas et al. 2009 [83]
		Adult, chronic, low → high	↓ movement at high dose	Yadav et al. 2009 [85]
		Adult, subchronic, high	↓ total movement	Rodriguez et al. 2010 [84]
	Grip strength	Adult, chronic, low → high	↓ total movement	Yadav et al. 2009 [85]
		Grip strength	Adult, subchronic, high	↓ strength

Exposure type:

Developmental: in utero to postnatal exposure (maternal consumption of arsenic)

Adult exposure: from weaning up to death

Exposure level:

High exposure: ppm (mg/L)

Medium exposure: above 100 ppb (µg/L)

Low exposure: below 50 ppb (µg/L)

Low → high; multiple doses examined

Exposure paradigm:

Acute: less than two weeks

Subchronic: less than one month

Chronic: more than one month

In particular, behavioral studies using hippocampal-dependent tasks, including the Morris Water Maze and fear conditioning, have corroborated epidemiological evidence of reduced cognition observed in humans with arsenic exposure. Table 2.3 details the behavioral paradigms and arsenic exposures used in these studies. Results from this body of literature suggest that regardless of dose, timing, or extent of exposure, arsenic induces hippocampal-dependent behavioral deficits in rodent models, suggesting impaired spatial,

working, long-term, and short-term memory (Martinez-Finley *et al.*, 2009; Jing *et al.*, 2012; Cronican *et al.*, 2013; Sharma & Sharma, 2013). Interestingly, the effect extends to nonmammalian species as arsenic exposure in *Danio rerio* (zebrafish) induces deficits in long-term memory as well (de Castro *et al.*, 2009). Several of these behavioral deficits can be ameliorated with treatment; for example, sodium butyrate, a histone deacetylase inhibitor, attenuated deficits seen in the Radial Arm Maze after two weeks of arsenic exposure of 100 µg/L during adulthood (Sharma & Sharma, 2013). More detail on therapeutic interventions after arsenic exposure will be discussed in later sections of this review. Overall, work with animal models has demonstrated that arsenic induces deficits in multiple learning paradigms, particularly those relying on proper hippocampal function. Yet, identifying the hippocampus as a sensitive area for arsenic's effects does not necessarily identify a molecular target. Alterations in multiple signaling pathways are localized to the hippocampal formation, but it should be noted that mossy fiber terminals in the hippocampus contain substantial amounts of zinc. Thus, the sensitivity of the hippocampus may be due the effect of arsenic on zinc either via displacement or substitution. To date, no studies investigating this mechanism have been published.

2.5.3 Glutamatergic signaling

Arsenic impacts the synaptic activity of neurons localized to the hippocampus. Slices obtained from young and adult rats exposed to 100 µM arsenite *in vitro* had reduced amplitudes of excitatory post synaptic potentials (EPSPs) in the Schaffer collateral/CA1 synapses. Exposure inhibited long-term potentiation (LTP), a form of synaptic plasticity, in hippocampal slices from adult but not young rats; however, this effect was reversible

after twenty minutes of washout. Arsenic exposure did not impair paired-pulse facilitation, indicative of presynaptic activity, suggesting that acutely applied arsenic does not affect presynaptic neurotransmitter release (Kruger *et al.*, 2006). However, components of NMDA receptors, specifically NR2A, were reduced after three months of arsenic exposure (2.72, 13.6, 68 mg/L arsenic) in mice. Additionally, these mice exhibited morphological changes in hippocampal neurons—reduced size with a condensed nucleus and cytoplasm—along with capillary edema and irregular vascular endothelial cell morphology (Luo *et al.*, 2009a). Similar morphological changes in hippocampal neurons were observed after a 3-month exposure of 8.2 mg/kg/day arsenic; both the striatum and hippocampus contained abnormally myelinated nerve fibers, while the hippocampus contained reduced mossy fiber terminals (Jing *et al.*, 2012). Brief, two week exposure to 100 μ M arsenic also altered expression levels of mRNA for synapse related genes, including increased Grin1, Syn2, and Stx6 expression, similar to results from our studies (Cronican *et al.*, 2013; Tyler & Allan, 2013).

In addition to altered synaptic activity and synapse-related gene expression, arsenic has been shown to impart alterations in central pathways involved in mediating learning and memory in the hippocampus. Using a three-month exposure model (from weaning until four months), arsenic-exposed animals had decreased NR2A expression, PSD-95, and p-CAMKIIa in the hippocampus with concurrent increased SynGAP expression, a known negative regulator of the Ras-MAPK pathway (Luo *et al.*, 2012). Reduced p-ERK1/2 in the hippocampus was also observed in the arsenic-exposed animals. These findings corroborate our own work demonstrating reduced ERK2 in the hippocampus after

perinatal exposure to arsenic (Martinez *et al.*, 2008). Thus, the Ras-MAPK/ERK pathway appears to be sensitive to arsenic damage; interestingly, zinc has been shown to alter this pathway as well (Klein *et al.*, 2006). It is possible that arsenic is acting in a similar fashion. Overall, altered Ras-MAPK/ERK signaling, LTP, and synaptic regulation in the hippocampus could underlie behavioral deficits suggesting arsenic-induced alterations in long-term episodic memory, associative learning, spatial learning, and working memory.

2.5.4 Glucocorticoid signaling

In addition to altered hippocampal-dependent behaviors, depressive-like symptoms have been observed in arsenic-exposed mice. We have demonstrated increased immobility in the forced swim task and increased latency for escape in the learned helplessness task, along with increased plasma corticosterone levels (Martinez *et al.*, 2008). Corticosterone (CORT), the rodent equivalent of cortisol, is a stress hormone that plays a role in mediating the effects of the hypothalamus-pituitary-adrenal (HPA) axis in response to “stressful” events ranging from fear to learning. In humans, significant alterations in the HPA axis have been connected to depressive-like symptoms, and patients with depression typically report stress a major factor in the onset of their depression. Thus, decreased behavioral ability and increased depressive-symptoms in arsenic-exposed animals correlate with the epidemiological data on reduced cognition in humans; as such, arsenic may be mediating not only cognitive impairments but also mood disorders in humans via the glucocorticoid signaling pathway.

CORT signaling is mediated through the corticosterone receptors, specifically the mineralocorticoid (MR) and glucocorticoid (GR) receptors. When activated, MR and GR translocate to the nucleus to allow for binding to response elements (MRE or GRE) on genes for transcriptional activation or repression. CORT signaling, via its receptors in the hippocampus, is responsible for imparting an inhibitory tone for the HPA axis. Our work has demonstrated nuclear levels of GR are much lower in adult mice perinatally exposed to arsenic than levels observed in controls in hippocampal tissue (Martinez-Finley *et al.*, 2009). Additionally, we have shown decreased GR binding to and expression of *H-Ras* and *Raf-1*, genes involved in modulating the MAPK pathway with GRE binding sites (Martinez-Finley *et al.*, 2011). *In vitro* studies have demonstrated that various levels of arsenic impact GR-mediated transcription in a bidirectional manner: high arsenic levels (1-3 mM) induce an inhibitory effect, while low levels (0.05–1 mM) seem to produce a stimulatory effect (Bodwell *et al.*, 2004). Arsenic's impact on GR is predicated on the DNA binding domain (DBD) within the GR. Other steroid receptors, including the MR and the progesterone receptor respond to arsenic in a similar biphasic manner as the GR (Bodwell *et al.*, 2006). Interestingly, arsenic's effects on transcriptional regulation of the estrogen receptor seem to be only inhibitory (Davey *et al.*, 2007). A recent study predicted that the GR pathway was a common mediator of metal-induced birth defects: indeed, arsenic-induced deficits, specifically neurodevelopmental toxicity, were prevented by inhibition of GR signaling in a chick embryo model (Ahir *et al.*, 2013). Oscillatory signaling of the HPA axis, in addition to proper GR localization for MAPK activation, is paramount for proper learning and memory. This signaling may be impaired in arsenic-exposed animals: while they have elevated circulating CORT, their ability to

initiate a proper HPA response is blunted after a stressor (Goggin *et al.*, 2012). Thus, the susceptibility of the GR to arsenic may play an important role in hippocampal-related deficits, including reduced learning and increased depressive-like symptoms (observed in rodent models) and may underlie mood and cognitive deficits seen in human studies as well.

2.5.5 Cholinergic signaling

Motor learning, cholinergic signaling, and locomotion are all affected by arsenic exposure in rodent models. Early studies demonstrated impaired motor coordination and delayed spontaneous alteration in rats chronically consuming arsenic (36 mg/L) for four months (Rodriguez *et al.*, 2002). However the increased locomotion reported in this study has been challenged by more recent work: while altered locomotion is a common behavior seen in arsenic-exposed mice, whether this behavior is hypo- or hyperlocomotion seems to be dependent on sex and the arsenic exposure paradigm (see Table 2.3 for studies). Low levels of arsenic exposure seem to induce hyperactivity in male mice, while high levels induce hypoactivity (Bardullas *et al.*, 2009), (Rodriguez *et al.*, 2010). Conversely, female mice consuming any level of arsenic display hyperlocomotion (Bardullas *et al.*, 2009). Subchronic exposures to arsenic increase locomotion but impair motor coordination in both sexes (Yadav *et al.*, 2009), but reports on *in utero* exposure are conflicting, with some suggesting impaired neural reflexes and others reporting no changes in locomotor behavior (Luo *et al.*, 2013), (Gandhi *et al.*, 2012). A similar dose relationship between locomotion and arsenic level occurs in the zebrafish model, where moderate arsenic levels reduced locomotion (line crossing) and

high levels increased the distance travelled (Baldissarelli *et al.*, 2012). Details on these studies, including dose, timing of exposure, and age of behavioral assessment are provided in Table 2.3. While parsing out verifiable locomotor actions from anxiety is difficult and likely to impact these studies, we can ascertain that high concentrations of arsenic induce hypolocomotion, moderate levels of arsenic induce hyperlocomotion, and low concentrations may induce no change in locomotion.

Altered motor coordination and locomotion could arise from aberrant cholinergic functioning. Several reports have noted reduced acetylcholinesterase (AChE) activity and choline acetyltransferase (ChAT) functioning after arsenic exposure. Female rats exposed to 20 mg/kg arsenic for 28 days displayed deficits in the transfer latency of the passive avoidance response and decreased labeling of muscarinic cholinergic receptors in the hippocampus and frontal cortex (Yadav *et al.*, 2011). These brain regions had reduced AChE activity and ChAT labeling after arsenic exposure as well. Interestingly, treatment with a bioactive component of the spice curcumin during arsenic exposure attenuated these observed effects in female rats (Yadav *et al.*, 2011). Exposure to less arsenic (5 mg/kg body weight) also inhibited AChE activity in the brain and was associated with poorer performance in operant learning (Nagaraja & Desiraju, 1994); another study demonstrated that AChE activity decreased with increasing arsenic concentrations in male rats after five days of exposure (Patlolla & Tchounwou, 2005). Our analyses have shown that mRNA of *AchE* is increased in the adult dentate gyrus after developmental arsenic exposure to 50 µg/L arsenic, suggesting a compensatory mechanism for altered AChE activity (Tyler & Allan, 2013). These studies provide support for arsenic in the etiology

of Alzheimer's disease, as more reports confirm reduced AchE and ChAT along with degeneration of cholinergic neurons in AD pathology (Mufson *et al.*, 2008).

2.5.6 Alzheimer's disease

Global cognitive impairments assessed after arsenic exposure have been associated with Alzheimer's disease (AD) in the human population; for an in-depth assessment of the relationship between arsenic and Alzheimer's disease, see (Gong & O'Bryant S, 2010). For the purposes of this review, we will discuss arsenic exposure in the context of experimental studies. *In vitro* research using a cholinergic neuronal cell line has demonstrated that sodium arsenite and dimethylarsenic acid (DMA) have different effects on APP protein levels, b-amyloid formation, and altered activity of AchE and ChAT (Zarazua *et al.*, 2011). These findings corroborate our own: we have found altered mRNA expression of genes associated with AD, including *Appb1* and *ApoE*, and *Ache* in the dentate gyrus of adult male mice after developmental exposure to 50 µg/L arsenic (Tyler & Allan, 2013).

For hallmark pathologies associated with AD, cell culture studies have demonstrated arsenic exposure increases b-amyloid protein and induces hyper-phosphorylation of tau protein, oxidative stress, inflammation, endothelial cell dysfunction, and angiogenesis (Giasson *et al.*, 2002). Interestingly, zinc has also been shown to induce tau phosphorylation through an ERK-sensitive mechanism (Kim *et al.*, 2011a); again, this effect may be due to a cation sensitive system (to zinc or arsenic) involving the Ras-MAPK/ERK pathway. In 2012, Piacentini and colleagues provided further evidence

supporting the role of arsenic in the etiology of AD. The authors found a significant correlation between a genetic polymorphism of the protein glutathione S-transferase *GSTO1-1* in a population of 120 AD patients compared to 114 healthy controls (OR=3.70); this transferase has been linked to both Alzheimer's and Parkinson's disease and is involved in arsenic metabolism, even in invertebrate systems (Whitbread *et al.*, 2005; Ortiz *et al.*, 2009; Piacentini *et al.*, 2012). While arsenic does not participate directly in REDOX reactions, it can induce oxidative stress via depletion of glutathione and impair REDOX reactions by inhibiting enzymes with sulfhydryl groups (Jomova & Valko, 2011); and oxidative damage is strongly associated with AD. In rodent models, arsenic exposure results in vascular endothelial dysfunction, impairment of learning and memory, altered nitrogen levels, and oxidative stress; all of which have been associated with symptoms of dementia (Sharma & Sharma, 2013). Treatment with a histone deacetylase inhibitor, sodium butyrate, attenuated these cognitive and vascular deficits observed in arsenic-exposed animals (Sharma & Sharma, 2013). Sodium butyrate has been used in the treatment of depression and improves cognitive skills in the Alzheimer's mouse model (APP/PS1-21) (Govindarajan *et al.*, 2011). Overall, we confidently state that arsenic imparts cognitive deficits associated with AD in rodent studies of exposure; however, very few epidemiological studies have investigated this relationship to date.

2.5.7 Monoaminergic signaling

Arsenic affects many transporter systems including the monoamines, dopamine (DA), serotonin (5-HT), and norepinephrine (NE). We have demonstrated that very low doses of arsenic (50 mg/L) during development increase 5-HT_{1A} receptors in the dorsal

hippocampus in adult offspring (Martinez *et al.*, 2008). Exposure to moderate levels of arsenic (1, 2, and 4 mg/L) in water for 60 days reduced levels of NE, DA, and 5-HT in both the cerebrum and cerebellum of 7-week old mice in a dose-dependent manner. mRNA levels of monoamine synthetases (including dopamine b-hydroxylase, tyrosine hydroxylase (TH), and tryptophan hydroxylase) were also all reduced after exposure to 4 mg/L arsenic (Liu *et al.*, 2013). Evidence from other studies using low to moderate doses of arsenic suggest differential effects on monoaminergic signaling based on sex, as observed in locomotion tasks. Four months of chronic exposure to 0.05, 0.5, 5.0 or 50 mg/L arsenic in drinking water decreased levels of DA in the striatum and hypothalamus in females (who also exhibited increased locomotion) but not in males (Bardullas *et al.*, 2009). TH and thioredoxin (Trx-1 A) levels were reduced in the striatum of males but not females; curiously, the opposite effect was observed in the nucleus accumbens where TH and Trx-1A were reduced in females but not males (Bardullas *et al.*, 2009). Conversely, in a different study on rats ingesting 50 mg/L arsenic in water, DA content in the striatum was increased in males, although no changes in DA, its metabolites, or serotonin were found in the prefrontal cortex or the nucleus accumbens (Rodriguez *et al.*, 2010). This group also reported alterations in mRNA of several antioxidant genes including superoxide dismutase (SOD), Trx-1, and NE and DA receptor genes in arsenic-exposed animals dependent on dose and region of interest in the brain (nucleus accumbens, prefrontal cortex, or striatum). Up-regulation of antioxidant factors (Trx-1 and SOD) indicate compensatory mechanisms to overcome the oxidative byproduct effects of arsenic toxicity on thiol containing enzymes related to arsenic metabolism (Rodriguez *et al.*, 2010). It is difficult to propose a mechanism of action for opposite effects based on

sex for different brain regions. However, evidence from these studies, while conflicting, suggest that arsenic may induce a complex dose-response relationship, which may not be linear, on locomotion and the nigrostriatal dopaminergic system as seen in other studies.

High levels of arsenic exposure, which have been reviewed elsewhere, produce more definitive deficits on dopaminergic and serotonergic signaling in the corpus striatum, hippocampus, and frontal cortex (Yadav *et al.*, 2009; Yadav *et al.*, 2010). Interestingly, treatment with curcumin in these studies attenuates deficits in monoamines and increases nitric oxide (NO) and TH expression, in both sexes. More details on therapeutic strategies to combat arsenic toxicity will be provided later.

2.5.8 Neurogenesis

Deficits in hippocampal-related behavioral tasks and increased depressive-like symptoms suggest that arsenic exposure induces changes in hippocampal morphology. Recent studies have demonstrated deficits in adult neurogenesis in the dentate gyrus of the hippocampus in both developmental and adult exposures to arsenic. Treatment for four months with 4 µg/L arsenic in drinking water reduced proliferation of neural progenitor cells and the number of mature neurons (Liu *et al.*, 2012). Developmental exposure of 50 mg/L arsenic (*in utero* and postnatal) altered differentiation but not proliferation of neural progenitor cells in the adult hippocampus at PND63 (Tyler & Allan, 2013). In both studies, deficits in adult neurogenesis were ameliorated either after cessation of the use of arsenic in water (Liu *et al.*, 2012) or with experience in an enriched environment (Tyler & Allan, 2013). *In vitro* studies using P19 pluripotent cells cultured with varying

concentrations of arsenic (7.5-75.0 µg/L) demonstrated that arsenic inhibited the formation of muscle and neuronal cells during P19 cell differentiation in a dose dependent manner. Deficits in neuronal differentiation may have resulted from reduced expression of transcription factors including neurogenin1, neurogenin 2, and NeuroD as compared to control P19 cultures. Further, *Nanog* expression increased during cell differentiation, suggesting that arsenic impacts differentiation but not proliferation (Hong & Bain, 2012a).

Altered hippocampal morphology in CA1, CA3 and the dentate gyrus was also observed during postnatal development after I.P. injection of 1, 1.5, and 2.0 mg/kg arsenic from PND4-11 (Kaler *et al.*, 2013). Two weeks of 100 µM exposure to arsenic reduced GFAP staining in the hippocampus in another study, indicative of less neural stem cells (Cronican *et al.*, 2013), while exposure to arsenic induced deficits in cell proliferation and increased apoptosis in the brains of zebrafish (Li *et al.*, 2009a). These studies suggest many different types of exposures to arsenic, including brief, chronic, adult, or developmental, can impact the morphology of the hippocampus. The effect of arsenic on neurogenesis may be mediated through a signaling mechanism already discussed: altered glucocorticoid and HPA axis function. Neurogenesis is particularly sensitive process in the brain, and as such, is prone insults like stress activity. Recent *in vitro* studies done in human hippocampal progenitor cells have demonstrated biphasic responses of adult neurogenesis to cortisol; of particular interest is that increased cortisol levels inhibit proliferation and differentiation possibly mediated by the GR (Anacker *et al.*, 2013). But the effect of the GR on adult neurogenesis is complex, as proposed by work showing

antidepressants mediate an increase in adult hippocampal neurogenesis by activating the GR (Anacker *et al.*, 2011b) as well. Arsenic has an impact on adult neurogenesis and hippocampal-dependent learning and memory and induces cognitive deficits and depressive-like symptoms; altered HPA axis regulation, and particularly GR signaling in the hippocampus could underlie all of these alterations seen in arsenic-exposed animals.

2.5.9 Summary of Mechanisms of Action

Arsenic can alter a multitude of systems in the brain. Of particular interest is HPA axis dysregulation that may underlie several behavioral deficits, particularly related to the hippocampus including deficits in adult neurogenesis and Ras-MAPK/ERK signaling. Additionally, arsenic seems to have an impact on cholinergic and monoaminergic signaling, though the mechanisms are not well understood at this point. Rodent studies have provided useful corroboration of the epidemiological evidence suggesting that a number of mechanisms could underlie cognitive deficits and mood disorders observed in human populations. More research focused on the dynamics of epigenetics, particularly on mechanisms of learning and memory and mood, will be important for understanding the impact of arsenic on the brain. While it is unlikely one common unifying mechanism for arsenic's effects will be identified, some clues on how naturally present cations, like zinc, interact with the system, may provide insight.

2.6 Therapeutics

2.6.1 Antidepressants

Some arsenic-induced deficits have been ameliorated with therapeutics. For example, in rodent studies, deficits in adult neurogenesis can be attenuated either by abolishment of exposure to arsenic water (Liu *et al.*, 2012), experience in an enriched environment (Tyler & Allan, 2013), or chronic antidepressant treatment (our unpublished observations). Reduced expression of monoamines after arsenic exposure can be reversed by treatment with curcumin (Yadav *et al.*, 2009) or taurine (Liu *et al.*, 2013). Studies described here have demonstrated that use of selenium, zinc, arsenic chelators, and environmental enrichment can attenuate systemic deficits due to arsenic exposure.

2.6.2 Selenium and zinc

The use of selenium as an anti-carcinogen and chemoprotective compound and its interaction with arsenic has been extensively studied in the cancer field. Selenium forms conjugates with arsenic using glutathione for excretion (Zeng *et al.*, 2005). A study on selenium concentrations in adults and children reported an inverse correlation between blood concentrations of selenium and urinary concentrations of arsenic (George *et al.*, 2013). In rodent studies, selenium concentrations in the cerebrum and cerebellum are reduced after exposure to 1-4 mg/L arsenic (Wang *et al.*, 2013). Treatment with selenium (Se) can attenuate some arsenic-induced deficits such as the inhibition of AchE in arsenic-exposed fish (*Channa punctatu*), particularly if Se treatment precedes arsenic exposure (Roy *et al.*, 2006). Another naturally occurring metal, zinc, is an important component of several enzymes involved in development and CNS function. Concurrent

exposure to 40 mg/kg arsenic and 4% w/v zinc in drinking water during pregnancy induced no teratological deficits or arsenic toxicity in male offspring, as seen in arsenic-only exposure. No changes were observed in sensory reflexes, glutathione, or motor behavior, suggesting that zinc treatment attenuated arsenic-induced deficits in mice (Ahmad *et al.*, 2013). Zinc has also been shown to be protective against arsenic-induced apoptosis in neurons *in vitro* (Milton *et al.*, 2004). The mechanism by which zinc prevents arsenic-induced toxicity has not been elucidated, but we hypothesize that the Ras-MAPK/ERK pathway is involved.

2.6.3 Arsenic chelators

In India, where efforts for intervention of arsenic-induced toxicity are at the forefront of research, the use of chelators has been suggested for attenuating the effects of chronic arsenic exposure, including thiol chelators that form insoluble complexes with arsenic (Flora & Pachauri, 2010). One such chelator is monoisoamyl meso-2-3-dimercaptosuccinic acid (MiADMSA), a lipophilic analog of DMSA and a strong thiol chelator. Five days of MiADMSA was provided for treatment of male rats after 10 weeks of exposure to 2 mg/kg arsenic. Compared to control animals, arsenic-exposed rats displayed decreased locomotor activity, fore and hind limb strength, reduced exploration, and impaired learning in the MWM. Additionally, reduced levels of malondialdehyde (MDA) and oxidative stress markers including superoxide dismutase, glutathione peroxidase, glutathione reductase, and glutathione transferase were measured in the cerebellum, hippocampus, and most notably, the frontal cortex. Treatment with

MiADMSA attenuated all of these effects but failed to restore performance back to control levels (Ram Kumar *et al.*, 2013).

Other notable interventions include high protein diet, antidepressants, curcumin, and exposure to enriched environments. Casein and pea protein, both of which have antioxidant proteins, protected against the effects of arsenic on reproductive measures in female rats (Mondal *et al.*, 2013), while curcumin in the diet attenuated the effects of arsenic on malonaldehyde (MDA), glutathione, and superoxide dismutase in several key brain regions, including decreased TH expression in the striatum (Yadav *et al.*, 2009). Our own studies of treatment with fluoxetine (Prozac) or exposure to an enriched environment after developmental arsenic exposure suggests that the insults arsenic imparts can be ameliorated (Tyler & Allan, 2013). Research on therapeutic approaches will become more pronounced as the number of people exposed to arsenic increases. However, efforts to implement safe water programs have been successful in reducing arsenic exposure in some parts of the world (Seow *et al.*, 2012). Arsenic remediation endeavors remain the most effective method for reducing exposure to this insidious toxic metalloid.

2.7 Conclusions concerning arsenic dysregulation in the brain

The most recent report provided by the ATSDR (Agency for Toxic Substances and Disease Registry) has suggested that toxic exposures to arsenic may result in memory loss and emotional instability in humans. Epidemiological studies from the past decade have provided a wealth of information supporting a strong correlation between arsenic

exposure and neurological and cognitive dysfunction in children and adults. These deficits seem to be dependent on concentration, timing, and duration of exposure, with cumulative arsenic inducing more severe insults. Developmental exposure to arsenic may impart damage on critical processes involved in programming in the brain. Research in rodent models has provided sufficient evidence to suggest that arsenic toxicity affects multiple systems and specific pathways involved in several aspects of learning, memory, movement, decision making, and mood. The next phases of research will delve deeper into mechanisms of action including epigenetics and specifically focus on therapeutics for the treatment of arsenic toxicity on the brain. More information on health and exposure during the intrauterine period could be helpful in ascertaining the true impact of arsenic exposure on the central nervous system. Additionally, more epidemiological studies, particularly in the United States, would be useful in determining if the current standard (10 µg/L) will be sufficient enough to block arsenic-induced neurotoxicity, and if not, what can be done, either legislatively or pharmaceutically to alleviate arsenic-mediated cognitive deficits.

3. Research Rationale, Hypothesis, and Specific Aims

3.1 Research Rationale

Major depressive disorder (MDD; depression) is the leading cause of disability worldwide, affecting more than 350 million people (Hyman *et al.*, 2006). A combination of factors, including genetic predisposition, individual experience, and environmental exposures contribute to the etiology of depression. Epidemiological studies in the United States and Bangladesh have shown that the incidence of depression in humans correlates with arsenic levels in drinking water. Arsenic, a common environmental contaminant, is a deadly toxin in high doses, a co-carcinogen in moderate doses and has been shown to negatively impact human cognition in low doses. Reports focusing on low doses of arsenic exposure demonstrate that even minimal amounts, previously considered safe by the Environmental Protection Agency (EPA), can have long-lasting cognitive consequences in adults and children. Prior work in our laboratory has demonstrated that perinatal exposure (during three trimester equivalents) to 50 parts-per-billion (ppb) arsenic induces deficits in the molecular constituents of the hypothalamic-pituitary-adrenal (HPA) axis, depressive-like symptoms, and deficits in hippocampal-dependent cognitive measures. The mechanism by which arsenic induces these alterations is still under investigation.

One particular mode of action may be aberrant adult neurogenesis. Patients with depression typically have hippocampal atrophy, and antidepressant treatment reverses this atrophy by increasing adult hippocampal neurogenesis (Boldrini *et al.*, 2013). It is

unclear if developmental insults, such as arsenic exposure, lead to depression due to impaired adult neurogenesis. Additionally, the regulation of adult neurogenesis is mediated by transcription factors and epigenetic modifications, which alter gene expression programs for either maintaining the neurogenic niche or stimulating maturation and integration of newborn neurons. Long-term exposure to arsenic has been shown to change the epigenome by reducing methyl donors (Zhao *et al.*, 1997), altering DNA methylation patterns in the hippocampus (Martinez *et al.*, 2011), and altering posttranslational modifications including histone methylation and acetylation in humans (Chu *et al.*, 2011; Chervona *et al.*, 2012b). Yet, the epigenetic link between developmental arsenic exposure and adult onset depression has not been elucidated to date.

3.2 Hypothesis

Based on this rationale, the overall goal of this dissertation has been to test the hypothesis that perinatal arsenic exposure induces epigenetic dysregulation of adult hippocampal neurogenesis leading to increased susceptibility to depression in adulthood.

3.3 Specific Aims

To test this hypothesis, I developed two specific aims with two sub aims to characterize the epigenetic and neurogenic link between arsenic exposure and depression. The first aim focuses on characterizing the impact of arsenic on the hippocampus, particularly molecular and morphological damage, and the potential reversibility of that damage. The

second aim focuses on determining the contribution of epigenetic dysregulation in the dentate gyrus as a potential molecular mechanism of action for arsenic exposure.

3.3.1 Aim 1: Determine the extent and reversibility of hippocampal damage induced by perinatal arsenic exposure in the adult mouse. My working hypothesis is that perinatal arsenic exposure results in deficits in adult neurogenesis and hippocampal-dependent behavioral tasks, both of which can be rescued by chronic antidepressant treatment. This first aim was developed to establish depression as an endophenotype of perinatal arsenic exposure by demonstrating etiologic and predicative validity of the behavior.

Aim 1A: Characterize depression as a behavioral endpoint after perinatal arsenic exposure with and without antidepressant treatment.

- Determine the stress response and behavioral outcomes of arsenic-exposed animals in the learned helplessness task and forced swim task after a mild stressor.
- Develop a chronic antidepressant treatment regime to increase neurogenesis.
- Determine the impact of chronic antidepressant treatment on the stress response and behavioral outcomes in each of the previous tasks.

Aim 1B: Assess adult neurogenesis in the hippocampus with and without antidepressant treatment.

- Determine the effect of a perinatal arsenic exposure on proliferation, differentiation, and survival of neural progenitor cells in the dentate gyrus using immunohistochemistry, confocal microscopy, and unbiased stereology.

- Determine the impact of an antidepressant on these components of hippocampal neurogenesis using the previously established treatment regime.
- Characterize the effect of perinatal arsenic exposure on a neurogenesis-dependent behavioral task and the potential amelioration of arsenic-induced deficits in this task.

3.3.2 Aim 2: Determine the impact of perinatal arsenic exposure on the epigenetic programming of neurogenesis-related genes. My working hypothesis is that perinatal arsenic exposure alters histone methylation and acetylation patterns on neurogenesis-related genes specifically in the dentate gyrus. This second aim was developed to determine if perinatal arsenic exposure induced long-lasting effects on the epigenome leading to altered gene regulation in adulthood.

Aim 2A: Characterize the effect of perinatal arsenic exposure on genetic programs associated with neurogenesis.

- Measure changes in gene expression of neurogenesis-related genes using qRT-PCR after perinatal arsenic exposure in the adult dentate gyrus.
- Determine the potential reversibility of genetic alterations by measuring the effect of a treatment regime that enhances neurogenesis (using qRT-PCR).

Aim 2B: Characterize the effect of perinatal arsenic exposure on the epigenetic landscape of the dentate gyrus.

- Determine the effect of perinatal arsenic exposure on histone posttranslational modifications by measuring global histone modifications specifically in the dentate gyrus via Western blot.
- Determine the effect of perinatal arsenic exposure on the protein expression of chromatin modifying proteins that impart histone modifications specifically in the dentate gyrus via Western blot.
- Assess the impact of perinatal arsenic exposure on the epigenetic regulation of neuronal genes by measuring the (histone) occupancy on genes expressed in the dentate gyrus (ChIP-Seq).

The expected outcomes from these aims include the identification of the molecular and morphological effects of development arsenic exposure and possible identification of novel epigenetic mechanisms that may be useful as therapeutic targets for treatment of depression. By evaluating the contribution of aberrant epigenetic regulation of adult neurogenesis to adult onset depression after a developmental environmental insult of arsenic, these studies will not only forward our understanding of environmental influences on epigenetics but also provide knowledge as to the impact developmental toxins have on adult depression, which may help to inform environmental toxin policy.

4.0 Adult hippocampal neurogenesis and mRNA expression are altered by perinatal arsenic exposure in mice and restored by brief exposure to enrichment

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Abstract

Arsenic is a common and pervasive environmental contaminant found in drinking water in varying concentrations depending on region. Exposure to arsenic induces behavioral and cognitive deficits in both human populations and in rodent models. The Environmental Protection Agency (EPA) standard for the allotment of arsenic in drinking water is in the parts-per-billion range, yet our lab has shown that 50 ppb arsenic exposure during development can have far-reaching consequences into adulthood, including deficits in learning and memory, which have been linked to altered adult neurogenesis. Given that the morphological impact of developmental arsenic exposure on the hippocampus is unknown, we sought to evaluate proliferation and differentiation of adult neural progenitor cells in the dentate gyrus after 50 ppb arsenic exposure throughout the perinatal period of development in mice (equivalent to all three trimesters in humans) using a BrdU pulse-chase assay. Proliferation of the neural progenitor population was decreased by 13% in arsenic-exposed mice, but was not significant. However, the number of differentiated cells was significantly decreased by 41% in arsenic-exposed mice compared to controls. Brief, daily exposure to environmental enrichment significantly increased proliferation and differentiation in both control and arsenic-exposed animals. Expression levels of 31% of neurogenesis-related genes including those involved in Alzheimer's disease, apoptosis, axonogenesis, growth, Notch signaling, and transcription factors were altered after arsenic exposure and restored after enrichment. Using a concentration previously considered safe by the EPA, perinatal arsenic exposure altered hippocampal morphology and gene expression, but did not inhibit the cellular neurogenic response to enrichment. It is possible that behavioral deficits observed during adulthood

in animals exposed to arsenic during development derive from the lack of differentiated neural progenitor cells necessary for hippocampal-dependent learning. This study is the first to determine the impact of arsenic exposure during development on adult hippocampal neurogenesis and related gene expression.

4.1 Introduction

Arsenic is a naturally occurring essential trace element found in soil deposits and water. Both natural and human sources contribute to the ubiquitous presence of arsenic in the environment; as such, several millions of people are exposed to this toxic metalloid in varying concentrations depending on location. Consistent exposure to arsenic results in a myriad of problems associated with almost every organ system in the body, including problems with renal, cardiovascular, reproductive, hepatic, and neurological systems (Brinkel *et al.*, 2009). It's well established that arsenic is a toxin in high doses and a co-carcinogen in moderate doses; the last decade of research has shown arsenic to be a neurotoxin in low doses. The current EPA standard for arsenic in water is 10 parts per billion; however, many people in rural areas and developing countries, where arsenic is not regulated, are exposed to much higher levels of arsenic in their water. Developmental and continuous arsenic exposure induces significant deficits in long-term memory in children, as measured by the Wechsler Intelligence Scale for Children (WISC) (Calderon *et al.*, 2001). Other research has revealed considerable deficits in learning and memory after several methods of arsenic exposure in both rodent models and in humans (Nagaraja & Desiraju, 1994; Calderon *et al.*, 2001; Chattopadhyay *et al.*, 2002; Rodriguez *et al.*, 2002; von Ehrenstein *et al.*, 2007; Wasserman *et al.*, 2007; Martinez-Finley *et al.*, 2009;

O'Bryant *et al.*, 2011). Additionally, developmental arsenic exposure alters cerebellar morphology in the brain and disrupts cell cycle dynamics of neuroepithelial cells *in vitro* (Sidhu *et al.*, 2006; Dhar *et al.*, 2007). Recently, we have demonstrated that developmental exposure to arsenic alters components of the hypothalamus-pituitary-adrenal (HPA) axis (Goggin *et al.*, 2012) including increased hypothalamic corticotrophin releasing hormone (CRH), altered corticosterone (CORT) secretion (both at baseline and in response to a stressor), decreased hippocampal 11 β -HSD 1, and altered subcellular glucocorticoid receptor (GR) distribution in the hypothalamus. We have also reported deficits in learning tasks, including 8-way radial arm, novel object exploration, learned helplessness, and forced swim tasks, some of which are hippocampal-dependent (Martinez *et al.*, 2008; Martinez-Finley *et al.*, 2009). The underlying mechanism responsible for these behavioral outcomes in adulthood after developmental exposure to arsenic has not been elucidated to date.

Of the two neurogenic regions of the brain capable of continual mitosis, adult neurogenesis in the dentate gyrus of the hippocampus has been implicated as an integral component of hippocampal-dependent learning and memory (Shors *et al.*, 2002; Deng *et al.*, 2010; Sahay *et al.*, 2011). Adult neurogenesis involves proliferation of a neural progenitor cell (NPC) population comprised of neural stem cells left over from development and transient amplifying cells, differentiation of daughter cells from NPC into either glia or neurons, and survival and integration of these new cells into the hippocampal circuitry. Research conducted over the last decade has shown that ablation of adult neurogenesis in the hippocampus results in substantial deficits in learning and

memory and that these new neurons are required for integration of new memories (Jessberger *et al.*, 2009; Shors *et al.*, 2012). This budding population of cells seems particularly sensitive to environmental factors, as evidenced by decreased adult neurogenesis after exposure of several different neurotoxins, including adult or fetal exposure to alcohol (Nixon & Crews, 2002; Klintsova *et al.*, 2007; Gil-Mohapel *et al.*, 2011) or nicotine (Abrous *et al.*, 2002; Shingo & Kito, 2005), and fetal exposure to lead (Gilbert *et al.*, 2005; Jaako-Movits *et al.*, 2005; Verina *et al.*, 2007) or methylmercury (Faustman *et al.*, 2002; Falluel-Morel *et al.*, 2007). This sensitivity in the hippocampus coupled with the vulnerability of the brain during development may result in an environment that is susceptible to damage even with low concentrations of toxin. However, the neurogenic environment shows plasticity to extrinsic factors like antidepressants, exercise, novelty, and environmental enrichment with toys and social interaction, all of which increase adult neurogenesis and learning (Kempermann *et al.*, 1997; van Praag *et al.*, 1999a; Malberg *et al.*, 2000). Environmental enrichment also enhances cognitive performance, decreases depression and anxiety, and increases proliferation and differentiation of adult neural progenitor cells (Malberg *et al.*, 2000; Koh *et al.*, 2007; Zhao *et al.*, 2008; Fabel *et al.*, 2009).

Based on our previously published behavioral data and research supporting the link between learning and memory and adult neurogenesis, we hypothesized that our environmentally relevant perinatal arsenic exposure model induces deficits in proliferation and differentiation of neural progenitor cells in the dentate gyrus of exposed animals. This deficit may be responsible for the behavioral outcomes we observe in these

animals (Martinez *et al.*, 2008; Martinez-Finley *et al.*, 2009). We found that developmental exposure to a low concentration of arsenic (50 parts per billion, ppb) over the first three trimester equivalents significantly decreased the number of differentiated neural progenitor cells but did not affect proliferation of these cells in adult mice. Additionally, perinatal arsenic exposure altered 31% of target neurogenesis-related genes as well, including several involved in growth and differentiation in adult animals. Brief exposure to an enriched environment increased both proliferation and differentiation of neural progenitor cells in both control and arsenic-exposed animals and significantly reversed mRNA expression of aberrantly expressed neurogenesis genes. Thus, it is possible that impaired hippocampal adult neurogenesis is a candidate mechanism by which developmental arsenic exposure induces deficits in learning and memory seen in adulthood.

4.2 Methods

Note: Arsenic is a known human carcinogen, and all arsenicals were handled with caution according to MSDS standards.

4.2.1 Ethics Statement

All experiments were performed in accordance with protocols approved by the University of New Mexico Institutional Animal Care and Use Committee (Protocol number: 11-100679 HSC).

4.2.2 Perinatal Arsenic Exposure Paradigm

The arsenic exposure paradigm was performed as previously described (Martinez *et al.*, 2008). Briefly, C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) were bred and maintained in the University of New Mexico (UNM) Animal Research Facility on a 12-hour reverse light/dark cycle with lights off at 0800 hr. Animals had *ad libitum* access to food and water in a temperature controlled (22°C) room. Female mice were assigned to either control (arsenic-free) or arsenic exposure groups and acclimated for seven days prior to mating. Fifty parts per billion (ppb) arsenic-treated water was prepared from sodium arsenate (Sigma-Aldrich, St. Louis, MO) and ultra pure water from a Milli-Q Plus purification system (Millipore, Billerica, MA). Dams were provided *ad libitum* access to 50 ppb arsenic (equivalent to 50 µg/L) or normal tap water during breeding and pregnancy until pups were weaned at approximately PD23–25. After weaning, pups were group housed, four per cage, with *ad libitum* access to food and normal tap water (Figure 4.1).

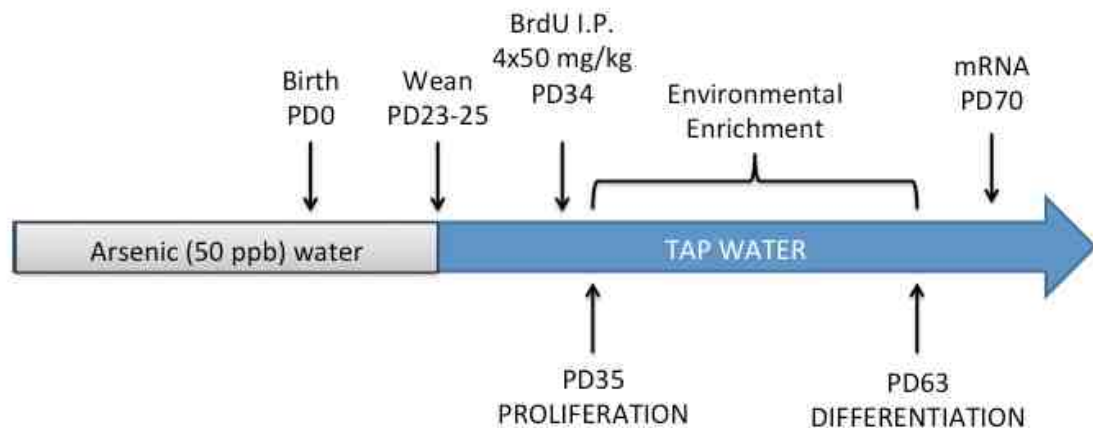


Figure 4.1. Arsenic exposure paradigm and subsequent BrdU injection strategy and experience in environmental enrichment

Male mice were used for all studies with only one animal from each litter to eliminate litter effects, $n = 7-9$ mice per group. The UNM Health Sciences Center Institutional Animal Care and Use Committee approved all the procedures described in these studies. As described in our previous papers, the arsenic concentrations were 56 ppb in the arsenic-treated water and 6 ppb in standard tap water (Martinez *et al.*, 2008; Martinez-Finley *et al.*, 2009). Average daily water consumption is not significantly different between arsenic-exposed and control groups. We have previously assessed total arsenic concentration in whole brains of animals: perinatal arsenic exposure results in 2.24 ± 0.2 ppb arsenic in whole brains at PD35, which is significantly greater than 1.0 ± 0.24 ppb arsenic determined in age-matched control animals on tap water (Martinez *et al.*, 2008; Martinez-Finley *et al.*, 2009).

