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Perinatal arsenic exposure inhibits binding ability of glucocorticoid receptors to nuclear response elements altering gene expression and affecting learning behavior

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PERINATAL ARSENIC EXPOSURE INHIBITS BINDING ABILITY OF GLUCOCORTICOID RECEPTORS TO NUCLEAR RESPONSE ELEMENTS ALTERING GENE EXPRESSION AND AFFECTING LEARNING BEHAVIOR

BY

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B.S., Biology, University of New Mexico, 2005
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DISSERTATION

Submitted in Partial Fulfillment of the
Requirements for the Degree of

Doctor of Philosophy
Biomedical Science

The University of New Mexico
Albuquerque, New Mexico

May, 2010

DEDICATION

To my parents, with love:

For my dad, Michael, who taught me to be self-reliant, showed me that hard work and dedication pay off, and who took the word ‘can’t’ out of my vocabulary; you are my favorite philosopher

and

For my mom, Annette, who taught me to read, encouraged my love of reading and involved me in theater as a child; little did you know those would be great tools for a scientist

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To all of my family, thank you for your support and for dreaming with me.

To my father-in-law, Tom, whose babysitting made the writing of this dissertation possible.

To my siblings, Justin, Audra and Dillon, you have been inspirations to me all of my life. The bar is set, surpass it.

To my husband, Will, for your love, support and encouragement, thank you for helping me pursue my dreams.

And finally, to my son, Maverik, you are my biggest accomplishment.

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ABSTRACT

Learning deficits in children following arsenic (As) exposure via drinking water have been epidemiologically described in the last decade. Arsenic is a persistent environmental toxin and exposure has been shown to perturb the hypothalamic-pituitary-adrenal (HPA) stress axis. The glucocorticoid receptors (GR) are an integral part of the HPA axis and are found throughout the central nervous system, particularly in the hippocampus, an area of the brain important

in learning and memory. The mitogen-activated protein kinase (MAPK) pathway is involved in learning and two kinases of the MAPK pathway, Ras and Raf are transcriptionally regulated by GRs. In the studies detailed in this dissertation the impact of perinatal exposure to 50 parts per billion (ppb) sodium arsenate on learning behavior, GRs and MAPK pathway genes in the C57BL/6J adolescent mouse were examined. Hippocampal-sensitive learning was assessed using a novel object task and eight-way radial arm maze (RAM). Arsenic-exposed offspring showed increased latency to the novel object and increased number of entry errors in the RAM compared to controls. Immunoblotting revealed that arsenic-exposed offspring had significantly lower levels of both GR and mineralocorticoid receptors in the activated nuclear subcellular fraction than controls. As-exposed mice also showed significantly lower Ras and Raf-1 mRNA levels, assessed by real-time RT-PCR, compared to controls. Binding of the glucocorticoid receptor (GR) to glucocorticoid response elements (GREs) in Ras and Raf genes was measured by chromatin immunoprecipitation (ChIP). ChIP revealed reduced binding of the GR to GREs in target genes in the As group. Electrophoretic mobility shift assay (EMSA) revealed intact binding ability in both control and arsenic-exposed offspring to a synthetic GRE. Results suggest that moderate exposures to As can significantly reduce GR levels in the hippocampus, affecting expression of genes that are under the control of the GR and impacting learning behavior. Overall, these data suggest that moderate levels of perinatal As can have a lasting impact on the brain and HPA axis of offspring.

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ABBREVIATIONS

As	Arsenic
ChIP	Chromatin Immunoprecipitation
CORT	Corticosterone
DEX	Dexamethasone
DMA	Dimethylarsinic acid
EMSA	Electrophoretic Mobility Shift Assay
ERK	Extracellular-regulated signaling kinase
GR	Glucocorticoid Receptor
HPA	Hypothalamic-Pituitary-Adrenal Axis
ICP-MS	Inductively Coupled Plasma-Mass Spectrometry
MAPK	Mitogen-activated-phosporylated kinase
MMA	Methylarsonic acid
MR	Mineralocorticoid Receptor
RAM	Eight-way Radial Arm Maze
RIA	Radioimmunoassay
SAM	S-adenosylmethionine
WISC	Wechsler Intelligence Scale for Children

Chapter 1. INTRODUCTION

1.1 Overview

Arsenic (As) is a naturally occurring metalloid and one of the most common environmental contaminants. Arsenic is the 20th most abundant element in the Earth's crust and originates from volcanic tuff. It has several oxidation states but those of greatest toxicologic importance are inorganic arsenite (AsIII) and arsenate (AsV), the two forms that are also most commonly found in drinking water. A common source of human exposure to inorganic arsenic is through groundwater and drinking water contamination. In 1942, the EPA maximum exposure limit of arsenic in drinking water was established at 50 parts per billion (ppb). However, due to the extensive research on arsenic and carcinogenesis the maximum contaminant level (MCL) was reduced to 10ppb (0.01mg/L) in 2006. Epidemiologic evidence links inorganic arsenic exposure to an increased risk of a variety of cancers (i.e., lung, bladder, skin, kidney), type 2 diabetes, vascular and cardiovascular disease, hypertension, genotoxicity and reproductive and developmental anomalies (NRC, 2001). Many of these disease states can be linked to problems with steroid or nuclear receptor-medicated gene regulation (Barr et al., 2009). Arsenic is also a neurotoxin. While most studies on arsenic have focused on its carcinogenic potential, a few studies suggest that arsenic can adversely affect cognitive development in both rodents (Rodriguez et al., 2001, 2002) and humans (Calderone et al., 2001). These studies and others have prompted the National Research Council (NRC) to recommended additional research in all areas of arsenic's impact on human health. Specifically, the NRC

stated, “A possible impact of arsenic exposure on neuro-cognitive development in children requires further investigation” (National Research Council, 1999). However, the effects of arsenic on nervous system function have received considerably less attention than its association with cancer, genotoxicity, and cellular disruption (Rodriguez et al., 2003). Literature describing the effects of arsenic in drinking water on cognitive development is sparse. A PubMed search for arsenic and any of the following key words cognitive development, cognition, learning, memory, prenatal arsenic, perinatal arsenic, revealed less than 40 articles. Arsenic neurotoxicity has been best characterized through epidemiological studies, often based on research from countries (e.g., Mexico, Taiwan, Bangladesh) with significantly higher arsenic concentrations than the United States (Calderone et al., 2001; Wasserman et al., 2004; von Ehrenstein et al., 2007). The majority of these studies have focused on adolescents; and deficits in learning and memory, particularly in hippocampal-dependent tasks, following acute and chronic arsenic exposure have been reported for both rodents (Rodriguez, 2001, 2002) and humans (Calderone et al., 2001; Wasserman et al., 2004; von Ehrenstein et al., 2007).

The mechanism through which it produces this damage is yet to be elucidated; however, arsenic has been shown to perturb components of the hypothalamic-pituitary-adrenal (HPA) stress axis, such as the glucocorticoid receptor (GR) (Bodwell et al., 2004), by altering GR-mediated transcription. Perturbation of this axis has been implicated in both cognitive damage and the promotion of carcinogenesis. Persistent activation of the HPA axis in the chronic stress

response probably impairs the immune response and contributes to the development and progression of some types of cancer (reviewed in Reiche et al., 2004). Studies on arsenic's role in carcinogenesis have found that genes under the control of the GR are sensitive to arsenic (Kaltreider et al., 2001) and they may provide the link to arsenic-induced deficiencies in cognitive development. De Kloet and colleagues showed that both the presence of glucocorticoids and a functional GR are critical to normal hippocampal function (1998). Others have also shown that, at intermediate levels, glucocorticoids enhance learning and memory whereas low or high levels adversely affect cognition (Lupien and McEwen, 1997). Glucocorticoid receptors are found throughout the central nervous system (CNS) but are particularly concentrated in the hippocampus, an area of the brain of central importance for learning and memory. A signaling pathway that has been shown to be important in the processes of learning and memory is the mitogen-activated protein kinase (MAPK) signaling pathway (Atkins et al., 1998; Blum et al., 1999; Selcher et al., 1999; Shalin et al., 2004). Two genes of the MAPK pathway, Ras and Raf, have promoters that contain glucocorticoid response elements (GREs) which are activated upon binding of GRs (Figure 1.1).

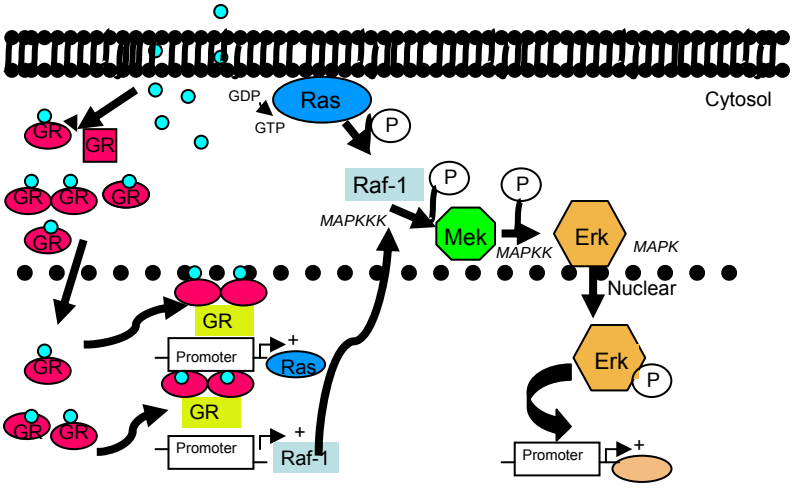

Figure 1.1 MAPK signaling in the absence of arsenic

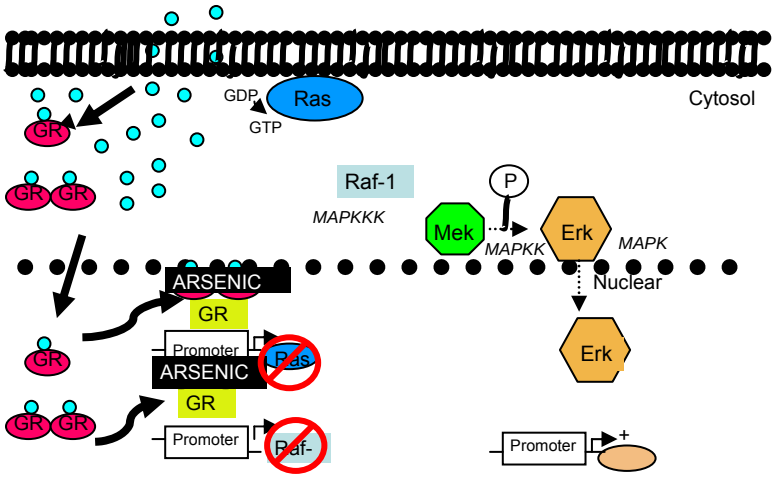

Figure 1.2 MAPK signaling in the presence of arsenic

Extracellular signal-regulated kinase 1/2 (ERK1/2) is the downstream target of Ras and Raf activation; phosphorylation of ERK1/2 facilitates its entry into the nucleus where its transcriptional targets play an important role in learning and memory (Shalin et al., 2004). Several studies demonstrate a requirement for the mitogen-activated protein kinase (MAPK)/extracellular signal regulated kinase (ERK) cascade in hippocampal-dependent associative and spatial learning (Sweatt, 2004). Glucocorticoid receptor activation is intimately coupled to ERK phosphorylation. Thus, disruption of GR/GRE binding by arsenic could lead to less Ras and Raf mRNA, ultimately leading to less phosphorylation of ERK1/2 (Figure 1.2).

The HPA axis and the MAPK signaling pathway may prove to be a common pathway for the diverse effects of arsenic. The overarching hypothesis for these studies was perinatal arsenic exposure alters HPA axis parameters and GR/GRE interactions, leading to impairments in the MAPK signaling pathway which are manifested as behavioral learning and memory deficits (Figures 1.1-1.2). The goal of this research was to test this hypothesis by determining the effects of moderate perinatal arsenic exposure on the HPA axis, the MAPK/ERK1/2 signaling pathway and cognition.

1.2 Introduction to Arsenic

The gamut of health effects after arsenic exposure can be attributed to whole body organ distribution of arsenic after an oral exposure, such as via drinking water. Inorganic arsenic is transformed in the body via one-carbon metabolism

through a series of methylation and reduction reactions, utilizing S-adenosylmethionine (SAM) as a donor of methyl groups. Methylarsonic acid (MMA) and dimethylarsinic acid (DMA) are the main metabolites of this pathway and are excreted in urine. Up until recently, this pathway was thought to be the detoxification pathway but several studies have shown that the metabolites themselves can also produce toxicity (Vahter, 2009). Both inorganic arsenic and its metabolites easily pass the placental and blood-brain barriers (Lindgren et al., 1984; Willhite and Ferm, 1984; Jin et al., 2006) Jin and colleagues showed that DMA was the major metabolite found in the brains of offspring whose mothers were exposed to 10 or 30ppm arsenate or arsenite in drinking water (2006).

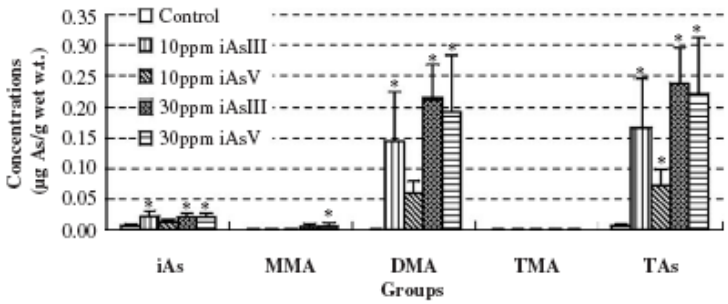

Figure 1.3 Arsenic speciation in brain of prenatally arsenic exposed pups (Jin et al., 2006)

The high levels of DMA in brains of offspring may be due to the fact that methylation of arsenic in women is increased during pregnancy (Concha et al., 1998; Hopenhyan et al., 2003). The methylation of arsenic is influenced however, by a number of factors including, but not limited to, genetic polymorphisms, nutrition, exposure to other chemicals and level of arsenic exposure (ATSDR

Arsenic, 2007). Although arsenic can readily cross the placental and blood-brain barriers it is not excreted at high levels in breast milk. A study of Andean women exposed to high concentrations (about 200ppb) of inorganic arsenic in drinking water detected concentrations of arsenic in their breast milk from 0.0008 to 0.008 ppm (Concha et al., 1998). Although this study was not conducted in rodents, it is generally accepted that the concentration of arsenic present in the breast milk is negligible.

1.3 HPA axis, GRs and Effects of Arsenic

Arsenic has been shown to produce a number of effects on the hypothalamic-pituitary-adrenal (HPA) stress axis (Baos et al., 2006), but there are no studies assessing perinatal arsenic effects on the developing HPA axis using an *in vivo* model. Research conducted in the last decade suggests that arsenic is a potent endocrine disruptor (Bodwell et al., 2006, 2006; Kitchin and Wallace, 2005; Kaltreider et al., 2001) and that moderate doses of arsenic can perturb glucocorticoid receptors (GRs), which are an integral part of the HPA axis. These receptors are not restricted to the brain but are distributed throughout the HPA axis, and the body. The HPA axis is also critical to the development process and anything that produces alterations in HPA axis during this time period is likely to have significant ramifications for later function.

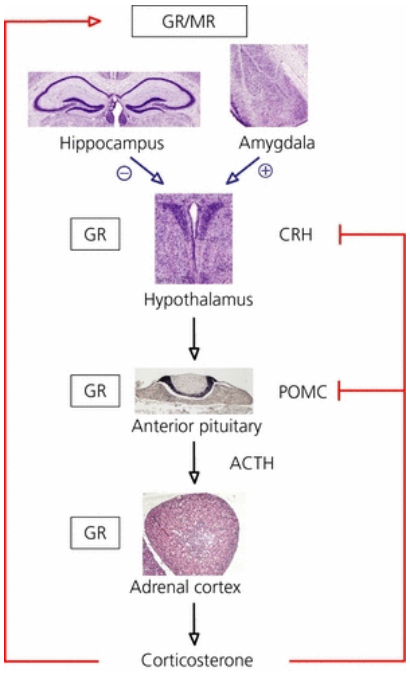

Figure 1.4 HPA Axis Regulation (Erdmann et al., 2008)

The HPA axis functions through negative feedback regulation at the levels of the hypothalamus, pituitary, and adrenals and additional regulation by the hippocampus (Figure 1.4). HPA responses are initiated by secretion of corticotrophin releasing factor (CRF) and arginine vasopressin (AVP) from neurons of the medial parvocellular paraventricular nucleus (PVN) of the hypothalamus through the portal blood system into the anterior lobe of the pituitary. These hormones stimulate the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary into the systemic circulation. ACTH then promotes the release of glucocorticoids from the adrenal cortex. Negative feedback regulation exists to dampen the stress response, and glucocorticoids, acting through GRs and MRs in the PVN and pituitary inhibit further

glucocorticoid release via inhibition of CRF/AVP and ACTH release, respectively. Glucocorticoids can also modulate HPA axis tone and activity by GR-mediated inhibition in the hippocampus and medial prefrontal cortex via projections to the PVN. Circulating glucocorticoids are required for proper control of gene expression during development and homeostasis (Sun et al., 2008). There are two types of receptors in the central nervous system that mediate the glucocorticoid response. Mineralocorticoid receptors, also referred to as Type 1 receptors, have an affinity for glucocorticoids that is ten times higher than glucocorticoid, or type 2 receptors. Therefore, in normal physiological states, MR is activated at very low concentrations of glucocorticoids whereas GR remains in the cytoplasm in its unactivated form until glucocorticoid concentrations reach threshold levels. Because of its higher affinity for glucocorticoids, MR, under normal physiological conditions, is thought to be fully occupied and thus, it is the GR that mediates the actions of circulating high glucocorticoids activating stress signaling. In the hippocampus there are abundant levels of glucocorticoids and coexpression of both MR and GR (Nishi and Kawata, 2006) in both neurons and glial cells. GRs can first be detected in rat brain on embryonic day (E17) (Kitraki et al., 1984) and increase gradually to adult levels by postnatal day 15 (PND 15) to PND30 (Olpe and McEwen, 1976; Clayton et al., 1977). Thus, the regulation of HPA axis activity has been assumed to be established during the embryonic development stage. MRs are located mainly in the hippocampus, whereas GRs have a fairly wide distribution throughout the brain and central nervous system. GRs and MRs regulate expression of genes involved in the maintenance of

homeostasis (Bagchi et al., 1992) and because of their strong expression in the limbic system of the brain they affect physiological processes such as neurogenesis, synaptic plasticity and behavioral responses including learning, memory, depression and anxiety (Erdmann et al., 2008).