4.2.3 Enriched Environment Paradigm

For enrichment studies, PD35 male mice were separated into two groups: one with daily exposure to enrichment (enriched environment, EE) and one without exposure to enrichment (no enrichment, NE). Mice used for proliferation assessment were exposed to an enriched environment (EE) for 2–4 hours per day for five days after which proliferation was assessed at PD40 for both EE and NE groups. Mice used for differentiation assessment were exposed to an enriched environment for 2–4 hours per day for 28 days (approximately one month post last-BrdU injection) after which differentiation was assessed at PD63 for EE and NE groups. No enrichment control groups consisted of both perinatally arsenic-exposed animals and unexposed animals in

which neither group had exposure to enrichment. The enriched environment consisted of toys (ladder, plastic house, chew toys) and a running wheel in a large housing container (48 cm × 27 cm × 20 cm). Toys were exchanged every other day, but the running wheel was kept throughout the exposure paradigm. The location of all the objects was changed every other day. All NE control groups (perinatal arsenic or control) and all enriched groups (perinatal arsenic or control) were maintained in standard housing (28 cm × 18 cm × 13 cm). Each standard housing cage contained two to three mice, while the enrichment cages contained four to six mice; the same four to six mice were exposed to enrichment together each day. All groups were given *ad libitum* access to food and tap water while in the enriched environment and in their own standard housing.

4.2.4 Histological Evaluation of Neurogenesis

To analyze adult neurogenesis, 5-bromo-2'-deoxyuridine (97% Sigma-Aldrich, St. Louis, MO) BrdU was used to label neural progenitor cells (NPC) in the dentate gyrus (DG) of the hippocampus of PD34 male mice for all groups. To reduce BrdU toxicity and double labeling of neural progenitor cells, a 12-hour, 4-injection paradigm was used with a low concentration of BrdU (50 mg/kg made in 0.9% sterile NaCl) (Hayes & Nowakowski, 2002). Assessment of proliferation or differentiation was performed either 12 hours (PD35) or 4 weeks (PD63) following the final BrdU injection, respectively. Brain slices were obtained according to an earlier published protocol (Guo *et al.*, 2011). Briefly, mice were euthanized with an i.p. injection of sodium pentobarbital followed by transcardiac perfusion with 4% PFA. Brains were dissected and post-fixed overnight in 4% PFA followed by equilibration in 30% sucrose for 48 hours. Brains were frozen in 30%

sucrose and 40 μm coronal sections were obtained using a sliding microtome; tissue was stored at -20°C in 96-well plates with cryoprotectant (1:2:2 by volume glycerol, ethylene glycol, and 0.1M PBS, pH 7.4). Immunohistological analysis for every 1-in-6 serial, floating, 40 μm section (240 μm apart) for the rostral-caudal extent of the hippocampus was performed. Free-floating sections were washed with 0.1 M TBS (0.15 M NaCl, 3 mM KCl, 30 mM Tris-base, pH 7.4) for 15 minutes. Sections were incubated for 30 minutes in 1 N HCl at 37°C , rinsed in 0.1 M borate buffer for 30 minutes, and rinsed several times in 0.1 M TBS. After one hour incubation in TBS-T (0.1 M TBS, 0.1% Triton X, and 3% normal goat serum), sections were incubated in primary antibodies BrdU (rat, 1:100, Abcam), Ki67 (mouse, 1:200, Vector), NeuN (mouse, 1:500, Millipore), doublecortin (rat, 1:500, Cell Signaling), and DAPI (1:1000, Sigma) in TBS-T for 72 hours at 4°C . Sections were washed in 0.1 M TBS and incubated for one hour in appropriate fluorescent secondary antibodies (Alexa 488; Cy5; Cy3). Sections were mounted, coverslipped with DABCO, and maintained at 4°C in the dark until analysis; a minimum of $n = 7$ was analyzed for each group.

4.2.5 Stereology

StereoInvestigator software (Microbrightfield, Wiliston, VT) was used for assessment of adult neurogenesis. Both proliferation and differentiation were scored for at least 10, 1-in-6 serial sections throughout the rostral-caudal extent of the granule cell layer of the dentate gyrus for at least seven animals per group. BrdU with Ki67 positive cell counts were limited to the granule cell layer and subgranular zone next to the hilus; this was accomplished using StereoInvestigator to outline digital contours around each dentate

gyrus for each section using a 10X objective. BrdU positive cells that were more than three nuclear diameters from the base of the granule cell layer or more than two nuclear diameters into the hilus were not counted, as only progenitor cells lie within this region. A two-micron guard zone was used with a 25 µm optical fractionator probe and a 40X objective for counting cells. As arsenic has been shown to induce DNA damage (Piao *et al.*, 2005), proliferation was assessed via colocalization of BrdU positive cells (BrdU⁺) with Ki67 positive (Ki67⁺) cells using unbiased stereology. Cells expressing only BrdU or only Ki67 were not counted; although, very few cells had BrdU⁺ labeling only. One month after the final BrdU injection, differentiation of progenitors was assessed via BrdU colocalized with either doublecortin (DCX) or BrdU colocalization with NeuN positive cells. Counts for both types of differentiated cells (immature DCX⁺ and mature NeuN⁺) were pooled together to determine the total impact of arsenic exposure on the differentiation capacity of NPC. Results are presented as cell counts directly from the StereoInvestigator analysis for the total number of cells throughout the entire dentate gyrus per animal. This assessment takes into account the section sampling fraction (SSF), the area subfraction (ASF), the thickness subfraction (TSF) and the total number of cells counted (Q): $N=Q/(SSF \times ASF \times TSF)$. Phenotype analysis was performed for 100 randomly selected BrdU positive cells per animal for differentiation studies. Phenotypes were counted as BrdU⁺, BrdU⁺DCX⁺, or BrdU⁺NeuN⁺.

4.2.6 Confocal Microscopy

Images used for stereological analysis were collected using an Olympus DSU spinning disk confocal inverted IX-81 microscope endowed with argon and HeNe lasers. A Z-

stack composite was obtained using a 40X objective throughout the orthogonal plane with appropriate filters for each secondary antibody for 10 slices per brain. A guard zone of 2 μm was used along with a probe of 25 μm . Cell bodies within the guard zone were not counted. Images for publication were acquired using a Zeiss LSM510 META confocal microscope endowed with a laser diode, one argon laser, and two HeNe lasers. Colocalization was visualized using a 63X oil objective (Figure 4.2A, 4.3A), while maximum intensity projections of Z-stacks were acquired with a 20X objective.

4.2.7 Real-time PCR analysis

The dentate gyrus of the hippocampus in PD70 male mice was isolated, microdissected as described by Hagihara and colleagues, snap frozen in liquid nitrogen, and stored at -80°C until use (Hagihara *et al.*, 2009; Brady *et al.*, 2013), $n = 6$ litters/group, where one mouse is used per litter to eliminate litter effects. Total RNA was extracted from homogenized frozen tissue using the QIAshredder homogenizer (Qiagen, Valencia, CA) and the RNeasy Mini Kit (Qiagen, Valencia, CA). The concentration of mRNA was assessed using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE); the 260/280 absorbance ratio for all RNA was ~ 2.0 . Isolated 750 ng mRNA was converted to cDNA using the RT2 First Strand Kit (SABiosciences, Frederick, MD). Real-time PCR analysis was performed using the RT2 Profiler Mouse Neurogenesis PCR Array (PAMM-404Z, SABiosciences) according to the manufacturer's instructions on an ABI 7300 Real-Time PCR System (Applied Biosystems). No template controls and RT controls were on the microarray. Gene of interest (GOI) CT values were normalized to the average CT value of four housekeeping genes (β -actin, B2m, GAPDH, Hsp90ab1),

and subsequent ΔCT values were assessed using the comparative CT method ($\Delta\Delta\text{CT}$) (Livak & Schmittgen, 2001). Results are expressed as fold change, $2^{-\Delta\text{CT}(\text{GOI-T})}/2^{-\Delta\text{CT}(\text{GOI-C})}$, where GOI-T refers to the gene of interest in a particular treatment group and GOI-C to the same gene of interest but in the control group. Fold change was measured as the ratio of the $2^{-\Delta\text{CT}}$ of the GOI under exposure conditions (e.g. arsenic or enrichment) to the $2^{-\Delta\text{CT}}$ under control conditions (e.g. tap water or no enrichment) for a particular GOI. Fold regulation was calculated as $-1/2^{-\Delta\text{CT}}$ for all fold changes less than one.

4.2.8 Statistical Analysis

Neurogenesis data was analyzed using one-way ANOVA (SPSS, v.19; Chicago, IL) with post-hoc analysis performed as needed and corrected for multiple comparisons with Bonferroni, $n = 7-9$ mice per group. Gene expression data was analyzed using the Student's t-test, $n = 6$ mice per group with Bonferroni corrections when multiple comparisons were performed.

4.3 Results

4.3.1 Perinatal arsenic exposure did not alter proliferation of neural progenitor cells

Proliferation of neural progenitor cells (NPC) was measured using colocalization of BrdU, an exogenous S-phase marker, and Ki67, an endogenous cell proliferation marker (Figure 4.2A). The use of two markers was necessary, as arsenic has been shown to induce DNA damage, and BrdU may label cells with DNA damage; thus, as Ki67 expression mimics that of BrdU within the proliferative zone, it was used to confirm the BrdU⁺ cell counts, validate the pulse chase assay, and to ensure measurement of an actively proliferating

population of neural progenitors. While it is more common to label with one or the other marker, BrdU⁺ only cell counts may overestimate proliferation of NPC and Ki67⁺ only cell counts do not give an accurate birthdate for cells. However, the disadvantage of double labeling with both BrdU and Ki67 is that cell counts do not include the very small quantity of BrdU⁺ labeled cells that exit the cell cycle within 12 hours of the injections; we found this amount to be negligible compared to the total counts of BrdU⁺Ki67⁺ for proliferation. The labeling method used here provides a depiction of a population of cells that includes neural stem cells and their progeny, transient amplifying cells, which can give rise to neurons. Four injections of 50 mg/kg BrdU ($n = 7-9$ mice/group) were given over a 12-hour period to PD34 male mice. Twelve hours after the last BrdU injection, the animals were sacrificed and adult neurogenesis was measured. Labeling with BrdU⁺ and Ki67⁺ resulted in an average of 3973 ± 497 NPC in the DG of control animals and 3465 ± 372 NPC in the arsenic-exposed animals (Figure 4.2B, 4.2C). Thus, arsenic-exposed mice have 13% less NPC than their control counterparts. Cells that were only labeled with BrdU or Ki67 or more than three nuclei away from the subgranular zone of the dentate gyrus were not counted as proliferating progenitor cells. One-way analysis of variance (ANOVA) assessing the effect of treatment (arsenic water versus tap water) on proliferation showed that average cell counts are not significantly different from one another. As such, perinatal exposure of 50 ppb arsenic (throughout all three trimester equivalents in mice) did not alter the proliferation of NPC in the DG of the hippocampus at PD35 (Figure 4.2D).

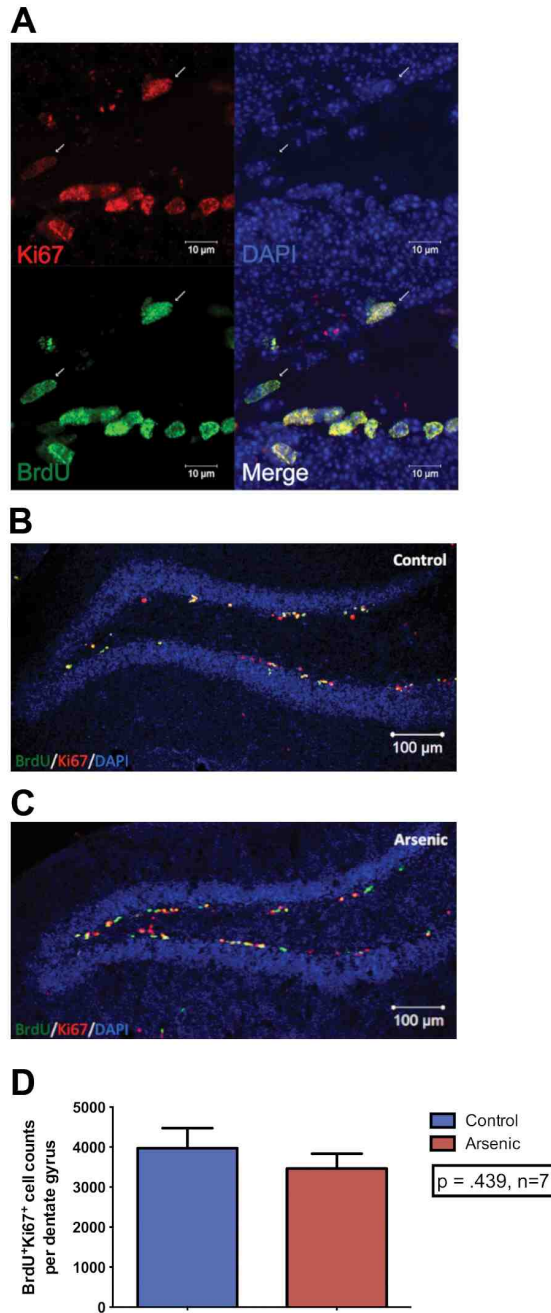


Figure 4.2. Perinatal arsenic exposure does not alter proliferation at PD35 in adult male mice.

Representative images of neural progenitor cell (NPC) proliferation at PD35 in the dentate gyrus: (A) Colocalization of Ki67 (endogenous mitotic marker) and BrdU (exogenous S-phase marker) determine the proliferating pool of neural progenitor cells in (B) control and (C) arsenic-exposed animals. (D) There was no significant effect of treatment on the proliferation of neural progenitor cells, $n = 7-9$ mice per group (each from different litters). Results are expressed as the mean \pm SEM. DAPI (blue), BrdU (green), Ki67 (red).

4.3.2 Perinatal arsenic exposure reduced the number of differentiated neural progenitor cells

Differentiation of NPC was measured using colocalization BrdU with either doublecortin (DCX), a marker of neuroblasts and immature neurons, or NeuN, a marker of mature neurons (Figures 4.3A, 4.3B). Since the impact of arsenic on the differentiation capacity of NPC is not known, we assessed overall differentiation, including the generation of neuroblasts and immature neurons (DCX⁺) and mature neurons (NeuN⁺). Differentiation was measured four weeks after the last BrdU injection to allow for maturation of BrdU-labeled cells ($n = 7-9$ mice/group). Figures 4.3A and 4.3B show representative images of colocalization of BrdU with DCX (BrdU⁺DCX⁺) and BrdU with NeuN (BrdU⁺NeuN⁺): both types of cell counts were used for assessment of total differentiation. Analysis of phenotype indicated that the relative amounts of each cell type were unchanged between groups: BrdU⁺ labeling (~25%), BrdU⁺DCX⁺ (~15%), and BrdU⁺NeuN⁺ (~60%). From the proliferation data, a significant quantity (approximately 3500) of NPC was labeled in both control and arsenic-exposed animals. In control animals, there were 1997 ± 178 BrdU⁺/DCX⁺ and BrdU⁺NeuN⁺ labeled cells (Figure 4.3C), while in the arsenic-exposed animals, there were 1178 ± 252 BrdU⁺DCX⁺ and BrdU⁺NeuN⁺ labeled cells. This translates into a 41% decrease in the number of differentiated cells in arsenic-exposed animals as compared to their control counterparts (Figure 4.3D). One-way ANOVA revealed a significant effect of arsenic exposure on differentiation of NPC in the DG ($F(1,13)=7.311, p=.018$). Thus, perinatal exposure of 50 ppb arsenic (throughout all three trimester equivalents in mice) reduced the number of differentiated NPC in the DG of the hippocampus at PD63 (Figure 4.3E).

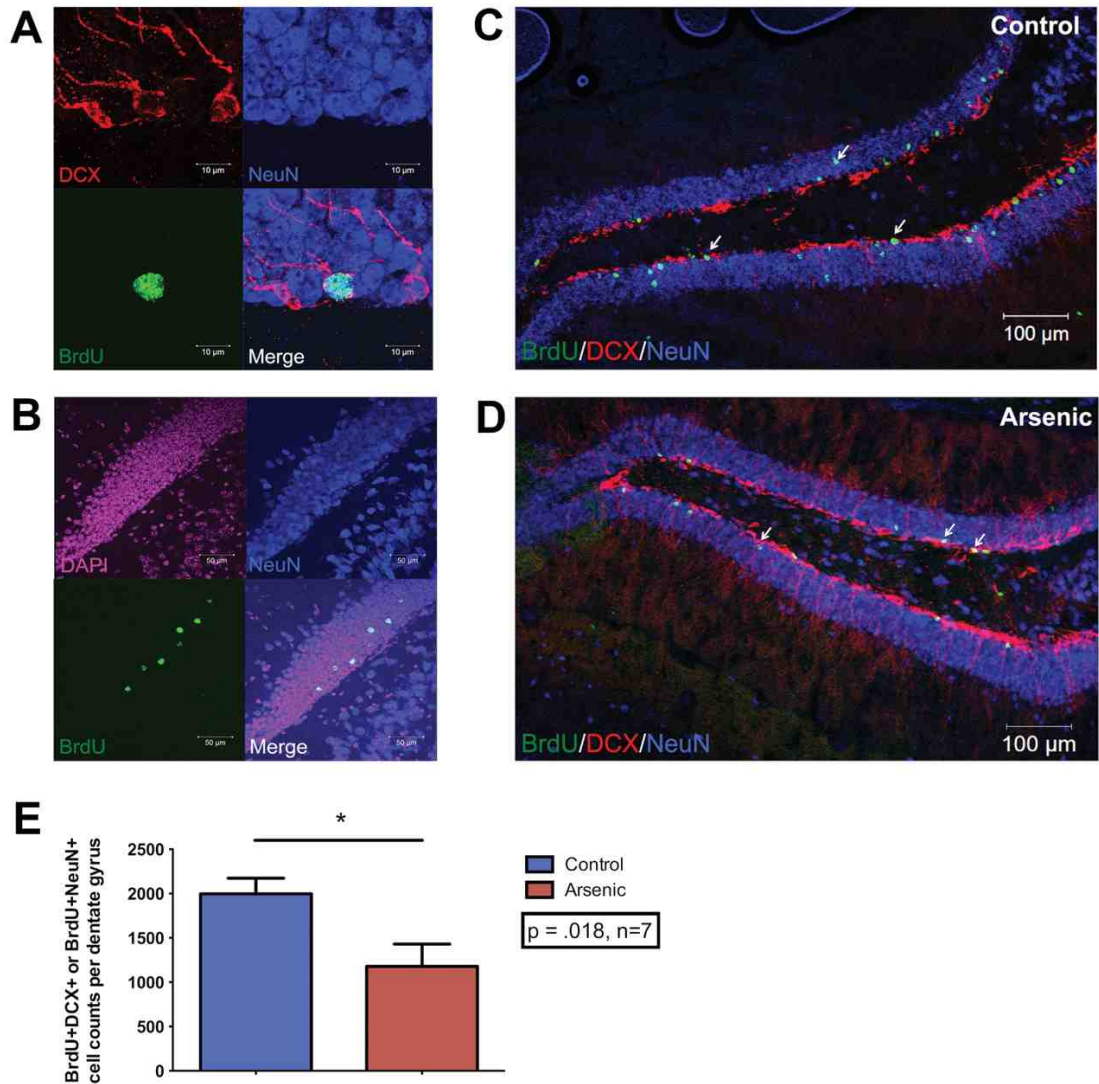


Figure 4.3. Perinatal arsenic exposure decreases the number of differentiated neural progenitor cells at PD63 in adult male mice (A) Colocalization of doublecortin (immature neuron marker DCX) or NeuN (mature neuron marker) and BrdU determine differentiated neurons derived from labeled neural progenitor cells. (B) Colocalization of BrdU with NeuN in the dentate gyrus reveals mature daughter cells of BrdU-labeled progenitor cells, (BrdU, green; DAPI, pink; NeuN blue). Arrows indicate colocalization of marks and cells that were counted. Representative images of neural progenitor differentiation at PD63 in the dentate gyrus of (C) control and (D) arsenic-exposed animals. (E) There was a significant effect of treatment on differentiation of neural progenitor cells; BrdU+DCX+ and BrdU+NeuN+ were significantly decreased after perinatal arsenic exposure, $n = 7-9$ mice per group (each from different litters). Results are expressed as the mean \pm SEM. BrdU (green), DCX (red), NeuN (blue). $*p < .05$.

4.3.3 Exposure to an enriched environment increased proliferation of neural progenitor cells in control animals

Using the same BrdU injection paradigm, NPC were labeled in four sets of PD34 mice: control and arsenic-exposed animals that were exposed to enrichment and control and arsenic-exposed animals that were not exposed to enrichment. After five days of daily, brief (2–4 hours) exposure to enrichment after which animals were returned to their home cages, proliferation of NPC was assessed via BrdU and Ki67 colocalization in the subgranular zone of the dentate gyrus of PD40 animals ($n = 7-9$ mice/group). Figure 4.2A shows a representative image of colocalization of BrdU with Ki67 for proliferation assessment. Assessment of proliferation resulted in 6753.64 ± 1372.1 BrdU⁺Ki67⁺ labeled cells in control animals exposed to enrichment: this constitutes a 70% increase over control animals not exposed to enrichment. In arsenic-exposed animals exposed to enrichment, there were 5312.96 ± 1935.95 BrdU⁺Ki67⁺ labeled cells: this constitutes a 53% increase over arsenic-exposed animals not exposed to enrichment and a 33% increase over control animals not exposed to enrichment (Figure 4.4A, 4.4C). Figure 4.4A shows a representative image of the DG from an arsenic-exposed animal with experience in enrichment. One-way ANOVA indicated a significant effect of enrichment on proliferation regardless of perinatal treatment ($F(1,21)=13.87$, $p=.001$). Using a Student's t-test with Bonferonni correction, brief exposure to enrichment slightly increased proliferation for arsenic-exposed animals but was not significant (Figure 4.4C, $p=.061$); however, exposure to enrichment did significantly increase proliferation for control mice (Figure 4.4C, $p=.008$). There is no statistically significant difference in the average cell counts between both groups exposed to enrichment. Regardless of perinatal

exposure, NPC proliferation in the DG is enhanced after five days of brief exposure to enrichment, though this increase is only statistically significant in control animals.

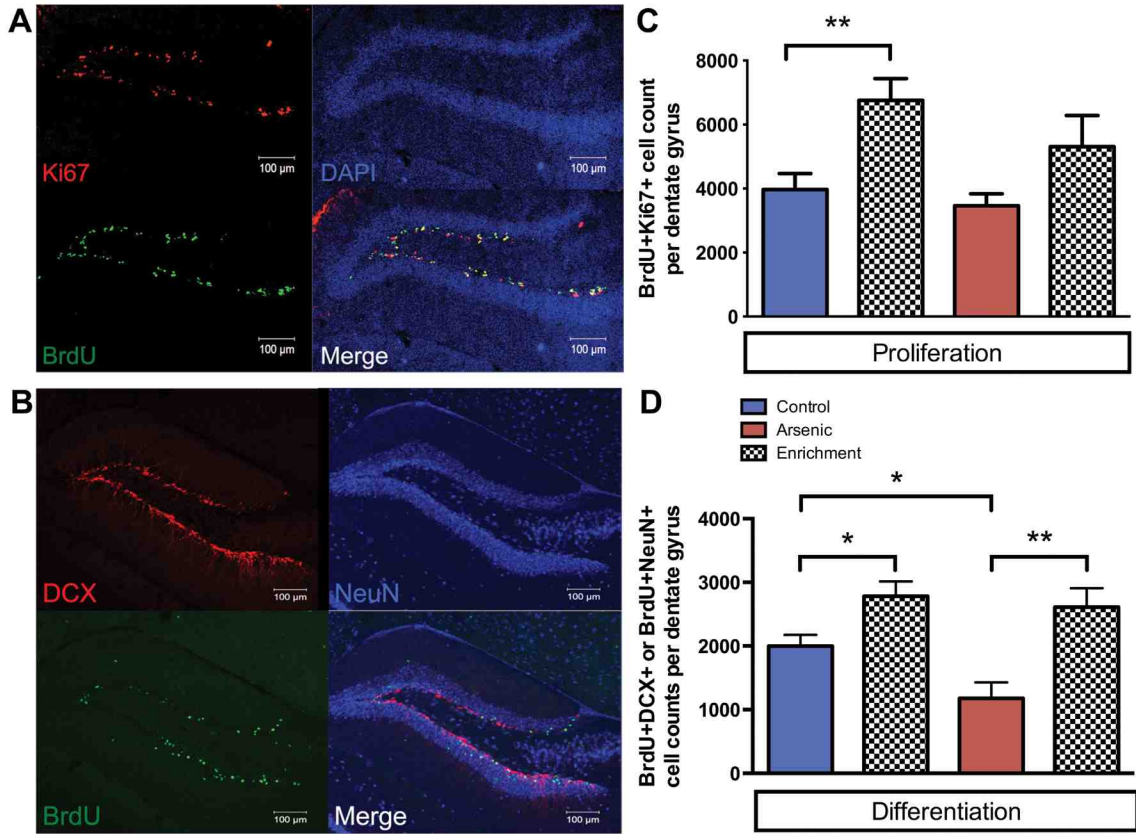


Figure 4.4. Brief, daily exposure to enrichment increases proliferation and differentiation in control and arsenic-exposed male mice. Representative images of increased (A) proliferation after brief exposure to enrichment for five days in arsenic-exposed animals at PD40 (BrdU (green), Ki67 (red), DAPI (blue)) and increased (B) differentiation after brief, daily exposure to enrichment for one month in arsenic-exposed animals at PD63 (BrdU (green), DCX (red), NeuN (blue)). (C) Perinatal arsenic exposure did not affect proliferation of NPC; exposure to enrichment for five days increased NPC proliferation at PD40 in both arsenic-exposed and control mice but was only significant in controls. (D) Perinatal arsenic exposure decreased the total number of differentiated cells (BrdU+DCX+ and BrdU+NeuN+); brief, daily exposure to enrichment for one-month increased differentiation in both arsenic-exposed and control animals at PD63, n=7–9 mice per group, each from different litters. (**p<.01, *p<.05).

4.3.4 Exposure to an enriched environment increased the number of differentiated neural progenitor cells in all animals

Using the same BrdU injection paradigm, NPC were labeled in four sets of PD34 mice: control and arsenic-exposed animals that were exposed to enrichment for one month and control and arsenic-exposed animals that were not exposed to enrichment ($n = 7-9$ mice/group). After brief (2-4 hours), daily enrichment for 28 days, colocalization of BrdU with DCX (BrdU⁺DCX⁺) and BrdU with NeuN (BrdU⁺NeuN⁺) in the DG was assessed at PD63 in all four groups (Figure 4.3A, 4.3B). One-way ANOVA showed a significant effect of enrichment on differentiation for both controls and arsenic-exposed animals regardless of perinatal exposure ($F(1,21)=14.710$, $p=.001$). Analysis of phenotype indicated no significant difference in the percent of cell types between the two groups exposed to enrichment (data not shown), similar to that seen in groups with no exposure to enrichment. For animals exposed to enrichment, colocalization revealed 2778 ± 232 BrdU⁺DCX⁺ and BrdU⁺NeuN⁺ labeled cells in control mice, a 39% increase over control animals without exposure to enrichment. In arsenic-exposed mice with exposure to enrichment, colocalization revealed 2613 ± 294 BrdU⁺DCX⁺ and BrdU⁺NeuN⁺ labeled cells, a 121% increase over arsenic-exposed animals without exposure to enrichment and a 31% increase over control animals not exposed to enrichment (Figure 4.4B, 4.4D). Figure 4.4B shows a representative image of the DG from an arsenic-exposed animal with one month of exposure to enrichment. Using a Student's t-test with a Bonferroni correction, brief exposure to enrichment significantly increased differentiation for arsenic-exposed mice (Figure 4.4D, $p=.006$) and control mice (Figure 4.4D, $p=.027$). These results show that regardless of perinatal exposure, the number of differentiated

NPC in the DG significantly increased after one month of brief exposure to enrichment in both control and arsenic-exposed animals.

4.3.5 Perinatal arsenic exposure altered mRNA expression of neurogenesis-related genes

To analyze several genes associated with neurogenesis, we compared gene expression profiles between control and arsenic-exposed animals using a microarray on microdissected dentate gyrus tissue from PD70 animals ($n = 6$ mice/group). All CT values of genes of interest (GOI) were normalized to the average CT value of four housekeeping genes (b-actin, B2m, GAPDH, Hsp90ab1), and subsequent Δ CT values were assessed using the comparative CT method ($\Delta\Delta$ CT) (Livak & Schmittgen, 2001). Fold change was measured as $2^{-\Delta\Delta\text{CT}}$ (arsenic or enrichment)/ $2^{-\Delta\text{CT}}$ (control or no enrichment) for each GOI. Fold regulation is expressed as the negative inverse for negative fold changes. A Student's t-test was used to compare exposure conditions to control conditions. Table 4.1 shows gene expression of arsenic-exposed adult males compared to age-matched control animals. Of the 82 genes on the microarray, 13.4% were significantly up-regulated ($p < .05$): these included Apbb1, Apoe, Ntn, Ache, and Adora1, which are involved in apoptosis and in Alzheimer's disease. Perinatal arsenic exposure resulted in down-regulation ($p < .05$) of 18.3% of the genes on the array. These included genes involved in axonogenesis and neurite growth (Dcx, Tnr, Robo1, Mtap2), growth factors (Ptn, Odz, Fgf2), transcription factors (Pax6 and Creb1), synaptic signaling molecules (Nf1 and Chrm2), and Notch and TGFb signaling molecules (Ascl1, Hey2, Nrg1, and Tgfb1) as seen in Figure 4.5.

Table 4.1 Aberrant expression of neurogenesis-related genes in perinatal arsenic-exposed mice compared to control age-matched animals.

Accession Number	Gene Title	Gene Symbol	Fold Regulation	p-value	Function
NM_009599	Acetylcholinesterase	Ache	1.676	<.01	Apoptosis; Synaptogenesis
NM_001008533	Adenosine A1 receptor	Adora1	1.145	<.05	Apoptosis; GPCR Signaling
NM_009685	Amyloid beta (A4) precursor protein-binding, family B, member 1	Apbb1	3.042	<.01	Apoptosis; Axonogenesis
NM_009696	Apolipoprotein E	Apoe	1.979	<.01	Synaptic Transmission
NM_008553	Achaete-scute complex homolog 1	Ascl1	-1.374	<.01	Notch Signaling
NM_203491	Cholinergic receptor, muscarinic 2, cardiac	Chrm2	-2.024	<.05	GPCR Signaling; Synaptic Transmission
NM_133828	CAMP responsive element binding protein 1	Creb1	-1.468	<.01	Synaptic Transmission; Transcription Factor
NM_010025	Doublecortin	Dcx	-1.230	<.05	Axonogenesis
NM_007865	Delta-like 1	Dll1	2.337	<.05	Notch Signaling; Cell Adhesion
NM_010077	Dopamine receptor D2	Drd2	1.649	<.05	Axonogenesis; GPCR Signaling; Synaptic Transmission
NM_010110	Ephrin B1	Efnb1	1.279	<.05	Cell Adhesion
NM_008006	Fibroblast growth factor 2	Fgf2	-1.371	<.01	Growth Factor; Synaptic Transmission
NM_010227	Filamin, alpha	Flna	1.748	<.01	Transcription Factor
NM_013904	Hairy/enhancer-of-split related with YRPW motif 2	Hey2	-1.536	<.05	Notch Signaling; Transcription Factor
NM_001039934	Microtubule-associated protein 2	Mtap2	-1.870	<.01	Axonogenesis
NM_010883	Norrie disease (pseudoglioma)	Ndp	1.312	<.01	Growth Factor; WNT Signaling
NM_010897	Neurofibromatosis 1	Nf1	-1.666	<.05	Synaptic Transmission
NM_008711	Noggin	Nog	1.653	<.05	Axonogenesis
NM_178591	Neuregulin 1	Nrg1	-1.608	<.01	Growth Factor; Notch Signaling
NM_008744	Netrin 1	Ntn1	1.913	<.05	Apoptosis
NM_011855	Odd Oz/ten-m homolog 1	Odz1	-1.256	<.05	Growth Factor
NM_013627	Paired box gene 6	Pax6	-1.390	<.05	Transcription Factor
NM_008973	Pleiotrophin	Ptn	-1.256	<.05	Cell Cycle; Cytokine; Growth Factor
NM_019413	Roundabout homolog 1	Robo1	-1.552	<.01	Cell Adhesion
NM_011577	Transforming growth factor, beta 1	Tgfb1	-1.320	<.05	TGFβ Signaling; Cytokine
NM_022312	Tenascin R	Tnr	-1.338	<.01	Cell Adhesion

Aberrant gene expression after perinatal arsenic exposure in adult male mice

** denotes genes with reversed expression after experience in enrichment*

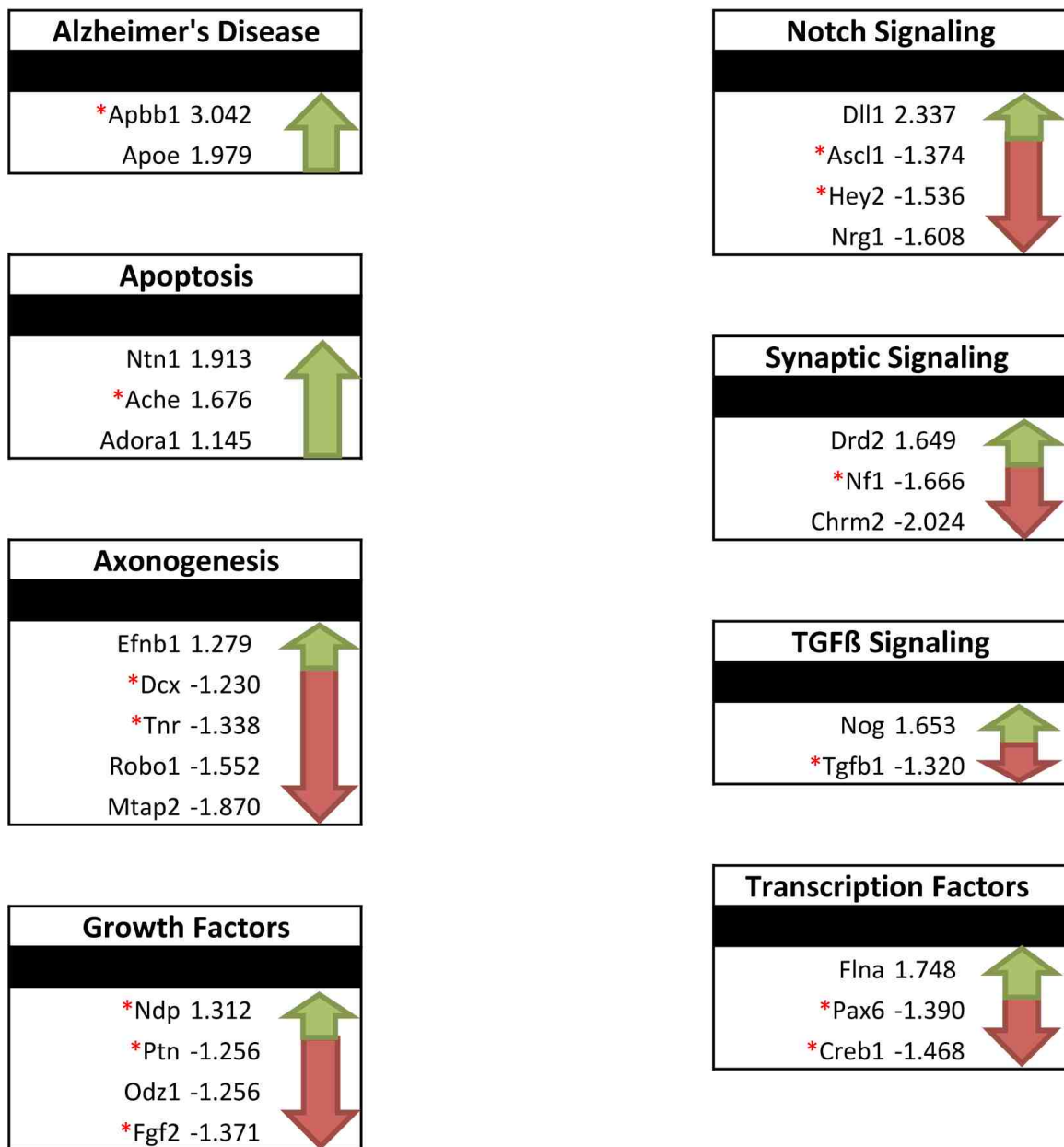


Figure 4.5. Perinatal arsenic exposure alters genes involved in several different pathways important in neurogenesis. Each box contains genes that have altered expression in the dentate gyrus derived from adult male mice exposed to 50 ppb arsenic during development. Arrows indicate direction of expression, while red asterisks indicate genes with reversed expression after exposure to environment enrichment for one month. Genes altered include those involved in Alzheimer’s disease, apoptosis, axonogenesis, growth, transcription, and Notch, synaptic, and TGFβ signaling. In each set of genes at least one aberrantly expressed gene has a reversal of expression after enrichment indicating restoration.

4.3.6 Exposure to an enriched environment reversed altered mRNA expression in arsenic-exposed animals

After one month of brief, daily exposure (2–4 hours per day) to an enriched environment, mRNA expression for neurogenesis-related genes was analyzed on dentate gyrus tissue derived from adult male mice aged PD70 ($n = 6$ mice/group). Genes that were significantly altered ($p < .05$) after this enrichment paradigm are displayed in Table 4.2 for control animals and Table 4.3 for arsenic-exposed animals. Enrichment induced several changes in both sets of animals in genes involved in growth; axonogenesis; cell cycle dynamics; transcription factors; and Notch, WNT, and TGF β signaling. Enrichment in the arsenic-exposed mice compared to their arsenic-exposed counterparts without exposure to enrichment included significant up-regulation of 23 genes (Table 4.3); 12 of those 23 genes were previously down-regulated in arsenic-exposed animals prior to enrichment, indicating a reversal of expression due to the enrichment exposure (Table 4.4 column 2). These same 12 genes were up-regulated in control animals with exposure to enrichment to a greater extent than in the arsenic-exposed animals (Table 4.4). Figure 4.6 shows all genes that had altered expression after exposure to environmental enrichment in arsenic-exposed animals and control animals, including those genes that were common to both groups. Complete data sets for gene expression from the array are provided in Table 4.5 (arsenic-exposed), Table 4.6 (control animals with exposure to enrichment), and Table 4.7 (arsenic-exposed animals with exposure to enrichment).

Table 4.2. Exposure to enrichment for one month altered gene expression in control adult male mice compared to age-matched animals without exposure to enrichment, $p < .05$.

Accession Number	Gene Title	Gene Symbol	Fold Regulation	Function
NM_009599	Acetylcholinesterase	Ache	1.188	Apoptosis; Synaptogenesis
NM_009630	Adenosine A2a receptor	Adora2a	-1.527	Apoptosis; GPCR Signaling
NM_007471	Amyloid beta (A4) precursor protein	App	-1.203	Notch Signaling
NM_145990	CDKS regulatory subunit associated protein 2	Cdk5rap2	-5.006	Cell Cycle
NM_009871	Cyclin-dependent kinase 5, regulatory subunit 1 (p35)	Cdk5r1	-1.349	Cell Cycle
NM_007864	Discs, large homolog 4	Dlg4	-1.310	Synaptic Transmission
NM_010025	Doublecortin	Dcx	1.193	Axonogenesis
NM_010227	Filamin, alpha	Flna	1.426	Transcription Factors
NM_008169	Glutamate receptor, ionotropic, NMDA1	Grin1	-1.155	Synaptic Transmission
NM_013905	Hairy/enhancer-of-split related with YRPW motif-like	Heyl	-1.494	Notch Signaling
NM_207225	Histone deacetylase 4	Hdac4	-1.341	Cell Cycle
NM_010784	Midkine	Mdk	1.934	Cell Cycle
NM_025282	Myocyte enhancer factor 2C	Mef2c	1.198	Transcription Factors
NM_010894	Neurogenic differentiation 1	Neurod1	2.344	Transcription Factors
NM_176930	Neuron-glia-CAM-related cell adhesion molecule	Nrcam	1.374	Synaptogenesis
NM_010939	Neuropilin 2	Nrp2	-1.188	Cell Adhesion
NM_008742	Neurotrophin 3	Ntf3	1.427	Synaptic Transmission
NM_008711	Noggin	Nog	-1.962	Axonogenesis
NM_010883	Norrie disease	Ndp	1.326	WNT Signaling
NM_008714	Notch gene homolog 1	Notch1	-1.422	Notch Signaling
NM_016967	Oligodendrocyte transcription factor 2	Olig2	-2.664	Growth Factor
NM_008900	POU domain, class 3, transcription factor 3	Pou3f3	-1.267	Transcription Factors
NM_009237	SRY-box containing gene 3	Sox3	-1.537	Transcription Factors
NM_011313	S100 calcium binding protein A6 (calcyclin)	S100a6	2.193	Axonogenesis

Table 4.3. Exposure to enrichment for one month altered gene expression in arsenic-exposed adult male mice compared to arsenic-exposed age-matched animals without exposure to enrichment, $p < .05$.