At the cellular level, glucocorticoids cross the membrane by simple diffusion and enter the cytosol of the cell, where they bind to the cytosolic GR. Once bound, the hormone-receptor complex is translocated to the nucleus and regulates gene transcription of a subset of genes. Distinct subsets of glucocorticoid target genes are regulated differentially by MR or GR homodimers or MR/GR heterodimers (Conway-Campbell et al., 2007). Transcriptional regulation is mediated through binding to specific sequences of DNA named glucocorticoid response elements (GREs). The outcome of transcription can either be enhancement or repression of gene expression depending on whether GR binds to positive or negative GREs (nGREs). It is not necessary to have direct interaction between GRs and GREs in order to regulate gene transcription. Transcription can occur via direct GR/GRE interaction or as GR tethered to other DNA-bound proteins (Webster and Cidlowski, 1999). Other transcription factors known to be involved in the regulation include NF-kB, AP-1 and CRE (Diamond et al., 1990). Gene activation usually requires a 10-fold higher glucocorticoid concentration than gene repression, even though the same GR-steroid complex is involved (Sun et al., 2008). It has been estimated that approximately 1000-2000 genes in rat cortical neuronal cells are under the control of glucocorticoids, with an equal number being repressed and induced (Kino et al., 2007). Kaltreider and colleagues have

shown that nontoxic doses of arsenite can interact directly with GR complexes and selectively inhibit GR-mediated transcription (Kaltreider et al., 2001). Concentrations of arsenic in the 0.1-0.7uM range were shown to stimulate transcription of the tyrosine aminotransferase gene and the reporter genes containing tyrosine aminotransferase glucocorticoid response elements in rat hepatoma cells. At higher concentrations (1-3uM), the effects of arsenic on GR-mediated gene activation are inhibitory (Bodwell et al., 2006). Hamilton and colleagues showed that a single, low dose of arsenite (100μmol/kg sodium arsenite in 100uL H2O) significantly altered basal and inducible mRNA expression of the GRE-controlled phosphoenolpyruvate carboxykinase gene in cell culture and in a whole-animal model (1998). They also showed that arsenite had no effect on expression of GRE-non-inducible or constitutively expressed genes (Hamilton et al., 1998). Micromolar amounts of inorganic arsenic can inhibit transcription mediated by other members of the superfamily of nuclear receptors including the mineralocorticoid receptor, the progesterone receptor, the androgen receptor, the estrogen receptor, the thyroid hormone receptor and the retinoic acid receptor (Kaltreider et al., 2001; Davey et al., 2007; Bodwell et al., 2004; Davey et al., 2008). Based on these studies and the lack of receptor specificity, it is possible that there is a common target for arsenic, but this mechanism is yet to be elucidated. All of these receptors use a common mechanism of activation of gene transcription following ligand binding, allowing them to respond to a broad range of stimuli.

These studies have yet to characterize the effects of arsenic in an in vivo rodent model. Although there is information about arsenic in these various cell culture and chick embryo models there is no information about perinatal exposure to arsenic and its effects on GR-mediating gene transcription. Thus we are aware of potential damage produced by arsenic but these models don't account for what is happening when the whole system is intact and allowed to compensate for the damage.

1.4 Role of GR in Hippocampal-dependent Learning and Memory and Deficits in Arsenic-exposed populations

The connection between the hippocampus and learning and memory has been well established for decades (O'Keefe and Nadel, 1978). De Kloet and colleagues showed that both the presence of corticosteroids and a functional GR are critical to normal hippocampal function (De Kloet et al., 1998). Deficits on plasticity or memory in rats and humans are correlated with a high increases in plasma glucocorticoid (i.e. cortiocosterone and cortisol, respectively) that activate GRs (Lupien and McEwen, 1997; Belanoff et al., 2001, Diamond et al., 1992). Others have also shown that, at intermediate levels, corticosteroids enhance learning and memory but low or high levels adversely affect cognition (Lupien and McEwen, 1997). GRs have been shown to be involved in consolidation of learned information (De Kloet et al., 1999) and MRs involved in interpretation of novel information (Berger et al., 2006), memory retrieval (Conrad et al., 1997), and visuospatial learning (Yau et al., 1999). Animal studies have shown that intracerebroventricular injection of a rather selective MR antagonist in rats

influenced corticosterone-induced behavioral reactivity to spatial novelty (Oitzl et al., 1994). Mice with loss of limbic MR, via Cre-loxP inactivation, showed normal open field behavior and normal radial arm maze performance but impairments in behavioral reactivity to novelty (Berger et al., 2006), suggesting the MRs control behavioral response to novelty. GR is implicated in memory consolidation processes, demonstrated by using GR-agonists and GR-antagonists (Oitzl et al., 2001; Donley et al., 2005; Oitzl and deKloet, 1992; Roozendaal., 2003; Sandi and Rose, 1994). Intracerebral ventricular injection of a GR antagonist after training in the water maze task resulted in increases in latencies to find the platform (Oitzl and deKloet, 1992). Oitzl and colleagues showed that mice deficient in GR DNA-binding showed selective impairment of spatial memory in the water maze (Oitzl et al., 2001). Donley and colleagues showed hippocampal administration of GR antagonist RU 38486 decreased freezing in a 24 hour contextual fear conditioning retention test while post-shock levels of freezing were intact (Donley et al., 2005). We assessed MR and GR levels in our perinatal model due to their involvement in learning and memory and the fact that arsenic neurotoxicity has been best characterized through epidemiological studies of learning and memory deficits (Calderone et al., 2001; Wasserman et al., 2004; von Ehrenstein et al., 2007). One potential setback with these studies is that they are all retrospective and there may be a time interval between the exposure and the production of learning and memory deficits. In addition there may be covariants that are not reported such as other chemical exposures or nutrition which can not be controlled. One cross-sectional study conducted in Mexico

examined the effect of chronic arsenic and lead exposure on learning and memory. This study examined two populations with a total of 80 children, ages 6-9, one living close to a smelter and the other living in a lower arsenic concentration area. They compared test scores of both groups using the Wechsler Intelligence Scale for Children revised for Mexico (WISC-RM) and reported that higher levels of urinary arsenic were correlated with poorer performance on tests examining long-term memory after correcting for lead exposure levels (Calderone et al., 2001). Another cross-sectional study conducted in Taiwan, with 109 adolescents, reported significant effects of long-term cumulative exposure to arsenic on memory and switching attention after adjusting for education and sex (Tsai et al., 2003). A study conducted in Bangladesh, with 201 children, age 10, using the WISC, reported that the children's intellectual function was reduced in relation to exposure to arsenic in drinking water. This study corrected for manganese, which was also present in high amounts in their water source and socioeconomic factors. With covariate adjustment they found that children with arsenic levels above 50ppb achieved significantly lower performance and full-scale test scores than children with arsenic levels below 5ppb (Wasserman et al., 2004). Another study conducted in India with 351 children, ages 5 to 15 years, using the WISC, in conjunction with the Colored Progressive Matrices test, and the Total Sentence Recall test reported significant associations between urinary arsenic levels and performance in the vocabulary test, the object assembly test and the picture completion test. These studies provide a basis for arsenic toxicity and neurobehavioral effects.

Cross-sectional studies analyze the relationship between different variables at a point in time and since exposure and disease status are measured at the same point, it may not always be possible to distinguish whether the exposure preceded or followed the disease. Thus, we have limited information on sensitive time periods of exposure and the mechanisms involved in the deficits. Our perinatal arsenic mouse model is useful for determining if the neurobehavioral outcomes could be the result of exposure to arsenic during the sensitive perinatal time period. Our rodent model also provides a way to measure outcomes without confounds such as socioeconomic status, nutrition, intelligence and other chemical exposures. In addition, it is possible to look at the brain molecularly and biochemically to help establish a mechanism for the behavioral learning and memory deficits.