Accession Number	Gene Title	Gene Symbol	Fold Regulation	Function
NM_009599	Acetylcholinesterase	Ache	-1.407	Apoptosis; Synaptogenesis
NM_008553	Achaete-scute complex homolog 1	Ascl1	1.265	Notch Signaling
NM_009685	Amyloid beta (A4) precursor protein-binding, family B, member 1	Apbb1	-1.969	Axonogenesis
NM_007554	Bone morphogenetic protein 4	Bmp4	1.616	TGF β Signaling
NM_007540	Brain derived neurotrophic factor	Bdnf	1.251	Growth Factor
NM_133828	CAMP responsive element binding protein 1	Creb1	1.470	Transcription Factor
NM_145990	CDKS regulatory subunit associated protein 2	Cdk5rap2	2.722	Cell Cycle
NM_009871	Cylin-dependent kinase 5, regulatory subunit 1	Cdk5r1	1.196	Cell Cycle
NM_010025	Doublecortin	Dcx	1.278	Axonogenesis
NM_010113	Epidermal growth factor	Egf	1.670	Growth Factor
NM_008006	Fibroblast growth factor 2	Fgf2	1.657	Growth Factor
NM_008169	Glutamate receptor, ionotropic, NMDA1	Grin1	1.160	Synaptic Transmission
NM_013904	Hairy/enhancer-of-split related with YRPW motif 2	Hey2	1.426	Notch Signaling
NM_207225	Histone deacetylase 4	Hdac4	1.331	Cell Cycle
NM_010556	Interleukin 3	Il3	3.422	Cytokine
NM_001039934	Microtubule-associated protein 2	Mtap2	1.654	Axonogenesis
NM_001081049	Myeloid/lymphoid or mixed-lineage leukemia 1	MLL1	1.238	Cell Cycle
NM_010897	Neurofibromatosis 1	Nf1	1.579	Synaptic Transmission
NM_010883	Norrie disease	Ndp	-1.264	WNT Signaling
NM_013627	Paired box gene 6	Pax6	1.487	Transcription Factor
NM_008973	Plectrophenin	Ptn	1.374	Cell Cycle
NM_008900	POU domain, class 3, transcription factor 3	Pou3f3	1.272	Transcription Factor
NM_019413	Roundabout homolog 1	Robo1	1.618	Cell Adhesion
NM_022312	Tenascin R	Tnr	1.402	Cell Adhesion
NM_011577	Transforming growth factor, beta 1	Tgfb1	1.357	TGF β Signaling
NM_009505	Vascular endothelial growth factor A	Vegfa	1.467	Growth Factor

Table 4.4. Reversal of altered gene expression after one month exposure to an enriched environment compared to arsenic-exposed animals without exposure to enrichment, $p < .05$.

Gene Symbol	Perinatal Arsenic Exposure compared to controls	Perinatal Arsenic Exposure with EE compared to column A	Control (no perinatal exposure) with EE compared to column A
Apbb1	3.042	-1.969	-3.697
Ache	1.676	-1.407	-1.287
Ndp	1.312	-1.264	no change
Dcx	-1.230	1.278	1.608
Ptn	-1.256	1.374	1.547
Tgfb1	-1.320	1.357	1.399
Tnr	-1.338	1.402	1.365
Fgf2	-1.371	1.657	1.417
Ascl1	-1.374	1.265	1.555
Pax6	-1.390	1.487	1.760
Creb1	-1.468	1.470	1.547
Hey2	-1.536	1.426	1.684
Robo1	-1.552	1.618	1.528
Nf1	-1.666	1.579	1.781
Mtap2	-1.870	1.654	1.942

Column A

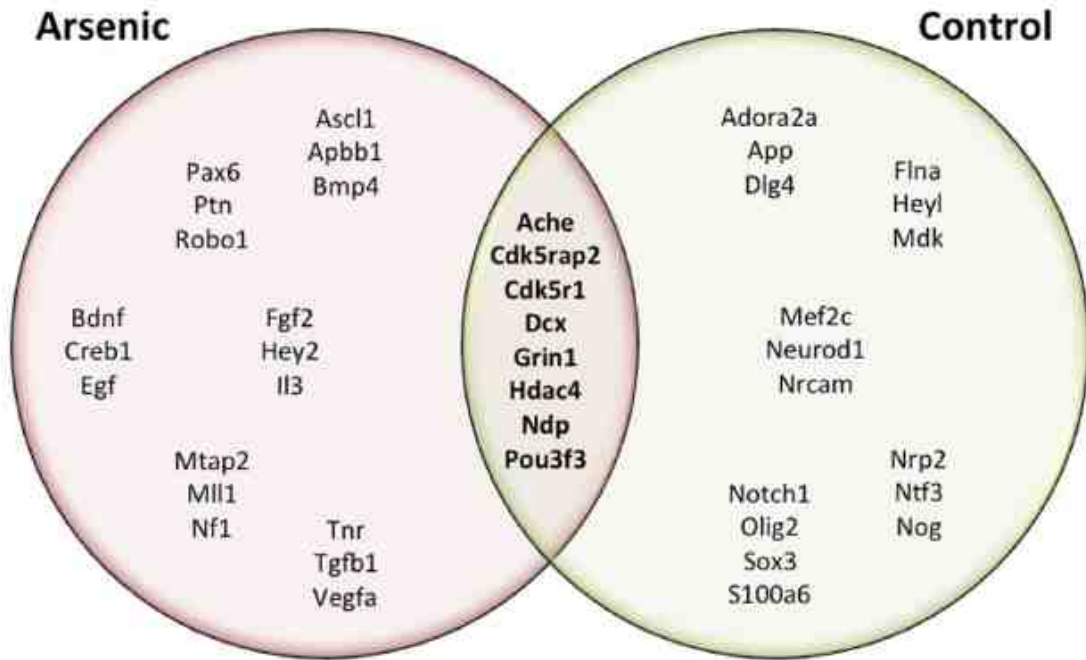


Figure 4.6 One month of brief, daily exposure to enrichment alters gene expression in both arsenic-exposed and control animals.

A Venn diagram depicts gene expression alterations after brief, daily exposure to enrichment for one month in control animals (right) and in arsenic-exposed animals (left). Fold changes of displayed genes include up and down regulation, $p < .05$ (Tables 4.2, 4.3). Gene expression changes common to both sets of animals are displayed in the middle. These include genes involved in cell signaling, axonal growth, histone maintenance, receptors, and cell cycle dynamics.

Table 4.5. Expression of neurogenesis-related genes in arsenic-exposed adult male mice compared to control age-matched animals, entire data set including non-significant changes.

Accession Number	Gene Title	Gene Symbol	Fold Change	p-value
NM_009599	Acetylcholinesterase	Ache	1.68	0.001
NM_001008533	Adenosine A1 receptor	Adora1	1.14	0.03
NM_009630	Adenosine A2a receptor	Adora2a	1.25	0.27
NM_007439	Anaplastic lymphoma kinase	Alk	1.49	0.06
NM_009685	Amyloid beta (A4) precursor protein-binding, family B, member 1	Apbb1	3.04	0.00
NM_009696	Apolipoprotein E	ApoE	1.98	0.01
NM_007471	Amyloid beta (A4) precursor protein	App	1.13	0.22
NM_009711	Artemin	Artn	1.03	0.90
NM_008553	Achaete-scute complex homolog 1 (Drosophila)	Asc1	0.73	0.002
NM_009741	B-cell leukemia/lymphoma 2	Bcl2	0.86	0.13
NM_007540	Brain derived neurotrophic factor	Bdnf	0.82	0.41
NM_007553	Bone morphogenetic protein 2	Bmp2	1.21	0.48
NM_007554	Bone morphogenetic protein 4	Bmp4	0.79	0.09
NM_007559	Bone morphogenetic protein 8b	Bmp8b	0.80	0.38
NM_009871	Cyclin-dependent kinase 5, regulatory subunit 1 (p35)	Cdk5r1	0.87	0.08
NM_145990	CDK5 regulatory subunit associated protein 2	Cdk5rap2	0.47	0.16
NM_203491	Cholinergic receptor, muscarinic 2, cardiac	Chrm2	0.49	0.05
NM_133828	CAMP responsive element binding protein 1	Creb1	0.68	0.01
NM_008176	Chemokine (C-X-C motif) ligand 1	Cxcl1	0.86	0.69
NM_010025	Doublecortin	Dcx	0.81	0.02
NM_007864	Discs, large homolog 4 (Drosophila)	Dlg4	1.11	0.34
NM_007865	Delta-like 1 (Drosophila)	Dll1	2.34	0.02
NM_010077	Dopamine receptor D2	Drd2	1.65	0.02
NM_007889	Dishevelled 3, dsh homolog (Drosophila)	Dvl3	1.14	0.29
NM_010110	Ephrin B1	Efnb1	1.28	0.05
NM_010113	Epidermal growth factor	Egf	0.93	0.70
NM_177821	E1A binding protein p300	Ep300	0.78	0.07
NM_001003817	V-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian)	ErbB2	1.06	0.80
NM_008006	Fibroblast growth factor 2	Fgf2	0.73	0.001
NM_010227	Filamin, alpha	Flna	1.75	0.01
NM_010275	Glial cell line derived neurotrophic factor	Gdnf	0.96	0.88
NM_008155	Glucose phosphate isomerase 1	Gpi1	1.24	0.06
NM_008169	Glutamate receptor, ionotropic, NMDA1 (zeta 1)	Grin1	1.13	0.14
NM_207225	Histone deacetylase 4	Hdac4	0.86	0.29
NM_008235	Hairy and enhancer of split 1 (Drosophila)	Hes1	1.09	0.64
NM_010423	Hairy/enhancer-of-split related with YRPW motif 1	Hey1	1.21	0.13
NM_013904	Hairy/enhancer-of-split related with YRPW motif 2	Hey2	0.65	0.03
NM_013905	Hairy/enhancer-of-split related with YRPW motif-like	Heyl	0.96	0.81
NM_010556	Interleukin 3	Il3	0.69	0.59
NM_010784	Midkine	Mdk	1.48	0.20
NM_025282	Myocyte enhancer factor 2C	Mef2c	1.32	0.07
NM_001081049	Myeloid/lymphoid or mixed-lineage leukemia 1	Mll1	0.99	0.92
NM_001039934	Microtubule-associated protein 2	Mtap2	0.53	0.01
NM_010882	Necdin	Ndn	1.84	0.14
NM_010883	Norrie disease (pseudoglioma) (human)	Ndp	1.31	0.00
NM_010894	Neurogenic differentiation 1	Neurod1	1.05	0.87
NM_010896	Neurogenin 1	Neurog1	2.19	0.06
NM_009718	Neurogenin 2	Neurog2	1.97	0.09

Table 5. Cont.

Accession Number	Gene Title	Gene Symbol	Fold Change	p-value
NM_010897	Neurofibromatosis 1	Nf1	0.60	0.04
NM_008711	Noggin	Nog	1.65	0.05
NM_008714	Notch gene homolog 1 (Drosophila)	Notch1	0.98	0.92
NM_010928	Notch gene homolog 2 (Drosophila)	Notch2	1.08	0.46
NM_013708	Nuclear receptor subfamily 2, group E, member 3	Nr2e3	1.17	0.66
NM_176930	Neuron-glia-CAM-related cell adhesion molecule	Nrcam	1.10	0.30
NM_178591	Neuregulin 1	Nrg1	0.62	0.01
NM_008737	Neuropilin 1	Nrp1	0.86	0.62
NM_010939	Neuropilin 2	Nrp2	1.02	0.79
NM_008742	Neurotrophin 3	Ntf3	1.09	0.46
NM_008744	Netrin 1	Ntn1	1.91	0.02
NM_011855	Odd Oz/ten-m homolog 1 (Drosophila)	Odz1	0.80	0.05
NM_016967	Oligodendrocyte transcription factor 2	Olig2	1.58	0.07
NM_013625	Platelet-activating factor acetylhydrolase, isoform 1b, subunit 1	Pafah1b1	0.99	0.97
NM_033620	Par-3 (partitioning defective 3) homolog (C. elegans)	Pard3	0.97	0.71
NM_008781	Paired box gene 3	Pax3	Undetermined	N/A
NM_008782	Paired box gene 5	Pax5	1.13	0.65
NM_013627	Paired box gene 6	Pax6	0.72	0.02
NM_008900	POU domain, class 3, transcription factor 3	Pou3f3	0.92	0.47
NM_011143	POU domain, class 4, transcription factor 1	Pou4f1	1.30	0.47
NM_008973	Pleiotrophin	Ptn	0.80	0.02
NM_009007	RAS-related C3 botulinum substrate 1	Rac1	1.04	0.91
NM_019413	Roundabout homolog 1 (Drosophila)	Robo1	0.64	0.01
NM_194053	Reticulon 4	Rtn4	0.97	0.76
NM_011313	S100 calcium binding protein A6 (calcydin)	S100a6	1.43	0.25
NM_009115	S100 protein, beta polypeptide, neural	S100b	1.08	0.72
NM_009170	Sonic hedgehog	Shh	0.74	0.12
NM_178804	Slit homolog 2 (Drosophila)	Slit2	0.86	0.39
NM_011434	Superoxide dismutase 1, soluble	Sod1	1.04	0.87
NM_011443	SRY-box containing gene 2	Sox2	1.59	0.07
NM_009237	SRY-box containing gene 3	Sox3	1.00	0.99
NM_011486	Signal transducer and activator of transcription 3	Stat3	0.90	0.21
NM_011577	Transforming growth factor, beta 1	Tgfb1	0.76	0.03
NM_009377	Tyrosine hydroxylase	Th	0.90	0.69
NM_022312	Tenascin R	Tnr	0.75	0.001
NM_009505	Vascular endothelial growth factor A	Vegfa	0.78	0.08

Table 4.6. Expression of neurogenesis-related genes in control adult male mice after brief, daily exposure to enrichment for one month compared to control age-matched animals without exposure to enrichment

Accession Number	Gene Title	Gene Symbol	Fold Change	p-value
NM_009599	Acetylcholinesterase	Ache	1.19	0.03
NM_001008533	Adenosine A1 receptor	Adora1	0.94	0.26
NM_009630	Adenosine A2a receptor	Adora2a	0.65	0.01
NM_007439	Anaplastic lymphoma kinase	Alk	0.68	0.07
NM_009685	Amyloid beta (A4) precursor protein-binding, family B, member 1	Apbb1	0.75	0.49
NM_009696	Apolipoprotein E	ApoE	0.85	0.54
NM_007471	Amyloid beta (A4) precursor protein	App	0.83	0.05
NM_009711	Artemin	Artn	1.17	0.45
NM_008553	Achaete-scute complex homolog 1 (Drosophila)	Ascl1	1.03	0.64
NM_009741	B-cell leukemia/lymphoma 2	Bcl2	1.05	0.54
NM_007540	Brain derived neurotrophic factor	Bdnf	1.66	0.10
NM_007553	Bone morphogenetic protein 2	Bmp2	0.96	0.86
NM_007554	Bone morphogenetic protein 4	Bmp4	1.39	0.09
NM_007559	Bone morphogenetic protein 8b	Bmp8b	0.90	0.51
NM_009871	Cyclin-dependent kinase 5, regulatory subunit 1 (p35)	Cdk5r1	0.74	0.0003
NM_145990	CDK5 regulatory subunit associated protein 2	Cdk5rap2	0.20	0.03
NM_203491	Cholinergic receptor, muscarinic 2, cardiac	Chrm2	0.70	0.19
NM_133828	CAMP responsive element binding protein 1	Creb1	0.96	0.57
NM_008176	Chemokine (C-X-C motif) ligand 1	Cxcl1	0.63	0.12
NM_010025	Doublecortin	Dcx	1.19	0.04
NM_007864	Discs, large homolog 4 (Drosophila)	Dlg4	0.76	0.03
NM_007865	Delta-like 1 (Drosophila)	Dll1	0.65	0.30
NM_010077	Dopamine receptor D2	Drd2	0.76	0.28
NM_007889	Dishevelled 3, dsh homolog (Drosophila)	Dvl3	1.01	0.92
NM_010110	Ephrin B1	Efnb1	0.84	0.08
NM_010113	Epidermal growth factor	Egf	1.30	0.13
NM_177821	E1A binding protein p300	Ep300	1.01	0.91
NM_001003817	V-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian)	ErbB2	0.92	0.44
NM_008006	Fibroblast growth factor 2	Fgf2	0.94	0.62
NM_010227	Filamin, alpha	Flna	1.43	0.01
NM_010275	Glial cell line derived neurotrophic factor	Gdnf	0.91	0.72
NM_008155	Glucose phosphate isomerase 1	Gpi1	1.02	0.71
NM_008169	Glutamate receptor, ionotropic, NMDA1 (zeta 1)	Grin1	0.87	0.05
NM_207225	Histone deacetylase 4	Hdac4	0.75	0.04
NM_008235	Hairy and enhancer of split 1 (Drosophila)	Hes1	1.15	0.15
NM_010423	Hairy/enhancer-of-split related with YRPW motif 1	Hey1	0.96	0.64
NM_013904	Hairy/enhancer-of-split related with YRPW motif 2	Hey2	1.00	1.00
NM_013905	Hairy/enhancer-of-split related with YRPW motif-like	Heyl	0.67	0.02
NM_010556	Interleukin 3	Il3	0.62	0.54
NM_010784	Midkine	Mdk	1.93	0.04
NM_025282	Myocyte enhancer factor 2C	Mef2c	1.20	0.04
NM_001081049	Myeloid/lymphoid or mixed-lineage leukemia 1	Mll1	1.15	0.07
NM_001039934	Microtubule-associated protein 2	Mtap2	0.95	0.70
NM_010882	Necdin	Ndn	0.98	0.94
NM_010883	Norrie disease (pseudoglioma) (human)	Ndp	1.33	0.003
NM_010894	Neurogenic differentiation 1	Neurod1	2.34	0.03
NM_010896	Neurogenin 1	Neurog1	1.09	0.88
NM_009718	Neurogenin 2	Neurog2	0.55	0.19

Table 6. Cont.

Accession Number	Gene Title	Gene Symbol	Fold Change	p-value
NM_010897	Neurofibromatosis 1	Nf1	0.97	0.88
NM_008711	Noggin	Nog	0.51	0.04
NM_008714	Notch gene homolog 1 (Drosophila)	Notch1	0.70	0.01
NM_010928	Notch gene homolog 2 (Drosophila)	Notch2	1.09	0.37
NM_013708	Nuclear receptor subfamily 2, group E, member 3	Nr2e3	0.50	0.09
NM_176930	Neuron-glia-CAM-related cell adhesion molecule	Nrcam	1.37	0.01
NM_178591	Neuregulin 1	Nrg1	1.05	0.75
NM_008737	Neuropilin 1	Nrp1	1.46	0.10
NM_010939	Neuropilin 2	Nrp2	0.84	0.06
NM_008742	Neurotrophin 3	Ntf3	1.43	0.004
NM_008744	Netrin 1	Ntn1	1.43	0.20
NM_011855	Odd Oz/ten-m homolog 1 (Drosophila)	Odz1	0.87	0.18
NM_016967	Oligodendrocyte transcription factor 2	Olig2	0.38	0.02
NM_013625	Platelet-activating factor acetylhydrolase, isoform 1b, subunit 1	Pafah1b1	1.60	0.13
NM_033620	Par-3 (partitioning defective 3) homolog (C. elegans)	Pard3	1.15	0.12
NM_008781	Paired box gene 3	Pax3	Undetermined	N/A
NM_008782	Paired box gene 5	Pax5	0.90	0.69
NM_013627	Paired box gene 6	Pax6	1.15	0.25
NM_008900	POU domain, class 3, transcription factor 3	Pou3f3	0.79	0.002
NM_011143	POU domain, class 4, transcription factor 1	Pou4f1	0.74	0.47
NM_008973	Pleiotrophin	Ptn	1.12	0.26
NM_009007	RAS-related C3 botulinum substrate 1	Rac1	1.65	0.08
NM_019413	Roundabout homolog 1 (Drosophila)	Robo1	0.90	0.29
NM_194053	Reticulon 4	Rtn4	0.94	0.37
NM_011313	S100 calcium binding protein A6 (calcyclin)	S100a6	2.19	0.02
NM_009115	S100 protein, beta polypeptide, neural	S100b	1.45	0.11
NM_009170	Sonic hedgehog	Shh	0.81	0.21
NM_178804	Slit homolog 2 (Drosophila)	Slit2	0.72	0.07
NM_011434	Superoxide dismutase 1, soluble	Sod1	1.37	0.21
NM_011443	SRY-box containing gene 2	Sox2	0.81	0.45
NM_009237	SRY-box containing gene 3	Sox3	0.65	0.01
NM_011486	Signal transducer and activator of transcription 3	Stat3	0.93	0.26
NM_011577	Transforming growth factor, beta 1	Tgfb1	0.97	0.79
NM_009377	Tyrosine hydroxylase	Th	0.59	0.13
NM_022312	Tenascin R	Tnr	0.93	0.32
NM_009505	Vascular endothelial growth factor A	Vegfa	0.82	0.02

Table 4.7. Expression of neurogenesis-related genes in arsenic-exposed adult male mice after brief, daily exposure to enrichment for one month compared to arsenic-exposed age-matched animals without exposure to enrichment

Accession Number	Gene Title	Gene Symbol	Fold Change	p-value
NM_009599	Acetylcholinesterase	Ache	0.71	0.001
NM_001008533	Adenosine A1 receptor	Adora1	1.04	0.49
NM_009630	Adenosine A2a receptor	Adora2a	1.11	0.62
NM_007439	Anaplastic lymphoma kinase	Alk	0.95	0.77
NM_009685	Amyloid beta (A4) precursor protein-binding, family B, member 1	Apbb1	0.51	0.01
NM_009696	Apolipoprotein E	ApoE	0.76	0.18
NM_007471	Amyloid beta (A4) precursor protein	App	0.98	0.84
NM_009711	Artemin	Artn	1.10	0.57
NM_008553	Achaete-scute complex homolog 1 (Drosophila)	Ascl1	1.27	0.03
NM_009741	B-cell leukemia/lymphoma 2	Bcl2	1.15	0.23
NM_007540	Brain derived neurotrophic factor	Bdnf	1.25	0.05
NM_007553	Bone morphogenetic protein 2	Bmp2	1.08	0.69
NM_007554	Bone morphogenetic protein 4	Bmp4	1.62	0.001
NM_007559	Bone morphogenetic protein 8b	Bmp8b	2.04	0.10
NM_009871	Cyclin-dependent kinase 5, regulatory subunit 1 (p35)	Cdk5r1	1.20	0.02
NM_145990	CDK5 regulatory subunit associated protein 2	Cdk5rap2	2.72	0.05
NM_203491	Cholinergic receptor, muscarinic 2, cardiac	Chrm2	1.65	0.15
NM_133828	CAMP responsive element binding protein 1	Creb1	1.47	0.01
NM_008176	Chemokine (C-X-C motif) ligand 1	Cxcl1	0.66	0.44
NM_010025	Doublecortin	Dcx	1.28	0.03
NM_007864	Discs, large homolog 4 (Drosophila)	Dlg4	1.19	0.21
NM_007865	Delta-like 1 (Drosophila)	Dll1	0.69	0.21
NM_010077	Dopamine receptor D2	Drd2	0.77	0.25
NM_007889	Dishevelled 3, dsh homolog (Drosophila)	Dvl3	1.01	0.96
NM_010110	Ephrin B1	Efnb1	0.89	0.37
NM_010113	Epidermal growth factor	Egf	1.67	0.03
NM_177821	E1A binding protein p300	Ep300	1.28	0.09
NM_001003817	V-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian)	ErbB2	1.27	0.19
NM_008006	Fibroblast growth factor 2	Fgf2	1.66	0.00
NM_010227	Filamin, alpha	Flna	0.86	0.34
NM_010275	Glial cell line derived neurotrophic factor	Gdnf	1.38	0.33
NM_008155	Glucose phosphate isomerase 1	Gpi1	0.90	0.19
NM_008169	Glutamate receptor, ionotropic, NMDA1 (zeta 1)	Grin1	1.16	0.04
NM_207225	Histone deacetylase 4	Hdac4	1.33	0.05
NM_008235	Hairy and enhancer of split 1 (Drosophila)	Hes1	0.90	0.51
NM_010423	Hairy/enhancer-of-split related with YRPW motif 1	Hey1	0.96	0.77
NM_013904	Hairy/enhancer-of-split related with YRPW motif 2	Hey2	1.43	0.05
NM_013905	Hairy/enhancer-of-split related with YRPW motif-like	Heyl	1.09	0.56
NM_010556	Interleukin 3	Il3	3.42	0.04
NM_010784	Midkine	Mdk	0.70	0.20
NM_025282	Myocyte enhancer factor 2C	Mef2c	0.80	0.25
NM_001081049	Myeloid/lymphoid or mixed-lineage leukemia 1	Mll1	1.24	0.02
NM_001039934	Microtubule-associated protein 2	Mtap2	1.65	0.01
NM_010882	Necdin	Ndn	0.71	0.18
NM_010883	Norrie disease (pseudoglioma) (human)	Ndp	0.79	0.03
NM_010894	Neurogenic differentiation 1	Neurod1	0.85	0.44
NM_010896	Neurogenin 1	Neurog1	0.88	0.75
NM_009718	Neurogenin 2	Neurog2	0.77	0.40

Table 7. Cont.

Accession Number	Gene Title	Gene Symbol	Fold Change	p-value
NM_010897	Neurofibromatosis 1	Nf1	1.58	0.01
NM_008711	Noggin	Nog	0.82	0.40
NM_008714	Notch gene homolog 1 (Drosophila)	Notch1	1.24	0.21
NM_010928	Notch gene homolog 2 (Drosophila)	Notch2	1.11	0.45
NM_013708	Nuclear receptor subfamily 2, group E, member 3	Nr2e3	1.40	0.48
NM_176930	Neuron-glia-CAM-related cell adhesion molecule	Nrcam	0.91	0.08
NM_178591	Neuregulin 1	Nrg1	1.24	0.16
NM_008737	Neuropilin 1	Nrp1	1.18	0.35
NM_010939	Neuropilin 2	Nrp2	1.22	0.22
NM_008742	Neurotrophin 3	Ntf3	0.88	0.24
NM_008744	Netrin 1	Ntn1	0.80	0.22
NM_011855	Odd Oz/ten-m homolog 1 (Drosophila)	Odz1	1.26	0.12
NM_016967	Oligodendrocyte transcription factor 2	Olig2	0.89	0.62
NM_013625	Platelet-activating factor acetylhydrolase, isoform 1b, subunit 1	Pafah1b1	0.85	0.48
NM_033620	Par-3 (partitioning defective 3) homolog (C. elegans)	Pard3	1.29	0.09
NM_008781	Paired box gene 3	Pax3	Undetermined	N/A
NM_008782	Paired box gene 5	Pax5	0.93	0.76
NM_013627	Paired box gene 6	Pax6	1.49	0.02
NM_008900	POU domain, class 3, transcription factor 3	Pou3f3	1.27	0.03
NM_011143	POU domain, class 4, transcription factor 1	Pou4f1	1.33	0.48
NM_008973	Pleiotrophin	Ptn	1.37	0.0003
NM_009007	RAS-related C3 botulinum substrate 1	Rac1	0.87	0.54
NM_019413	Roundabout homolog 1 (Drosophila)	Robo1	1.62	0.01
NM_194053	Reticulon 4	Rtn4	1.13	0.14
NM_011313	S100 calcium binding protein A6 (calcyclin)	S100a6	0.69	0.12
NM_009115	S100 protein, beta polypeptide, neural	S100b	0.97	0.88
NM_009170	Sonic hedgehog	Shh	0.91	0.21
NM_178804	Slit homolog 2 (Drosophila)	Slit2	1.04	0.72
NM_011434	Superoxide dismutase 1, soluble	Sod1	0.87	0.38
NM_011443	SRY-box containing gene 2	Sox2	0.89	0.63
NM_009237	SRY-box containing gene 3	Sox3	1.37	0.14
NM_011486	Signal transducer and activator of transcription 3	Stat3	1.18	0.10
NM_011577	Transforming growth factor, beta 1	Tgfb1	1.36	0.0003
NM_009377	Tyrosine hydroxylase	Th	1.61	0.27
NM_022312	Tenascin R	Tnr	1.40	0.001
NM_009505	Vascular endothelial growth factor A	Vegfa	1.47	0.01

4.4 Discussion

It is widely recognized that high levels of arsenic produce deleterious effects on the brain. The results presented here demonstrate that our developmental arsenic exposure model, which represents a low and environmentally relevant concentration previously considered safe, significantly impacts adult neurogenesis, specifically the number of differentiated NPC in the SGZ. Given that our labeling method is more likely to underestimate the total

degree of neurogenesis (Hayes & Nowakowski, 2002) (as it does not include BrdU⁺ only cells that have exited the cell cycle after 12 hours for proliferation assessment), the fact that we were able to discern a difference of 13% for proliferation (though not significant) and 41% for differentiation after perinatal arsenic exposure in the adult animal is substantive. Our subsequent studies will further elucidate arsenic's impact on the immature cells (DCX⁺) and mature cells (NeuN⁺) separately and on NPC survival. Because adult neurogenesis has been shown to be integral for certain forms of learning and memory, the hippocampal damage measured in this study may be responsible for the cognitive deficits previously observed in this arsenic exposure model.

Other heavy metals similar to arsenic have been implicated in reduced adult neurogenesis and deficits in cognition. A single injection of 5 µg methylmercury per gram body weight at PD7, resulting in 500 ppb concentration in the brain, was enough to reduce adult neurogenesis and hippocampal size and induce hippocampal-dependent learning and memory deficits specifically in a spatial learning task (Falluel-Morel *et al.*, 2007). Using tritiated thymidine incorporation at PD7, the authors were also able to show inhibited hippocampal DNA synthesis, degradation of cyclin E, and reduced cyclin D1 and D3 during postnatal development in which neurogenesis is highly active in the hippocampus (Falluel-Morel *et al.*, 2007). Mercury is a potent neurotoxin and elevated exposures may lead to mental retardation; yet, this brief exposure resulted in significant morphological changes and learning deficits similar to the results we report here using an arsenic concentration an order of magnitude lower than that used in the methylmercury study. Similarly, postnatal exposure to low levels of lead (0.2% lead acetate resulting in 6 ng

Pb/g brain by PD80) from PD1 to PD30 results in increased anxiety behavior, deficits in contextual fear conditioning, reduced adult neurogenesis (specifically differentiation), and preferential astroglial lineage in PD80 Wistar rats (Jaako-Movits *et al.*, 2005). Another study using the same low level of lead acetate exposure from GD16 to PD21, resulting in 35-40 ppm Pb in blood, showed altered differentiation of adult neurogenesis at PD110 but no learning deficits in the Morris water maze (Gilbert *et al.*, 2005). Chronic exposure during development using an environmentally relevant dose of lead (1500 ppm lead acetate resulting in 258 ppm Pb in blood) results in significantly decreased proliferation and survival of granule cells and reduced mossy fiber input into CA3 region of the hippocampus from the dentate, possibly accounting for the deficits in synaptic plasticity and learning seen in lead-exposed animals (Verina *et al.*, 2007). Our results reveal that like mercury and lead, developmental arsenic exposure can induce potent morphological damage in the adult hippocampus even at a low concentration, and these deficits may be linked to altered behavioral changes seen in our model as we have previously reported (Martinez *et al.*, 2008; Martinez-Finley *et al.*, 2009).

In vitro studies investigating the effects of developmental arsenic exposure in the parts per billion range (up to 4 μ M) have shown altered cell cycling including reduced viability, minimal apoptosis, and an increase in caspase 3/7 resulting in inhibition of cell cycle progression in primary embryonic rat midbrain neuroepithelial cells (Sidhu *et al.*, 2006). Other *in vitro* work with P19 mouse embryonic stem cells (ESC) has demonstrated that low concentrations of arsenic in the ppb range (0.1 up to 1.0 μ M sodium arsenite) suppress the differentiation, but not proliferation, of ESC into neurons indicated by

reduced Tuj1, neurogenin 1, neurogenin 2, and NeuroD expression in arsenic-treated cells (Hong & Bain, 2012a). The results from these studies concur with our *in vivo* developmental model of arsenic exposure and the work presented here, suggesting that arsenic impacts the number of differentiated cells among several cell types both *in vivo* and *in vitro*.

Exposure to higher concentrations of arsenic (ppm range up to 68 mg/L) during adulthood results in changes in synaptic plasticity components, including the expression of the NMDA receptor subunit NR2A, PSD-95, and pCaMKIIa in the hippocampus, all of which are important for learning and memory (Luo *et al.*, 2012). In accordance with our own previous results (Martinez-Finley *et al.*, 2011), increased SynGAP, a negative regulator of Ras/MAPK activity, along with decreased pERK1/2 activity is also seen after chronic low dose arsenic exposure in the adult. Two months of 4.0 mg/L As₂O₃ (ppm range) exposure beginning at four months of age decreases both proliferation and differentiation of newly labeled cells in the dentate gyrus in mice (Liu *et al.*, 2012). This deficit was ameliorated after two months of drinking distilled water, suggesting that the effects of arsenic are transient. Our model indicates that significant changes in differentiation can be measured several weeks after developmental 50 ppb arsenic exposure, suggesting that the effect is not transient. It is possible that because arsenic is present during development of the brain, arsenic's effects are much longer lasting than those seen during adulthood exposure due to arsenic-induced molecular changes. For example, low concentrations of arsenic during development impact the epigenetic status of histones and DNA, which play an important role in gene expression regulation; these

epigenetic changes are concurrent with learning deficits seen in certain behavior tasks (Reichard & Puga, 2010; Martinez *et al.*, 2011). Thus, we hypothesize that behavioral deficits seen during adulthood of mice developmentally exposed to arsenic are due, in part, to lack of differentiated cells necessary for hippocampal-dependent learning.

To support the differences in adult neurogenesis we observed in arsenic-exposed animals, we sought to elucidate if there was transcriptional dysregulation of genes involved in adult neurogenesis in the dentate gyrus. In accordance with our morphological findings, arsenic exposure during the perinatal period resulted in down-regulation of 15 target genes (18%) and up-regulation of 11 target genes (13%) in adult animals.

Up-regulation of genes included those involved with apoptosis (Ntn1, Adora1, and Ache) and Alzheimer's disease (ApoE, Apbb1). In particular, acetylcholinesterase (Ache) is an important regulator of apoptosis; increased expression, while not initiating apoptosis, has been correlated with apoptotic cells (Xie *et al.*, 2011). Adult arsenic exposure (20 mg/kg body weight for 28 days in rats) results in decreased activity of acetylcholinesterase in the hippocampus, but also decreased binding of (3)H-QNB, a label of muscarinic-cholinergic receptors (Yadav *et al.*, 2011). While the increase in Ache mRNA is not indicative of greater activity of the enzyme, it could be overcompensating for a disruption in cholinergic signaling as suggested by the significantly reduced Chrm2 mRNA, a muscarinic cholinergic receptor (Table 4.1). The hippocampus receives several cholinergic inputs that may contribute to the formation of new neurons; additionally, cholinergic transmission is important for learning and memory and altered acetylcholine

levels have been associated Alzheimer's-related cognitive deficits (Veena *et al.*, 2011). Thus, aberrant cholinergic transmission induced by arsenic could play a part in reduced adult neurogenesis seen in our studies, along with up-regulation of the genes *Apbb1* and *ApoE*, both of which are associated with Alzheimer's disease and have been shown to impair adult neurogenesis (Ghosal *et al.*, 2010).

Additionally, while adult exposure to low concentrations of arsenic does not induce apoptosis in the hippocampus (Liu *et al.*, 2012), developmental exposure to arsenic increases apoptosis in conjunction with decreased Nissl body staining in neuronal bodies in the hippocampus (Fan *et al.*, 2013). In addition to increased apoptosis after developmental arsenic exposure, a study demonstrated significant down-regulation of nerve growth factor (NGF) and GAP-43 mRNA (Fan *et al.*, 2013) in response to arsenic, similar to the results from our arrays showing significant down-regulation of mRNA from *Ptn*, *Odz1*, and *Fgf2*. These growth factors are also involved in axonogenesis and neurite outgrowth, which arsenic exposure can impair (Aung *et al.*, 2013). Similarly, several important genes responsible for proper maturation and axonal growth including doublecortin, tenascin R, *Mtap2*, and *Robo1* are all significantly down-regulated in arsenic-exposed animals (Table 4.1). This further supports the notion that arsenic is impacting the ability of NPC to develop into mature neurons, possibly leading to the decrease in differentiated neurons in the dentate gyrus. It should be noted that the number of surviving neural progenitors does affect the number of differentiated cells as well; however, NPC survival was not assessed after four weeks. Thus, it is unclear whether this lack of differentiated cells is due to deficits in the surviving NPC population or issues

with differentiation program; however, the gene expression data do suggest that arsenic exposure is impacting neurogenesis programs in the dentate gyrus.

Several pathways, including Notch, TGFb, and WNT, all had factors that were aberrantly expressed in the arsenic-exposed animals (Table 4.1). Interestingly, arsenic induced an up-regulation of Dll1, a ligand that promotes Notch signaling, which enhances proliferation of progenitor cells. However, increased Dll1 also drives the down-regulation of Ascl1, which was also down-regulated in arsenic-exposed animals. Ascl1, or Mash1, is a basic helix-loop-helix (bHLH) transcription factor that increases expression of neurogenins to promote neuronal differentiation programs (Ables *et al.*, 2011). Thus, the Notch signaling program may be enhanced at the expense of the differentiation of progenitors as reflected in the deficits in differentiation of NPCs in arsenic-exposed animals (Shimojo *et al.*, 2011). Arsenic exposure resulted in down-regulation of other factors important for differentiation including Odz and Nrg1 (also involved in Notch signaling) and a trend toward down-regulation of neurogenins 1 and 2, though not significant. Thus, all of these changes in genes important for proper differentiation could be contributing to the lack of differentiated cells we observed after developmental arsenic exposure.

It's well established that exposure to an enriched environment can lead to increased adult neurogenesis, thereby enhancing certain forms of learning and memory (Kempermann *et al.*, 1997; Zhao *et al.*, 2008). Yet, our previous publications have demonstrated that developmental toxins, such as alcohol, reduce the ability of the neurogenic environment

in the hippocampus to respond to a continuous enrichment paradigm (Choi *et al.*, 2005). Other studies reveal that brief exposure to enrichment is adequate to improve behavior and adult neurogenesis (Rampon *et al.*, 2000; Frick & Fernandez, 2003; Veena *et al.*, 2009). To reduce the likelihood that animals habituate to the enriched housing environment, we decided to use a “playground” enrichment paradigm in which animals spend two to four hours per day in the enrichment cages (with toys, ladders, running wheels, housing, and other animals) and are subsequently returned to their standard home cages. We found that this model of brief, daily exposure to enrichment sufficiently increased both proliferation and differentiation in both control and arsenic-exposed animals regardless of perinatal exposure. The effect on control animals was as expected: significantly increased proliferation of 70%, likely due to exercise from use of the running wheel as suggested by work from van Praag, and significantly increased differentiation of 39%, likely due to exposure to novelty, other animals, and toys (van Praag, 2008). The use of both a running wheel and toys for enrichment has additive effects on adult neurogenesis and may likely account for the robust increase seen in both sets of animals (Fabel *et al.*, 2009). While the increase in proliferation of arsenic-exposed animals after enrichment was not significant, we consider a 53% increase to be considerable. Qualitative evidence suggests that a substantial difference exists even if not statistically significant (Figure 4.4C). Two reasons for this lack of significance include 1) the inherent variability in measuring neurogenesis and 2) the fact that, while arsenic may influence NPC proliferation, this impact may only be observed as the lack of significant neurogenic response to the enrichment, as seen in other studies using teratogens (e.g., alcohol) (Choi *et al.*, 2005). Surprisingly, we found that brief exposure to enrichment led

to increased differentiation (121%) in perinatal arsenic-exposed animals compared to their arsenic-exposed counterparts without exposure to enrichment. Cell counts show that arsenic-exposed animals have increased levels of differentiation after enrichment similar to those seen in control animals without exposure to enrichment. As expected, exposure to enrichment also increased mRNA levels of genes involved in proliferation and differentiation, including several growth and transcription factors (Table 4.3, 4.4; Figure 4.5). However, in control enriched animals versus control, no enrichment animals (Table 4.2), enrichment induces suppression of factors like *Noggin*, *Cdkr1*, *Notch1*, and *Sox3*, suggesting a shift from proliferation to differentiation in response to enrichment (Fabel *et al.*, 2009). Exposure to enrichment significantly reversed aberrant gene expression in arsenic-exposed animals (Tables 4.3, 4.4; Figure 4.5), inducing up-regulation of several genes important for axonogenesis, cell cycle dynamics, Notch signaling, and transcription factors. These results suggest that even though arsenic interferes with the number of differentiated cells in the dentate gyrus, the neurogenic morphological and genetic response to enrichment both is not impaired when using this particular playground model.

Evidence elucidating the importance of intact adult neurogenesis suggests that it is crucial for some types of hippocampal-dependent learning and memory, and ablation of adult neurogenesis results in learning and memory deficits similar to those observed in our arsenic model. We have demonstrated that exposure to 50 ppb arsenic, which is metabolized more efficiently in the mouse (and more closely models an exposure of 10 ppb arsenic in humans), during all three trimesters of gestational and postnatal development induces hippocampal-dependent learning and memory deficits (Martinez *et*

al., 2008; Martinez-Finley *et al.*, 2009). Results presented here indicate it is likely that altered neurogenesis is one mechanism responsible for these deficits. This study is the first to show that developmental exposure to a low concentration of arsenic induces changes the number of differentiated adult neural progenitors in the subgranular zone of the hippocampus and aberrant expression of neurogenesis-related genes. Further studies are needed to elucidate mechanisms that produce these genetic and morphological changes we have observed.

5.0 Fluoxetine treatment ameliorates depression induced by perinatal arsenic exposure via a neurogenic mechanism

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Abstract

Several epidemiological studies have reported an association between arsenic exposure and increased rates of psychiatric disorders, including depression, in exposed populations. We have previously demonstrated that developmental exposure to low amounts of arsenic induces depression in adulthood along with several morphological and molecular aberrations, particularly associated with the hippocampus and the hypothalamic-pituitary-adrenal (HPA) axis. The extent and potential reversibility of this toxin-induced damage has not been characterized to date. In this study, we assessed the effects of fluoxetine, a selective serotonin reuptake inhibitor antidepressant, on adult animals exposed to arsenic during development. Perinatal arsenic exposure (PAE) induced depressive-like symptoms in a mild learned helplessness task and in the forced swim task after acute exposure to a predator odor (2,4,5-trimethylthiazoline, TMT). Chronic fluoxetine treatment prevented these behaviors in both tasks in arsenic-exposed animals and ameliorated arsenic-induced blunted stress responses, as measured by corticosterone (CORT) levels before and after TMT exposure. Morphologically, chronic fluoxetine treatment reversed deficits in adult hippocampal neurogenesis (AHN) after PAE, specifically differentiation and survival of neural progenitor cells. Protein expression of BDNF, CREB, the glucocorticoid receptor (GR), and HDAC2 was significantly increased in the dentate gyrus of arsenic animals after fluoxetine treatment. This study demonstrates that damage induced by perinatal arsenic exposure is reversible with chronic fluoxetine treatment resulting in restored resiliency to depression via a neurogenic mechanism.

5.1 Introduction

In a recent report by the ATSDR (Agency for Toxic Substances and Disease Registry), arsenic was singled out as a considerable threat to human health compared to other toxicants, outranking even mercury and lead. While the World Health Organization standard remains at 10 parts per billion (ppb), it is estimated that over 100 million people worldwide are exposed to arsenic in amounts that well exceed this standard (in the ppm range) (Tyler and Allan, 2014). Research over the past few decades has identified pathologies resulting from chronic arsenic exposure, demonstrating that this metalloid disrupts many organ systems in the body, including the brain (Brinkel *et al.*, 2009; Farzan *et al.*, 2013). Epidemiological studies report that cumulative long-term exposure to low doses of arsenic correlates with learning and memory deficits in children and increased rates of depression in adults (Zierold *et al.*, 2004; Rosado *et al.*, 2007; O'Bryant *et al.*, 2011; Roy *et al.*, 2011). Additionally, in almost every country assessed, individuals residing in arsenic-affected areas report a poorer quality of life, poorer mental health, and are more likely to have a mood disorder, such as anxiety or depression, than individuals without significant arsenic exposure (Fujino *et al.*, 2004b; Sen & Sarathi Biswas, 2012; Syed *et al.*, 2012).

Research investigating the role of environmental factors, including toxin exposure, in the etiology of depression has gained momentum, as heritability studies report high discordance of the disorder and individual variability in gene expression (Byrne *et al.*, 2013). The early environment during development has been demonstrated to exert profound effects on the modulation behavior and susceptibility to psychiatric disorders in

adulthood (Hochberg *et al.*, 2011). In several rodent studies, pre- and postnatal toxin exposure results in the behavioral endophenotypes of anxiety and depression (Johansson *et al.*, 2007; Onishchenko *et al.*, 2008; Haider *et al.*, 2013). These toxin-induced behaviors are often accompanied by concurrent abnormalities in the regulation of the hypothalamus-pituitary-adrenal (HPA) axis (Hellemans *et al.*, 2008; Hellemans *et al.*, 2010; Bolton *et al.*, 2013). Individuals with robust homeostatic responses to stress are often considered resistant to depression (Krishnan & Nestler, 2008), thus dysregulation of the HPA axis after toxin exposure may be a mechanism by which toxins, like arsenic, increase susceptibility to psychiatric disorders. Indeed, we have previously demonstrated that perinatal (pre- and postnatal developmental) arsenic exposure (PAE) alters glucocorticoid signaling within the HPA axis, blunts stress responses, increases stress reactivity (Goggin *et al.*, 2012), and results in depressive-like behavior in adulthood (Martinez *et al.*, 2008, Martinez-Finley *et al.*, 2009). In addition to these aberrations, we have also observed altered hippocampal morphology and deficits in adult hippocampal neurogenesis after PAE (Tyler & Allan, 2013). Yet, the pathophysiology of arsenic-induced depression and the response to antidepressants after arsenic exposure has not been fully explored.

Fluoxetine, selective serotonin reuptake inhibitor (SSRI) antidepressant, is the most commonly prescribed treatment for depression. In addition to attenuation of stress-induced depressive-like behaviors, fluoxetine contemporaneously increases adult hippocampal neurogenesis (AHN) in rodents (Wang *et al.*, 2008, Malberg and Duman, 2003, David *et al.*, 2009, Rainer *et al.*, 2009, (David *et al.*, 2009; Mahar *et al.*, 2014).

Impaired AHN has been intimately linked with the onset of depression: recent clinical research reported that depressed patients treated with fluoxetine do not exhibit hippocampal atrophy or reduced cell numbers as those patients without treatment, further supporting the “neurogenic hypothesis of depression” (Boldrini *et al.*, 2013). Fluoxetine treatment has also been shown to normalize the corticosterone (CORT) response associated with aberrant HPA signaling after prenatal stress (Ishiwata *et al.*, 2005); restoration of HPA axis function is associated with robust increases in neurogenesis in cell culture as well (Anacker *et al.*, 2013). However, the molecular and behavioral responses to fluoxetine in a rodent model with arsenic-induced hippocampal morphological and behavioral deficits have not been determined to date.

To investigate the reversibility of arsenic-induced damage, we evaluated the effect of chronic fluoxetine treatment on behavioral, morphological, and molecular impairments resulting from PAE. To this end, we measured survival and differentiation of neural progenitor cells (NPCs) along with expression of several proteins involved in neurogenesis specifically in the dentate gyrus to determine fluoxetine’s mechanism of action in arsenic-exposed animals. We further characterized the vulnerability to stress-induced depression generated by arsenic exposure by evaluating behavior and corticosterone (CORT) before and after brief exposure to a predator odor. Chronic fluoxetine treatment reversed deficits in the learned helplessness task; attenuated blunted stress responses and immobility after predator exposure; increased NPC survival and differentiation; and increased expression of brain-derived neurotrophic factor (BDNF), the glucocorticoid receptor (GR), histone deacetylase 2 (HDAC2), and cAMP response

element binding protein (CREB) in the DG of arsenic-exposed animals but not in controls. This study suggests that impairments induced by developmental exposure to an environmental toxin like arsenic may be altered with pharmacological intervention in adulthood, leading to normalized hippocampal morphology and behavior.

5.2 Materials and Methods

5.2.1 Perinatal arsenic exposure paradigm

All procedures were conducted in accordance with protocols approved by the Institutional Animal Care and Use Committee at the University of New Mexico. Animals were maintained in a 22°C vivarium on a 12-hour reverse light/dark cycle with lights off at 0800 and *ad libitum* access to water and food. Exposure to arsenic during all three trimesters of brain development was performed as previously described (Tyler & Allan, 2013). Briefly, singly housed female C57BL/6J mice aged 55 days (Jackson Laboratories, Bar Harbor, ME) were acclimated to drinking 50 ppb arsenic-laced water (sodium arsenate, Sigma-Aldrich, St. Louis, MO) for seven to ten days prior to mating. Dams were provided either 50 ppb arsenic water or tap water during breeding and pregnancy until offspring were weaned (Figure 5.1A). Offspring were housed two per cage with tap water for the remainder of the study. The number of dams used in this study is reflected in the number of litters provided for each experimental analysis (i.e. n = 8-10 indicates that only one animal was used from each of 8-10 litters from 8-10 different dams.) Adult male offspring (postnatal day 70) from dams drinking arsenic water were euthanized by rapid decapitation for tissue dissection or by overdose with sodium pentobarbital followed by transcardiac perfusion with 4% paraformaldehyde for neurogenesis

assessment.

5.2.2 Fluoxetine treatment paradigm and plasma analysis

Fluoxetine hydrochloride (Sigma-Aldrich, St. Louis, MO) was provided for 35 days in drinking water containing 0.066% saccharin to mask any taste of the drug beginning at postnatal day (PD) 35. Male offspring from dams drinking arsenic or tap water were separated into three groups: those drinking 50 mg/L fluoxetine, 100 mg/L fluoxetine, and control (only 0.066% saccharin) as seen in Figure 5.1A. Fluoxetine solutions were shielded from the light and changed every other day. Weight gain and fluoxetine ingestion were monitored weekly.

5.2.3 Predator odor exposure and stress response

Exposure to 2,3,5-trimethyl-3-thiazoline (TMT) (Contech, Victoria, BC, Canada; #300000368) was performed as previously described (Goggin *et al.*, 2012). Animals were individually transferred to an exposure cage containing filter paper saturated with 100 μ L of 3% (v/v) TMT for ten minutes. Group-housed animals were exposed concurrently in parallel and returned to home cages for twenty minutes after the ten-minute exposure. Several cohorts of animals were exposed to TMT, some of which were placed into the forced swim task 20 minutes after exposure, while others were rapidly decapitated for trunk blood collection using Safe-t-Fill EDTA capillary tubes. Samples were centrifuged at $1000 \times g$ for 10 min at 4°C. Plasma was removed and stored at -80°C. Corticosterone (CORT) levels in plasma were determined using the DetectX Corticosterone EIA Kit (Arbor Assays, K014-H1) per manufacturer instructions with a 1:100 dilution of plasma

prior to analysis. Optical density absorbance was determined by an Infinite M200 instrument and data were analyzed using 4PLC ReaderFit online software.

5.2.4 Open field

Adult male mice (PD70) were permitted to explore a novel open arena, measuring 40 cm × 40 cm × 35 cm and constructed of white Plexiglas, for fifteen minutes for two consecutive days. Dim red overhead lighting was used along with white spotlights to illuminate the corners of the open field at 45-60 lumen and the center at 75-90 lumen. Total distance traveled was measured with the aid of an Ethovision video tracking system using the coordinates of the animal's center of mass image compared against a template of the arena dimensions. Locomotor activity in a novel environment was assessed the first day, and locomotor activity in a familiar environment was assessed on the second day.

5.2.5 Forced swim

The forced swim task (FST) was conducted essentially as previously described (Caldwell *et al.*, 2008; Martinez *et al.*, 2008) with a critical difference in the time of behavior assessment. Our prior studies used a conventional light/dark cycle (lights *on* at 0800); however, our current studies use a reverse light/dark cycle (lights *off* at 0800). Thus, all behavioral assessments occurred during the nocturnal (active) phase of the animals' circadian cycle (Kelliher *et al.*, 2000). Adult male mice (PD70) were placed in a clear Plexiglas cylinder (46 cm height × 30 cm diameter) filled with warm water (27°C) to a depth such that the animal's tail did not touch the bottom of the container. Both latency to cease active swimming and total immobility (cessation of escape directed movement)

were scored for five minutes by an experimenter blind to group designations (Porsolt *et al.*, 1977). The forced swim test was conducted with and without exposure to predator odor in separate cohorts of animals 20 minutes after cessation of the TMT exposure.

5.2.6 Learned helplessness

The learned helplessness (LH) task was conducted essentially as previously described (Caldwell *et al.*, 2008; Martinez *et al.*, 2008) with critical differences including the time of behavioral assessment and a more mild inescapable shock training. As previously stated, all behavioral assessment were performed during the nocturnal (active) phase of the animals' circadian cycle, as this has been shown to affect stress-related behavior. Animals were trained in the Coulbourn™ Habitest© shuttle box apparatus with 20 unpredictable foot shocks (0.5 mA, 2-sec) over the course of ten minutes for one session. Twenty-four hours later, animals were placed in the shuttle box and given 28 trials of shocks with a 30-second intertrial interval. Escape latency was measured as the time between opening and closing of the shuttle box door. The criterion for determining the number of trials to acquisition was set as three consecutive escape latencies of 10 seconds or less. Failure to escape after 24 seconds resulted in shock termination and door closure.

5.2.7 Assessment of adult neurogenesis and confocal stereology

Differentiation and survival of neural progenitor cells (NPC) in the dentate gyrus was assessed as previously described (Tyler & Allan, 2013). Briefly, 5-bromo-2'-deoxyuridine (97% Sigma-Aldrich, St. Louis, MO) BrdU was used to label NPCs in a pulse-chase assay using four injections (I.P.) of 50 mg/kg BrdU over a 12-hour period at

PD40. Thirty days later, coronal sections (40 μm) of the rostral-caudal extent of the hippocampus were obtained from 4% paraformaldehyde perfused adult male mice (PD70). Immunohistochemistry was performed for 1:6 serial sections over the dorsal extent of the dentate gyrus. Free-floating sections were incubated for 72 hours in primary antibodies for BrdU (1:300; Abcam, ab6326) and NeuN (1:1500; Millipore, MAB377) and subsequently incubated in secondary antibodies Alexa 488 (1:500; Invitrogen, A11006) and Alexa 647 (1:500; Invitrogen, A21236) and DAPI (1:300 nM; Sigma) for 2 hours at room temperature. Colocalization of BrdU and NeuN was determined for measurement of differentiated NPCs, while BrdU-only labeled cells were indicative of survival of NPCs. While it should be noted that some BrdU cells might be of glial origin (Steiner *et al.*, 2004), this approach is congruent with other recent studies assessing differentiation and survival (Klempin *et al.*, 2010; Hamilton *et al.*, 2014; Jiang *et al.*, 2014). Confocal stereology was performed using an Olympus DSU spinning disk inverted IX-81 microscope; a 40X objective through the orthogonal plane of the tissue was used to visualize colocalization of markers. Quantification of the number of cells with colocalization for 10 slices per brain was performed using StereoInvestigator software; specifications include a guard zone of 2 μm and a probe of 20 μm . Images for publication were acquired using a Zeiss LSM510 META confocal microscope with a 100X oil objective or a 10X objective to obtain maximum intensity projections of Z-stacks.

5.2.8 Protein expression quantification via immunoblotting

Immunoblotting was conducted essentially as previously described using established protocols (Caldwell *et al.*, 2014). Microdissected dentate gyrus tissue derived from one

animal (Hagihara *et al.*, 2009) was homogenized in a Biomasher II disposable microhomogenizer (Kimble Chase) with homogenization buffer containing 20 mM Tris-HCl, 1 mM EDTA, 320 mM sucrose, protease inhibitor cocktail (1:1000; Sigma, P8340), and 200 μ M sodium orthovanadate, pH 7.4. The nuclear fraction was obtained via centrifugation at $1000 \times g$ for 6 minutes at 4°C followed by resuspension in homogenization buffer and further centrifugation at $1000 \times g$ for 10 minutes; the supernatant was collected as the post-nuclear lysate and stored at -80°C . The nuclear pellet was resuspended in homogenization buffer containing 1% (v/v) Triton X-100, 75 mM NaCl, and 75 mM KCl; the nuclear envelope was lysed via sonication and centrifuged at $15,000 \times g$ for 10 minutes. The supernatant was collected as the nuclear fraction and stored at -80°C . Target proteins were assessed in the nuclear (CREB: 20 μ g, GR: 10 μ g, HDAC2: 10 μ g) and post-nuclear (BDNF: 21 μ g) subcellular fractions of the dentate gyrus. Samples from each of four groups (arsenic and control animals drinking only 0.066% saccharin solution or 100 mg/L fluoxetine and 0.066% saccharin solution) were run on the same blots for each target protein. Samples ($n = 7-10$ per group) were loaded in NuPAGE 4 to 12% Bis-Tris gels (Invitrogen, NP0336), separated by electrophoresis, and transferred to PVDF membranes (Millipore Corporation, IPFL00010). Membranes were incubated overnight using the following primary antibodies in 1:1000 dilutions: anti-CREB (Cell Signaling, 9104), anti-GR (Santa Cruz, sc-1004), anti-HDAC2 (Santa Cruz, sc-81599), and anti-BDNF (Santa Cruz, sc-546). All primary antibodies were diluted in PBS-T (1.06 mM KH_2PO_4 , 2.97 mM Na_2HPO_4 , 155 mM NaCl, 0.1 % (v/v) Tween-20, pH 7.4). Membranes were incubated for 45 minutes in respective secondary antibodies dilute to 1:15,000 in PBS-T: IRDye 680RD (LiCOR

Biosciences, 926-68073) and IRDye 800CW (LiCOR Biosciences, 926-32212). Quantification of immunoreactivity was performed using two-channel infrared detection Odyssey® Imaging System (LI-COR Biosciences); Image Studio (LI-COR Biosciences, version 3.1) was used for quantification assessment. For normalization, all blots were stained with IRDye® Blue Protein Stain (LI-COR Biosciences, 3343C056); Coomassie staining was demonstrated to be linear within the range of protein concentrations used in these studies. All blots were normalized to their respective Coomassie stain for quantification.

5.2.9 Statistical Analysis

Global analysis of variance (ANOVA) for two factors (arsenic, fluoxetine) was used to assess all data; further analysis of significant main effects was performed using post hoc Student's *t*-test with Bonferonni correction. Homogeneity of variance was confirmed using Levene's test. Data are presented as mean \pm SEM, and *P* value $<.05$ was deemed statistically significant for all experiments. The reported *n* refers to the number of litters used in each experiment, with only one animal per litter used in each experimental group to avoid litter effects. Separate animals were used for each behavioral experiment, and tissue derived from animals without behavioral assessment was used for all molecular and neurogenesis studies. All studies contain at least three different breeding rounds per assay; *n* = 7–11 and is reported for each assay.

5.3 Results

5.3.1 Perinatal arsenic exposure does not interfere with consumption of fluoxetine in adulthood

Fluoxetine consumption was monitored every other day for five weeks (Figure 5.1A) beginning on PD35. Average intake was calculated for each group: 9.79 ± 0.21 and 9.97 ± 0.21 mg/kg/day for arsenic and control groups consuming 50 mg/L fluoxetine, and 16.6 ± 0.66 and 17.1 ± 0.47 mg/kg/day for arsenic and control groups consuming 100 mg/L fluoxetine. No differences among exposure groups in consumption of fluoxetine were found (Figure 5.1B). Weight gain was monitored weekly in all animals; no differences in weight were observed among exposure or treatment groups (Figure 5.1C). Average weight gain per week was 1.44 ± 0.13 mg/week/animal. ($n = 8-10$ animals per group where $n =$ number of litters)

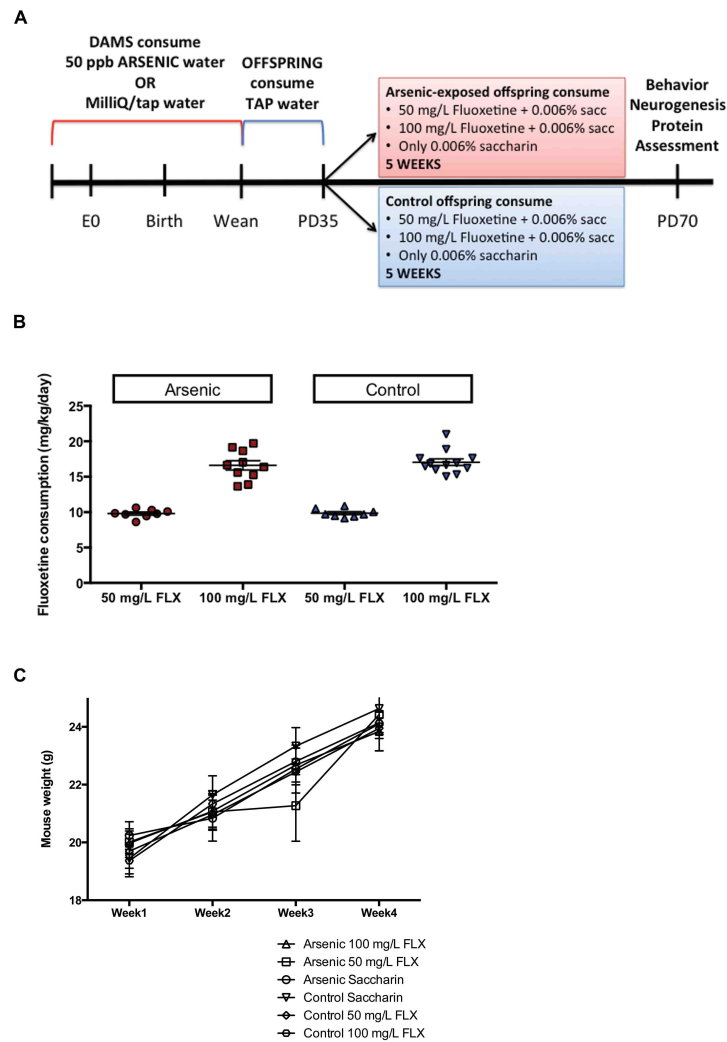


Figure 5.1. Perinatal arsenic exposure paradigm followed by chronic fluoxetine treatment.

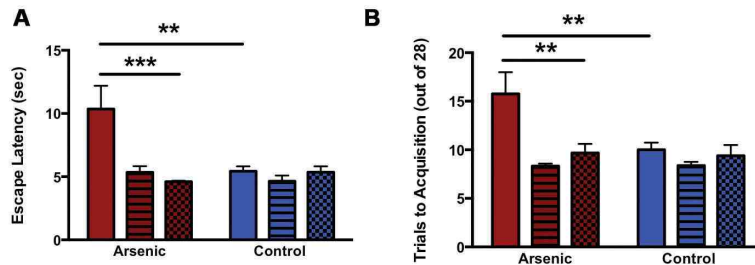
(a) Dams were provided water with 50 parts-per-billion (ppb) arsenic 10 days prior to and throughout breeding and pregnancy until weaning of offspring at postnatal day 23 (PD23). Offspring were placed on tap water until PD35. Male offspring were split into 6 groups: arsenic-exposed or control animals consuming either 50 mg/L fluoxetine with 0.066% saccharin, 100 mg/L fluoxetine with 0.066% saccharin, or 0.066% saccharin only for 35 days (approximately PD35-PD70). BrdU (5-bromo-2'-deoxyuridine) was used to label neural progenitor cells in a pulse-chase assay using four injections (I.P.) of 50 mg/kg BrdU over a 12-h period at PD40. Assessments of neurogenesis, behavior, and protein expression were conducted at PD70. (b) Male offspring from either the arsenic-exposed or control groups consume the same quantities of fluoxetine in drinking water. Average consumption for the arsenic-exposed offspring was 9.79 ± 0.21 mg/kg/day and 16.6 ± 0.66 mg/kg/day for the 50 mg/L and 100 mg/L fluoxetine groups, respectively. Average consumption for the control groups was not significantly different from arsenic-exposed groups: 9.97 ± 0.21 mg/kg/day and 17.1 ± 0.47 mg/kg/day for the 50 mg/L and 100 mg/L fluoxetine groups, respectively. (c) Fluoxetine consumption did not alter weight gain in the exposure groups; weight gain was monitored weekly. All animals were approximately the same weight (after week 4) during assessments.

5.3.2 Fluoxetine treatment prevents depressive-like symptoms induced by perinatal arsenic exposure in a mild learned helplessness task

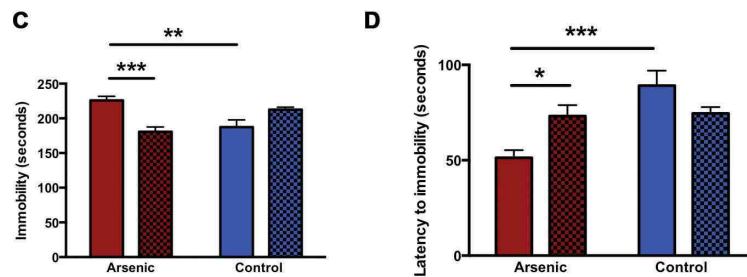
We have previously demonstrated that arsenic induces depressive-like symptoms in a robustly stressful learned helplessness (LH) task consisting of one hour of training with 120 uncontrollable foot shocks. In this experiment, we sought to assess the response to mild stress, which does not result in depressive-like symptoms in control animals. To validate the construct validity of such a task, chronic fluoxetine treatment (for one month prior to the LH task) was used to assess if depressive-like symptoms could be prevented. A mild learned helplessness task consisting of 20 uncontrollable foot shocks given over 10 minutes and subsequent assessment of latency 24 hours later, revealed a significant main effect of arsenic ($F_{(1,47)} = 6.58; p = .01$) and of fluoxetine ($F_{(2,47)} = 9.19; p = .0004$) with a significant interaction ($F_{(2,47)} = 7.24; p = .002$). Results are provided graphically in Figure 5.2A for average escape latency and in Figure 5.2B for trials to acquisition. Average escape latency for arsenic-exposed animals was 10.4 ± 1.8 seconds which was reduced by 52% with fluoxetine treatment (4.9 ± 0.6 seconds), ($p < .001$) independent of dose (50 mg/L versus 100 mg/L). Average escape latency for control animals was 5.4 ± 0.4 seconds (a 48% reduction compared to arsenic-exposed animals, $p < .01$) with no effect of fluoxetine consumption, suggesting lack of depressive-like symptoms. Assessment of trials to acquisition suggests a similar pattern: significant main effects of arsenic ($F_{(1,47)} = 4.39; p = .03$) and of fluoxetine ($F_{(2,47)} = 8.77; p = .0006$) with a significant interaction ($F_{(2,47)} = 4.28; p = .02$) yet no differences among fluoxetine treatment groups in control animals. Due to a lack of dose-dependent effects, all

subsequent treatments used 100 mg/L fluoxetine in drinking water. ($n = 8-10$ animals per group where $n =$ number of litters)

Learned Helplessness



Forced Swim



Stress Response

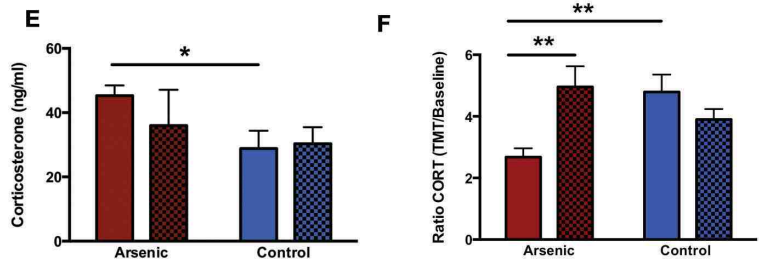


Figure 5.2. Chronic fluoxetine treatment enhances the resiliency to stress-induced depression and restores the stress response in arsenic-exposed animals at PD70. (a) Perinatal exposure to arsenic increases escape latency in the learned helplessness (LH) task at PD70 ($p < .01$). Chronic treatment with 50 mg/L or 100 mg/L fluoxetine decreases latency in arsenic-exposed animals ($p < .001$) in a dose-independent manner. Escape latency of control animals with and without fluoxetine treatment was not altered in this mild LH task. (b) Perinatal arsenic exposure increases the number of trials to criteria in the LH task at PD70 ($p < .01$). The criterion for determining the number of trials to acquisition was set as three consecutive escape latencies of 10 s or less. Chronic treatment with 50 mg/L or 100 mg/L fluoxetine decreases the number of trials to acquisition in arsenic-exposed animals ($p < .01$) in a dose-independent

manner. Average number of trials to acquisition for control animals with and without fluoxetine treatment was not altered in this mild LH task. (c) Total immobility in the forced swim test (FST) of arsenic-exposed animals is increased compared to controls ($p < .01$) 30 min after the onset of a 10-min predator odor exposure (TMT). Chronic treatment with 100 mg/L fluoxetine decreased immobility time of arsenic-exposed animals ($p < .001$), but had no effect on immobility time of control animals. (d) Latency to immobility in the FST is decreased in arsenic-exposed animals compared to controls 30 min after the onset of a 10-min predator odor exposure (TMT) with a significant main effect of arsenic ($F(1,28) = 12.7$; $p = .001$). Chronic treatment with 100 mg/L fluoxetine increased latency to immobility of arsenic-exposed animals ($p < .05$), but had no effect on the latency duration in control animals. (e) Basal corticosterone (CORT) levels in serum are elevated in arsenic-exposed animals at PD70 compared to controls ($p < .05$). Chronic treatment with 100 mg/L fluoxetine had no effect on basal CORT levels in either perinatal exposure group. (f) The percent CORT response, measured as CORT levels 30 min post-onset of the 10-min exposure to TMT divided by the basal CORT levels, was reduced by perinatal arsenic exposure ($p < .01$). Chronic treatment with fluoxetine restored the percent CORT response ($p < .01$) in arsenic-exposed animals, but had no effect on control animals. *** $p < .001$, ** $p < .01$, * $p < .05$.

5.3.3 Fluoxetine treatment prevents depressive-like behavior induced by perinatal arsenic exposure after TMT predator odor exposure in the forced swim task

We sought to assess stress-induced depressive-like behavior during the active nocturnal cycle in a forced swim task (FST) using warm water (25-27°C) to prevent the hypothermia confound observed in several FST paradigms (Petit-Demouliere *et al.*, 2005). We have previously shown adult males perinatally exposed to arsenic exhibit depressive-like behaviors during the diurnal cycle. To determine the resiliency of arsenic-exposed animals and the efficacy of fluoxetine, we assessed depressive-like symptoms in the forced swim task 20 minutes after a 10-minute exposure to a predatory odor (trimethylthiazoline, TMT, the main component of fox urine) for which we have previously shown arsenic-exposed animals do not respond appropriately. Results are provided graphically in Figure 5.2C for total immobility and in Figure 5.2D for latency. Analysis of immobility revealed no significant main effects; however, post hoc analysis indicates that arsenic exposure increases immobility ($p < .01$) and treatment with 100 mg/L fluoxetine ameliorates this depressive-like behavior in the arsenic-exposed group ($p < .001$). Measurement of latency to cease active swimming revealed a significant main

effect of arsenic ($F_{(1,28)} = 12.7; p = .001$), no significant main effect of fluoxetine, and an interaction ($F_{(1,28)} = 10.99; p = .0025$), suggesting that arsenic reduces resiliency in the FST. Fluoxetine treatment (100 mg/L) significantly increased latency in arsenic-exposed animals ($p < .05$) indicating restoration of resiliency, but had no effect on control animals. ($n = 8-10$ animals per group where $n =$ number of litters)

5.3.4 Neither perinatal arsenic exposure nor fluoxetine treatment alters locomotion in the open field task

To determine if altered behavior in the LH and FST behavioral tasks was due to impairment of locomotion, we assessed total movement in the open field test (OFT). Data analysis indicated no significant main effects of arsenic or fluoxetine at PD70 on Day 1 of the OFT. Total movement decreased in all groups on Day 2 of the OFT as expected (data not shown). ($n = 8-10$ animals per group where $n =$ number of litters)

5.3.5 Fluoxetine treatment restores the blunted stress response to predator odor induced by perinatal arsenic exposure

We have previously demonstrated that arsenic-exposed mice fail to activate a normal corticosterone (CORT) response to TMT (Goggin *et al.*, 2012); to determine if fluoxetine attenuates this effect, CORT levels at baseline and after TMT exposure were determined in all animals. As seen in Figure 5.2E, perinatal arsenic exposure increases the baseline levels of CORT: 45.3 ± 3.2 ng/ml compared to 28.8 ± 5.6 ng/ml in control animals ($p < .05$) as expected. Fluoxetine treatment did not alter baseline CORT levels in arsenic-exposed animals or in control animals. Plasma was obtained 30 minutes after the onset of

a 10-minute TMT exposure; subsequent analysis of change in CORT levels was determined using average baseline amounts ($CORT_{TMT} - CORT_{Baseline}$) as seen in Figure 5.2F. Two-way ANOVA analysis indicated no significant main effects of arsenic or fluoxetine, but a significant interaction ($F_{(1,27)} = 10.97, p=.003$). Perinatal arsenic exposure decreased the CORT response compared to controls ($p<.01$). Treatment with fluoxetine did not impact control animals and increased the CORT response in arsenic-exposed animals up to levels measured in the control animals ($p<.01$). These data suggest that arsenic-exposed animals have a blunted but prolonged response to TMT (stress) and fluoxetine treatment attenuates this effect. ($n = 7-10$ animals per group where $n =$ number of litters)

5.3.6 Fluoxetine treatment ameliorates deficits in survival and differentiation of neural progenitor cells after perinatal arsenic exposure

To determine if chronic treatment with 100 mg/L fluoxetine induces alterations in adult neurogenesis in adult males perinatally exposed to arsenic, the number of BrdU+ and BrdU+NeuN+ cells were measured four weeks after a pulse chase BrdU assay. Figure 3A shows triple staining (DAPI+BrdU+NeuN+) used for assessment of the number of differentiated neural progenitor cells (NPCs). All cells containing BrdU were counted for assessment of survival of NPCs. Figures 5.3D and 5.3F are representative images from arsenic-exposed animals at PD70 with and without five weeks of fluoxetine treatment, respectively. Figures 5.3E and 5.3G are representative images from control animals at PD70 with and without five weeks of fluoxetine treatment, respectively. For the number of BrdU+NeuN+ cells, two-way ANOVA data analysis indicates significant main effects

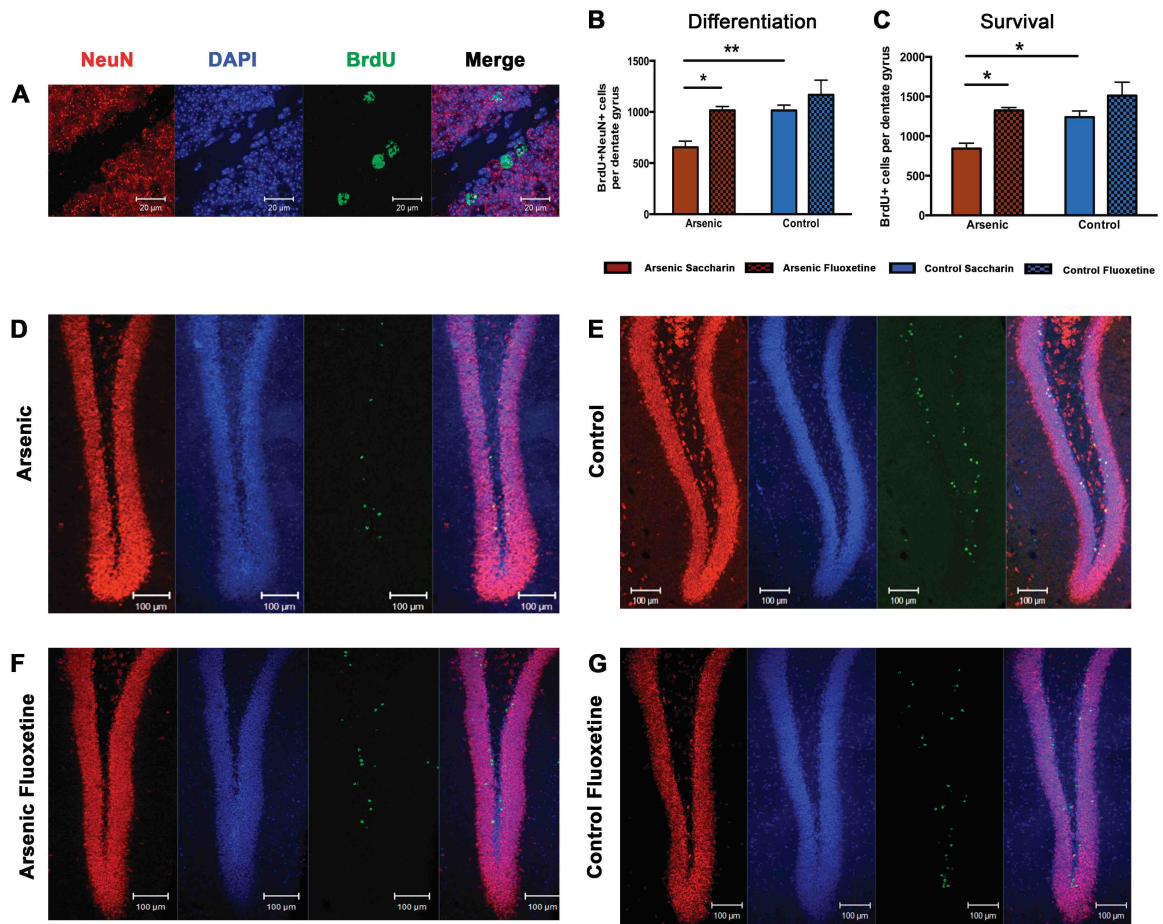


Figure 5.3. Chronic fluoxetine treatment restores deficits in adult hippocampal neurogenesis in arsenic-exposed animals at PD70. (a) Representative images of the dentate gyrus were taken with 100_ objective to demonstrate colocalization of markers to identify neurons (BrdU+NeuN+) and neural progenitor cells (BrdU+). NeuN (red) mature marker of neurons; DAPI (blue) nuclear counterstain; BrdU (green) exogenous mitotic marker; Merge, all three markers (b). Perinatal arsenic exposure decreases the number of BrdU+NeuN+ cells in the dentate gyrus compared to controls at PD70 ($p < .01$). Chronic treatment with 100 mg/L fluoxetine increases the number of BrdU+NeuN+ cells in arsenic-exposed animals ($p < .05$) but not in controls. (c) Perinatal arsenic exposure decreases the number of BrdU+ cells in the dentate gyrus compared to controls at PD70 ($p < .05$). Chronic treatment with 100 mg/L fluoxetine increases the number of BrdU+ cells in arsenic-exposed animals ($p < .05$) but not in controls. *** $p < .001$, ** $p < .01$, * $p < .05$. The following representative images were taken with 10_ objective on a META confocal microscope. NeuN (red) mature marker of neurons; DAPI (blue) nuclear counterstain; BrdU (green) exogenous mitotic marker; Merge, all three markers represented. (d) Representative image of the dentate gyrus at PD70 from an arsenic-exposed animal. (e) Representative image of the dentate gyrus at PD70 from a control animal. (f) Representative image of the dentate gyrus at PD70 from an arsenic-exposed animal chronically treated with 100 mg/L fluoxetine. (g) Representative image of the dentate gyrus at PD70 from a control animal chronically treated with 100 mg/L fluoxetine.

of arsenic ($F_{(1,26)} = 9.675$; $p = .005$) and fluoxetine ($F_{(1,26)} = 9.82$; $p = .004$) but no significant interaction. Arsenic-exposed animals had 654 ± 60 BrdU+NeuN+ labeled

cells; fluoxetine exposure resulted in 1016 ± 37 BrdU+NeuN+ labeled cells, a significant increase of 55% ($p < .05$). Control animals had 1014 ± 52 BrdU+NeuN+ labeled cells (also a 55% increase over arsenic-exposed animals $p < .01$); fluoxetine resulted in 1167 ± 144 BrdU+NeuN+ labeled cells in control animals which was not a significant increase. A similar trend was observed for BrdU+ cells: two-way ANOVA analysis revealed a significant main effect of arsenic ($F_{(1,28)} = 8.33$; $p = .007$) and fluoxetine ($F_{(1,28)} = 13.9$; $p = .001$) but no significant interaction. Arsenic-exposed animals had 843 ± 68 BrdU+ labeled cells, significantly 53% less than the 1240 ± 78 BrdU+ labeled cells in control animals ($p < .05$). Fluoxetine treatment in arsenic-exposed animals resulted in a 57 % increase ($p < .001$), 1325 ± 36 BrdU+ labeled cells. Fluoxetine treatment increased the number of BrdU+ cells by 22% (1511 ± 169 BrdU+ labeled cells) in control animals but this effect was not significant. ($n = 7-9$ animals per group where $n =$ number of litters)

5.3.7 Fluoxetine treatment increases expression of several proteins associated with neurogenesis in the dentate gyrus after perinatal arsenic exposure

To determine the mechanism by which fluoxetine rescues deficits in survival and differentiation of NPCs in perinatally arsenic exposed animals, protein expression of several factors that contribute to adult neurogenesis was assessed in dentate gyrus tissue. As we have previously demonstrated reduced expression of the glucocorticoid receptor (GR) in the hippocampus of PD35 arsenic-exposed animals (Martinez-Finley *et al.*, 2009), GR expression in the nuclear fraction of the dentate gyrus at PD70 was assessed. Representative blots and data are shown in Figure 5.4A for the GR. There were no significant main effects of arsenic but a significant main effect of fluoxetine ($F_{(1,38)} = 8.5$;