1.5 GRs as Transcriptional Regulators of Genes in the MAPK/ERK Signaling Pathway

While there are several neurochemical pathways involved in learning and memory processes, the mitogen-activated protein kinase (MAPK) pathway appears to provide a final common pathway for the many neurotransmitter systems involved in memory. Several studies support a requirement for the MAPK/extracellular signal regulated kinase (ERK) cascade in hippocampal-dependent associative and spatial learning (Sweatt, 2004). MAPKs are proline-directed, serine/threonine kinases that regulate a variety of cellular functions. MAPKs are converted to the active, phosphorylated form by dual-specificity kinases, MAP kinase kinases or MAPK/ERK kinases (MEKs), which

phosphorylate threonine and tryrosine residues in the enzymes. Two of the genes of importance in regulating MAPK phosphorylation, Ras and Raf, have been shown to be activated by GR binding to GREs (Revest et al., 2005). Ras and Raf expression activates the MAPK signaling pathway ultimately leading to phosphorylation and activation of ERK1/2. Phosphorylated ERK (p-ERK) is translocated to the nucleus where it activates a number of genes. There is evidence that ERK1/2 activation occurs in rodents after training in a spatial learning task, such as the hidden platform version of the Morris Water Maze (Blum et al., 1999), as well as after fear conditioning (Atkins et al., 1998). Intrahippocampal infusions of MAPK pathway inhibitors, prior to training, impair memory retention after training in the Morris Water Maze (Blum et al., 1999; Selcher et al., 1999). Shalin and colleagues used transgenic mice to show that neuronal MEK and subsequent ERK1/2 activation has an important role in hippocampal-dependent learning (Shalin et al., 2004). These transgenic mice were constructed to express a dominant-negative form of MEK, specifically in the hippocampus and thus they also had decreased levels of ERK1/2 activity in the hippocampus (Shalin et al., 2004). The transgenic mice showed normal performance in a battery of control tasks, such as open field, rotarod and shock threshold, but displayed significant impairments in contextual fear conditioning compared to their wild type littermates (Shalin et al., 2004) indicating an impairment in higher order functions. MEK activation and rapid ERK activation is critical for induction of long-term potentiation (LTP), the basis for learning at the synaptic level. ERK activation is also important in delayed gene expression

responses that come into play at a later stage of LTP in the hippocampus (Mailliet et al., 2008). To our knowledge no one has characterized the effect of perinatal arsenic exposure on the MAPK signaling pathway in the brain and its association with learning and memory deficits.

1.6 Summary

Based on the epidemiological studies that reported decrements in learning and memory in children exposed to arsenic via their drinking water (Calderone et al., 2001; Tsai et al., 2003; Wasserman et al., 2004; von Ehrenstein et al., 2007) we postulated that learning and memory would be impaired in mouse offspring born to arsenic-exposed mothers. In chapter three, we report nonspatial learning and memory using the novel object task and spatial learning and memory using the 8-way radial arm maze in 35 day old male mice exposed perinatally to 50 ppb arsenic in drinking water (Martinez-Finley et al., 2009). The 35 day time period was chosen because at this time point mice are equivalent in age to children/adolescents which were reported in the epidemiology studies. Figure 1.5 shows an age equivalency in mice and humans.

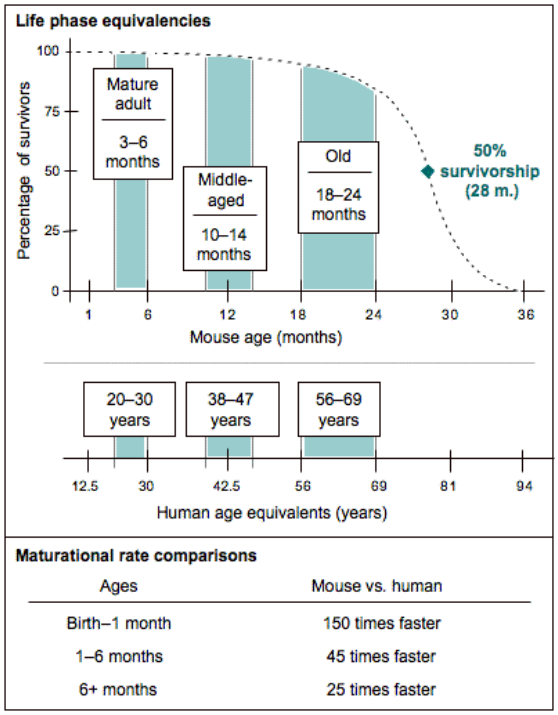

Figure 1.5 Human and Mouse Age Equivalencies
(http://research.jax.org/faculty/harrison/ger1vLifespan1.html)

Chapter three shows the level of arsenic found in the whole brains of our mice after perinatal exposure (Martinez-Finley et al., 2009). Arsenic levels in the hippocampal region were below the limit of detection in both control and perinatal arsenic-exposed offspring (unpublished observation). Chapter two reports the results of measurements of basal levels of corticosterone (CORT) using radioimmunoassay techniques from perinatally arsenic exposed mice along with their respective controls (Martinez et al., 2008). To address the status of the receptors mediating the CORT response and the learning and memory we report MR and GR protein levels within hippocampal tissue from 35 day old mice perinatally exposed to 50 ppb arsenic or control tap water (Martinez-Finley et al.,

2009) in chapter three. The appendicies contain information on the developmental vs. acute postweaning time periods. Chapter four is the assessment of the functional status of the GR-associated MAPK signaling pathway determined by measuring Ras and Raf mRNA levels using Real-Time PCR. We also measured the impact of reduced levels of upstream kinases, Ras and Raf, by assessing the ratio of phosphorylated ERK (p-ERK) to total ERK using western blot. We hypothesized that arsenic inhibits GR binding to the GREs in the promoter regions of Ras and Raf, thereby inhibiting transcription of these genes. To evaluate the level of GR binding to the GRE site on the Ras and Raf promoters we utilized Chromatin Immunoprecipitation of GR in arsenic-treated and control animals. Co-immunoprecipitated genomic DNA sequences were subjected to real-time PCR with primers for the promoter sequence of Ras and Raf.

Chapter 2. MODERATE PERINATAL ARSENIC EXPOSURE ALTERS NEUROENDOCRINE MARKERS ASSOCIATED WITH DEPRESSION AND INCREASES DEPRESSIVE-LIKE BEHAVIORS IN ADULT MOUSE OFFSPRING

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2.1 ABSTRACT

Arsenic is one of the most common heavy metal contaminants found in the environment, particularly in water. We examined the impact of perinatal exposure to relatively low levels of arsenic (50 parts per billion) on neuroendocrine markers associated with depression and depressive-like behaviors in affected adult C57BL/6J mouse offspring. Whereas most biomedical research on arsenic has focused on its carcinogenic potential, a few studies suggest that arsenic can adversely affect brain development and neural function.

Compared to controls, offspring exposed to 50 parts per billion arsenic during the perinatal period had significantly elevated serum corticosterone levels, reduced whole hippocampal $CRFR_1$ protein level and elevated dorsal hippocampal serotonin $5HT_{1A}$ receptor binding and receptor-effector coupling. $5HT_{1A}$ receptor binding and receptor-effector coupling were not different in the ventral hippocampal formation, entorhinal or parietal cortices, or inferior colliculus. Perinatal arsenic exposure also significantly increased learned helplessness and measures of immobility in a forced swim task.

Taken together, these results suggest that perinatal arsenic exposure may disrupt the regulatory interactions between the hypothalamic-pituitary-adrenal axis and the serotonergic system in the dorsal hippocampal formation in a manner that predisposes affected offspring to depressive-like behavior. These results are the first to demonstrate that relatively low levels of arsenic exposure

during development can have long-lasting adverse effects on behavior and neurobiological markers associated with these behavioral changes.

Key Words: Arsenic, Corticosterone, Hippocampal Formation, CRF Receptor, Serotonin Receptor, Learned Helplessness

2.2 INTRODUCTION

Failure to appropriately respond to external stressors has been implicated in a number of mental health disorders, particularly Major Depressive Disorder (Herman et al., 2003; Barden, 2004). Several lines of evidence suggest that HPA axis hyperactivity, due to excessive corticotrophin-releasing factor (CRF) release, combined with deficits in the negative feedback regulation of CRF release (Barden, 2004), may be a causative factor in the pathogenesis of depression (Pariente, 2004). In addition, elevated corticosterone levels have been associated with depression in a rodent model (Zhao et al, 2008). Elevated CRF release in response to stress is associated with onset of depression (Nemeroff 1992; Reul and Holsboer, 2002) and elevated cerebral spinal fluid levels of CRF have been reported in depressed patient populations (Nemeroff, 1988).

Several studies indicate functional interactions between CRF and the serotonergic system and have linked these interactions to depression (Leonard, 2005; Waselus and Van Bockstaele, 2007). In addition to CRF receptors present in the raphe nucleus (Sakanaka *et al,* 1987; Swanson *et al,* 1983; Ruggiero *et al*, 1999; Kirby *et al*, 2000; Lowry *et al*, 2000; Valentino *et al*, 2001), CRF exerts a

stimulatory effect on hippocampal serotonin (5HT) levels (Linthorst *et al*., 2002; Peñalva *et al*., 2002; Oshima *et al*., 2003). CRF receptor antagonists reduce 5HT levels in rat hippocampal formation (Isogawa *et al*, 2000; Linthorst *et al*, 2002). Homozygous $CRFR_1$ knockout mice showed enhanced hippocampal 5-hydroxyindoleacetic acid (5-HIAAA) under basal conditions and a greater rise in hippocampal 5HT during a forced swim task compared to wild type mice (Peñalva *et al*., 2002). The most abundant 5HT receptor in the hippocampus is the $5HT_{1A}$ receptor and its expression is regulated by HPA axis activity (Lopez *et al*., 1998); with expression being elevated during low corticosteroid conditions and decreased with high corticosteroid levels. Similarly, rats bred for high anxiety-related behavior (HAB) have lower $5HT_{1A}$ expression compared to the low anxiety-related behavior rats (Keck *et al*., 2004).