$p = .006$) and no significant interaction; however, treatment with fluoxetine increased expression of the GR in both arsenic-exposed and control groups ($p < .05$, Figure 5.4A). Histone deacetylase 2 (HDAC2) has been implicated in depression and has a known GR response element in its promoter. Perinatal arsenic exposure did not alter protein quantification of HDAC2 in the nuclear fraction of the dentate gyrus (Figure 5.4B); however, there was a significant main effect of fluoxetine treatment ($F_{(1,36)} = 6.1$; $p = .02$) with significantly increased expression of HDAC2 in arsenic-exposed animals ($p < .05$) but not in control animals. Brain derived neurotrophic factor (BDNF) is regulated by the GR, HDAC2, and cAMP response element binding protein (CREB); both BDNF and CREB are paramount to proper maturation and differentiation of neural progenitor cells (Gass & Riva, 2007). Protein quantification of the mature form of BDNF in the post-nuclear lysate fraction of the DG and of CREB in the nuclear fraction of the DG was determined. Representative blots and data are shown in Figure 5.4C for BDNF and Figure 5.4D for CREB. A significant main effect of fluoxetine on BDNF was determined ($F_{(1, 36)} = 6.2$; $p = .012$). While PAE did not alter BDNF expression, arsenic-exposed animals treated with fluoxetine had higher immunoreactivity to BDNF than their non-treated counterparts ($p < .05$), with no effect on control animals. A similar pattern was observed for CREB protein expression: a significant main effect of fluoxetine was determined ($F_{(1,31)} = 8.5$; $p = .0065$), with a significant difference among arsenic-exposed animals with and without fluoxetine treatment ($p < .05$) but not among control animals. ($n = 8-11$ animals per group where $n =$ number of litters)

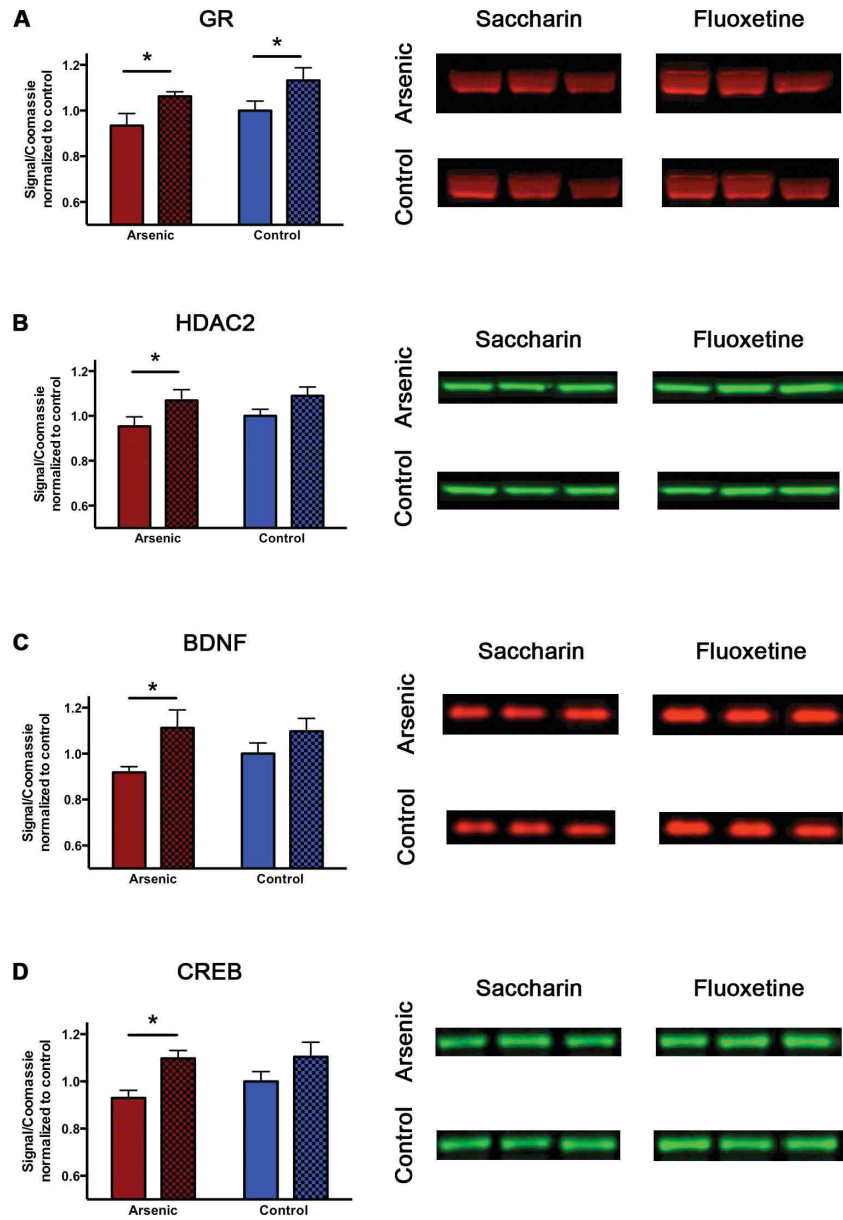


Figure 5.4. Chronic fluoxetine treatment increases expression of proteins associated with neurogenesis in the dentate gyrus of arsenic-exposed animals at PD70. (a) In (A)–(D), representative images of immunoblots are shown for arsenic-exposed and control animals consuming 0.066% saccharin or 100 mg/L fluoxetine with 0.066% saccharin. The immunoreactivity for each protein antibody was normalized to its respective Coomassie stain for quantification (see Supplementary Figure). (b) Perinatal arsenic exposure does not alter the protein expression of the glucocorticoid receptor (GR) in the nuclear fraction of the dentate gyrus at PD70 compared to controls. Chronic treatment with 100 mg/L fluoxetine increases expression of the GR in both arsenic exposed animals ($p < .05$) and control animals ($p < .05$) at PD70. (c) Perinatal arsenic exposure does not alter the protein expression of histone deacetylase 2 (HDAC2) in the nuclear fraction of the dentate gyrus at PD70 compared to controls. Chronic treatment with 100 mg/L fluoxetine increases expression of HDAC2 in arsenic-exposed animals ($p < .05$) but not in control animals at PD70. (d) Perinatal arsenic exposure does not alter the protein expression of the mature form of brain-derived neurotrophic factor (BDNF) in the post-nuclear lysate fraction of the dentate gyrus at

PD70 compared to controls. Chronic treatment with 100 mg/L fluoxetine increases expression of mature BDNF in arsenic-exposed animals ($p < .05$) but not in control animals at PD70. (e) Perinatal arsenic exposure does not alter the protein expression of cAMP responsive element binding protein (CREB) in the nuclear fraction of the dentate gyrus at PD70 compared to controls. Chronic treatment with 100 mg/L fluoxetine increases expression of CREB in arsenic-exposed animals ($p < .05$) but not in control animals at PD70. *** $p < .001$, ** $p < .01$, * $p < .05$.

5.4 Discussion

Toxins such as lead, methylmercury, organophosphates, and arsenic have been associated with psychiatric disorders, including depression, in a number of epidemiological studies (Zierold *et al.*, 2004; Sen & Sarathi Biswas, 2012; Beard *et al.*, 2013; Freire & Koifman, 2013; Malekirad *et al.*, 2013; Ojo *et al.*, 2013; Weisskopf *et al.*, 2013). Yet, the impact of environmental factors, particularly toxins, on the susceptibility to depression has not been investigated in great detail. We have previously shown several behavioral deficits, morphological abnormalities, and molecular aberrations induced by developmental arsenic exposure in adult mice (Martinez *et al.*, 2008; Martinez-Finley *et al.*, 2009; Martinez-Finley *et al.*, 2011; Tyler & Allan, 2013). In the present study, we determined the extent to which chronic fluoxetine treatment was able to reverse these effects and restore resiliency to stress-induced depression in arsenic-exposed animals.

Animals perinatally exposed to arsenic likely exhibit depressive-like symptoms and negatively process congruent information due to hyperactivity of the HPA axis, as observed in depressed patients and other rodent models (Vollmayr & Gass, 2013). Here, we have demonstrated that exposure to mild stress on the first day of a learned helplessness (LH) task was sufficient to induce depressive-like symptoms in arsenic-exposed mice, but not in controls, suggesting a lack of resiliency to stress. This particular LH paradigm is minimal (low foot shock and few inescapable exposures) such that it is

unlikely to induce depressive-like behaviors in controls as other LH paradigms do. Additionally, arsenic-exposed mice exhibit depressive-like behavior in the forced swim task (FST) after a 10-minute exposure to predator odor, while control animals do not. This effect may be mediated through alterations in the HPA axis signaling leading to an aberrant stress response. To support this assertion, we determined that corticosterone (CORT) levels are elevated under basal conditions in arsenic-exposed animals; and while hyperlocomotion due to elevated CORT can confound activity measured in depression tasks (Strekalova *et al.*, 2005), we found no difference in total locomotor activity in the open field test (data not shown). The blunted CORT response we measured after predator odor exposure in arsenic-exposed animals is conceivably due to the elevated CORT before the stressor and the lack of sufficient CORT activation. Reduced HPA axis responses have been observed in animals prenatally exposed to stress with behavioral deficits in adulthood due to HPA hyperactivity (Darnaudery & Maccari, 2008). Furthermore, abnormal HPA axis function has been measured in depressed patients, along with increased release of cortisol and blunted HPA axis feedback; these alterations are diminished after chronic treatment with antidepressants, typically preceding alleviation of behavioral symptoms (Wainwright & Galea, 2013). Thus, perturbation the HPA axis may be required to elucidate distinctions in depressive-like behavior between arsenic and control animals, yet these behaviors may be impermanent as resiliency to depressive-like symptoms was restored after chronic treatment with fluoxetine in a dose-independent manner.

Recent studies have demonstrated a link between the early environment, development of HPA axis feedback, and adult hippocampal neurogenesis (Loi *et al.*, 2014). Results presented here suggest that perinatal arsenic exposure induces unambiguous but reversible deficits in adult neurogenesis by reducing the survival (BrdU+ cells; 53% less) and differentiation (BrdU+NeuN+; 55% less) of neural progenitor cells four weeks after BrdU injection at PD70. Chronic treatment with 100 mg/L fluoxetine, resulting in 17 mg/kg/day consumption, attenuated these deficits in the arsenic-exposed animals: we measured a 57% increase in survival of BrdU+ cells and a 55% increase in the number of differentiated BrdU+NeuN+ cells. Region specificity was not determined; however all measurements occurred in the dorsal hippocampus. Additionally, BrdU+ cells may include a small population of glial cells, but this effect may be negligible (Steiner *et al.*, 2004). These results concur with our previous observations of the robust effect of intermittent interval exposure to enrichment on neurogenesis after arsenic exposure (Tyler & Allan, 2013). This increase in adult neurogenesis coincident with restored resiliency to stress-induced depression suggests that fluoxetine is ameliorating arsenic-induced deficits via a neurogenic mechanism.

Recent genomic analysis has demonstrated that genes associated with adult hippocampal neurogenesis are impacted by SSRI treatment (Malki *et al.*, 2012). Additionally, HPA axis stress responses in the dentate gyrus are mediated in part through the glucocorticoid receptor (GR) and can impact gene expression (de Kloet *et al.*, 2005). For its part, arsenic exposure induces deficits in mRNA expression of neurogenesis-related genes, including *Nrc31* (gene for GR protein), *Creb1*, and *Hdac2* (Goggin *et al.*, 2012; Tyler & Allan,

2013), though the mechanism by which this damage occurs is unclear. Thus to determine the pathways involved in fluoxetine's neurogenic actions and in arsenic-induced morphological damage, we assessed expression of proteins involved in AHN, including the GR, CREB, HDAC2, and BDNF. We would have expected all of these proteins to be reduced in arsenic-exposed animals, but arsenic exposure did not result in altered levels for any of these proteins. Our results may be specific to the dentate gyrus: studies demonstrating reduced CREB and BDNF, in response to either prenatal stress or toxins such as lead or mercury, have done so using mRNA expression in the dentate gyrus (similar to our previous observations) or protein quantification in the whole hippocampus, but never protein expression in the dentate gyrus (Onishchenko *et al.*, 2008; Feng *et al.*, 2012; Boersma *et al.*, 2013; Guan *et al.*, 2013; Sanchez-Martin *et al.*, 2013; Boersma *et al.*, 2014). While arsenic exposure in the parts-per-million range has been shown to reduce CREB and associated proteins (Wang *et al.*, 2009), the arsenic concentration in our study is in the parts-per-billion range, closer to the levels humans are exposed to, and likely too low to impair CREB signaling. Additionally, reduced *Nr3c1* and *Hdac2* mRNA levels in the hippocampus or reduced *Creb1* mRNA in the dentate gyrus may not translate to reduced protein levels, as seen in several other studies. While these proteins may not be altered, their downstream signaling, including ERK from the BDNF pathway, may lead to deficits in AHN or lead to greater susceptibility to stress-induced depression (Numakawa *et al.*, 2013). Thus, reduced AHN in arsenic-exposed animals does not derive from alteration of these target proteins, and elucidating an arsenic-specific mechanism requires further investigation.

While arsenic does not impact the expression of CREB, HDAC2, GR, or BDNF, chronic fluoxetine treatment increases expression of all target proteins, particularly in the arsenic-exposed animals. These results suggest that the mechanism by which fluoxetine enhances AHN is separate from the pathways damaged by arsenic exposure. Increased signaling of 5-HT receptors after fluoxetine administration ultimately leads to a steady increase in cAMP, protein kinase A, and CREB (Pinnock *et al.*, 2010). CREB, a histone acetyltransferase, then allows for increased transcription of BDNF, which has antidepressant effects when released and feedback on CREB. Thus, increased BDNF and CREB expression in the dentate gyrus after fluoxetine treatment was expected in these studies, as others have demonstrated similar results, particularly in rodent models with impaired stress responses (Blom *et al.*, 2002; Vinet *et al.*, 2004). Fluoxetine also enhances functionality of the GR (Pariante *et al.*, 2003) allowing for termination of HPA signaling via improved negative feedback (Sapolsky *et al.*, 1984), as seen in studies presented here using the predator odor and FST challenge. This enhanced GR signaling directly increases AHN (Pariante *et al.*, 2003; Anacker *et al.*, 2011b) and uses neurogenesis-dependent mechanisms to restore control over the HPA after dexamethasone administration (Surget *et al.*, 2011). The GR has also been demonstrated to increase expression of HDAC2 via the *Hdac2*-GRE (Graff *et al.*, 2012), while others have shown HDAC2 is necessary for GRE-mediated gene activation (Luo *et al.*, 2009b). However, HDAC2 negatively regulates BDNF (Guan *et al.*, 2009), though we are the first to show these alterations specifically in the dentate gyrus; as such, the interaction among these proteins after fluoxetine treatment remains to be elucidated. Overall, fluoxetine treatment is ameliorating depressive-like behavior after PAE by enhancing

AHN in the dentate gyrus likely mediated by these increased proteins. This effect is particularly robust in arsenic-exposed animals, as neither protein expression nor AHN was significantly increased in control animals after fluoxetine treatment.

A recent study reported that fluoxetine does not increase BDNF levels in males after chronic psychosocial isolation stress (Mitic *et al.*, 2013), and several others have demonstrated that in control animals not exposed to stress, BDNF does not increase after fluoxetine treatment (Nibuya *et al.*, 1995; Dias *et al.*, 2003; De Foubert *et al.*, 2004). While we anticipated increased protein expression and AHN in control animals after fluoxetine treatment, our results are consistent with these studies and the lack of altered behavior and stress response we observed in control animals. However, AHN is a sensitive system easily influenced by experience, stress, and pharmacological manipulation. As such, it is likely that our findings for control animals can be attributed to the method of fluoxetine administration. A similar pattern was reported in animals consuming fluoxetine via drinking water after developmental methylmercury exposure: three weeks of chronic fluoxetine treatment (80 mg/L) ameliorated deficits in total neuronal number in exposed animals but did not significantly impact cell numbers in unexposed control animals (Bose *et al.*, 2012). Other delivery methods of fluoxetine generate different results: animals given 18 mg/kg fluoxetine via gavage for 28 days exhibit increased proliferation and maturation of cells in the hippocampus (Wang *et al.*, 2008a), while interperitoneal (IP) administration of 5 or 10 mg/kg/day fluoxetine for 28 days increases survival of neural progenitor cells one month (Malberg *et al.*, 2000) and 24 hours after BrdU injection (Santarelli *et al.*, 2003) in control animals, respectively.

Both the IP and the gavage procedures provide instant delivery of the antidepressant, which should accelerate the time to achieving peak blood levels and may affect downstream mechanisms. It should also be noted that both methods of administration (injection and gavage) are stressful and may reduce AHN in the vehicle control animals not provided antidepressants. Similarly, fluoxetine increases proliferation of NPCs after mild chronic stress (Surget *et al.*, 2011) and survival and maturation of NPCs after corticosterone injection (David *et al.*, 2009) but this effect is not observed in control animals without exposure to stress. Since arsenic-exposed animals have alterations in the hypothalamic-pituitary-adrenal-axis (HPA) resulting in aberrant stress signaling (Goggin *et al.*, 2012), we sought to provide the antidepressant using the least stressful method.

The evidence provided here strengthens the connection between AHN and depression, as arsenic-exposed mice have decreased AHN and depressive-like symptoms; however, this association is correlational. It is unknown if alterations in the HPA axis result in deficits in AHN that ultimately lead to the onset of depression or if altered AHN is a byproduct of stress-induced depression (David *et al.*, 2009; Anacker *et al.*, 2013). In this study, increased AHN via fluoxetine in arsenic-exposed animals is concurrent with increased resiliency to stress-induced depression in the mild stressor LH task and in the FST following predator odor exposure. This effect may be mediated by increased AHN, possibly via increased nuclear GR in the dentate gyrus, as demonstrated in the hippocampus in other studies (Anacker *et al.*, 2011b), or via reduced basal levels of CORT and a subsequent proper response to stress. The serotonergic system may also contribute to the efficacy of fluoxetine: arsenic-exposed mice have decreased serotonin

levels and increased 5HT_{1A} receptors in the dorsal hippocampus (Martinez *et al.*, 2008), and these receptors have been implicated in the efficacy of fluoxetine as an antidepressant (Riad *et al.*, 2004). It is likely that fluoxetine alters morphology, behavior, and molecules in the arsenic-exposed animals via all of these pathways: increased AHN, restored stress response, and serotonergic signaling via 5HT_{1A} receptors, although we have yet to measure the latter.

5.5 Conclusion

Several epidemiological and rodent studies have demonstrated a correlation between exposures during gestation or early development and increased risk for developing psychiatric disorders (Hellemans *et al.*, 2010; Davis *et al.*, 2013; Talati *et al.*, 2013); indeed, arsenic exposure has been associated with depression in epidemiological studies (Zierold *et al.*, 2004). While it is unclear if the damage resulting from developmental exposures is amenable to intervention, we have provided evidence that arsenic-induced deficits can be reversed with fluoxetine treatment. Using a perinatal arsenic exposure model in mice, we have demonstrated that arsenic reduces resiliency to stress-induced depression, induces a blunted CORT response, and decreases adult hippocampal neurogenesis. These effects were ameliorated and behavior restored with chronic fluoxetine treatment, along with increased levels of proteins associated with neurogenesis in the dentate gyrus. Based on these findings, fluoxetine restores resiliency to depression via a neurogenic mechanism. However, the mechanisms through which arsenic exposure reduces AHN are likely not the same mechanism by which fluoxetine reverses these effects. While fluoxetine increases expression of neurogenesis-related proteins, deficits in

these proteins were not found after arsenic exposure. Thus, fluoxetine is likely bypassing arsenic-induced damage to increase AHN. As such, while alterations in morphology and behavior after PAE are ameliorated by chronic fluoxetine treatment, the mechanism by which arsenic produces deficits in AHN is still under investigation.

6.0 Developmental exposure to 50 ppb arsenic influences histone modifications and associated epigenetic machinery in a region- and sex-specific manner in the adult mouse brain

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Abstract

Epidemiological studies have demonstrated that arsenic exposure has a profound effect on the epigenetic landscape, particularly on histone posttranslational modifications (HPTMs), which are critical for programming of cellular function. We have previously shown that exposure to a low level of arsenic (50 parts-per-billion) during the perinatal period (three trimester equivalent) induces deficits in adult neurogenesis, depressive-like symptoms, and alterations in gene expression. As epigenetic processes control these outcomes, we assessed the influence of our developmental arsenic exposure paradigm (DAE) on global HPTMs and the expression of associated chromatin-modifying proteins in the dentate gyrus (DG) and frontal cortex (FC) of adult male and female mice using western blotting techniques. DAE preferentially altered histone methylation levels dependent on sex, with increased levels of trimethylation of histone 3 lysine 4 (H3K4me3) in both the FC and DG of adult males and decreased H3K4me3 levels in the female DG. The chromatin-modifying protein responsible for this modification, MLL, exhibited a similar expression profile in arsenic-exposed animals. Conversely, DAE influenced histone acetylation dependent on both tissue type and sex, with increased H3K9ac levels in the male and female DG and decreased levels in the male FC. Associated histone acetyltransferase expression of GCN5 and PCAF was similarly impacted by DAE without alterations in CREB-binding protein (CBP). Levels of H3, H3ac, H3K9me3 were not influenced by DAE in either brain region of either sex. Surprisingly, arsenic exposure reduced expression of CREB and p-CREB in the male frontal cortex with concurrent increased expression of the repressive complex proteins REST/NRSF and Co-REST. These findings suggest that developmental exposure to a

very low level of arsenic leads to long-lasting changes in histone methylation, acetylation, and associated chromatin modifying proteins in a region- and sex-dependent manner in the adult mouse brain.

6.1 Introduction

Arsenic exposure continues to be a worldwide health concern as several millions of people are exposed to this environmental toxicant via natural and anthropogenic sources (Naujokas *et al.*, 2013). The Environmental Protection Agency (EPA) and World Health Organization (WHO) have deemed 10 µg/L (parts-per-billion, ppb) arsenic in drinking water safe for consumption; however, in several countries around the world (including in the U.S. prior to 2006) 50 ppb arsenic remains the standard allotment in drinking water (WHO, 2008). Additionally, there are places where access to drinking water containing arsenic within the WHO limits is simply not possible, and populations within these regions are exposed to excessive arsenic, bordering on mass poisoning (Jiang *et al.*, 2013). Exposure to arsenic can also occur via consumption of arsenic-contaminated food, which is also a significant problem. It is well established that high levels of arsenic exposure (in the parts-per-million range) can induce disease states (including cancer) in almost every organ in the body including the heart, lungs, liver, and brain (Bustaffa *et al.*, 2014). Epidemiological studies have demonstrated that even low levels of arsenic exposure can negatively impact the body, including increasing the propensity toward developing a psychiatric disorder and cognitive dysfunction (Zierold *et al.*, 2004; Brinkel *et al.*, 2009). Importantly, *in utero* and developmental arsenic exposure results in learning and memory deficits in children and may underlie long-lasting susceptibility to disease later in life

(reviewed in (Tyler & Allan, 2014). However, relatively little is known about the long-term influence of low levels of arsenic exposure, particularly in the brain.

Research over the past decade has provided evidence demonstrating that arsenic alters the epigenetic landscape in various cell types. The epigenome consists of DNA methylation and histone modifications that collectively constitute chromatin structure and ultimately chromatin function, conferring regulation of gene expression (Kouzarides, 2007). Of particular interest are studies on histone posttranslational modifications (HPTM), as histone modifications can be dynamic in response to the extrinsic environment and are paramount for proper differentiation of neural stem cells in the brain (Hsieh & Eisch, 2010; Day & Sweatt, 2011). Indeed, epigenetic dysregulation HPTMs has been postulated as a molecular mechanism underpinning psychiatric disorders such as depression (Mateus-Pinheiro *et al.*, 2011; Sun *et al.*, 2013). However, the impact of arsenic on the epigenetic status of the brain has not been thoroughly investigated, particularly in the context of developmental exposure. To date, there are three studies on the effects of developmental exposure to arsenic in the brain, suggesting an impact of arsenic on histone acetylation and DNA methylation with concurrent deficits in proteins that may underlie learning and memory deficits (Zarazua *et al.*, 2010; Martinez *et al.*, 2011; Cronican *et al.*, 2013). Conversely, the literature on the effect of arsenic on the epigenome is quite extensive in the context of cancer. *In vitro* studies have demonstrated arsenic exposure influences histone methylation, acetylation, and phosphorylation along with the expression chromatin modifying proteins that impart these modifications in human carcinoma cell lines (Zhou *et al.*, 2008; Ren *et al.*, 2011; Chervona *et al.*, 2012a).

Additionally, a prospective epidemiological study reported an effect of arsenic exposure (via drinking water) on HPTMs in peripheral blood mononuclear cells from exposed individuals, demonstrating a particularly strong correlation between arsenic exposure and histone methylation and acetylation, with a differential influence of arsenic dependent on sex (Chervona *et al.*, 2012b). To our knowledge, there have been no reports on the long-term epigenetic consequences of developmental arsenic exposure in the brain, particularly of HPTMs and their associated chromatin modifying enzymes.

We are interested in the mechanisms that potentially mediate the long-lasting toxicity of developmental arsenic exposure into adulthood when the presence of arsenic is quite low. Using a perinatal exposure paradigm to 50 ppb sodium arsenate (through all three trimesters of fetal/neonatal development), we have previously demonstrated that arsenic reduces adult hippocampal neurogenesis (AHN) leading to greater susceptibility to developing stress-induced depressive-like symptoms in adult male mice (Tyler & Allan, 2013; Tyler *et al.*, 2014). Adult neurogenesis is comprised of several processes that include the continual mitotic activity of dentate gyrus neural progenitor cells and their subsequent maturation and integration into the hippocampal circuitry (Ming & Song, 2005). These processes are finely orchestrated by several epigenetic factors, including histone modifications (Ma *et al.*, 2010). We have provided evidence that developmental arsenic exposure (DAE) alters the expression of many neurogenesis-related genes in the adult dentate gyrus (Tyler and Allan, 2013); however, the mechanism by which arsenic induces this damage is currently not known. Based on the extensive literature investigating the effects of arsenic on the epigenome in relation to cancer, we hypothesize

that DAE leads to altered epigenetic processes, specifically histone modifications, in the dentate gyrus of adult male mice.

Here, we report an influence of perinatal arsenic exposure on levels of trimethylation of H3K4 and acetylation of H3K9 and on expression of associated chromatin-modifying proteins including MLL, GCN5, PCAF, CREB, p-CREB, and REST/NRSF, and Co-REST in a sex- and region-dependent manner. No changes in total H3, H3K9me3, total H3 acetylation or CBP expression in either tissue of either sex were observed. This is the first report to demonstrate that low-level arsenic exposure during development imparts damage on the epigenetic landscape of the brain in adulthood.

6.2 Materials and Methods

Chemical Hazards. Arsenic is classified as a human co-carcinogen; all arsenicals were handled with caution in accordance with MSDS standards.

6.2.1 Perinatal arsenic exposure paradigm

The Institutional Animal Care and Use Committee at the University of New Mexico (UNM) approved the animal protocols, including the arsenic exposure paradigm, used in this study. C57BL/6 mice obtained from Jackson Labs were maintained on a reverse light/dark cycle (lights off at 0800) with *ad libitum* access to food and water in the Animal Resource Facility at UNM. Arsenic exposure was performed as previously described (Tyler *et al.*, 2014), as depicted in Figure 1. Briefly, singly-housed female mice aged 55 days were acclimated to drinking 50 ppb arsenic water (sodium arsenate, Sigma

Aldrich) for 10 days prior to mating. Arsenic water was prepared weekly using standard tap and MilliQ water. Control mice were administered tap water from UNM, which contains approximately 2-5 ppb arsenic. Mating occurred for five days; dams continued to drink arsenic-laced water throughout pregnancy until offspring were weaned at postnatal day (PD) 23. Offspring were group housed, four per cage, with *ad libitum* access to food and tap water. At PD70, animals were euthanized via rapid decapitation, and the frontal cortex and dentate gyrus from both male and female animals were microdissected and snap frozen and stored at -80°C until further analysis. Female animals were exposed to male bedding prior to euthanizing. Synchronicity of cycle was confirmed via vaginal cytology, and only brain tissue from females in the proestrus phase was used, as some biochemical and behavioral measures are impacted by the phase of the estrous cycle (Warren & Juraska, 1997). Multiple rounds of breeding were performed for sufficient numbers of litters for arsenic or control groups. For each experiment, at least 6 different litters from different dams were used; e.g. $n = 6$ represents the number of different litters used with one animal per litter to avoid litter effects.

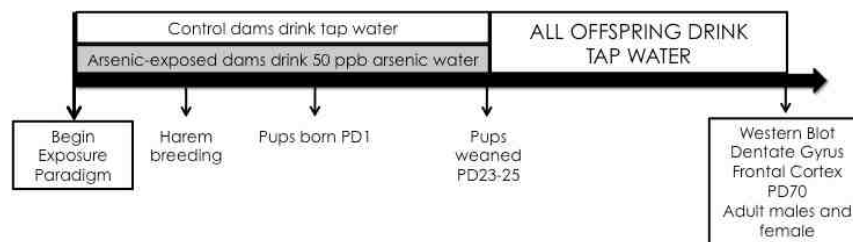


Figure 6.1. Perinatal Arsenic Exposure Paradigm

Exposure to 50 parts-per-billion arsenic via drinking water occurs throughout the perinatal period, which includes all three trimesters of development in rodents. The arsenic exposure paradigm is initiated 10 days prior to mating, and arsenic consumption is monitored. Control dams drink tap water from UNM, which contains approximately 2-5 ppb arsenic. Dams drink arsenic-laced water until pups are weaned at approximately postnatal day (PD) 23. Both control and arsenic-exposed offspring are group housed with *ad libitum* access to food and tap water; brain tissue is retrieved via microdissection at PD70 for analysis.

6.2.2 Evaluation of histone methylation and acetylation

Histone extraction. For preparation of extracted histones, tissue derived from one animal, either the dentate gyrus or the frontal cortex, was homogenized in a Biomasher II disposable microhomogenizer (Kimble Chase) using PBS buffer containing 0.5% Triton-X 100 (v/v), 2 mM phenylmethylsulfonyl fluoride (PMSF), 0.02% (w/v) sodium azide (NaN_3), 5 mM sodium butyrate (NaB), and 1 $\mu\text{g}/\mu\text{l}$ protease inhibitor cocktail (Sigma, #P8340). Tissue homogenates were centrifuged at $6500 \times g$ for 10 minutes at 4°C ; nuclei were washed and centrifuged as before. The pellet was resuspended in 1 N HCl and acid extraction was allowed to occur overnight at 4°C ; 1 N NaOH was added the following day to neutralize the acid. Lysates were centrifuged as before and the supernatant saved for protein quantification. Bradford assays were performed to determine the concentration of histone protein. Aliquots were stored at -80°C until further use.

Histone assessment. Approximately 8-12 μg histone protein, determined by antibody optimization, was separated using NUPAGE 10% bis-tris gels (Invitrogen, NP0316) and transferred to a PVDF membrane (Millipore Corporation, IPFL00010). Membranes were incubated overnight at 4°C using the following primary antibodies diluted in PBS-T: H3 (1:1000 in DG and FC; Cell Signaling, 3638), H3K4 trimethyl (1:1000 in DG and FC; Abcam, ab8580), H3 acetyl (1:1000 in DG and FC; Millipore, 06-599), H3K9 trimethyl (1:2000 in DG; 1:1000 in FC; Epigentek, A-4036), H3K9 dimethyl (1:2000 in DG; 1:1000 in FC; Epigentek, A-4035), H3K9 acetyl (1:500 in DG and FC; Epigentek, A-4022), or H3K27 trimethyl (1:2000 in DG; 1:1000 in FC; Epigentek, A-4039). Membranes were incubated for 45 min in their respective secondary antibodies

(1:15,000) from LiCOR: rabbit IRDye 680RD and mouse IRDye 800CW. Quantification of protein expression was performed using Image Studio, and values are expressed as each individual histone mark normalized to H3. Evaluation of each histone mark was performed on separate gels and blots to avoid confounds. The $n = 6-10$ litters depending on histone mark and will be expressed for each individual blot in the results.

6.2.3 Evaluation of chromatin modifying proteins

Immunoblotting for chromatin-modifying proteins was conducted as essentially described using our established protocols (Goggin *et al.*, 2012; Tyler *et al.*, 2014). Dentate gyrus or frontal cortex tissue lysates from one animal were prepared and the nuclear fraction was isolated for protein expression analysis. Total protein concentration was determined by Bradford assay. Antibody and protein optimization was performed for each target protein in both types of tissues. Membranes were incubated overnight at 4°C using the following primary antibodies diluted in PBS-T: MLL (1:1000 in DG, 1:500 in FC; Santa Cruz, sc-20153); GCN5 (1:000 in DG and FC; Santa Cruz, sc-20698); PCAF (1:000 in DG and FC; Santa Cruz, sc-13124); CREB binding protein, CBP (1:500 in DG, 1:1000 in FC; Cell Signaling, cs-9104); CREB (1:1000 in FC; Cell Signaling, cs-9104), phosphorylated CREB, p-CREB (1:1000 FC; Cell Signaling, cs-9198), REST/NRSF (1:500 in DG and FC; Santa Cruz, sc-15118); Co-REST (1:500 in DG and FC; Santa Cruz, sc-23449), and KDM5B (1:500 in DG and FC; Abcam, ab-181089). Membranes were incubated for 45 min in their respective secondary antibodies (1:15,000) from LiCOR: rabbit IRDye 680RD and mouse IRDye 800CW. Quantification of protein expression was performed using Image Studio; Coomassie staining was demonstrated to be linear for all protein

concentrations used. Values are presented as protein expression normalized to Coomassie and renormalized to controls.

6.2.4 Statistical Analysis

H3 expression levels were determined to be similar among exposure groups in males and females in both tissue types and was normalized to Coomassie staining. Histone expression levels, as measured via western blotting, were normalized to H3 levels for each sample, and each histone mark was run on a separate gel. Chromatin modifier protein expression was normalized to the average of total protein expression evaluated by Coomassie staining. All data are presented as mean \pm SEM normalized to control values, and a p value of $<.05$ was set for statistical significance. All data were analyzed by t -test using GraphPad software (GraphPad Software, v. 6.0; San Diego, CA). The number of litters is reported for each experiment, with only one animal per litter used to avoid litter confounds. All studies contain at least three different breeding rounds per assay, with at least $n = 6$ per assay.

6.3 Results

6.3.1 Validation of H3 for normalization of histone proteins

To validate histone 3 as a proper control, we evaluated the impact of perinatal arsenic exposure on H3 levels in dentate gyrus and frontal cortex tissue derived from adult male and female mice. We observed no effect of arsenic exposure on H3 levels (Figure 2A: Male DG, $t(11) = .4555$, $p = .6576$; Fig 2B: Male FC, $t(10) = .2597$, $p = .8003$; Fig 2C: Female DG, $t(11) = .2950$, $p = .7735$; Fig 2D: Female FC, $t(12) = .7512$, $p = .4670$). H3

expression was used to normalize expression for all subsequent histone modifications. ($n = 6-7$ per assay)

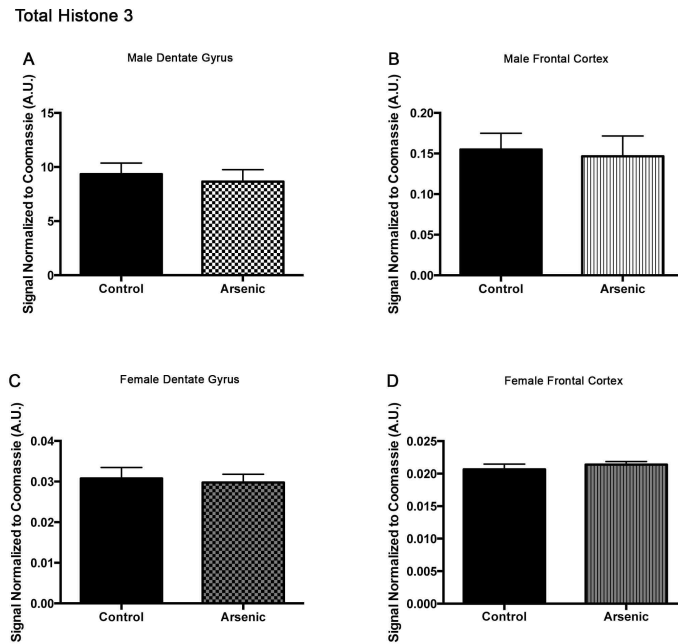


Figure 6.2. Developmental arsenic exposure does not impact H3 in the adult mouse brain.

To establish validity as a control for immunoblotting, H3 levels were assessed in PD70 control and arsenic-exposed male and female offspring in both tissue types. Perinatal arsenic exposure does not alter the expression levels of total histone protein 3 in either the dentate gyrus (A and C) or the frontal cortex (B and D) in male or female adult mice, respectively.

6.3.2 Perinatal arsenic exposure does not influence methylation of H3K9

To determine the long-term epigenetic consequences of perinatal arsenic exposure (Figure 1), we assessed histone methylation at postnatal day (PD) 70 in the dentate gyrus (DG) and the frontal cortex (FC) of both male and female mice. Perinatal arsenic exposure did not alter levels of histone 3 lysine 9 trimethylation (H3K9me3), a repressive posttranslational modification, in either brain region of either sex (Figure 3A: Male DG, $t(14) = .056$, $p = .956$; Fig 3C: Female DG, $t(16) = .2288$, $p = .822$; Fig 3D: Female FC, $t(12) = .5396$, $p = .5994$), although there was a modest increase of expression in the adult

male frontal cortex, though this was not significant (Fig 3B: Male FC, $t(14) = 1.596$, $p = .13$). ($n = 7-10$ per assay)

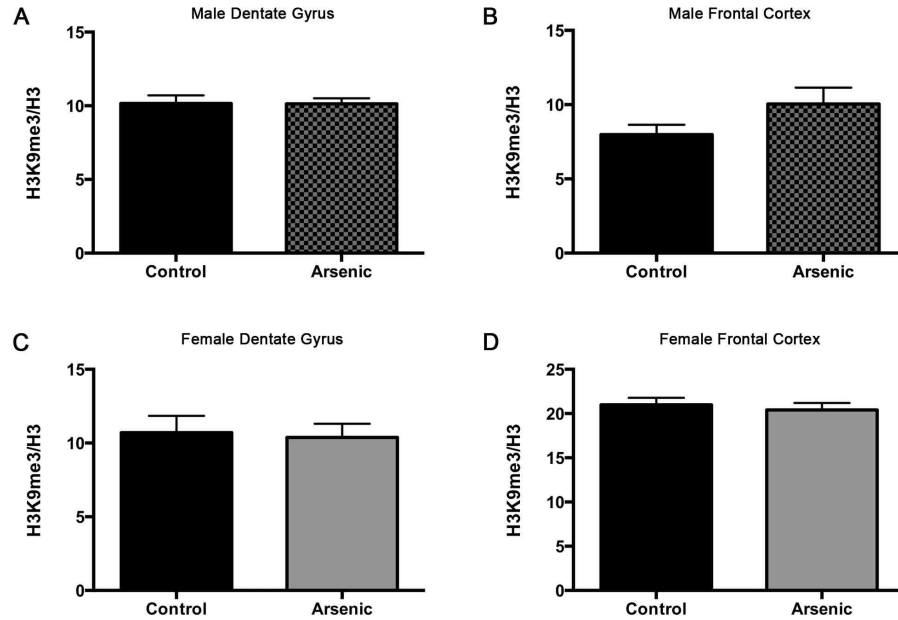


Figure 6.3. Developmental arsenic exposure does not impact H3K9me3 in the adult mouse brain.

Global analysis of trimethylation of histone 3 lysine 9 (H3K9me3) indicated that perinatal arsenic exposure does not significantly alter the levels of H3K9me3 in the (A) male dentate gyrus, (B) male frontal cortex, (C) female dentate gyrus, or (D) female frontal cortex at PD70.

6.3.3 Perinatal arsenic exposure alters methylation of H3K4 dependent on sex

Perinatal arsenic exposure differentially influenced the level of histone 3 lysine 4 trimethylation (H3K4me3), an activating HPTM, in adult mice (Figure 4). We observed increased expression of H3K4me3 in the dentate gyrus and the frontal cortex of adult male mice (Figure 4A: Male DG, $t(14) = 2.24$, $p = .04$; and Figure 4B: Male FC, $t(14) = 2.408$, $p = .027$), and decreased expression of H3K4me3 in the dentate gyrus of adult female mice (Figure 4C: Female DG, $t(15) = 2.184$, $p = .045$) with no change in the

female frontal cortex (Figure 4D: Female FC, $t(12) = 0.8357$, $p = .4197$). ($n = 7-10$ per assay)

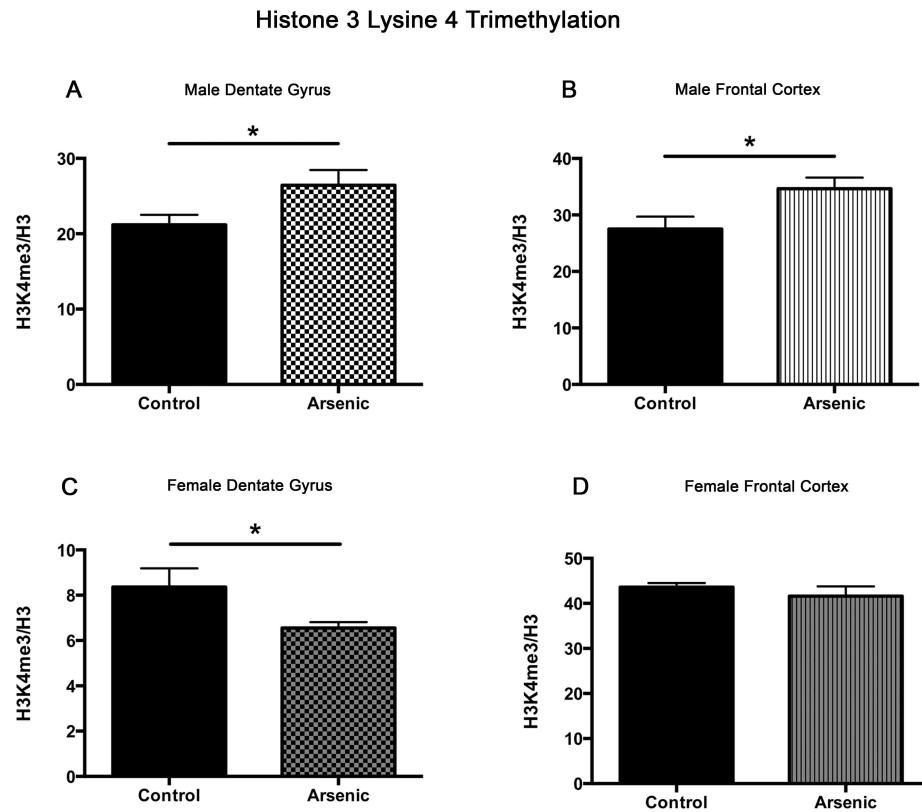


Figure 6.4. Developmental arsenic exposure alters H3K4me3 levels based on sex and brain region.

Global analysis of trimethylation of histone 3 lysine 4 indicated that perinatal arsenic exposure alters levels of H3K4me3 in a sex-specific manner. DAE increases H3K4me3 levels in the (A) male dentate gyrus, $p < .05$ and (B) male frontal cortex, $p < .05$ and decreases H3K4me3 levels in the (C) female dentate gyrus, $p < .05$, with no effect on (D) the female frontal cortex, $p > .05$. * $p < .05$

6.3.4 Perinatal arsenic exposure alters H3K9 acetylation dependent on brain region and sex

Histone acetylation at PD70 in the DG and FC of both male and female mice was assessed. Overall histone acetylation was not impacted by perinatal arsenic exposure in either brain regions of either sex (Figure 5A: Male DG, $t(11) = .3730$, $p = .7162$; Fig 5B: Male FC, $t(18) = .3777$, $p = .7101$; Fig 5C: Female DG, $t(12) = .4905$, $p = .6326$; Fig 5D:

Female FC, $t(12) = .3675$, $p = .7197$). ($n = 7-11$ per assay) Perinatal arsenic exposure differentially impacted specific lysine acetylation based on brain region and sex: we observed an increased in histone 3 lysine 9 acetylation (H3K9ac) in the dentate gyrus of adult male mice (Figure 6A: Male DG, $t(13) = 2.215$, $p = .05$) and decreased H3K9ac in the frontal cortex of adult male mice (Figure 6B: Male FC, $t(14) = 2.474$, $p = .0268$). We did measure a modest increase in H3K9ac in the female dentate gyrus, but this effect was not significant (Figure 6C: Female DG, $t(6) = 1.659$, $p = .1483$); H3K9ac in the female frontal cortex was unaffected by perinatal arsenic exposure (Figure 6D: Female FC, $t(11) = .9750$, $p = .3505$). ($n = 7-9$ per assay)

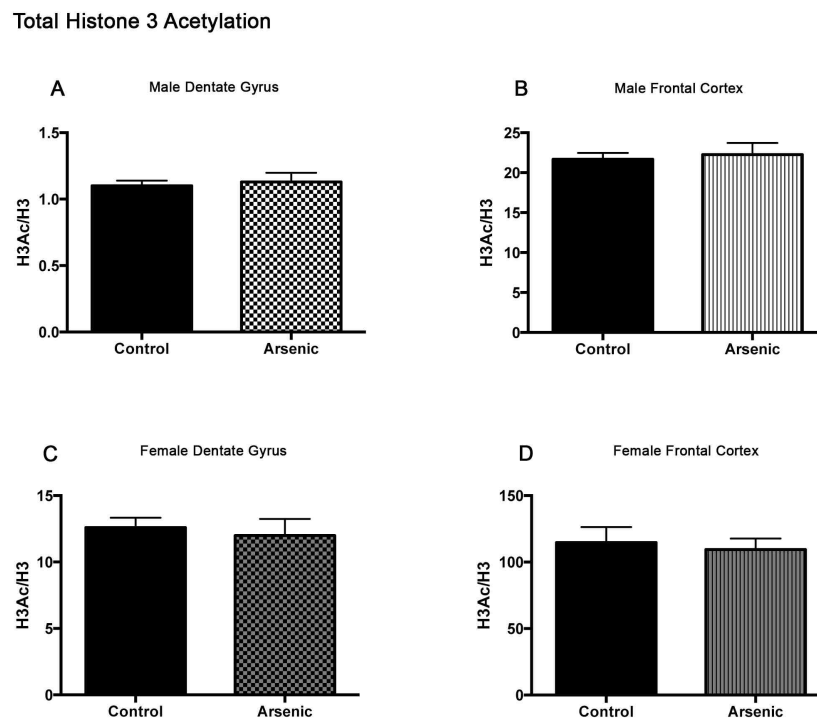


Figure 6.5. Developmental arsenic exposure does not impact total acetylation of H3 in the adult mouse brain.

Global analysis of total histone acetylation indicated that perinatal arsenic exposure does not impact levels of H3ac in the (A) male dentate gyrus, (B) male frontal cortex, (C) female dentate gyrus, or (D) female frontal cortex, $p > .05$ for all assessments.

Histone 3 Lysine 9 Acetylation

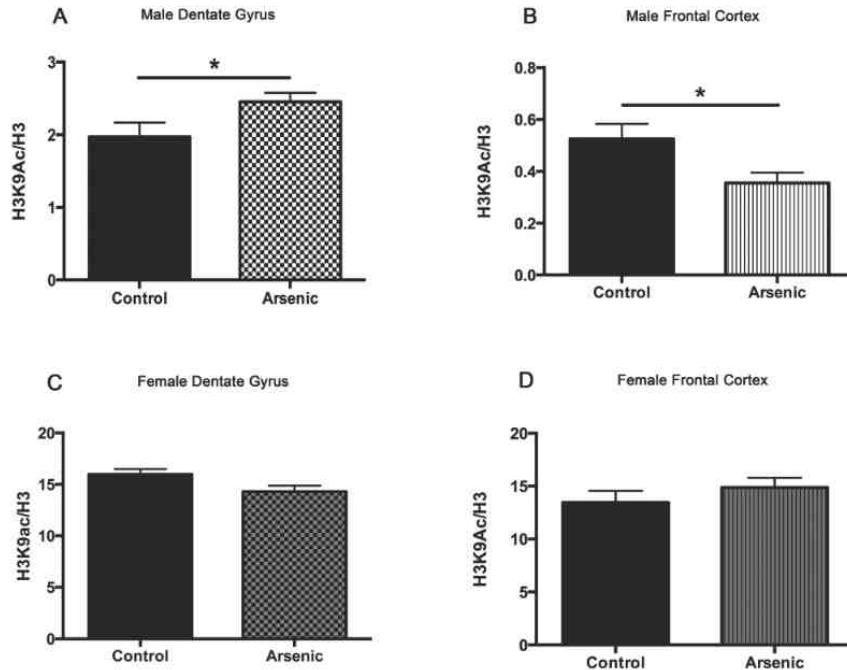


Figure 6.6. Developmental arsenic exposure alters H3K9ac based on brain region.

Global analysis of acetylation of histone 3 lysine 9 indicated that perinatal arsenic exposure alters levels of H3K9ac in a sex- and region-specific manner. DAE increases H3K9ac levels in the (A) male dentate gyrus, $p < .05$, but decreases levels in the (B) male frontal cortex, $p < .05$. There is a slight, but not significant trend of increased H3K9ac in the (C) female dentate gyrus, $p = .1$, but DAE has no effect on (D) the female frontal cortex, $p > .05$. * $p < .05$

6.3.5 Perinatal arsenic exposure influences expression of epigenetic machinery in a similar pattern to their respective histone modifications

6.3.6 Histone methyltransferase modifier: MLL1

To determine the effect of arsenic on the enzymes responsible for imparting histone modifications, we assessed the expression several chromatin-modifying proteins in the same brain regions in which we found altered histone modifications. The MLL family (mixed lineage leukemia factor) is comprised of several histone methyltransferase

enzymes responsible for imparting three methyl groups on H3K4. Perinatal arsenic exposure increased expression of MLL1 in both the dentate gyrus (Figure 7A: Male DG, $t(19) = 2.123, p = .0471$) and the frontal cortex of adult male mice (Figure 7B: Male FC, $t(22) = 2.732, p = .0122$). Conversely, we observed a decrease in MLL1 expression in the dentate gyrus of female mice (Figure 7C: Female DG, $t(15) = 3.092, p = .0074$), and no influence of perinatal arsenic exposure in the female frontal cortex (Figure 7D: Female FC, $t(12) = .01323, p = .9897$). Thus, perinatal arsenic exposure impacts the expression of the MLL1 protein similar to the pattern observed for the H3K4me3 modification in both sexes and brain regions. ($n = 9-12$ per assay)

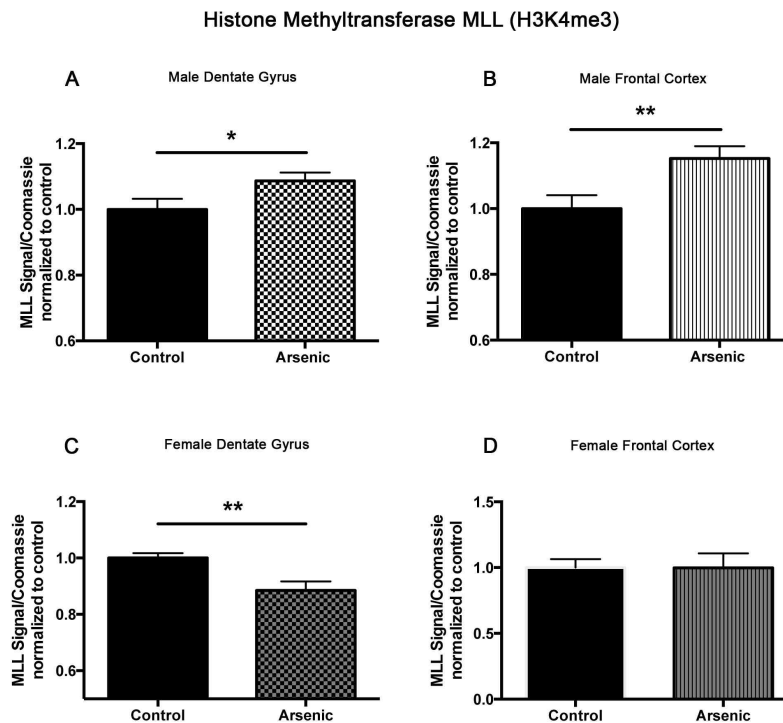


Figure 6.7. Developmental arsenic exposure alters expression of the histone methyltransferase, MLL1, based on sex and brain region.

Assessment of the chromatin-modifying protein, MLL1, which is responsible for the trimethylation of H3K4me, indicated that perinatal arsenic exposure alters levels of this enzyme in a similar pattern as levels of H3K4me3, in a sex-specific manner. DAE increases MLL1 expression the (A) male dentate gyrus, $p < .05$ and (B) male frontal cortex, $p < .05$ and decreases expression in the (C) female dentate gyrus, $p < .01$, with no effect on (D) the female frontal cortex, $p > .05$. * $p < .05$, ** $p < .01$

6.3.7 Histone Acetyltransferase Modifiers: GCN5, PCAF, CBP, and CREB

There are three groups of histone acetyltransferase enzymes responsible for imparting acetyl HPTM; these include the GNAT, MYST, and CBP/p300 families (Bannister & Kouzarides, 2011). Exposure to high concentrations of arsenic has been shown to alter expression of chromatin-modifying proteins responsible for histone acetylation and deacetylation, including members of the GNAT family, specifically GCN5 (Nelson *et al.*, 2009). GCN5 and PCAF are also responsible for H3K9 acetylation. Members of the CBP/p300 family of histone acetyltransferases are paramount for proper learning and memory and have been implicated in the response to stress and depressive-like behaviors (Maurice *et al.*, 2008). As such, expression of three histone acetyltransferase enzymes, GCN5, PCAF, and CREB binding protein (CBP) and the transcription factor, CREB, was assessed. Perinatal arsenic exposure increased GCN5 expression (Figure 8A: Male DG, $t(18) = 2.461, p = .0242$) but did not impact expression of PCAF or CBP in the dentate gyrus of adult males (Figure 8C: Male DG PCAF, $t(23) = .4144, p = .6924$; Fig 8E: Male FC CBP, $t(22) = .4370, p = .6664$). The impact of arsenic exposure on CREB and phospho-CREB in the dentate gyrus of adult males has previously been assessed; we reported no effect of arsenic exposure (Tyler *et al.*, 2014). Conversely, perinatal arsenic exposure decreased expression of the following proteins in the adult male frontal cortex: GCN5 (Figure 8B: Male FC, $t(20) = 2.279, p = .0338$), PCAF (Figure 8D: Male FC, $t(24) = 3.607, p = .0014$), with no impact on CBP (Figure 8F: Male FC, $t(22) = .6452, p = .5255$).

Histone Acetyltransferase Proteins

GCN5

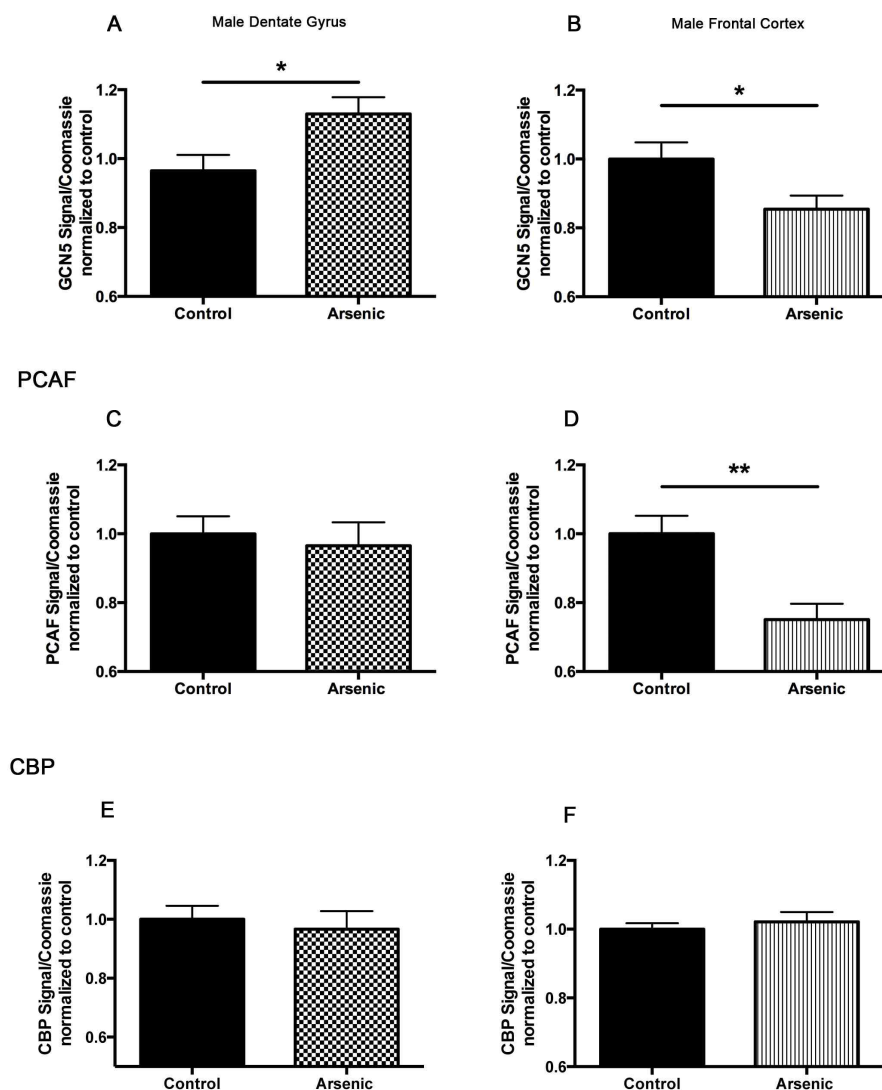


Figure 6.8. Developmental arsenic exposure alters the expression of some histone acetyltransferase proteins in both brain regions in adult male mice.

Assessment of the chromatin-modifying proteins, GCN5, PCAF, and CREB binding protein (CBP), all acetyltransferase enzymes, indicated that DAE increases GCN5 expression in the (A) male dentate gyrus, $p < 0.05$ and decreases expression of GCN5 in the (B) male frontal cortex, $p < 0.05$. DAE has no impact on PCAF expression in the (C) male dentate gyrus, and decreases expression of PCAF in the (D) male frontal cortex, $p < 0.01$. DAE has no effect on the expression of CBP in the (E) male dentate gyrus or the (F) male frontal cortex, $p > 0.05$. * $p < 0.05$, ** $p < 0.01$

Perinatal arsenic exposure decreased expression of CREB and phosphorylated CREB (Figure 9A: Male FC, $t(22) = 2.012$, $p = .05$), and p-CREB (Figure 9B: Male FC, $t(21) = 2.036$, $p = .05$).

Chromatin Associated Transcription Factors

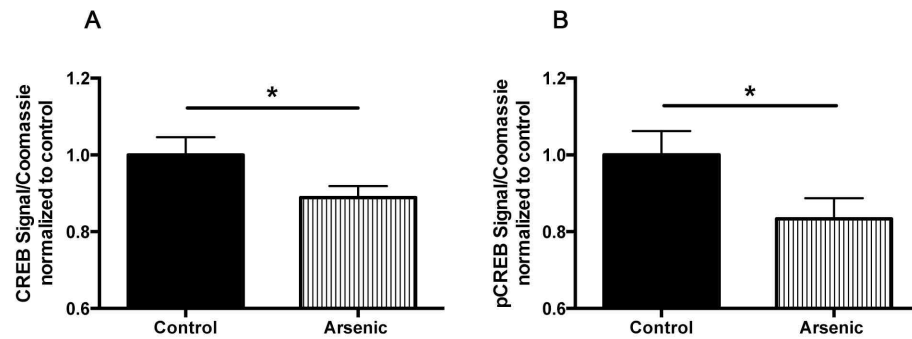


Figure 6.9. Perinatal arsenic exposure alters CREB proteins in the frontal cortex of adult male mice. Assessment of the chromatin associated transcription factors indicated that perinatal arsenic exposure reduces the expression of both (A) CREB and (B) pCREB in the male frontal cortex, $p < .05$. * $p < .05$

Expression of GCN5 was not altered in the female DG or FC (Figure 10A: Female DG, $t(11) = .1402$, $p = .8910$; Figure 10B: Female FC, $t(12) = .3423$, $p = .7381$); however there was a modest increase in PCAF expression in the female DG similar to the increase observed for H3K9ac (Figure 10C: Female DG, $t(12) = 1.452$, $p = .1722$), though PCAF was not impacted by arsenic in the female frontal cortex (Figure 10D: Female FC, $t(12) = .878$, $p = .3972$). CREB and CBP expression were not assessed in female brain tissue. Perinatal arsenic exposure influences the expression of GCN5 and PCAF proteins similar to the pattern observed for H3K9ac HPTM in both brain regions of both sexes. Additionally, perinatal arsenic exposure influences CREB and p-CREB expression but only in the adult male frontal cortex. ($n = 11-13$ per assay)

Histone Acetyltransferase Proteins: Females

GCN5

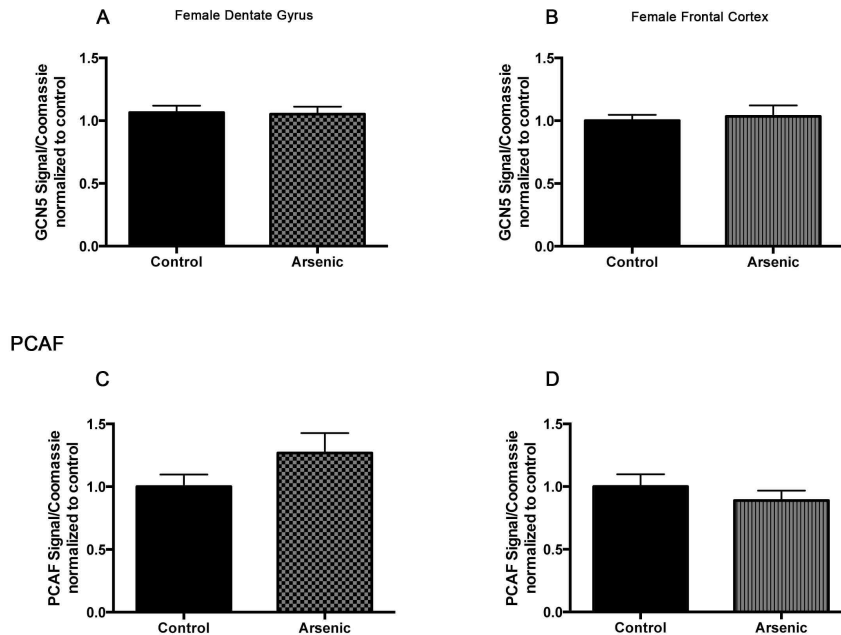


Figure 6.10. Developmental arsenic exposure does not alter histone acetyltransferase expression in adult female mice.

Assessment of the chromatin-modifying proteins, GCN5 and PCAF, in female brain regions indicates that perinatal arsenic exposure does not influence GCN5 expression in the (A) female dentate gyrus, $p > .05$, or the (B) female frontal cortex, $p > .05$, but does slightly increase expression of PCAF in the (C) female dentate gyrus, $p = .1$, with no effect on PCAF in the (D) female frontal cortex. $*p < .05$

6.3.8 Repressive chromatin complex: REST/NRSF and Co-REST

Recent studies have suggested that there is extensive interaction among various epigenetic factors, including the machinery and the modifications themselves, in order to control gene expression (Jobe *et al.*, 2012). Reduced histone acetylation has been associated with repressive complexes, including the REST/NRSF complex, known for its role in silencing neuronal programs (Ooi & Wood, 2007). In neural stem cells, REST/NRSF inhibits programs responsible for neural fate specification and differentiation (Gao *et al.*, 2011). As such, we assessed the expression of REST/NRSF and its co-repressor Co-REST in the dentate gyrus and frontal cortex of adult male mice.

Surprisingly, perinatal arsenic exposure did not impact the expression of REST/NRSF or Co-REST in the dentate gyrus of adult males (Figure 11A: Male DG REST, $t(22) = .4959$, $p = .6249$; Fig 11B: Male DG Co-REST, $t(24) = .04476$, $p = .9647$). However, we did observe an increase in the expression of both REST and Co-REST in the male frontal cortex (Figure 11C: Male FC REST, $t(20) = 2.612$, $p = .0167$; Fig 11D: Male FC Co-REST, $t(20) = 2.861$, $p = .0097$). This increased REST/NRSF and Co-REST expression parallels decreased histone acetylation observed in the frontal cortex. ($n = 11-14$ per assay)

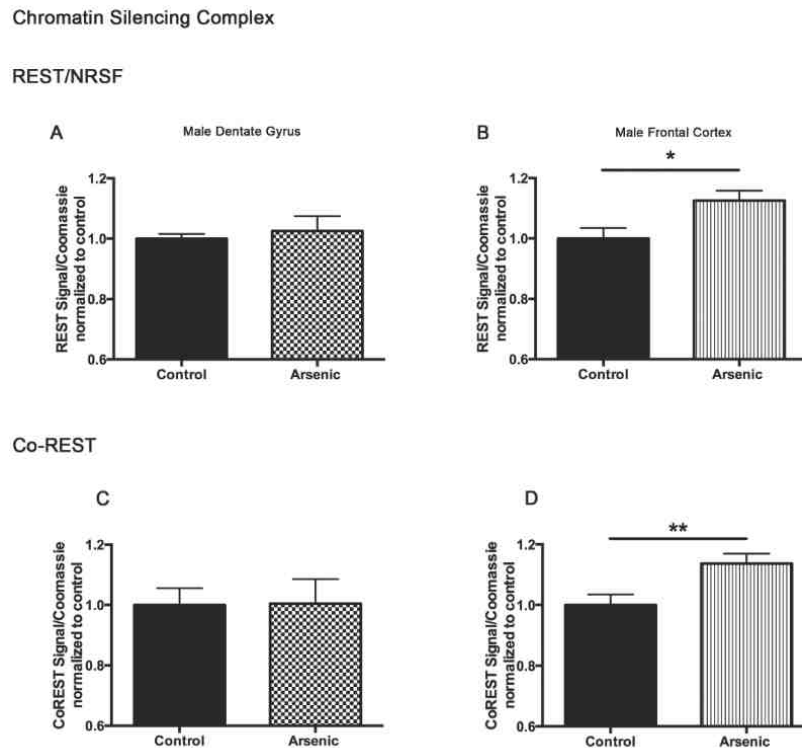


Figure 6.11. Perinatal arsenic exposure alters proteins in the REST chromatin silencing complex based on sex and brain region.

Assessment of chromatin associated factors, responsible for silencing of genes associated with neural lineages, indicated that perinatal arsenic exposure does not impact the expression of (A) REST in the male dentate gyrus or (C) Co-REST in the male dentate gyrus. However, REST expression is increased in the (B) male frontal cortex, $p < .05$ and Co-REST expression is increased in the (D) male frontal cortex, $p < .01$. * $p < .05$, ** $p < .01$

6.4 Discussion

Many researchers are interested in how toxins impact epigenetic processes in order to elucidate potential mechanisms of action in the body. While the impact of arsenic exposure on the epigenome has been extensively studied in the context of cancer research, the influence of this toxicant in the brain, particularly as it relates to epigenetics, is not well understood. Additionally, as the developing brain can be vulnerable to several insults, including arsenic, the genetic programs that are initiated, executed, and subsequently terminated via epigenetic processes may be sensitive as well (Calkins & Devaskar, 2011). Thus, determining the long-term impact of fetal exposures on the status of factors associated with epigenetic control of genetic programs could provide a window into how fetal programming leads to disease susceptibility in adulthood. This study provides the first step in demonstrating that perinatal exposure to 50 ppb arsenic, throughout all three trimesters of development, influences histone modifications and the expression of chromatin-modifying proteins in the adult mouse brain long after subjection to arsenic has diminished. Notably, the impact of this exposure paradigm is related to the type of histone modification (e.g. methylation or acetylation), the location of the modification (e.g. the dentate gyrus or frontal cortex), and the sex in which the modification is present (e.g. male or female arsenic-exposed offspring). Such specification of arsenic toxicity may shed light on its potential mechanisms of action in altering the adult brain.

Data provided here demonstrate a differential effect of perinatal arsenic exposure dependent on brain region. We have previously shown that our exposure model alters

expression of several genes associated with neurogenesis in the adult dentate gyrus (Tyler & Allan, 2013). The genetic programs involved in the processes of adult neurogenesis are highly orchestrated by epigenetic factors (Covic *et al.*, 2010). As such, to evaluate the regulation of neurogenesis, we sought to first demonstrate that arsenic impacts epigenetic processes in this brain region, possibly leading to aberrant gene expression. We chose to assess H3K4me3 and H3K9me3, as both of these modifications are influenced by arsenic (Zhou *et al.*, 2008) and each is present on either actively transcribed or repressed genes, respectively (Kouzarides, 2007). Surprisingly, we found no differences in H3K9me3 levels, as anticipated based on arsenic cell culture studies; potential reasons for this include the dose and timing of arsenic exposure or the cell type, as *in vitro* studies use direct application of high concentrations of arsenite on cancer cells (Chervona *et al.*, 2012a; Hong & Bain, 2012b). We did observe an increase in H3K4me3 levels in the dentate gyrus of adult males, and a decrease in levels in adult females in the same region, opposite of the expression patterns for this HPTM measured in human PBMCs (Chervona *et al.*, 2012b). To determine a possible mechanism for this altered level of modification, we analyzed the expression of MLL1, a histone methyltransferase responsible for trimethylation of H3K4 (Bannister & Kouzarides, 2011). We found increased expression of MLL1 in arsenic-exposed adult males and decreased expression in females (both in the dentate gyrus); this demonstrates that the level of histone modification is possibly due to the level of expression of its modifier. MLL1 has been implicated in subventricular zone neurogenesis and mediates the conversion of silent bivalent domains to active domains containing H3K4me3 (Lim *et al.*, 2009); as we have previously demonstrated deficits in subgranular zone neurogenesis in the dentate gyrus in adult males, the increased

expression of MLL1 and H3K4me3 could be a compensatory mechanism to counteract the deleterious effects of arsenic, which are perpetuated into adulthood.

Histone trimethylation of lysine 4 has been associated with histone acetylation, as it is thought that histone modifications create crosstalk and a particular “code” for regulation of expression (Kouzarides, 2007; Jobe *et al.*, 2012). We found no differences in overall histone acetylation in the dentate gyrus of either sex, but we did observe an increase in H3K9 acetylation levels in the male dentate gyrus with a slight increased trend in the female dentate gyrus. This was unexpected, as human studies have demonstrated that arsenic exposure is inversely correlated with H3K9ac levels (Chervona *et al.*, 2012b); however, a different study demonstrated that arsenic exposure via inhalation was directly correlated to H3K9ac levels in leukocytes (Cantone *et al.*, 2011). Additionally, increased acetylation occurs in response to oxidative stress induced by arsenic exposure *in vitro*; as such, the expression profile of H3K9ac in the dentate gyrus may be a residual response to arsenic toxicity during the embryonic period, though this has yet to be assessed (Li *et al.*, 2001; Sun *et al.*, 2009b). GNC5 and PCAF are histone acetyltransferase enzymes found in multi-subunit mammalian complexes, SAGA (STAGA, TFTC) and ATAC, and either complex can use either protein as the acetyltransferase for particular acetyl modifications *in vitro* (Wang *et al.*, 2008b). Conditional deletion of GCN5/PCAF together significantly reduces H3K9ac (Jin *et al.*, 2011); as such we assessed the expression of both GCN5 and PCAF to determine their correlation to H3K9ac levels. Interestingly, increased H3K9ac in the male and dentate gyrus was concurrent with increased expression of GCN5 (but not PCAF) and the trend of increased H3K9ac in the female dentate gyrus was concurrent

with increased PCAF (but not GCN5), though arsenic has been shown to alter GCN5 expression (Nelson *et al.*, 2009). While the histone acetyltransferase proteins are segregated into distinct families, extensive crosstalk between PCAF and CREB binding protein (CBP) has demonstrated to play a role in acetylation (Liu *et al.*, 1999; Nagy & Tora, 2007); however, perinatal arsenic exposure did not impact CBP expression in the male dentate gyrus, though there was an effect in the male frontal cortex.

Arsenic can accumulate in particular brain regions, including the hippocampus (in which the dentate gyrus is located) and the frontal cortex (Sanchez-Pena *et al.*, 2010). To demonstrate region specificity of arsenic toxicity for the dentate gyrus, we assessed histone modifications in the frontal cortex of both males and females. Surprisingly, we observed increased expression of both H3K4me3 and associated MLL1 in the male frontal cortex, similar to the pattern observed in the dentate gyrus in the males. MLL1 has been found in GABAergic and cortical neurons, and remodeling mechanisms in the frontal cortex that involve both H3K4me3 and MLL1 have been implicated in the etiology of schizophrenia (Huang *et al.*, 2007). However, the role of MLL1 and H3K4me3 in the frontal cortex, especially as it pertains to depression and metal toxicity like arsenic, is unknown. Interestingly, we found the perinatal arsenic exposure influences acetylation in a region- and sex-specific manner similar to that of methylation. Acetylation of H3K9 was reduced in the adult male frontal cortex, with no effect in the female frontal cortex; GCN5 and PCAF expression followed suit (decreased expression of both in the male frontal cortex), suggesting again that histone modifications are dependent on the expression of their modifiers long after arsenic exposure has essentially

ceased, though there is approximately 5 ppb arsenic in tap water. While there was no observable change in overall histone acetylation or CBP expression, perinatal arsenic exposure reduced both CREB and p-CREB in the male frontal cortex (unlike the dentate gyrus). Histone acetylation facilitates the binding of transcription factors (such as CREB) to increase gene transcription, and CREB, known to be paramount for learning and memory, has also been shown to be neuroprotective and to mediate the efficacy of antidepressants (Sakamoto *et al.*, 2011). Interestingly, aberrant epigenetic processes have been implicated in the etiology of psychiatric disorders, such as depression, but have been specific to measurements in the hippocampus and the amygdala (Sun *et al.*, 2013). Thus, it is possible that concurrent decreased acetylation and CREB/p-CREB in the frontal cortex may be one molecular mechanism by which arsenic increases susceptibility to depression. We are currently assessing the falsifiability of this new hypothesis.

Currently, a consensus on the “epigenetic orchestration” of adult neurogenesis has yet to be determined, yet there is one chromatin remodeling factor thought to play a paramount role: repressor element 1 (RE1) silencing transcription factor or neuron restrictive silencing factor, REST/NRSF. This factor has been termed the “master negative regulator” of neurogenesis (Gao *et al.*, 2011). As perinatal arsenic exposure reduces adult neurogenesis in the dentate gyrus of adult mice, we assessed the expression of REST/NRSF and its cofactor, Co-REST. We observed no changes in REST/NRSF or Co-REST expression in the dentate gyrus. MLL1 has been shown to antagonize the expression of REST/NRSF; thus, it is possible that increased H3K4me3 and MLL1 expression observed in differentiated dentate granule neurons is associated with reduced

or normalized levels of REST/NRSF (Ballas *et al.*, 2005; Ballas & Mandel, 2005). Conversely, we report increased levels of REST/NRSF and Co-REST in the frontal cortex of adult males. Increased REST/NRSF expression has been found as a normal part of aging and is reduced in mild cognitive impairment and Alzheimer's disease (Lu *et al.*, 2014). However reduced expression of REST/NRSF is required for differentiation, and in mature neurons, REST/NRSF is present but not active (Gao *et al.*, 2011). Activation of REST/NRSF expression can occur in response to neuronal insult (Kaneko *et al.*, 2014). While these studies demonstrate that the precise role of REST/NRSF in the brain is still under investigation, our findings suggest that REST/NRSF expression in the brain may be vulnerable to arsenic toxicity. Overall, we provide results establishing that arsenic impacts the molecular expression of proteins important for neuronal programs in the frontal cortex; this raises more questions as to the deficits in specific frontal cortex functioning in our perinatal arsenic exposure model.

It should be noted that sex-dependent changes in molecular components, including histone modifications in response to arsenic exposure, have been demonstrated in prior studies. In humans exposed to low to moderate levels of arsenic (50-500 ppb), high urinary arsenic levels are correlated with high levels of H3K4me3 and H3K27me3 in peripheral blood mononuclear cells from females, but the reverse of this expression is present in males (Chervona *et al.*, 2012b). *In utero* arsenic exposure alters global DNA methylation profiles in cord blood with a greater influence in males than females (Broberg *et al.*, 2014). Indeed, many of the changes that we observed using our perinatal arsenic exposure paradigm have been demonstrated in males (Martinez-Finley *et al.*,

2011; Goggin *et al.*, 2012). Finally, several sex-dependent alterations in cognition and intelligence measures have been observed among males and females, suggesting that arsenic may disrupt endocrine function, possibly mediating differences observed in these studies (Vahter, 2009; Hughes *et al.*, 2011). As such, our future studies, particularly as they pertain to endocrine function and epigenetic processes will include females.

In conclusion, we have provided evidence suggesting the perinatal arsenic exposure impacts histone modifications in the brain during adulthood. We have shown that levels of H3K4me3 and H3K9ac are altered in a region- and sex-specific manner, and that the chromatin modifying proteins responsible for these modifications also have altered expression profiles that parallel histone modification levels. Additionally, we observed sex-specific influences of arsenic that are antithetical to prior observations in human studies, likely due to cell type in which histone modifications were measured and the extent of arsenic exposure. Indeed, we did not observe changes in many histone modifications, including H3K9me3 or H3ac, as expected, suggesting that the timing and dose of exposure used in cell culture studies as compared to our *in vivo* studies plays a role in the damage arsenic elicits. Importantly, data demonstrating region specificity suggest a new avenue of research for arsenic toxicity: epigenetic regulation of frontal cortex functionality. Overall, these findings are the first step in analyzing the effect of developmental arsenic exposure on epigenetic programming of gene expression in the adult brain; further studies will evaluate the impact of histone modifications on gene expression and the potential aberrant fetal programming of these epigenetic modifications that may be perpetuated into adulthood.

7.0 General Discussion

7.1 Summary of Findings

Over 100 million people worldwide are exposed to levels of arsenic that well exceed the World Health Organization limit of 10 µg/L (parts per billion, ppb); as such, arsenic contamination and subsequent exposure via drinking water is considered a major public health epidemic. Exposure to this metalloid affects almost every organ system in the body, including the brain. Several epidemiological studies have provided evidence suggesting a link between arsenic exposure and increased rates of psychiatric disorders, including depression in adult populations (reviewed in Chapter 2). Additionally, children with arsenic exposure have deficits in intelligence measures and difficulty with certain forms of learning and memory (Calderon *et al.*, 2001; Calderon *et al.*, 2013). It is currently unknown if these exposed children will also have an increased risk of developing a psychiatric disorder, but prospective studies are currently underway. Research using rodent models for assessing the effect of arsenic has demonstrated that developmental exposure has long-lasting consequences into adulthood including deficits in learning and memory, molecular aberrations in the hypothalamic-adrenal-pituitary (HPA) axis, and depressive-like symptoms (Martinez *et al.*, 2008; Martinez-Finley *et al.*, 2009; Goggin *et al.*, 2012). However, the mechanistic link between arsenic exposure and these behavioral outcomes is still not well understood.

One neurobiological mechanism that underlies deficits in hippocampal learning and memory and increased risk of psychiatric disorders, such as anxiety and depression, is that of aberrant adult hippocampal neurogenesis (AHN). The studies described in this

dissertation were designed to characterize the impact of perinatal arsenic exposure (50 ppb during all three trimester equivalents) on the susceptibility to depression, with a particular focus on adult neurogenesis as a candidate mechanism of action. Two overall aims were developed to test the hypothesis that perinatal arsenic exposure leads to a depressive-like endophenotype via aberrant epigenetic regulation of neuronal genes involved in AHN in adult mice. This hypothesis is supported by literature demonstrating that in both human and rodent studies, depressive-like symptoms correlate with reduced hippocampal volume associated with reduced AHN and that the efficacy of antidepressants is dependent on intact AHN (Santarelli *et al.*, 2003; Boldrini *et al.*, 2013). Additionally, studies have demonstrated that epigenetic processes intricately control gene expression for all components of AHN and that aberrant epigenetic regulation may contribute to the etiology of depression (Mateus-Pinheiro *et al.*, 2011). Control of AHN requires appropriate expression of several chromatin-modifying proteins to impose distinct histone modifications on particular genes to maintain the neurogenic niche or to implement maturation programs. Epidemiological and *in vitro* cell culture studies have separately provided evidence that arsenic exposure leads to aberrant histone modifications (Ramirez *et al.*, 2008; Zhou *et al.*, 2008; Zhou *et al.*, 2009; Chervona *et al.*, 2012b). Additionally, one study has shown that early developmental exposure to arsenic (in the ppm range) alters that DNA methylation patterns in the brain for genes that are important for neuronal plasticity (Martinez *et al.*, 2011). These observations support the overall hypothesis that arsenic exposure during development interferes with the epigenetic environment of the hippocampus leading to susceptibility to depression in adulthood (See Figure 7.1).