Arsenic is one of the most common environmental contaminants found in water, food and air (DeSesso *et al*., 1998). Human exposure has been associated with skin, lung, and bladder cancers, vascular diseases, hypertension, and diabetes (National Research Council, 2001; Kitchin, 2001). In addition, arsenic crosses the placental barrier (Jin et al, 2006) and can affect offspring during critical periods of brain development. Exposure has also been implicated in damaging the hypothalamic-pituitary-adrenal (HPA) axis (Delgado *et al*., 2000) leading to deficits in stress responding.

Given the reported effects of arsenic exposure on HPA axis responsiveness and the putative interactions between the HPA axis and the serotonergic system in

depression, we examined the impact of moderate perinatal arsenic exposure on neuroendocrine markers associated with depression and depressive-like behaviors in mice. Because the perinatal exposure period encompasses the developmental period of the offspring from conception through embryonic stages, gestational stages, and postnatal stages until weaning, it is likely that a number of systems in the brain that are developing during these critical periods are affected. For example, $5HT_{1A}$ immunoreactivity has been shown as early as embryonic day 16 (Patel and Zhou, 2005) suggesting that this system is organized early on and could be affected in our model. We hypothesized that moderate arsenic exposure during the perinatal period would alter markers of the HPA axis and serotonergic neurotransmission and that these changes would be consistent with the appearance of depressive-like behavioral responses in affected offspring.

2.3 MATERIALS and METHODS

2.3.1 Perinatal Arsenic Exposure Paradigm

The arsenic exposure paradigm and behavioral tasks employed in these studies were approved by the UNM Health Sciences Center Institutional Animal Care and Use Committee. All mice were bred and maintained on a 12-hour light/dark cycle (lights on from 0700 to 1900) with food and water ad libitum in a temperature controlled (22 ºC) room in the Animal Resource Facility. Female C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) were assigned to either a control arsenic-free water or 50 parts per billion (ppb) arsenic (sodium arsenate, Sigma) water treatment group. Arsenic-free water was prepared by

reverse osmosis followed by a MilliQ water purification step. After a two-week acclimation period on the treated waters, male breeder mice were introduced into each female's cage. Three days later, the males were removed from the cages and nesting material was placed in the female's cage. Mouse dam water consumption was monitored throughout pregnancy and the dams continued to drink the treated waters until their offspring were weaned. Litter sizes and neonatal body weights were measured on postnatal day 7. Offspring were weaned at 23 days of age and maintained in same-sex, litter-mate housed cages with ad libitum access to untreated tap water and standard mouse chow until they were used in experimental procedures at 75 to 90 days of age. Tap water at UNM contains approximately 2 to 5 parts per billion arsenic. Mice (one per litter; 5-10 litters total per treatment group) were assigned to either one of the biochemical assay procedures or one of the two behavioral testing paradigms. Offspring used in behavioral studies were not used in biochemical studies.

2.3.2 Plasma Corticosterone

Blood samples were collected between 0800 and 1000 hours. Mice were decapitated and trunk blood collected into plastic SAFE-T-FILL capillary tubes prepared with liquid EDTA Di Potassium Salt (Ram Scientific Inc, Yonkers, NY). Tubes were immediately placed on ice and the plasma obtained by centrifugation (4000 rpm x 15 minutes) at 4^{o}C. Supernatents were decanted into storage tubes and the samples frozen at -20 ^{o}C until assay. Plasma samples were then thawed and assayed for corticosterone using a rodent double antibody ^{125}I-corticosterone

radioimmunoassay kit (MP Biomedicals, Orangeburg, NY), according to the manufacturer's instructions. Samples were assayed in duplicate.

2.3.3 $CRFR_1$ Receptors

Tissue preparation. Adult offspring, 75 to 90 days of age, were sacrificed by decapitation and whole hippocampal formation rapidly dissected, rinsed, transferred to Dounce homogenizing tubes containing 0.5 mL homogenizing buffer (0.32 M sucrose, 1 mM EDTA, 20 mM Tris-HCL and 0.1 mM AEBSP; HB buffer) and homogenized by five loose and five tight up and down strokes. Homogenates were centrifuged at 1,000xg for 6 minutes, the supernatant fraction (S1) collected and centrifuged at 200,000xg for 30 min. The resulting pellet (P2) was resuspended with 0.5 mL HB buffer with 75 mM NaCl, 75 mM KCl and 1% Triton X-100 added, re-homogenized and centrifuged at 200,000xg for 30 min. to produce detergent-soluble proteins (S3). Aliquots of S3 were snap-frozen in liquid nitrogen and stored at -80 ºC until assay.

Western blotting for $CRFR_1$ receptor levels. The S3 membrane extracts were thawed on ice, diluted in 4X SDS-PAGE sample buffer and heated at 70 ºC for 10 minutes. Samples (1ug protein per well) were then separated using 7% NuPAGE Tris-acetate gels (Invitrogen, Carlsbad, CA) and transferred to 0.45-µm-think nitrocellulose membranes. The membranes were blocked with 5% nonfat dry milk in TBS-T (25 mM Tris-HCL pH 7.2, 150 mM NaCl and 0.05% Tween-20) overnight at 4 ºC. Blots were then incubated with a polyclonal primary antibody to $CRFR_1$ (1:200; Santa Cruz H-215) in 5% nonfat dry milk and

TBS-T for 2 hours at room temperature. The reaction was stopped with six consecutive 10-minute washes in TBS-T. A goat anti-rabbit IgG (H+L):HRP (1:5000; Pierce) was used for the secondary antibody incubation in 5% nonfat dry milk and TBS-T for one hour at room temperature. The reaction was stopped with six consecutive ten-minute washes in TBS-T. Membranes were then incubated in Supersignal West Pico Working Solution (Pierce) for five minutes and exposed to CL-Exposure film (Pierce). Film was developed in Kodak D-19 developer then washed and fixed in Kodak fixer. The developed film was scanned (Hewlett Packard Scan Jet 5P) and immunoreactivities quantified by measurements of optical densities using BioRad Quantity-One analysis software. Each protein sample was run in duplicate and the average optical density determined. B-actin was used as a loading control. The optical density measurements, obtained from internal standards, were linear over the range of 10 to 50 µg of whole brain homogenate. For experimental samples with optical densities in the linear range of the standard curve, the amount of immunoreactivity was defined by dividing the optical density of the sample by the optical density of one unit of immunoreactivity as determined by the standard curve. The antigen protein level in the sample was then determined by dividing the units of antigen present by the total µg of protein loaded on the SDS-PAGE gel.

2.3.4 Serotonin $5HT_{1A}$ Receptor Number and Receptor-Effector Coupling

Histological Sectioning. Adult offspring, 75 to 90 days of age, were sacrificed by decapitation. Whole brains were rapidly dissected, frozen in isopentane, chilled

in a dry ice/methanol bath and then stored in airtight containers at -80 ^{o}C until sectioning. Ten-µm-thick coronal sections were collected at atlas coordinates, interaural 2.10 mm, bregma -1.70 mm, according to mouse stereotaxic atlas of Franklin and Paxinos (1997; atlas Figure 45). The brain was then rotated ninety degrees to collect horizontal sections containing the ventral hippocampal formation, entorhinal cortex and inferior colliculus. The level of sectioning in each plane was verified by examination of Nissl-stained sections prior to the collection of sections for the binding assays. The sections were thaw-mounted onto precleaned Superfrost-Plus® microscope slides (VWR Scientific, West Chester, PA) and stored at -80 ^{o}C in airtight containers until assay.

[^{3}H]-DPAT Binding Assay. The binding of [^{3}H]-(+)-8-Hydroxy-2-dipropylaminotetralin hydrobromide ([^{3}H]-DPAT) to $5HT_{1A}$ receptors was conducted according the methods reported by Hensler et al., (1991). Sections were preincubated for 30 minutes in incubation buffer (150 mM Tris-HCl, pH 7.6 at 25 ^{o}C). Sections were then incubated with 2 nM [^{3}H]-DPAT (200 Ci/mmole, Amersham, Piscataway, NJ) for 60 minutes at 25 ^{o}C in the absence or presence of 20 µM unlabelled DPAT. After incubation, sections were rinsed twice for five minutes each in ice-cold incubation buffer, quickly dipped in ice-cold distilled water, dried under a stream of cool air, vacuum desiccated overnight and then loaded into x-ray cassettes along with tritium standards. A 20.3 x 25.4 cm sheet of Biomax MR Film (Kodak, Rochester, NY) was apposed to the standards and sections and exposed for eight weeks. After exposure, the film was developed in Kodak D-19 developer (1:1) at 18 ^{o}C for 5 minutes, fixed for 5 minutes in Kodak

Fixer, rinsed and dried. For ease of handling and protection of the emulsion, the film was cut into 2 x 5 cm strips and mounted onto clean microscope slides with Permount.

DPAT-Stimulated [^{35}S]-GTPγS Binding Assay. Agonist-stimulated [^{35}S]-guanosine 5'-(γ-thio) triphosphate ([^{35}S]-GTPγS) binding was conducted using the methods originally reported by Sim et al. (1995). Tissue sections were preincubated for 10 minutes in incubation buffer (50 mM Tris-HCl, 100 mM NaCl, 5 mM $MgCl_2$, pH 7.4 at 25 ^{o}C) containing 1 mM DL-dithiothreitol, 0.2 mM EGTA and 2 mM guanosine 5'-diphosphate. Sections were then incubated with 100 pM [^{35}S]-GTPγS (specific activity = 1250 Ci/mmole; Perkin Elmer Life Sciences, Boston, MA) for 90 minutes in the absence or presence of 10 μM unlabeled GTPγS). DPAT-stimulated [^{35}S]-GTPγS binding was assessed by incubating sections in the presence of 2 μM or 20 μM unlabelled DPAT. After incubation, sections were rinsed twice for 15 seconds each in fresh incubation buffer at 4 ºC, dipped for one second in 4 ºC distilled water, dried under a stream of cool air and then vacuum desiccated overnight. Sections were then loaded into x-ray cassettes along with ^{14}carbon standards and a sheet of Biomax MR Film was apposed to the standards and sections and exposed for four days. Film development, fixation and subsequent handling were the same as described for the [^{3}H]-DPAT assay above.

2.3.5 Quantitative microdensitometry

Microdensitometry was performed using Media Cybernetics Image Pro Plus® (Silver Spring, MD) on an Olympus BH-2 microscope at a total image magnification of 3.125X. In each assay, an optical density standard curve, expressed in picoCuries/10^5 μm^2, was established based on the autoradiograms of the standards. In the coronal sections, optical density measurements of ligand binding were made in two brain regions, the dorsal hippocampal CA1 and in the parietal cortex (Layers I-III) immediately above the dorsal hippocampal region. In horizontal sections, binding measurements were made in the ventral hippocampal CA1, the medial entorhinal cortex (Layers I-III) and in the inferior colliculus.

In the [^{3}H]-DPAT binding assay, total [^{3}H]-DPAT binding in each brain region of interest was measured in quadruplicate sections incubated in the absence of excess unlabelled DPAT. Non-specific [^{3}H]-DPAT binding was measured in duplicate sections incubated in the presence of 20 µM unlabelled DPAT. Specific [^{3}H]-DPAT binding in each brain region of interest was defined as the difference between total and non-specific [^{3}H]-DPAT binding.

In the [^{35}S]-GTPγS binding assay, total [^{35}S]-GTPγS binding in each brain region of interest was measured in quadruplicate sections incubated in the absence of excess unlabelled GTPγS. Non-specific [^{35}S]-GTPγS binding was measured in duplicate sections incubated in the presence of 10 µM unlabelled GTPγS. Basal [^{35}S]-GTPγS binding in each brain region of interest was defined as the difference

between total and non-specific [^{35}S]-GTPγS binding. DPAT-stimulated [^{35}S]-GTPγS binding was measured in triplicate sections incubated in the presence of one of two concentrations of unlabelled DPAT. DPAT-stimulated [^{35}S]-GTPγS binding was defined as the difference between [^{35}S]-GTPγS binding in the presence of DPAT and basal [^{35}S]-GTPγS binding.

In a preliminary [^{35}S]-GTPγS binding study, brain sections were incubated with twelve different concentrations of DPAT ranging from 50 nM to 30 μM. DPAT increased [^{35}S]-GTPγS binding in a concentration-dependent manner between 200 nM and 20 μM DPAT (data not shown). At 20 μM DPAT, [^{35}S]-GTPγS binding in the dorsal hippocampal formation was seven-fold higher than basal [^{35}S]-GTPγS binding. The apparent EC_{50} for DPAT-stimulated [^{35}S]-GTPγS binding was 2 μM. Based on this study, 2 μM and 20 μM DPAT were selected as EC_{50} and EC_{100} concentrations for the study of $5HT_{1A}$ agonist-stimulated [^{35}S]-GTPγS binding in perinatal arsenic-exposed offspring.

2.3.6 Learned Helplessness Task

The learned helplessness task was performed in a Coulbourn™ Habitest© shuttle box apparatus using training and testing procedures modified from a method described by Caldarone et al. (2000). Each experimental group consisted of five animals (one mouse per litter per group) in each of eight experimental groups: Two perinatal treatments (control or arsenic) X two genders (male or female) X two training groups (shock or no shock). Mice in the learned helplessness (LH-trained) groups received 120 uncontrollable and unpredictable foot shocks (0.5

mA, 5 second duration) over the course of one hour. The probability for delivery of a foot shock was 50% for every 15 second interval. The mouse was removed from the shuttle box 30 seconds after the delivery of the 120th footshock. The non-shocked (NS-trained) groups were placed into the shuttle box apparatus for one hour during which no foot shocks were delivered. Two mice were trained at a time, one LH-trained and one NS-trained. Mice were returned to their home cages after the one-hour training session. Twenty-four hours later, escape latencies were measured using an active avoidance procedure. Animals were placed into the shuttle box and given 30 trials with an inter-trial interval of 30 seconds. Latency for the mouse to escape through the door was measured as the time from the door rising to the time it closed. If no escape was made 24 seconds after the start of the trial, the shock terminated and the door closed.

2.3.7 Forced Swim Task

Eight animals from each group (arsenic-exposed and control) were tested. Mice were placed in a 30 cm diameter, 46 cm tall cylinder of water (22-25°C, depth 26 cm) and forced to swim for 3 minutes as described previously (Sunal et al., 1994; Porsolt et al, 1977). The combination of floating, twitching and paddling behaviors represented immobility indicative of depressive-like behavior. Time spent in immobile behaviors was scored during the three minute period. The sum of thrashing, climbing and swimming behaviors were defined as escape-directed behaviors and were not actively scored.

2.3.8 Statistical Analysis

All data were analyzed either by t-test (Table 1 and Figures 2.1, 2.2, and 2.7) or one or two-way ANOVA (Figures 2.5 and 2.6) using SPSS Version 14 (Chicago, IL). When an ANOVA was performed, a t-test was performed as the post-hoc test.

2.4 RESULTS

2.4.1 Perinatal Arsenic Exposure Paradigm

The perinatal arsenic exposure paradigm produced no significant differences in litter size or offspring body weight at seven days of age (Table 1). Furthermore offspring body weight, within the same gender, were not different between the treatment groups ($t(15) = 1.92$; ns).

2.4.2 Plasma Corticosterone Levels

Plasma corticosterone levels were significantly elevated compared to control offspring. Figure 2.1 represents the results from the RIA analysis, showing that corticosterone levels in arsenic offspring were higher than controls by approximately a 2-fold increase ($t(9) = 2.491$; $p = 0.03$).

2.4.3 $CRFR_1$ Receptor Protein

Figure 2.2A illustrates a representative Western blot analysis of hippocampal $CRFR_1$ receptor protein in whole hippocampal homogenates from arsenic-exposed and control offspring. As shown in Figure 2.2A, the optical densities of the bands from the arsenic-exposed mice were lower than the controls. A summary of the Western blot data is provided in Figure 2.2B. Perinatal arsenic

exposure significantly reduced the quantity of $CRFR_1$ receptor protein by approximately 32% in hippocampal formation compared to the control water group (33 +/- 1.8 for control vs. 22 +/- 1.8 for arsenic samples). Asterisk denotes data significantly different compared to the control water group ($t(22) = 3.64$, $p<0.001$).

2.4.4 Specific [^{3}H]-DPAT Binding and DPAT-stimulated [^{35}S]-GTPγS Binding

Figures 2.3 and 2.4 illustrate representative autoradiograms depicting the distribution of total and non-specific [^{3}H]-DPAT binding to $5HT_{1A}$ receptors (panels A & B) and 2 μM DPAT-stimulated and basal [^{35}S]-GTPγS binding (panels C & D) in coronal and horizontal sections from the brain of an untreated control mouse. Total [^{3}H]-DPAT binding site densities were highest in the hippocampal formation and cortical brain regions (Figures 2.3A & 2.4A). The distribution of DPAT-stimulated [^{35}S]-GTPγS binding (Figures 2.3C & 2.4C) was similar to the distribution of [^{3}H]-DPAT binding (Figures 2.3A & 2.4A).