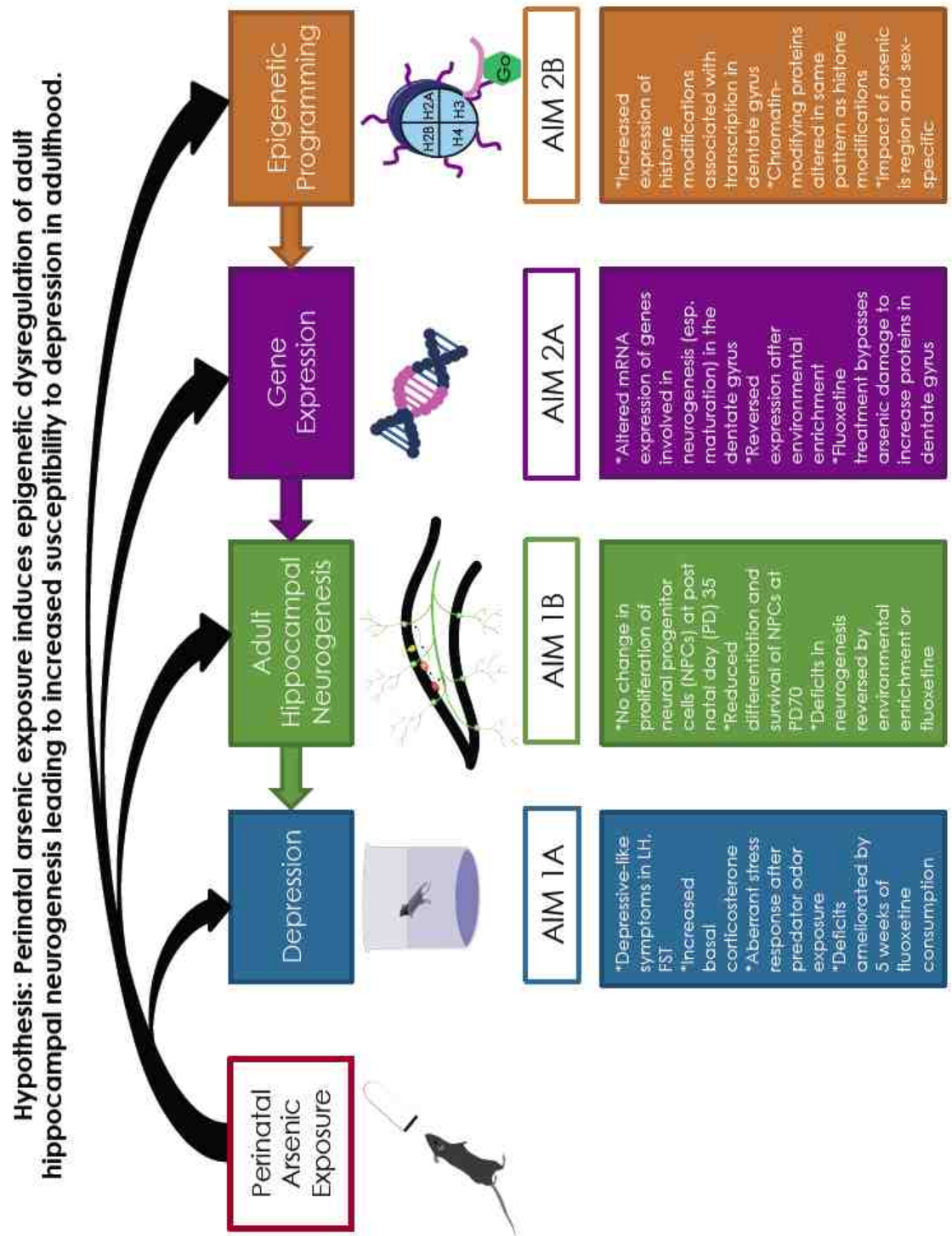


Figure 7.1 Overall model of processes impacted by perinatal arsenic exposure

Perinatal arsenic exposure damages epigenetic and neurogenic processes associated with depression. Aims were developed to assess hypothesis that perinatal arsenic exposure increases susceptibility to depression in adulthood by inducing deficits in adult neurogenesis via altered epigenetic mechanisms, like histone modifications. Boxes under each aim present findings from the studies described in this dissertation.

Aim 1 experiments in this dissertation were designed to assess the hypothesis that perinatal arsenic exposure results in deficits in adult neurogenesis and hippocampal-dependent behavioral tasks, both of which can be rescued by chronic antidepressant treatment. Thus, the first set of experiments established arsenic-induced morphological and functional damage to the hippocampus: maternal consumption of 50 ppb arsenic in drinking water resulted in reduced differentiation and survival of neural progenitor cells in the dentate gyrus of the hippocampus in adult male offspring concurrent with increased susceptibility to stress-induced depression as measured by learned-helplessness and forced-swim tasks after exposure to a predator odor (Chapters 4 and 5). Additionally, stress responses, which are altered in depressed patients, were blunted in arsenic-exposed animals as measured by plasma corticosterone levels (CORT) after TMT exposure.

A second set of experiments for Aim 1 were performed to determine the reversibility of arsenic-induced damage, thereby demonstrating causality of aberrant AHN as a mechanism of action. Deficits in AHN were ameliorated using a novel chronic, intermittent environmental enrichment approach in which animals experience enrichment for a few hours daily and are returned to their home environment (Chapter 4). This model allows for novelty of the environment and restoration of the stress response, and while this enrichment paradigm is important for animal studies, it does not translate directly for human therapies. A chronic regime of fluoxetine, a selective serotonin reuptake inhibitor antidepressant commonly prescribed as Prozac®, was provided for one month via drinking water to adult male animals perinatally exposed to arsenic. Fluoxetine treatment prevented depressive-like behaviors in all behavioral tasks in arsenic-exposed animals,

and ameliorated arsenic-blunted stress responses and deficits in neural progenitor cell differentiation and survival (Chapter 5). These studies demonstrated that hippocampal morphological and behavioral damage induced by perinatal arsenic exposure is reversible with chronic fluoxetine treatment, resulting in restored resiliency to depression via a neurogenic mechanism.

Aim 2 experiments were designed to determine the validity of a second hypothesis: perinatal arsenic exposure alters histone methylation and acetylation patterns on neurogenesis-related genes. Thus, these studies were performed to investigate arsenic's effect on the molecular components of AHN in the dentate gyrus and the epigenetic regulation of neuronal genes. Deficits in AHN were associated with abnormal neuronal gene expression within the pathways involved in Alzheimer's disease, apoptosis, axonogenesis, growth, Notch signaling, and transcription factors in the dentate gyrus. Additionally, some of this aberrant gene expression was ameliorated with chronic intermittent exposure to enrichment, suggesting reversibility of arsenic damage (Chapter 4). To identify potential epigenetic mechanisms by which arsenic imparts dentate-specific damage, global post-translational histone modifications (PTHM) were assessed along with the expression of several chromatin-modifying proteins. Perinatal arsenic exposure resulted in increased histone 3 lysine 4 trimethylation and histone 3 lysine 9 acetylation in the dentate gyrus of adult males with altered expression of their respective histone methyl- and acetyltransferase modifiers, Mll1 and GCN5/PCAF. However, arsenic did not impact overall acetylation of H3 or trimethylation of H3K27 or H3K9, as seen in previous studies. These effects were specific to the adult male dentate gyrus, as the effect

of arsenic exposure on histone modifications was dependent on region of interest and sex, as described in Chapter 6. Additionally, chromatin-modifying proteins including REST, Co-REST, CREB binding protein (CBP), and factors involved in neurogenesis including BDNF and CREB were not altered in the dentate gyrus of arsenic-exposed mice, implying that further analysis of the mechanism of arsenic-induced damage in the dentate is required. Sequencing analysis (ChIP-Seq) using the dentate gyrus of adult males after developmental arsenic exposure (PD70) will demonstrate which genes have histone 3 lysine 4 trimethylation occupancy, and thus, may be considered, up-regulated in response to arsenic; this analysis is currently underway.

Data presented in this dissertation demonstrates that developmental arsenic exposure alters hippocampal morphology by inducing deficits in AHN, alters expression of genes associated with neurogenesis, and increases activating posttranslational modifications of histones in the dentate gyrus. Deficits in hippocampal morphology and gene expression were associated with behaviors indicative of depression and aberrant responses to stress. These alterations were restored using two therapeutic approaches: a novel environmental enrichment paradigm and chronic antidepressant administration, confirming that aberrant adult neurogenesis is one mechanism by which arsenic alters the brain. Arsenic likely impacts AHN by altering epigenetic programming during development that is perpetuated into adulthood, resulting in the phenotypes measured here. Thus, future studies might aim to further characterize the epigenetic modifications present on neural progenitor cells in the embryonic brain when arsenic is present.

7.2 Interpretation and Significance of Results

7.2.1 Significance of depressive-like symptoms and altered stress responses in adult male mice perinatally exposed to arsenic

The contribution of environmental factors in the etiology of depression, while widely acknowledged, has not been thoroughly investigated. Epidemiological studies of toxin-induced depression have suggested an association between exposure and an increased risk of development of a psychiatric disorder (Zierold *et al.*, 2004; Sen & Sarathi Biswas, 2012; Beard *et al.*, 2013; Freire & Koifman, 2013; Malekirad *et al.*, 2013; Ojo *et al.*, 2013; Weisskopf *et al.*, 2013). Additionally, the fetal origins of disease hypothesis postulates that the early environment plays a role in the development of diseases in adulthood; thus, it is likely that toxin exposure during development may also contribute to a predisposition for depression or other psychiatric disorders (Bale *et al.*, 2010).

As discussed in Chapter 5, exposure to a mild stress (footshock) on the first day of a learned helplessness (LH) task was sufficient to induce depressive-like symptoms in adult arsenic-exposed mice, but not in control animals. Chronic LH has been used to generate depression in other rodent models; therefore, we wanted to evaluate a mild LH paradigm in which the stress is minimal and does not induce a depressive-like phenotype in control animals. This finding suggests that arsenic “primes” a depressive-like response to a mild stressor. Indeed, arsenic-exposed mice exhibited depressive-like symptoms on the forced swim task after a 10-minute exposure to a predator odor (TMT) (Chapter 5). Therefore, adult male mice perinatally exposed to arsenic will have a depressive-like phenotype after

the stress response is activated, suggesting a lack of resiliency to stress. Resiliency, while difficult to measure, has been implicated in depression, as individuals with robust homeostatic responses to stress are often considered resistant to depression (Krishnan & Nestler, 2008).

Chronic stress paradigms are used to produce depressive-like symptoms in rodents and create hyperactivity of the HPA axis, as described in the Introduction. The Allan lab has previously published findings demonstrating that arsenic exposure during development increases circulating corticosterone (CORT) levels in adolescent mice (PD35) and reduces expression of the glucocorticoid receptor in the hippocampus, likely impeding the inhibitory tone the hippocampus provides for the HPA axis (Goggin *et al.*, 2012). As such, an additional stress like that provided in each of the behavioral tasks presented here, would facilitate a stress response leading to depressive-like behavior. Indeed, these findings were corroborated by demonstrating that in adult male mice, CORT levels are elevated, and the stress response to TMT is diminished compared to control animals (Chapter 5). Blunted HPA axis feedback has been measured in depressed patients, along with increased circulating cortisol (human equivalent of CORT), validating the depression endophenotype observed in the arsenic-exposed animals (Wainwright & Galea, 2013). Thus, these results provide greater evidence that arsenic may predispose an individual for developing depression via lack of resiliency to stress. By demonstrating that arsenic exposure leads to aberrant stress responses that may result in greater risk for developing a psychiatric disorder like depression, these findings have provided more evidence of the contribution of the environment in the etiology of depression.

7.2.2 Significance of altered adult hippocampal neurogenesis after perinatal arsenic exposure

As explained in the Introduction, adult hippocampal neurogenesis is comprised of several processes including proliferation of neural progenitor cells (NPCs), differentiation/maturation of these cells into neurons, and the survival and incorporation of these cells in the hippocampal circuitry. Assessment of proliferation of NPCs demonstrated that perinatal exposure to arsenic only reduced proliferation by 13%, which was not significant (Chapter 4). However, one caveat to this finding is that the BrdU labeling method that was employed may have underestimated that total degree of neurogenesis. The 12-hour pulse chase assay labels a small percentage of the proliferation progenitors as a representation of the total quantity of the progenitor niche. This method of labeling is considered the gold standard of the field; however, if arsenic alters cell cycle dynamics, as seen in cell culture studies (Sidhu *et al.*, 2006), then it is possible that the timing of proliferation is different from that of control animals (not exposed to arsenic). This change in cycle dynamics would not be evident from the lineage tracer assay employed in these studies; thus, it is possible that the molecular mechanisms of proliferation are altered without this being represented with immunohistochemistry (IHC) measurement. The use of environmental enrichment increased proliferation of both control and arsenic-exposed animals (Chapter 4); however, the extent of increased proliferation in arsenic animals was not significant. Thus, if the proliferation potential of neural progenitor cells after arsenic exposure is indeed not different from that of controls, we would have expected the response to enrichment to be

equivalent among the two. Since the response was blunted in arsenic-exposed animals, it may be proposed that arsenic impacts proliferation in a manner that is not measurable using methods employed in these studies. It would be important to determine if arsenic impacted cell cycle dynamics; this could be accomplished using flow cytometry for measuring G₀/G₁, S, and M/G₂ to definitively state that arsenic does not impact proliferation. However, it should be noted that the lack of impact on proliferation does corroborate other findings in cell culture and animals studies demonstrating that arsenic preferentially impacts differentiation and not proliferation of stem cells (Hong & Bain, 2012a).

Perinatal arsenic exposure reduced the number of differentiated progenitor cells (BrdU+NeuN+) by 55% and the number of surviving cells (BrdU+) by 53% one month after BrdU administration (Chapters 4 and 5). Other toxins found in water, including heavy metals like mercury and lead, have been shown to impact AHN, specifically differentiation (Jaako-Movits *et al.*, 2005). Indeed, *in vitro* assessment of neurogenesis suggests that arsenic suppresses differentiation but not proliferation of neural stem cells, concurrent with reduced expression of pro-neural genes such as neurogenins and NeuroD1 (Hong & Bain, 2012a). The reduction in differentiation but not proliferation may underlie the learning and memory deficits observed in arsenic-exposed animals. It is thought that deficits in AHN lead to altered synaptic plasticity and are associated with deficits in learning and memory paradigms. Indeed, there is a critical window of maturation for newborn neurons from 1-2 weeks (after proliferation has been assessed but prior to maturation assessment) (Aasebo *et al.*, 2011); as such, the lack of maturing

newborn neurons during learning experiences may underlie both the learning deficits and the depressive-like symptoms observed in this arsenic model.

The mechanism by which arsenic reduces the number of differentiated cells may be twofold: the first is that of epigenetic regulation of genes responsible for neurogenesis as discussed later. The second is that the arsenic may impact the stages of maturation, particularly including increased excitability (glutamatergic inputs) and synaptic plasticity (lower threshold for long-term potentiation) that are required for these newborn neurons to become integrated into the hippocampal circuitry. Other prenatal toxin exposures like maternal alcohol consumption have been shown to alter the synaptic connections among older dentate granule cells and newborn neurons later in life. Glutamatergic and GABAergic inputs into the newborn neurons are affected by prenatal alcohol exposure, resulting in inappropriate firing of excitatory and inhibitory field potentials (Brady *et al.*, 2013; Kajimoto *et al.*, 2013). This has yet to be measured in developmental or adult arsenic exposure studies.

The data demonstrating that perinatal arsenic results in long-lasting deficits in differentiation and survival of neural progenitor cells in the dentate gyrus of adult males with depressive-like symptoms provides yet another study elucidating the link between neurogenesis and depression. There are some conflicting results in the literature concerning the connection between adult neurogenesis and depression. Ablation of AHN does not always produce a depressive-like phenotype in rodent models, suggesting the validity of the neurogenic hypothesis of depression is questionable; however, the

variability in results reported is likely due to the method, extent, and timing of ablation of AHN (Petrik *et al.*, 2012). A second facet of the neurogenic hypothesis purports that AHN is required for the efficacy of antidepressant therapy. While both stress and antidepressant treatment regulate neurogenesis, demonstrating that AHN is causal in mediating the effects of stress and antidepressants, thereby inducing depression, is still under investigation (Mahar *et al.*, 2014). Some studies have shown that intact AHN is not necessary for mediating the response to antidepressants (Jedynak *et al.*, 2014); however, again the method of ablation likely contributes to these results. The findings presented in this dissertation would further support the neurogenic hypothesis of depression, suggesting that animals with depressive-like symptoms have concurrent reduced AHN and yet have sufficient quantities of newborn neurons (not complete ablation) for the action of an antidepressant, such as fluoxetine. However, these findings are correlational, and causality has yet to be demonstrated.

7.2.3 Significance of reversibility of arsenic-induced behavioral and molecular damage

The potential to restore resiliency to stress-induced depression in arsenic-exposed animals was assessed using pharmacological treatment regime, as described in Chapter 5. It is known that fluoxetine, a selective serotonin reuptake inhibitor, leads to alleviation of depression in some patient populations after chronic intake. Additionally, as described in the Introduction, antidepressants can increase AHN with concurrent reversal of depressive-like symptoms in rodents that are chronically stressed.

As presented in Chapter 5, arsenic-exposed animals were provided a chronic treatment regime of 100 mg/L of fluoxetine in their drinking water for one month. Treatment with fluoxetine prevented depressive-like behaviors in the behavioral tasks described above, namely the LH and FST after exposure to a stressor; this not only demonstrates the predictive validity of these tasks in this depression model but also the reversibility of arsenic-induced damage. Additionally, fluoxetine ameliorated blunted HPA axis stress responses, providing further evidence of the link between stress, neurogenesis, and depression. Fluoxetine treatment likely repaired behavioral and morphological damage by increasing the expression of several proteins involved in neurogenesis, including brain derived neurotrophic factor (BDNF), the cAMP response element binding protein (CREB), the glucocorticoid receptor (GR), and histone deacetylase 2 (HDAC2). Interestingly, the protein expression in the dentate gyrus of these four targets was not impacted by arsenic exposure (Chapter 5); this finding suggests that fluoxetine bypasses damage induced by arsenic to restore AHN and prevent a depressive-like phenotype in mice developmentally exposed to arsenic.

Exposure to an enriched environment has also been demonstrated to increase neurogenesis; as discussed in Chapter 4, arsenic-exposed animals were provided access to an enriched environment for one month. This access was daily but intermittent in the time of day and in the allotment of time in the enrichment; this variation allowed for novelty of the environment. Previous studies have shown that with developmental toxin exposure (prenatal alcohol), the neurogenic response to experience in enrichment is blunted (Choi *et al.*, 2005; Kajimoto *et al.*, 2013); therefore, this new “playground” model of

enrichment was developed. It was hypothesized that since arsenic induces alterations in stress responding and part of the experience in enrichment requires mild activation of the stress response, a period of “nonstressful” home activity may be required for efficacy of the enrichment. Animals also experienced social interaction with other animals not from their home cage; toys were periodically moved to activate spatial learning, and a running wheel was provided, as exercise has been shown to enhance proliferation. Interestingly, experience in enrichment (EE) increases both proliferation and differentiation in arsenic-exposed animals (though only significantly for differentiation). Additionally, aberrant gene expression (discussed below) was reversed in some cases in the EE arsenic-exposed mice compared to non-EE arsenic-exposed mice, again suggesting the reversibility of arsenic-induced damage. It is unclear if reversibility of these deficits would have appeared if animals had been in a different enrichment paradigm (See Limitations). However, it can be discerned that both environmental and pharmacological interventions are suitable to reverse deficits in AHN induced by perinatal arsenic exposure. This may be useful for therapeutics, as many people have an adverse reaction to the notion of taking antidepressant medications.

The fact that both pharmacological and environmental paradigms were able to reverse the behavioral endophenotypes and deficits in AHN suggest that interventions for developmental arsenic exposure are possible. The mechanism by which these treatments reverse deficits is interesting but not entirely clear. Fluoxetine was able to reverse deficits in AHN and did so, likely, by increasing GR, BDNF, HDAC2, and CREB. However, there were no molecular deficits induced by arsenic for these four proteins. Additionally,

EE reversed gene expression of some genes that were altered after arsenic exposure, including *Creb1*, but these gene sets did not completely overlap with the genes altered in EE control animals (see below). This suggests that fluoxetine and EE may possibly bypass the damage done by arsenic to improve AHN and behavioral outcomes; yet this begs the question as to actual damage imparted by arsenic exposure.

7.2.4 Significance of genetic outcomes in arsenic-exposed adult male mice

To elucidate if there was transcriptional dysregulation after perinatal arsenic exposure, we assessed mRNA expression using a microarray of genes specifically associated with neurogenic programs (Chapter 4). Arsenic exposure resulted in up-regulation of several genes associated Alzheimer's disease; this finding suggests a molecular mechanism supporting epidemiological studies showing a correlation between arsenic exposure and Alzheimer's disease (O'Bryant *et al.*, 2011). Additionally, arsenic exposure resulted in down-regulation of growth factors involved in axonogenesis, neurite outgrowth, and maturation (including doublecortin), all of which are important and necessary for proper differentiation. This molecular evidence corroborates morphological evidence: arsenic likely impedes the newborn neuron's ability to develop into a mature neuron, possibly leading to decreased numbers of differentiated neurons and decreased survival but not proliferation. Indeed, while cell death was not directly assessed, perinatal arsenic exposure did up-regulate factors associated with apoptosis. In other studies, developmental arsenic exposure increases apoptosis in other parts of the hippocampus (not in the dentate gyrus) (Fan *et al.*, 2013); however, adult exposure does not result in increased apoptosis in the dentate gyrus (Liu *et al.*, 2012). Studies investigating the effect

of perinatal arsenic exposure on apoptosis would aid in determining the fate of newborn neurons that do not functionally mature and integrate into the hippocampal circuitry. It is possible that reduced differentiation could occur due to either 1) increased apoptosis or 2) lack of activation of neuronal programs in favor of self-renewal gene expression. Interestingly, Dll1 was up-regulated in arsenic-exposed animals in the dentate gyrus; this ligand promotes Notch signaling, which enhances proliferation of progenitor cells. Down-regulation of Ascl1, a target of Dll1 was also observed; this bHLH transcription factor promotes differentiation. As such, increased Notch signaling may confer a genetic program for maintenance of the stem cell niche as a result of arsenic exposure but at the expense of differentiation. Thus, it can be further hypothesized that ChIP-Seq will reveal increased H3K4me3 occupancy of Notch pathway components in the dentate gyrus.

It should be noted that some aberrant mRNA expression as a result of perinatal arsenic exposure was reversed when animals experienced enrichment for one month (Chapter 4). This exposure paradigm resulted in increased expression of both proliferation and differentiation factors to levels previously measured in control animals; our findings suggest that enrichment shifts the focus from proliferation to differentiation by inhibiting pro-proliferative factors such as Noggin, Notch, and Sox3. Additionally, in arsenic-exposed animals, axonogenesis and transcription factor gene expression was up-regulated in response to enrichment. This is yet another piece of evidence suggesting that arsenic-induced damage is reversible and that genes associated with neurogenesis are dynamically expressed and responsive to the external environment. This finding furthered

the concept that epigenetic regulation of neurogenesis likely plays a role in the response of the dentate gyrus to arsenic.

7.2.5 Significance of epigenetic outcomes in arsenic-exposed adult mice

The findings from Chapters 4 and 5, that enrichment and fluoxetine were able to reverse arsenic-induced morphological and behavior damage, lead to us to further question the mechanism by which arsenic alters neurogenesis. Toxin exposure outcomes may be mediated through modifications of genetic programs, but the mechanism is not well understood. In one study, arsenic exposure increased DNA methylation on genes associated with plasticity, but the response of histone modifications in the adult brain to developmental arsenic toxicity had yet to be determined. We found that perinatal arsenic exposure altered some global histone posttranslational modifications (HPTM) in the dentate gyrus. The levels of two activating marks, H3K4 trimethylation and H3K9 acetylation, were increased in the dentate gyrus of arsenic-exposed adult males. Yet, these two particular marks were decreased in the frontal cortex of the same adult males. Additionally, female mice seemed to be robustly unaffected by arsenic exposure, with only H3K4 trimethylation decreased in the dentate gyrus. These findings suggest that the response to arsenic is likely both region and sex-dependent (see Future Directions). To determine the mechanism by which arsenic alters histone modifications, the expression of chromatin modifying proteins that are responsible for imparting these particular HPTMs were assessed. Expression of MLL1, the histone methyltransferase for H3K4me3, matched that of the histone modification: increased in the dentate gyrus of males, and decreased in the frontal cortex of males and the dentate gyrus of females. A similar

pattern was observed for GCN5 and PCAF, both histone acetyltransferase proteins responsible for H3K9 acetylation (among other marks). This result is specific to the lysine residue on which histone acetylation occurs as overall acetylation and expression of the histone acetyltransferase, CREB binding protein (CBP) was not affected by arsenic exposure in either brain region. Additionally, the master negative regulator of neuronal gene expression, REST and its cofactor, Co-REST, which we would have expected to be altered, as both are involved in neurogenesis, were unaffected by perinatal arsenic exposure in the adult dentate gyrus. Both proteins negatively regulate the neurogenic program in neural stem cells; yet, as described later, the heterogeneous population of the dentate gyrus is predominantly comprised of mature granule neurons with low expression of REST/CoREST. As such, further analysis of adult NPCs specifically, may provide a different outcome. Interestingly, we found REST and Co-REST expression were increased in the frontal cortex of arsenic-exposed males. At this point, the consensus in the literature suggests that the particularly distinct roles that REST/Co-REST play may be tissue and context dependent (Gao *et al.*, 2011). The results described here offer the first insight into how arsenic impacts the epigenetic landscape of histone modifications in the brain, suggesting that dysregulation of chromatin modifying factors may be the mechanism by which arsenic impairs gene expression. Relating these epigenetic modifications to behavior will require the use of epigenetic therapeutics and silencers to demonstrate causality.

7.3 Limitations and Critique of Studies

The studies presented in this dissertation are not without limitations. For each of the experiments and results outlined above, there are caveats to the interpretation of findings and possibly other techniques that can be used to answer these questions.

For example, there are several other measures for assessment of a depressive-like endophenotype in rodent models. We did not employ the sucrose preference test due to lack of face validity or the tail suspension task due to the inherent confound in using that particular assessment for C57BL/6 mice (as described in the Introduction). However, it may have been useful to assess animals' behavior in all possible behavioral measurements, including those related to depression and anxiety, to create a broad picture of the behavioral endophenotype elicited by perinatal arsenic exposure. Indeed, anxiety assessments would help further characterize the extent of arsenic-induced damage in the susceptibility to psychiatric disorders overall. This model uses very low doses of arsenic exposure, and while observationally, there are behavioral differences among control and exposed animals, characterizing these differences using the appropriate behavioral tasks has been difficult (See Future Directions).

Additionally, one design flaw is the administration of fluoxetine: several groups have used either gavage or i.p. injection for direct administration of fluoxetine in assessment of adult neurogenesis to directly control the amount of antidepressant (Malberg *et al.*, 2000; Malberg & Duman, 2003; Wang *et al.*, 2008a). However, one seminal paper that demonstrated the requirement of adult neurogenesis for the efficacy of antidepressant

actions did use oral administration via drinking water (Santarelli *et al.*, 2003). As neurogenesis is highly sensitive to stress and arsenic-exposed animals display HPA axis hyperactivity, we designed the fluoxetine administration to be as “stress-free” as possible. While, we feel, the results from the fluoxetine studies on AHN are robust, we can only assess the approximate amount of fluoxetine required to increase AHN as 20 mg/kg/day based on the oral consumption of the fluoxetine solutions (Chapter 5). Therefore, this administration model is not sufficient for deriving a dose-response relationship between fluoxetine consumption and degree of increased AHN. Indeed, the same could be true for the use of environmental enrichment: many studies determining the impact of EE require that the animals live continuously in the enrichment. Our intermittent design was based on previous studies demonstrating that the neurogenic response to EE is diminished with exposure to prenatal toxins when animals live in enrichment (Choi *et al.*, 2005). Based on these findings, we developed a “playground” model of enrichment to provide novelty and increased social interaction, which have been shown to be important for differentiation of AHN (Fabel *et al.*, 2009). However, from our model we cannot determine if “normal” EE would have elicited the same response of increased AHN after developmental arsenic exposure. Additionally, our findings do not aid in determining which component of EE is most important for ameliorating arsenic-induced deficits in AHN: novelty, social interaction, exercise, or toys.

The assessment of neurogenesis, while compelling and robust, also has its own limitations. The use of BrdU has been called into question over the last few years of research, as the antibody concentration and particular thymidine analog selection has

been shown to affect the cell counts, possibly providing a window into the inherent variability in assessment of AHN and the conflicting results reported in the neurogenesis literature (Leuner *et al.*, 2009). Additionally, while we implemented a pulse-chase assay considered to be the optimal paradigm for BrdU labeling, there are transgenic models that label neural stem cells more efficiently without the toxic side effects, such as cell death (Lagace *et al.*, 2007). However, replacing C57BL/6 mice with Nestin-CreER^{T2}/YFP mice would not only require re-characterizing the arsenic exposure paradigm, but would also require tamoxifen injections to activate the CRE recombinase for Nestin⁺ neural stem cells. Thus, the BrdU injection scheme for only 12 hours was the least stressful process of labeling cells. It should be noted that some groups prefer to use only Ki67, an endogenous marker of cell cycle activity, to label proliferating cells (Kee *et al.*, 2002); however, it is difficult to determine the number of cells that are maturing from the time of birth without using a thymidine label. One possible alternative that is less toxic and still provides robust labeling is the use of 5-ethyl-2'-deoxyuridine (EdU); this label is mostly used for pulse chase assays in conjunction with BrdU but can be used alone as well (Bradford & Clarke, 2011).

Finally, it should be noted that the dentate gyrus is a very heterogeneous tissue with several different cell types all undergoing different processes and thus different gene expression and regulation. As such, a limitation to these findings is that gene and protein expression analysis was performed on this heterogenous cell population of the dentate gyrus. For example, global histone modifications as measured by western blot analysis do not provide specificity for the modification present in specific cell types; this can be

overcome using immunohistochemistry methods, which would provide resolution for the type of cell containing the histone modification. However, western blot analysis was chosen to answer the question of the impact of arsenic exposure on the overall expression of the histone modification. Additionally, it is likely multiple histone modifications that were assessed are present in every cell type; using double-labeling IHC for several histone modifications may be more useful to demonstrate colocalization of expression levels. We were concerned with the degree of expression of histone modifications for the purposes of knowing which modifications to assess in chromatin immunoprecipitation next generation sequencing. However, the extent to which arsenic exposure impacts histone modifications on particular cell types thus possibly leading to deficits in AHN cannot be deduced from our findings (See Future Directions).

7.4 Future Directions

As with any study, there are many remaining questions that require further investigation. I have condensed the seemingly endless possibilities for future studies into distinct sets of inquiry: the effect of arsenic on embryonic programming, restoration of function, the frontal cortex, female mice, and micro-RNA expression and regulation. First, alternative methods of measurements that should be considered for future directions will be discussed.

7.4.1 Alternative strategies for measurements

As described in the limitations section above, one caveat to the molecular findings presented in this dissertation is that analysis was performed on the heterogeneous dentate

gyrus tissue (though this tissue is less heterogeneous than the entire hippocampus). Additionally, even measurements of embryonic neurogenesis (discussed below), would only provide morphological assessment of the impact of perinatal arsenic exposure, not gene or protein expression in specific cell types. Consequently, for this research, methods to separate cell populations would be useful. This could be accomplished with flow cytometry, which would use cell membrane identification for either endogenous or exogenous markers for neurons and neural stem cells. However, the current methods for isolation of NPCs from the dentate gyrus use cell culture coupled with flow cytometry (Gilley *et al.*, 2011). Laser capture microdissection of labeled neural stem cells or neurons in the adult or postnatal brain, followed by single cell transcriptomics would provide information as to the effect of arsenic on particular cell populations (Kohen *et al.*, 2014). This could be accomplished using the transgenic model, the Nestin:CreER^{T2}:YFP transgenic mouse model that with tamoxifen injection can birthdate neural stem cells (Kajimoto *et al.*, 2013); however, the arsenic exposure paradigm would need to be characterized in this new model.

7.4.2 Embryonic programming during arsenic exposure

Assessment of the effect of arsenic during embryonic brain development would answer many questions, but most importantly, are deficits observed in adulthood remnants of developmental alterations? This question could pertain to embryonic neurogenesis or to epigenetic programming.

7.4.2.1 Developmental neurogenesis

It is estimated that almost 95% of the granule cells of the dentate gyrus are generated during postnatal development; thus, the contribution of newborn neurons from NPCs in the adult is quite small (Shors *et al.*, 2012). Thus, it is likely that the granule neurons present during development of the dentate gyrus are impacted by perinatal arsenic exposure. Molecular aberrations measured in the adult dentate gyrus may be altered genetic programs of these older granule cells rather than the newly differentiated newborn neurons from adult neurogenesis. Additionally, arsenic could impact neurogenic programs in neural stem cells that are left over from development, which reside in the subgranular zone to form the adult neural progenitor pool. Further, aberrant early programming of genetic programs due to environmental factors, can lead to increased risk of psychiatric disorders (and other adverse health outcomes) in adulthood (Stolp *et al.*, 2012). Thus, it would be useful to characterize the impact of arsenic exposure on components of embryonic neurogenesis, providing a possible mechanism for these perpetuated deficits in adulthood. Embryonic neurogenesis can be difficult to measure, as several areas of the brain have neural progenitor cells that are dividing, maturing, and creating the circuitry of the brain; assessment between embryonic day 14.5 and 16.5, at the peak period of cortical neurogenesis and again at postnatal day 23 after the dentate gyrus is fully formed, would provide the most compelling results.

7.4.2.2 Epigenetic regulation during embryonic development

The results provided in Chapter 6 suggest that arsenic exposure during development leads to aberrant epigenetic programming in the adult dentate gyrus. It is possible that arsenic

induces an imbalance between proliferation and differentiation: up-regulation of MLL1 is important for proliferation and was found to be increased in arsenic-exposed adult males in the dentate gyrus, and deficits in AHN present as reduced numbers of differentiated neural progenitor cells. The brain may respond to arsenic toxicity by upregulating self-renewal and proliferative factors at the expense of neural factors for differentiation, as suggested by the results presented in Chapter 4. To determine the validity of this assertion, the molecular programs in proliferating neural progenitors versus neurons would need to be determined in both the embryonic and adult brain. This would require one of the two strategies mentioned before, flow cytometry or laser cell capture. Separation of two cell populations followed by assessment of gene expression, histone modifications, chromatin modifying proteins, and eventually the epigenetic regulation of altered gene expression (ChIP-qPCR) would test the hypothesis that arsenic alters the balance between proliferation and differentiation via aberrant epigenetic programming during development. It is possible that this altered epigenetic programming due to arsenic exposure is either 1) simply damage induced by arsenic that reverses once arsenic exposure ceases or 2) a compensatory response to arsenic that is perpetuated into adulthood. Results from these studies regardless of outcome (similar or opposite expression profiles from that measured in adulthood), would provide an answer to the response of the brain to arsenic exposure.

7.4.3 The regulation and expression of micro-RNAs after developmental arsenic exposure

The findings presented in Chapter 6 are the first to demonstrate the long-lasting effects of perinatal arsenic exposure on histone modifications and chromatin modifying proteins in

the brain. One particular set of observations described in Chapter 6 raises questions of how arsenic impacts the expression, function, and regulation of micro-RNAs. A recent study demonstrated that REST and CREB were responsible for the modulation of miR-9-2 expression during *in vitro* neuronal differentiation (Laneve *et al.*, 2010); developmental arsenic exposure altered expression of CREB and REST/NRSF and Co-REST in the frontal cortex in adult males, suggesting that miR-9 may be influenced by arsenic exposure. Indeed, inhibition of miR-9 has been shown to decrease neuronal differentiation (Krichevsky *et al.*, 2006), while loss of miR-9 has been reported in Alzheimer's disease (a disease that has been associated with arsenic exposure) (Packer *et al.*, 2008). miR-9 is highly expressed in the brain, along with miR-124, and cell cultures studies have reported its involvement with embryonic neural progenitor maintenance demonstrating that miR-9 targets some neurogenesis-related genes (Coolen *et al.*, 2013). Additionally, there is extensive crosstalk among epigenetic factors in controlling cell function; for example, miR-137 inhibits expression of EZH2, thereby maintaining the self-renewal of the neural stem cell pool, while miR-124 reduces expression of REST, allowing for the differentiation and maturation of neurons (Shi *et al.*, 2010). And while the effect of arsenic on micro-RNAs has not been fully explored, especially in the context of the brain, it has been demonstrated that arsenic may act through miR-9 to promote abnormal angiogenesis in carcinoma cell culture studies (Bailey & Fry, 2014). It is plausible that damage generated by arsenic exposure in the brain could be mediated by micro-RNAs, as such, the effect of developmental arsenic exposure on micro-RNA expression and function, particularly miR-9 and miR-124, should be investigated.

7.4.4 Mechanisms of therapeutic interventions as a window into the toxicity of arsenic exposure

The findings of the reversal of arsenic-induced damage after chronic fluoxetine treatment or experience in enrichment lead to further questions on the mechanism of therapeutics after arsenic exposure. How does enrichment impact the stress response after perinatal arsenic exposure? Are deficits in AHN ameliorated after “normal” enrichment? How does chronic fluoxetine impact the gene expression in arsenic-exposed animals? How does enrichment or fluoxetine impact the expression of epigenetic modifiers or histone modifications? Answering these questions was beyond the scope of the experiments for this dissertation; however, understanding the mechanism of reversal of arsenic-induced damage may be essential in characterizing how arsenic impacts the brain. For example, if fluoxetine was found to restore proper histone acetylation in the frontal cortex after perinatal arsenic exposure, this might lead to the use of more specific epigenetic therapeutics, like histone deacetylase inhibitors (HDACis) for treating patients with arsenic exposure to restore proper brain function. Indeed, the use of HDAC inhibitors for alleviating cognitive deficits and depressive-like symptoms is gaining traction; to demonstrate causality in arsenic-mediated alterations in histone acetylation, treatment with an HDACi such as sodium butyrate or valproate and subsequent assessment of cognitive function and histone acetylation would be useful.

7.4.5 The contribution of the frontal cortex in arsenic-induced depression

The studies presented in this dissertation largely focused on the damage induced by arsenic in the dentate gyrus of the hippocampus. The frontal cortex (FC) is responsible

for “executive functioning”, or the ability to plan, control and direct appropriate behaviors. Deficits in FC functioning have been implicated in the etiology of depression: the perceived lack of control over a situation and the subsequent passive behavior and maladaptive response to stress is likely mediate through the FC in depressed patients (Holmes & Wellman, 2009). The dentate gyrus receives inputs from the FC via the entorhinal cortex; FC signaling may be partially responsible for sending inappropriate messages to the dentate gyrus thereby contributing to the altered AHN after perinatal arsenic exposure. Additionally, FC tissue was used in the histone modifications studies for Aim 2B to demonstrate the specificity of arsenic-induced damage on the dentate gyrus (Chapter 6). We did not expect to have findings specific to the FC; as such, these new observations lead to the question of how arsenic impacts the behavioral, morphological, and molecular components of the frontal cortex. It would be interesting to assess the impact of perinatal arsenic exposure on frontal cortex function using FC-specific behavioral tasks, especially since epidemiological studies have shown an association between arsenic-exposure and attention deficits in human populations. Cognitive flexibility (and thus FC functioning) can be assessed using reversal learning, attentional set shifting, or a combination of the two. In reversal learning, the animal learns to associate a reward with certain cues and lack of a reward with a different set of cues; after attaining the initial association, the reward and cue coupling are reversed, such that the animal has to learn a different association for the reward. Reversal learning can be implemented in many tasks for working memory like the 8-way radial arm or spatial memory like the Morris Water Maze. However, the use of these behaviors requires hippocampal functioning; therefore, it would be more appropriate to assess reversal

learning in a different context, for example in a buried food pellet test or in a Y-maze. The ability to shift attention from one set of cues to another for proper reversal learning is predominately mediated by the frontal cortex; assessing this behavior in the arsenic-exposed animals would provide useful information as to the effect of perinatal arsenic exposure on frontal cortex functioning. Additionally, prefrontal cortex neurons have been shown to be highly sensitive to stress and undergo synaptic remodeling. Assessment of dendritic arborizations or synapses in the FC (using either IHC or western blotting for PSD-95), if the behavioral deficits are found, would provide another piece of evidence demonstrating FC dysfunction in this toxin exposure model.

7.4.6 Are female mice resistant to arsenic toxicity?

Experiments from Aim 2B are the first to suggest that perinatal arsenic exposure preferentially impacts protein expression in adult males and that adult female mice may be more resilient to arsenic toxicity. Indeed, this discrepancy among sex is common in toxin exposures, particularly when the HPA axis is involved. As such, new questions as to the effect of arsenic on females, including behavioral, morphological, and molecular outcomes are raised from these findings. Thus, future studies on the impact of perinatal arsenic exposure, particularly during embryonic development, should include females. A previous finding from the Allan lab determined that female mice perinatally exposed to arsenic exhibit depressive-like behavior in a stressful learned helplessness task. Thus, it would be useful to characterize the depressive-like endophenotype in female offspring in greater detail, as females are preferentially impacted by depression in the human population.

7.5 Summary

The studies outlined in this dissertation were designed to determine if adult hippocampal neurogenesis is a candidate mechanism by which perinatal arsenic exposure induces greater risk of developing depression in adulthood. The experiments initially sought to determine the behavioral and morphological damage imparted by perinatal arsenic exposure on the hippocampus. In this way, experiments performed characterized stress-induced depression as a behavioral endophenotype of perinatal arsenic exposure in adult male mice and ascertained the potential reversibility of depressive-like behavior using pharmacotherapy and environmental enrichment. Concurrent measurement of proliferation, differentiation, and survival of neural progenitor cells in the dentate gyrus was assessed, providing greater evidence of the connection between aberrant AHN and depression. Additionally, reversal of these morphological deficits using two different therapeutic methods demonstrates the potential transient nature of arsenic-induced hippocampal damage and provides insight suggesting that an intact progenitor niche may afford the efficacy of antidepressant treatment. Further studies were developed to determine the epigenetic mechanism by which arsenic impairs adult hippocampal neurogenesis; dentate-gyrus specific alteration of gene expression specifically associated with neurogenesis provided information demonstrating the potential opposing genetic programs of progenitor maintenance and neuronal maturation. Increased expression of active transcriptional histone modifications were found in arsenic-exposed adult males, along with aberrant expression of particular chromatin-modifying proteins. Subsequent analysis of an active transcriptional mark on gene expression, specifically in the dentate gyrus, will further provide evidence of genes up-regulated in response to developmental

arsenic exposure. It is possible that increased expression of certain genes in the dentate gyrus may inadvertently impact adult neurogenesis by promoting self-renewal and maintenance over maturation of neural progenitor cells, leading to deficits in AHN and increased depressive-like symptoms. However, further studies elucidating the causality of this potential mechanism are required. Overall, results provided in this dissertation imply that toxin exposure to an environmentally-relevant dose of arsenic that is present in non-municipal areas, rural well water, and in developed and developing countries alike, can have a profound impact on brain structure and signaling, leading to increased susceptibility to psychiatric disorders, such as depression. This may provide implications for either 1) alterations in water treatment regimens or 2) regulation of arsenic exposure from multiple sources.