The effects of perinatal arsenic exposure on specific [^{3}H]-DPAT binding, basal and DPAT-stimulated [^{35}S]-GTPγS binding in adult offspring are summarized in Figure 2.5. Specific [^{3}H]-DPAT binding was significantly increased by approximately 77% in the dorsal hippocampal formation of arsenic-exposed mice (0.57 +/-0.06) compared to the control group (0.31+/- 0.05) (Figure 2.5A). In contrast, specific [^{3}H]-DPAT binding was not different in the ventral hippocampal formation, the parietal cortex, entorhinal cortex or the inferior colliculus. Basal [^{35}S]-GTPγS binding was not different in any of the brain regions measured

(Figure 2.5B). In the presence of a half-maximally effective (EC_{50}) concentration of DPAT (2 μM) dorsal hippocampal DPAT-stimulated [^{35}S]-GTPγS binding was 64% greater in arsenic-exposed offspring (0.35 +/-0.07) than control (0.23 +/-0.03) (Figure 2.5C). Asterisks denote data significantly increased compared to the untreated control group (multivariate, one-way ANOVA $F(1,12)=9.8$ $p<0.001$ in Figure 2.5A; $F(1,10)=6.6$ $p<0.03$ in Figure 2.5C). As was the case for specific [^{3}H]-DPAT binding (Figure 2.5A), 2 μM DPAT-stimulated [^{35}S]-GTPγS binding was not different between the two groups in the other four brain regions examined (Figure 2.5C). In the presence of a maximally effective concentration of DPAT (20 μM), DPAT-stimulated [^{35}S]-GTPγS binding was not different in any of the five brain regions examined (Figure 2.5D).

2.4.5 Learned Helplessness Task

Perinatal arsenic exposure produced a significant increase in escape latencies in both male and female adult mice (Figure 2.6). There was a significant effect of learned helplessness training ($F(1,40) = 783$, $p<0.0001$), an effect of perinatal arsenic ($F(1,40) = 194$ $p<0.0001$) and an interaction between perinatal treatment and learned helplessness training ($F(1,40)= 63.6$ $p<0.001$). No differences were observed between the groups in a pain threshold test measuring vocal response to footshock intensity (data not shown).

2.4.6 Forced Swim Task

Perinatal arsenic exposure produced a significant increase in immobility in exposed mice compared to controls (Figure 2.7). There was a significant effect of arsenic on immobility $t(14)=8.14$, $p<0.001$.

2.5 DISCUSSION

The present findings demonstrate that perinatal exposure to relatively low levels (50 ppb) of arsenic in drinking water can have long-lasting biochemical and behavioral effects on adult offspring. Perhaps the most striking effect observed in these studies was the impact of perinatal arsenic exposure on plasma corticosterone (Figure 2.1) which was assessed under baseline, non-stressed circumstances (Figure 2.1). Qualitatively similar elevations in corticosterone levels have been noted also in perinatal lead-exposed rats (Cory-Slechta et al, 2008). Increased serum corticosterone levels suggest that the HPA axis is overactive in these arsenic-exposed offspring.

While it is not possible to discern from these experiments whether elevated plasma corticosterone is a primary teratogenic effect of perinatal arsenic exposure or a secondary, perhaps compensatory consequence of effects on systems that regulate the HPA axis, it is noteworthy that the other biochemical alterations (Figures 2.1, 2.2, 2.5) and behavioral effects (Figures 2.6, 2.7) observed in these studies are consistent with sustained elevations in HPA axis activity. For example, perinatal arsenic exposure decreased hippocampal $CRFR_1$ levels compared to controls (Figure 2.2). While hippocampal CRF levels

were not measured in this study, it is reasonable to speculate that if CRF levels are elevated to a similar degree as plasma corticosterone (Figure 2.1), the decrease in hippocampal $CRFR_1$ in the perinatal arsenic mice may represent a compensatory down-regulation in response to heightened CRF activation. Several studies have reported increased CRF in response to intense or prolonged stress. Four hours of restraint stress in mice resulted in a significant increase of CRF mRNA expression within the paraventricular nucleus (Nomura et al., 2003) and similar increases in CRF were seen using in an earlier study using crowded rearing conditions as a stressor (Albeck et al., 1997) in rats.

Perinatal arsenic exposure elevated specific [^{3}H]-DPAT binding to $5HT_{1A}$ receptors (Figure 2.5A) in dorsal hippocampal formation. This study utilized a [^{3}H]-DPAT concentration near the half-maximally saturating (Kd) concentration (Hensler et al., 1991). This effect of perinatal arsenic exposure on specific [^{3}H]-DPAT binding was accompanied by increased DPAT-stimulated [^{35}S]-GTPγS binding at a half-maximally effective concentration of agonist (Figure 2.5C), but not at a saturating concentration of the agonist (Figure 2.5D). Taken together, these data suggest that perinatal arsenic exposure increases the sensitivity of dorsal hippocampal $5HT_{1A}$ receptors to serotonin without altering the total number of receptors present. Further, these results suggest that basal [^{35}S]-GTPγS binding (Figure 2.5B) and $5HT_{1A}$ receptor-effector coupling were not affected in perinatal arsenic exposed offspring. More detailed saturation of specific [^{3}H]-DPAT binding and DPAT-stimulated [^{35}S]-GTPγS binding would be required to substantiate this interpretation. However, the inability to collect an

adequate number of histological sections in specific regions of mouse brain limits more extensive saturation of binding studies.

It is noteworthy that elevations in $5HT_{1A}$ receptor sensitivity were not observed in other brain regions (Figure 2.5A and 2.5C) and were observed only in the dorsal hippocampal formation. This suggests that the hippocampal formation is at least one of the brain regions most susceptible to the consequences of perinatal arsenic exposure. Further, while differences between the dorsal and ventral hippocampal formation have been identified (see Moser and Moser, 1998), the basis for differential dorsal-ventral hippocampal sensitivity in arsenic-exposed mice is not known. However, qualitatively similar outcomes have been observed in the study of hippocampal glutamate receptors in prenatal ethanol-exposed offspring (Farr et al., 1988; Savage et al., 1991). The mechanistic basis for elevated $5HT_{1A}$ receptor sensitivity in perinatal arsenic-exposed mice is not known. Chronic elevated HPA axis activity that has been associated with diminished firing of raphe serotonergic neurons and a reduction in serotonin turnover and release (Davis et al., 1995; Myint et al., 2007), including a decrease in $5HT_{1A}$ mRNA following 20 days of chronic unpredicted footshock stress (Xu et al., 2007) and decrease in $5HT_{1A}$ activity in the dorsal raphe, following chronic social defeat stress (Cornelisse et al., 2007). Thus, in our study a diminished serotonergic activity following the chronic elevation of corticosterone produced by perinatal arsenic may have resulted in a compensatory up-regulation of $5HT_{1A}$ receptor sensitivity in the hippocampus.

The effects of perinatal arsenic exposure on biochemical measures associated with depression were accompanied by alterations in two measures of depressive-like behaviors, learned helplessness and immobility during forced swim. Both male and female perinatal arsenic-exposed mice displayed greater learned helplessness behavior following inescapable shock exposure (Figure 2.6). No gender effects were noted between groups. Forced swim task data (Figure 2.7) revealed that arsenic-exposed animals show more immobility, which is indicative of depressive-like behavior. These data suggest an increased susceptibility to depression and are consistent with human studies suggesting that elevated cortisol in response to chronic stress is associated with increased depressive symptoms (see Southwick et al., 2005 for review). While perinatal arsenic may not be considered a typical stressor, our data suggest that the prolonged elevation of corticosterone produced by perinatal arsenic may have produced physiological state mimicking chronic stress.

While there are no studies directly demonstrating arsenic induced $5HT_{1A}$ receptor changes, arsenic does interfere with glucocorticoid signaling pathways (Stancato et al., 1993; Kaltreider et al., 2001; Bodwell et al., 2004) which, in turn, affect serotonin neurotransmission, acute stress increasing activity and chronic stress attenuating activity (Meijer and DeKloet, 1998). Arsenic directly interferes with the glucocorticoid receptor complex and inhibits steroid binding to glucocorticoid receptors (Simons et al., 1990). Our model suggests that this would prevent the normal operation of the negative feedback mechanism, increasing plasma corticosterone levels, a phenomenon we observed. We also observed lower

levels of $CRFR_1$ in the perinatal arsenic-exposed animals, perhaps as a compensatory response to elevated CRF. Lower activity by $CRFR_1$ would attenuate serotonin release in the hippocampus (Oshima et al., 2003) resulting in a compensatory increase in $5HT_{1A}$ receptor affinity.

Previous research has outlined a role for 5-HT_{1A} receptor in antidepressant response. Patients who respond to antidepressant treatment require a desensitization of 5-HT_{1A} receptor (Rausch et al., 2006). Studies also suggest that a polymorphism in the 5-HT_{1A} gene resulting in non-repression of the receptor has been associated with poor response to antidepressant therapy in unipolar and bipolar subjects (Lemonde et al., 2004; Serretti et al., 2004). Further evidence suggests that there is an increased expression of the 5-HT_{1A} receptor and mRNA in brains of suicide victims (Escriba et al., 2004). Recently, it has been suggested that an alteration of the hippocampal function may be involved in the etiology of depression (Warner-Schmidt and Duman, 2006) and that changes in the hippocampal serotonergic system are, in part, responsible for its pathogenesis (Iritani et al., 2006). All of the previous research on serotonin ties into our depressive-like behavioral analysis of perinatal arsenic-exposed mice.