Appendix A: Supplemental Data

A1: Context discrimination behavior assessment

Control and arsenic-exposed adult male C57BL/6 mice (PD65-70) were assessed for neurogenesis-dependent learning and memory in the context discrimination task, as described by (Sahay *et al.*, 2011), with minor modifications. Animals were trained for 7 consecutive days without odor cues in two Coulbourn Habitest chambers. Animals were habituated to the testing area for 1 hour prior to assessment each day; exposure to each chamber, labeled as Context A or Context B, occurred each day and was separated by 1-2 hours. The order of the contexts (A to B, B to A) was reversed for each animal each day. Context A and B were very similar except for floor texture, wall cues, and lighting. In Context A, animals received two, 2-second 0.8 mA foot shocks separated by 90 seconds. In Context B, animals did not receive foot shocks but were placed in the context for the same duration of time as Context A. Freezing behavior was recorded with a digital camera and scored as lack of deliberate movement during the first 90 seconds of experience in either context (CRT, AMA). Discrimination scores were calculated as the ratio of freezing in one context versus the other over the total freezing score: $((\text{Context A freezing} - \text{Context B freezing}) / (\text{Context A freezing} + \text{Context B freezing}))$. Developmental arsenic exposure resulted slower acquisition of the learning task (Standard). Experience in enrichment for one month rescued deficits in context discrimination in arsenic-exposed mice.

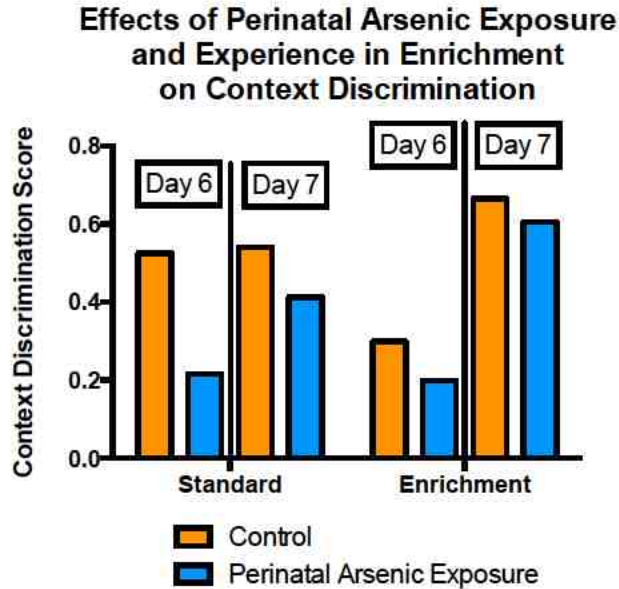


Figure A1. Developmental arsenic exposure reduces learning in the context discrimination task; experience in enrichment rescues this deficit

A2: Assessment of histone modifications

As described in Chapter 6, several histone modifications are altered in arsenic-exposed populations in peripheral blood monocytes. Trimethylation of H3K27 and dimethylation of H3K9 were two modifications that were originally tested in the dentate gyrus; no effect of developmental arsenic exposure was determined as analyzed by immunoblotting for A) H3K27me₃, ($t(7) = .4037$, $p = .69$) and B) H3K9me₂ ($t(4) = .86$, $p = .44$) in the male dentate gyrus.

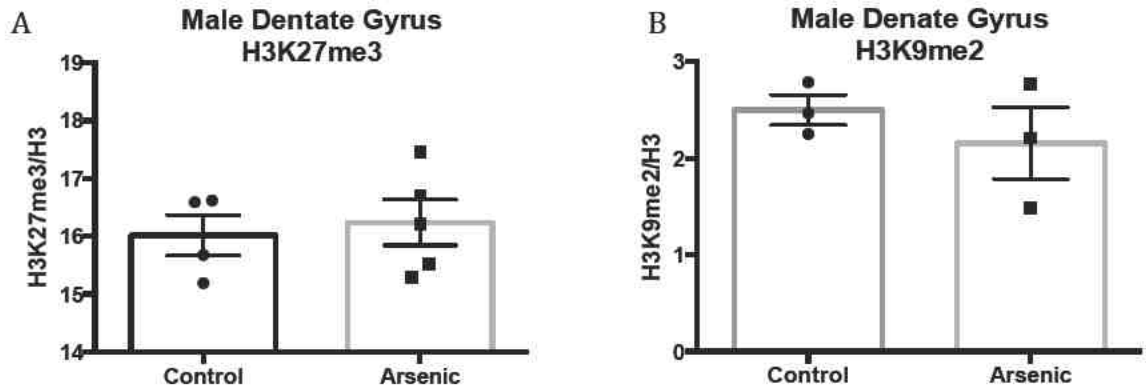


Figure A2. Developmental arsenic exposure does not alter A) H3K27me₃ or B) H3K9me₂ in the adult male dentate gyrus at PD70.

A3: The effect of developmental arsenic exposure on protein expression in the hippocampus

Prior to evaluation of BDNF in the dentate gyrus (Chapter 5), the effect of developmental arsenic exposure on expression of the pro and mature forms of BDNF and its receptor TrkB were assessed in the post-nuclear fraction of the hippocampus at PD35 in males. No significant effect of arsenic was determined; however, the trend of increased BDNF prompted further investigation of BDNF in the dentate gyrus ($t(8) = 2.01, p = .07$). Assessment of expression of the TrkB receptor in arsenic-exposed hippocampal tissue from PD35 male mice revealed a slight, almost significant, increase in expression ($t(10) = 2.029, p = .069$)

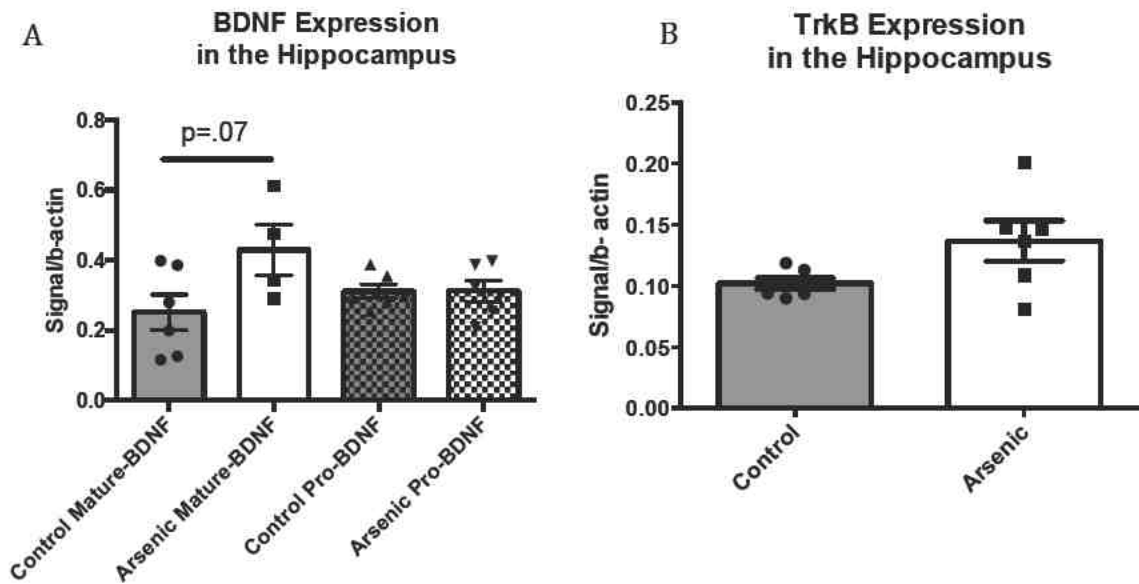


Figure A3.1. A) Developmental arsenic exposure slightly increases expression of the mature form of BDNF but has no effect on the expression of the B) TrkB receptor in the hippocampus of males at PD35.

Appendix A3 Continued:

Evaluation of the NR1 and NR2A subunits of the N-methyl-D-aspartate (NMDA) receptor were performed in the post-nuclear fraction of the hippocampus at PD35 after developmental arsenic exposure in males; a slight increase of expression of NR1 was determined, though not significant, ($t(9)=1.683$, $p = .12$), while DAE significantly decreased expression of the NR2A subunit, ($t(10) = 2.309$, $p = .0436$).

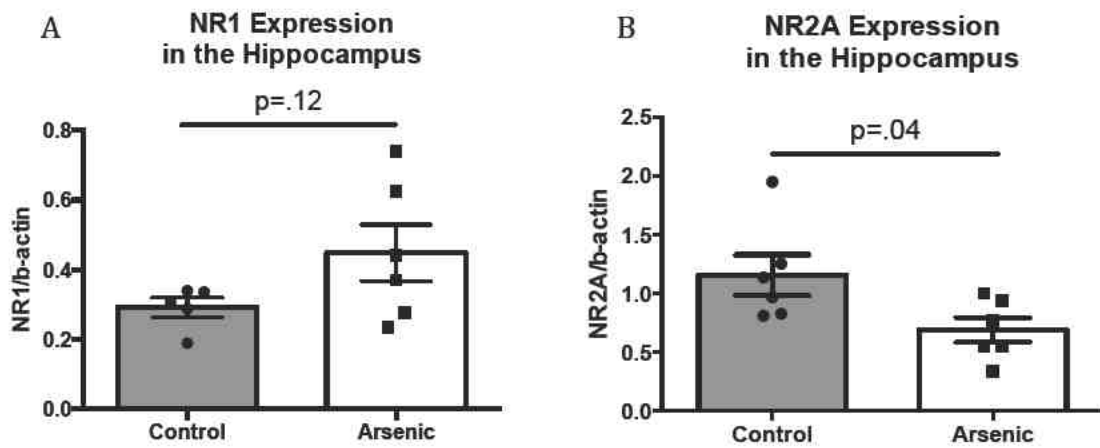


Figure A3.2. Developmental arsenic exposure slightly increases expression of the A) NR1 subunit and decreases expression of the B) NR2A subunit of the NMDA receptor in the hippocampus at PD35

A4: The effect of developmental arsenic exposure on protein expression in the dentate gyrus

The Wnt signaling pathway has been implicated in mediating many the various processes of adult hippocampal neurogenesis, including self-renewal, proliferation, and fate specification (Lie *et al.*, 2005). Wnt signaling is, in part, carried out via the inhibition of glycogen synthase kinase-3 β (GSK3 β), which when activated, suppresses β -catenin functioning via phosphorylation. Antidepressants may act to suppress the activity of GSK3 β in the hippocampus, while deficits in β -catenin activity, possibly due to increased GSK3 β expression, have been shown to reduce proliferation of neural stem cells (Kaidanovich-Beilin *et al.*, 2004). As such, the expression of GSK3 β and its phosphorylated form (p-GSK3 β) were assessed in the dentate gyrus after developmental arsenic exposure at PD70 in the dentate gyrus of adult males with and without one month of fluoxetine treatment (100 mg/L in water). Developmental arsenic exposure did not impact the expression of GSK3 β ($t(10) = .1537$, $p = .88$) nor did treatment with fluoxetine ($t(12) = .604$, $p = .557$). Interestingly, there was a trend of decreased expression of GSK3 β after fluoxetine treatment, but only in control animals, $t(11) = 1.839$, $p = .093$). Expression of the phosphorylated form of GSK3 β was not impacted by developmental arsenic exposure ($t(12) = .8254$, $p = .42$) or fluoxetine ($t(12) = .8514$, $p = .41$).

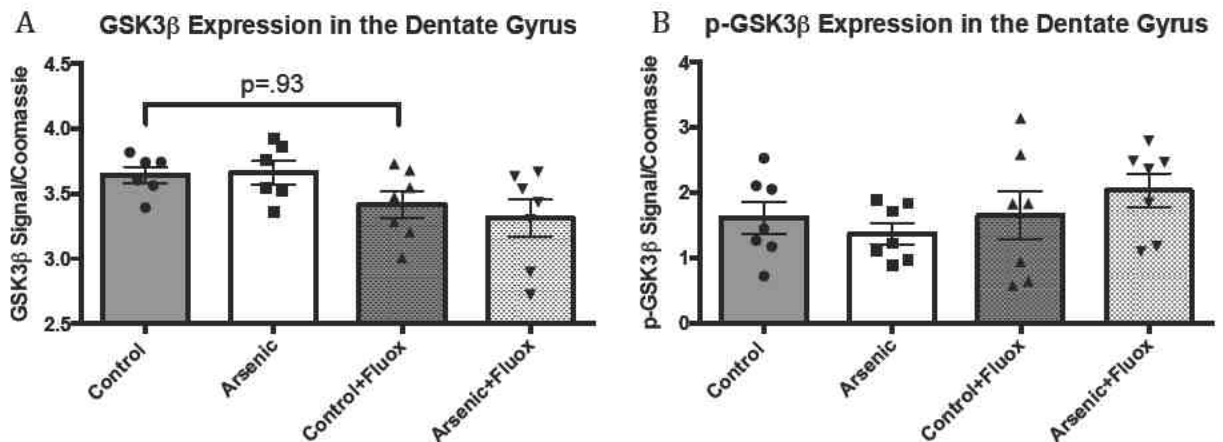


Figure A4.1. Developmental arsenic exposure and subsequent treatment with fluoxetine does not alter A) GSK3 β or B) p-GSK3 β expression in the male dentate gyrus at PD70.

Appendix A4 Continued:

Recent work by Eric Kandel's lab has implicated histone-binding protein RbAp48 in mediating deficits in learning and memory associated with aging, specifically in the dentate gyrus (Pavlopoulos *et al.*, 2013). Developmental arsenic exposure did not impact expression of RbAp48 in the nuclear fraction of the dentate gyrus from adult male mice (PD70), ($t(12) = .6632$, $p = .51$); treatment for one month with fluoxetine (100 mg/L in drinking water) did not alter expression of RbAp48 in control animals ($t(11) = .6027$, $p = .5589$), but fluoxetine did significantly decrease expression of RbAp48 in animals developmentally exposed to arsenic ($t(11) = 2.525$, $p = .0282$).

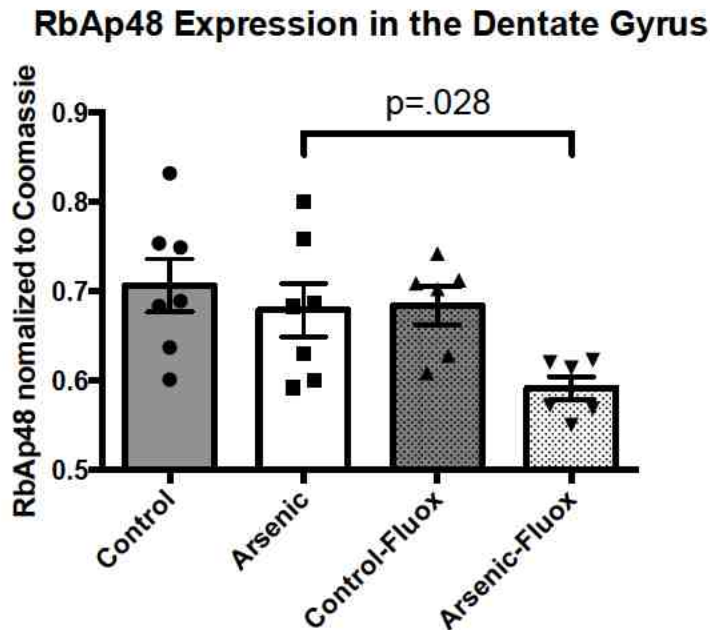


Figure A4.2: Developmental arsenic exposure does not alter RbAp48 expression in the male dentate gyrus at PD70, but treatment with fluoxetine for one month significantly decreases expression in arsenic-exposed mice.

A5: Treatment with imipramine ameliorates depressive-like symptoms but not neurogenesis deficits after developmental arsenic exposure

The first studies assessing the impact of an antidepressants on depressive-like behavior and adult neurogenesis after developmental arsenic exposure utilized the tricyclic antidepressant, imipramine, for treatment (Figure A5.1). Control and arsenic-exposed animals, aged PD35, were given daily I.P. injections of 15 mg/kg imipramine or vehicle for ten days. A pulse-chase BrdU assay was administered on PD46, as described in Chapter 4, with assessment of proliferation 24 hours after the final BrdU injection, on PD47. (See Figure A5.1) Assessment of depressive-like behavior in the learned helplessness task was performed essentially as described in Chapter 5, with minor alterations in the training (day 1) phase, including a more severe shock paradigm.

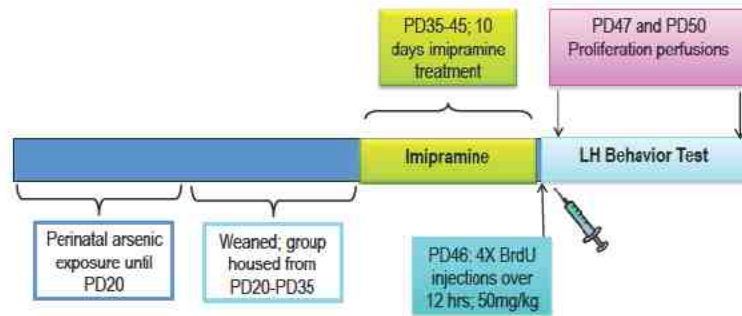


Figure A5.1. Imipramine treatment (15 mg/kg via i.p. injection) was given for 10 days prior to assessment of neurogenesis and depressive-like behavior in the learned helplessness task.

Initial assessment of the learned helplessness task demonstrated that the shock paradigm induced depression in both control and arsenic-exposed animals as indicated by trials to criteria of >20 out of 24. Treatment with imipramine reduced this effect but only in arsenic-exposed animals, $p < .05$ (Figure A5.2.A). The number of escape failures was increased in arsenic-exposed mice, though not significantly, compared to control animals, while imipramine significantly reduced the number of failed escapes in both groups ($p < .05$) (Figure A.5.4.B).

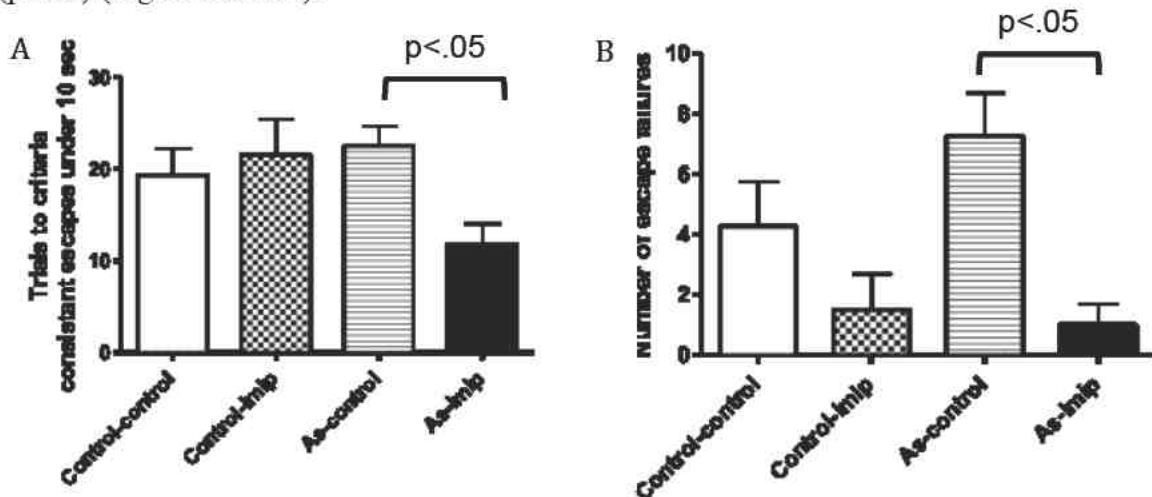


Figure A5.2. A) Imipramine treatment for 10 days reduced depressive-like symptoms in the learned helplessness task in arsenic-exposed animals as measured via trials to criteria ($p < .05$), and B) in both control and arsenic-exposed animals as measured by the number of escape failures, ($p < .05$).

Appendix A5 Continued:

Initial assessment of the effect of 10 days of imipramine treatment (15 mg/kg i.p.) from PD35-45 after developmental arsenic exposure revealed no significant effect of the antidepressant on proliferation of NPCs in arsenic-exposed animals at PD47.

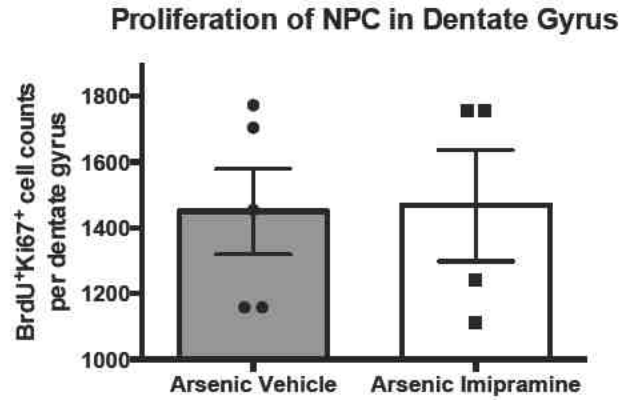


Figure A.5.3. Imipramine treatment for 10 days does not increase proliferation in the dentate gyrus of arsenic-exposed animals at PD47.

A6: Validation of altered gene expression in the dentate gyrus

Developmental arsenic exposure resulted in several changes in gene expression in the adult dentate gyrus at PD70 shown in Table 4.1, as measured using a microarray. Results from the microarrays were validated using qRT-PCR in triplicate. RNA (1 µg) was isolated from the dentate gyrus and stored at -80°C until cDNA conversion. Reverse transcription was performed using the Quantitect Reverse Transcription Kit (Qiagen; 205311) and a Peltier Thermal Cycler. Quantitative PCR was performed using FastStart Universal SYBR Green (Roche; 04913850001) on an ABI 7300 Real-Time PCR System (Applied Biosystems). Primer efficiencies of each primer set were assessed (presented as percentages below) to determine the quantity of DNA to use along with appropriate control primers. Primers were as follows:

Ache (F: 5'ATCGGTGTACCCCAAGCAAG, R:5'CTACCACTGCACTCATGGCA, 83%
 Apbb1 (F:5'AGCCAGTCGGCCATTAACG, R:5'GCACACTACCCTCTCCCATAG, 96%
 Ascl1 (F:5'GCAACCGGGTCAAGTTGGT, R:5'GTCGTTGGAGTAGTTGGGGG, 74%
 Fgf2 (F:5' GGCTGCTGGCTTCTAAGTGT, R:5' TCTGTCCAGGTCCCCTTTT, 96%
 Nrg1 (F:5'ATGGAGATTTATCCCCAGACA, R:5'GTTGAGGCACCTCTGAGAC, 91%
 Tgfb1 (F:5' GACTCTCCACCTGCAAGAC, R:5' GACTGGCGAGCCTTAGTTT, 89%
 Hprt (F:5'AGTCCCAGCGTCGTGATTAG, R:5'TTTCCAAATCCTCGGCATAATGA, 100%
 PPIA (F:5' TGCTGGACCAAACACAAACG, R:5' GCCTTCTTTCACCTTCCCAA, 95%
 B2M (F:5'TTCTGGTGCTTGTCTCACTGA, R:5' CAGTATGTTCCGGCTTCCCATTC, 87%

PCR cycles were as follows: 95°C for 5 min for step 1; 95°C for 10 sec, 60°C for 30 sec, 40 cycles for step 2, following by a 5 min dissociation step. Developmental arsenic exposure reduced expression of Ache, Apbb1, Fgf2, and Nrg1 and increased expression of Ascl1 and Tgfb1. However, some of these changes were in direct opposition to those reported in the microarray in Chapter 4. It was determined that tissue used for validation was derived from animals previously exposed to the learned helplessness task and the forced swim task, which may have altered mRNA expression compared to tissue derived from naïve mice used in the microarray.

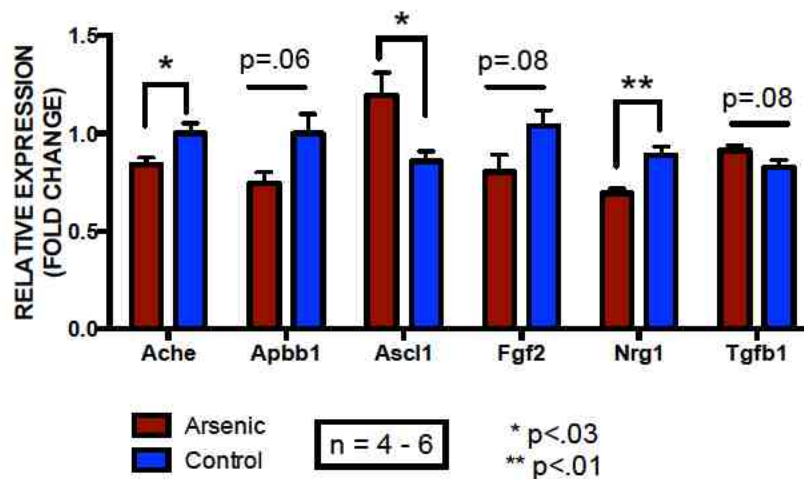


Figure A6.1. Validation of altered mRNA expression in the dentate gyrus after developmental arsenic exposure.

A7: Depressive-like Behavior in the Forced Swim Task

To corroborate findings previously reported by the Allan Lab, assessment of depressive-like behavior was performed at PD70 for control and arsenic-exposed mice and for mice treated with fluoxetine, as described in Chapter 5. As indicated by Figure A.7, developmental arsenic exposure did not elicit depressive-like behavior in the forced swim task, and fluoxetine treatment did not alter behavior. Previous assessment of depressive-like behavior in the FST task for both male and female mice perinatally exposed to arsenic indicated a main effect of arsenic (Martinez *et al.*, 2008); however, performance assessment occurred under diurnal conditions. The FST assessment described here was performed under nocturnal conditions, as animals are on a reverse light/dark cycle. Altered circadian rhythms have been associated with depression and shown to regulate CORT production and impact behavioral performance in the FST (Kelliher *et al.*, 2000; Darnaudery & Maccari, 2008). As such, we reassessed behavior in these tasks and determined the effect of fluoxetine after a stressor (Chapter 5).

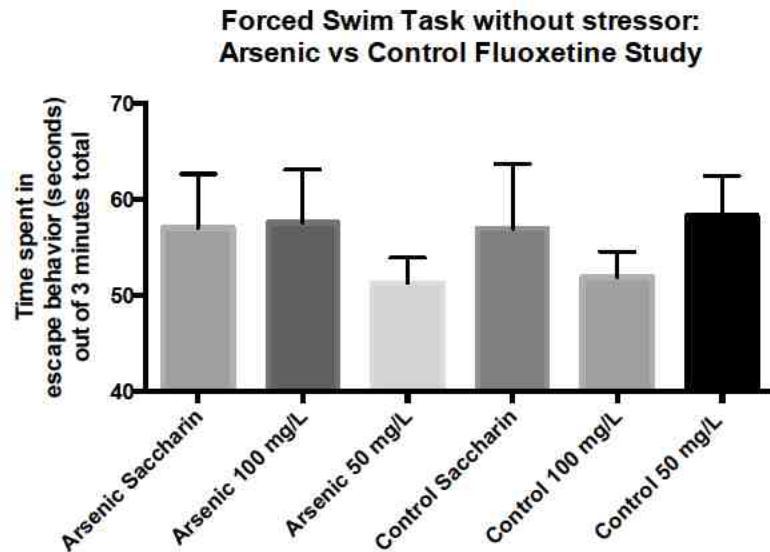


Figure A7. Developmental arsenic exposure and subsequent fluoxetine treatment does not alter behavior in the forced swim task without prior exposure to a stressor

A8: Chromatin Immunoprecipitation for Dentate Gyrus

To our knowledge, chromatin immunoprecipitation on the mouse dentate gyrus has not been performed to date. Since we observed changes in gene expression and histone modifications specifically in the dentate gyrus, assessment of the occupancy for particular histone modifications (H3K4me3) on genes in the dentate gyrus was determined via ChIP-Seq. The following is the protocol developed for ChIP on dentate gyrus tissue derived from adult male mice PD70.

Chromatin immunoprecipitation.

Dentate gyrus tissue obtained from adult male mice was homogenized in a Biomasher II disposable microhomogenizer (Kimble Chase) using a 1% formaldehyde crosslinking solution (1% formaldehyde, 1 mM EDTA, 0.5 mM EGTA, 50 mM HEPES, pH 8.0) for 15 minutes. Reagents from the ChIP-IT[®] Express Chromatin Immunoprecipitation Kits from Active Motif (53008) were used for some steps in the protocol. A 1X Glycine solution (Active Motif) was added to each reaction for 5 min. Homogenates were centrifuged at $1000 \times g$ for 6 min at 4°C and washed with 1X PBS containing 1 µg/µl protease inhibitor cocktail (Sigma, P8340). Cell pellets were resuspended in cell lysis buffer containing 0.5% Triton-X 100 (v/v), 85 mM KCl, 5 mM HEPES, and 2 µg/µl protease inhibitor cocktail and allowed to sit on ice for 30 min. Cells were centrifuged as before, resuspended in cell lysis buffer, and pelleted as before. The pellet was resuspended in nuclear lysis buffer containing 50 mM Tris, 10 mM EDTA, 1% SDS, and 2 µg/µl protease inhibitor cocktail. Crosslinked chromatin was sheared via sonication. Samples were sonicated 10 times for 10 s with 2 min on ice in between rounds to yield approximately 300-500 base pair chromatin. The size of the chromatin was determined using 10 µl of sample treated with 10 mg/ml proteinase K for 45 min at 50°C and run on 1.5% (w/v) agarose gel for 45 min. See Figure A.8.1. Samples were centrifuged at $14,000 \times g$ for 10 min at 4°C. At this point, the supernatant was snap frozen and stored at -80°C. The antibody of interest was incubated with magnetic beads for 4 hours at 4°C prior to the IP. ChIP-ready 800 µg chromatin was incubated overnight with magnetic beads (Active Motif) and the antibody of interest (RNA Polymerase II (Qiagen, GAM-111), H3K4me3 (Millipore 04-745) or IgG (Qiagen, GAM-8208). A small fraction of the chromatin was saved as input DNA. The following day, samples were washed at 4°C and the DNA eluted using the ChIP DNA Purification Kit from Active Motif (58002).

Library Preparation and ChIP-Seq

Library preparation of H3K4me3 immunoprecipitated DNA from two control and two arsenic samples was performed by Epigenome Center at the University of Southern California. Libraries were quantitated and size distribution determined using a Qubit fluorometer and an Agilent 2100 Bioanalyzer, respectively. Sequencing was performed using the on an Illumina Hi-Seq platform at the University of Southern California using 50 single end reads. Data quantification and analysis is currently underway.

The following table provides some information concerning the parameters of the chromatin immunoprecipitation protocol, including tissue type and amount used, preparation, chromatin amount, antibody type and concentration, and IP volume.

Table A8. Chromatin immunoprecipitation optimization

Tissue	Conc. Of Chromatin (ug/ul)	Chromatin Used (ug)	Total Volume of IP (ul)	Antibody Type	Amt Ab used (ul)	Magnetic beads (ul)	DNA conc. (ug/ul)	Total DNA	Preparation
2DG	0.60	100	341	Active Motif	5	25	0.0604	3.02	Tyler
2 DG	1.00	50	127	Active Motif	5	25	0.05	2.5	Active Motif
3 DG	0.66	200	611	Active Motif	8	50	0.14	7	Tyler
6 DG	0.74	200	552	Millipore	8	50	0.29	14.5	Tyler
3/6 DG	0.70	116	335	Millipore	5	25	0.107	5.35	Tyler
2 HIPP	1.94	700	450	Millipore	10	50	1.83	82.35	Active motif
4 HIPP	9.80	500	200	Active Motif	8	50	4.94	247	Active motif
4 HIPP	1.00	50	200	Active Motif	5	25	0.0324	1.62	Tyler
4 HIPP	9.80	500	200	Active Motif	8	50	7.62	381	Tyler/Active motif
6 DG	2.03	700	450	Millipore	10	50	1.29	58.05	Tyler/Active motif
12 DG	13.88	700	150	Millipore	10	50	3.83	172.35	Tyler/Active motif

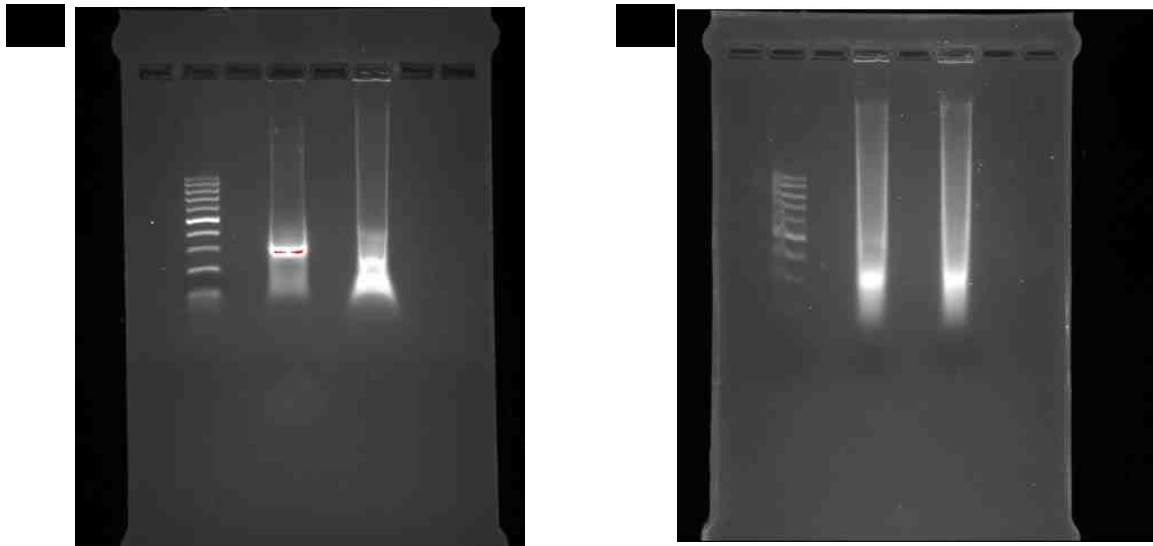


Figure A8.1. Agarose gel stained with ethidium bromide to demonstrated size of DNA in ChIP with 1kb DNA ladder. A) Hippocampal tissue with either 10 (lane 1) or 12 (lane 2) rounds of sonication, and B) Dentate gyrus tissue from arsenic (lane 1) or control (lane 2) with 12 rounds of sonication.

Antibody validation was performed using input DNA and the antibodies IgG and H3K4me3 (Qiagen) on hippocampal tissue for the genes Gapdh and Myod1. H3K4me3, an activation histone modification, should be present in sufficient quantities for detection in Gapdh (positive control) but not on Myod1 (negative control). An IgG antibody is used as a negative control for this validation. Figure A8.2 shows lower CT values (greater DNA expression) of Gapdh for the H3K4me3 ChIP (blue) compared to the IgG ChIP (green). Figure A.8.3 shows higher CT values (less DNA expression) of Myod1 for the H3K4me3 ChIP (blue) compared to the IgG ChIP (green).

Appendix A8 Continued:

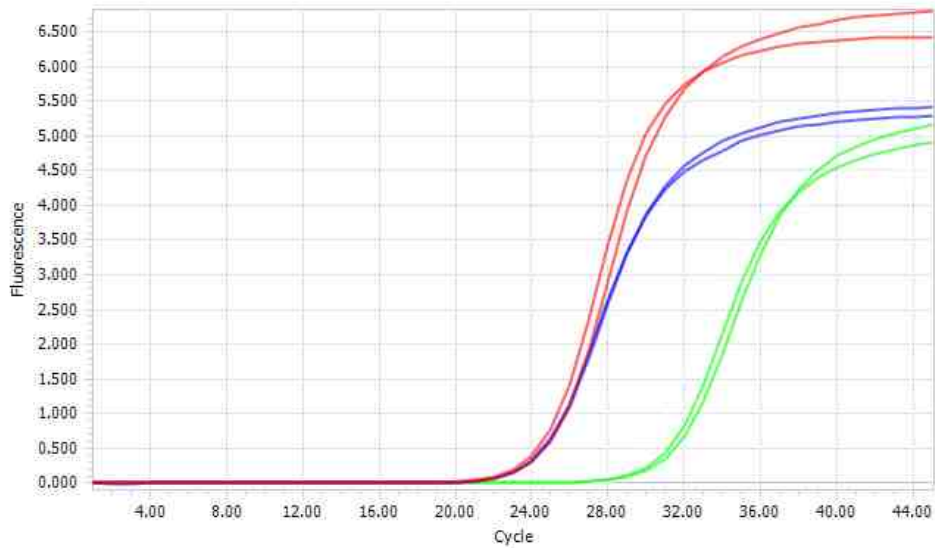


Figure A8.2. Cycle threshold plots for Input DNA (red), H3K4me3 (blue), and IgG (green) for Gapdh produced via qRT-PCR via hippocampal tissue.

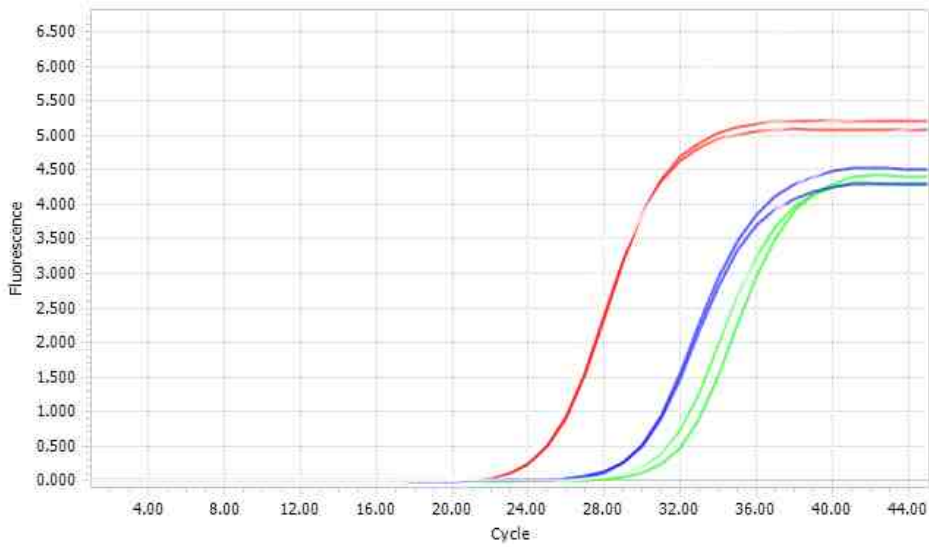


Figure A8.3. Cycle threshold plots for Input DNA (red), H3K4me3 (blue), and IgG (green) for Myod1 produced via qRT-PCR via hippocampal tissue.

Abbreviations Used

5HT _{A1}	serotonin 1A receptor
ACTH	adrenocorticotrophic hormones
AHN	adult hippocampal neurogenesis
AML	acute myeloid leukemia
APL	acute promyelocytic leukemia
BDNF	brain-derived neurotrophic factor
BrdU	bromodeoxyuridine
CARM1	cofactor associated arginine methyltransferase
CBP	CREB binding protein
ChIP-Seq	chromatin-immunoprecipitation followed by next generation sequencing
CNS	central nervous system
CORT	corticosterone
CREB	cAMP response element binding protein,
CRH	adrenocorticotrophin releasing hormone
DAE	developmental arsenic exposure
DAT	dopamine transporter
DCX	doublecortin
DEX	dexamethasone
DG	dentate gyrus
DNMT	the DNA methyltransferases
DSM-V	Diagnostic and Statistical Manual of Mental Disorders, version 5
ECS	Electroconvulsive seizure therap
EE	experience in enrichment; environmental enrichment
EPA	Environmental Protection Agency
FC	frontal cortex
Fgf1	fibroblast growth factor 1
FST	forced swim task
GCL	granule cell layer
GR	glucocorticoid receptor
GSH	glutathione
GWAS	genome-wide association studies
H3K27me3	histone 3 lysine 27 trimethylation
H3K4me3	histone 3 lysine 4 trimethylation
HAT	histone acetyltransferase
HDAC	histone deacetylase
HDACi	histone deacetylase inhibitor
HPA	hypothalamic-pituitary-adrenal
HPTM	histone posttranslational modifications
iAs	inorganic arsenic

LH	learned helplessness task
MAOi	monoamine oxidase inhibitors
MAPK	Mitogen-activated protein kinase
Mdb1	methyl CpG binding protein 1
MDD	major depressive disorder
MeCP2	methyl CpG binding protein 2
MLL	Mixed lineage leukemia factor
MR	mineralocorticoid receptors
MWM	Morris water maze
NaB	sodium butyrate
NET	norepinephrine transporter
NIMH	National Institutes of Mental Health
NPCs	neural progenitor cells
NRSF	neuron restrictive silencing factor
NSC	neural stem cells
OFT	open field test
PAE	perinatal arsenic exposure
PCAF	P300/CBP-associated factor
PcG	polycomb group
PD	postnatal day
PBMCs	peripheral blood mononuclear cells
PMSF	phenylmethylsulfonyl fluoride
ppb	parts-per-billion
ppm	parts-per-million
PRC1	polycomb repressive complex 1
PRC2	polycomb repressive complex 2
PRMT	protein arginine methyltransferase
PVN	paraventricular nucleus of the hypothalamus
REST	RE1-Silencing Transcription factor
SAHA	suberoylanilide hydroxamic acid
SAM	s-adenosyl methionine
SEM	standard error of the mean
SERT	serotonin transporter
SIRT	sirtuin deacetylase proteins
SNPs	single nucleotide polymorphisms
SSRI	selective serotonin reuptake inhibitors
TAPs	transiently amplifying cells
TCA	tricyclic antidepressant
TrxG	trithorax group
TST	tail suspension test
WHO	World Health Organization

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