Finally, a negative impact of perinatal arsenic on learning has previously been reported (Rodriguez et al. 2002). In a study using rats, they showed perinatal exposure to arsenate (36.70 mg/L in drinking water) from gestation day 15 (GD 15) resulted in increased spontaneous locomotor activity and an increased number of errors in a delayed alternation task in comparison to the control group

(Rodriguez et al., 2002). Although we are studying a mouse model, it is important to note that the whole body retention of inorganic arsenic in rats has been shown to be 20 times higher than that in similarly exposed mice due to retention of arsenic in erythrocytes, (Vahter, 1981) therefore the arsenic effects observed in our mouse model are at a dose that is much lower than those used in the Rodriguez study. Also, the Rodriguez study dosing schedule occurs at GD15 suggesting that effects we have seen at lower doses may be taking place during the embryonic stages when the brain is forming. One alternative interpretation of our data is that failure to escape in the learned helplessness task may be due to arsenic-induced deficits in learning and memory, and not an indication of increased depressive-like behavior. While this is possible, the arsenic animals failed to demonstrate escape-directed behaviors in the forced swim task which is not dependent upon learning or memory to perform. Further, there were no differences in non-shock escape latencies in the non-shock control groups between the two perinatal exposure conditions in either male or female mice (data not shown). These results suggest that the perinatal arsenic-exposure did not affect the ability to escape in the absence of learned helplessness training. Thus, while arsenic has been demonstrated to produce deficits in learning performance, the deficits in learned helplessness seen in our study were not the result of an inability to perform the task or to understand association between escape and the termination of the foot shock since the perinatal arsenic-exposed mice were as successful as control mice in escaping the shock in the non-shocked control condition.

These results demonstrate that there are neurochemical and behavioral consequences of heavy metal exposure during brain development. The perinatal period encompasses the key developmental periods in most species. Synaptic organization, which sets up functional development of neurotransmitter and neuromodulatory systems, is occurring during this time. This period is critical because it influences how the animals will respond to their external environment throughout their lives. Administration of toxins during this critical period can have lifelong consequences due to their deleterious effects during major brain maturation periods. Future research should aim to address the consequences of arsenic in the developing CNS.

2.6 ACKNOWLEDGEMENTS

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2.7 TABLES

Table 1. Effects of perinatal exposure to 50 parts per billion arsenic in drinking water on litter size and offspring body weight on postnatal day 7.

Experimental Group	# of Litters	Litter Size[a]	Body Weight[b]
Control	8	6.0 ± 0.78	4.66 ± 0.39
Sodium Arsenate	9	4.6 ± 0.86	4.29 ± 0.46

a – Mean + S.E.M. number of pups per litter

b – Mean + S.E.M. pup body weight in grams

2.8 FIGURES

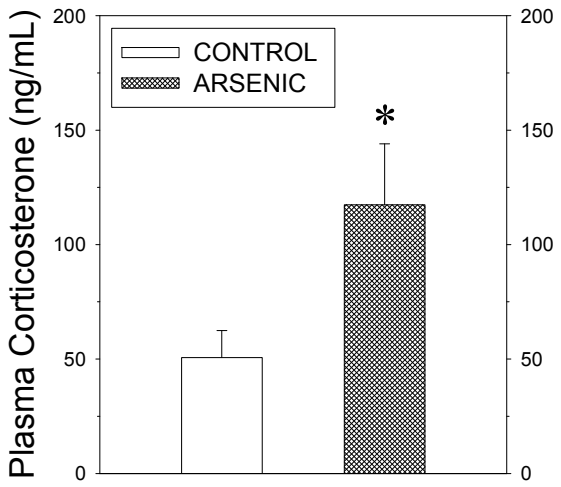

Figure 2.1 The effect of perinatal arsenic exposure on plasma corticosterone levels.

Data bars represent the mean ± SEM nanograms corticosterone per mL plasma from five or six mice per perinatal treatment group. Asterisk denotes data significantly elevated compared to the control water group (two-tailed t-test, $t(9) = 2.491$, $p = 0.03$).

A.

Cont Cont Con Ars Ars Ars 10 20 30 40 50

Standard Curve

B.

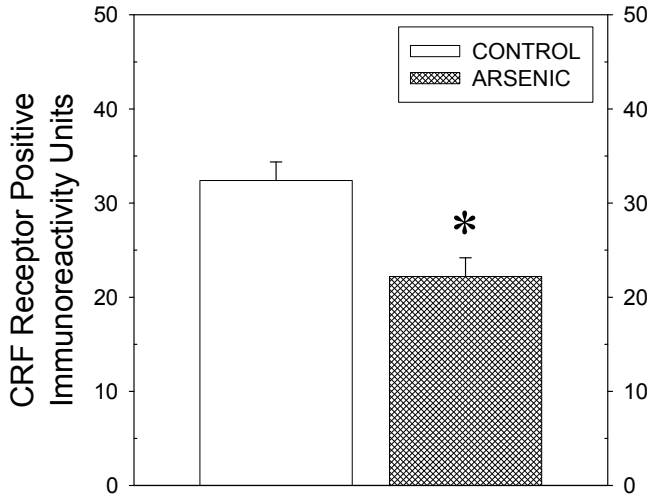

Figure 2.2 The effect of perinatal arsenic exposure on hippocampal CRF receptor protein levels in adult offspring.

A: Representative immunoblots of triplicate control and arsenic-exposed samples and a standard curve. B: Data expressed as immunoreactivity units calculated from a protein standard curve using control hippocampal tissue. Data bars represent the mean ± SEM of 12 mice in each perinatal treatment group. Beta-actin was used as a loading control. Asterisk denotes data significantly different compared to the control water group ($t(22) = 3.64$, $p<0.001$).

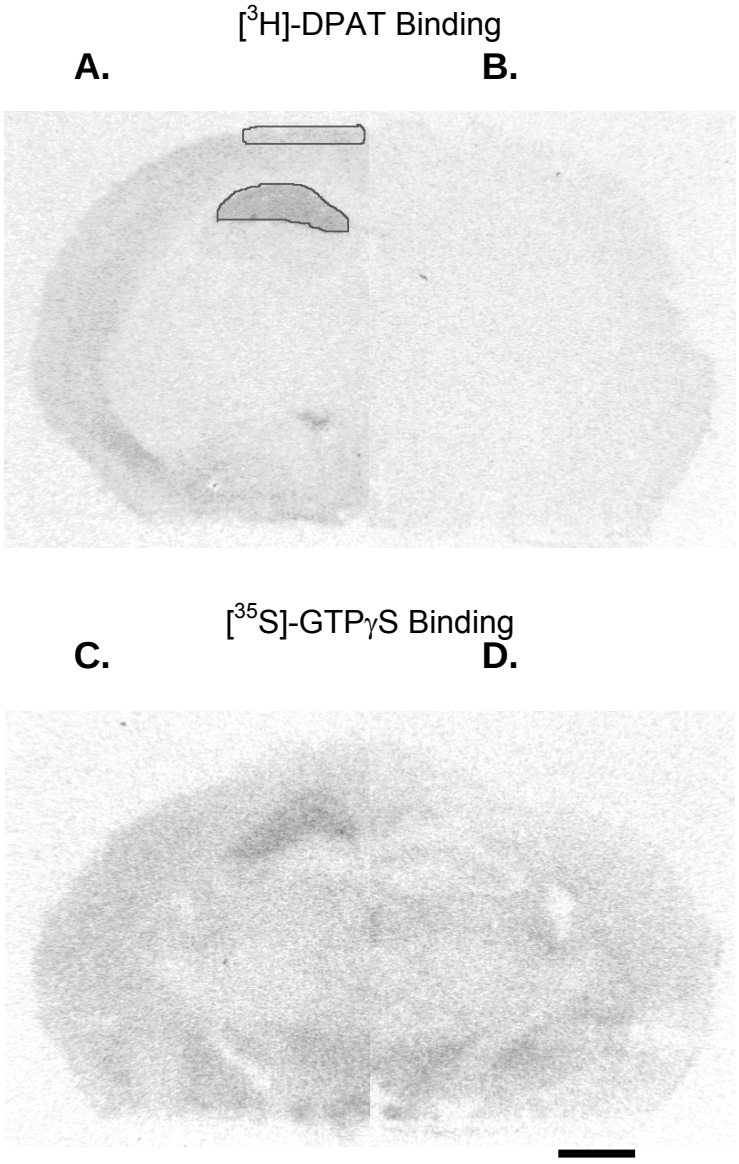

Figure 2.3 Autoradiograms of [^{3}H]-DPAT binding and DPAT-stimulated [^{35}S]-GTPγS binding in coronal sections of brain from an untreated control mouse.

A: Total [^{3}H]-DPAT binding. B: Non-specific [^{3}H]-DPAT binding (binding in the presence of 20 μM unlabelled DPAT). C: [^{35}S]-GTPγS binding in the presence of 2 μM DPAT. D: Basal [^{35}S]-GTPγS binding (binding in the absence of added DPAT). Binding measurements were made in the dorsal hippocampal CA1 *stratum radiatum* and the parietal cortex (Layers 1-3) immediately dorsal to the

hippocampal formation. The horizontal bar at the bottom of panel D denotes a distance of 1 mm.

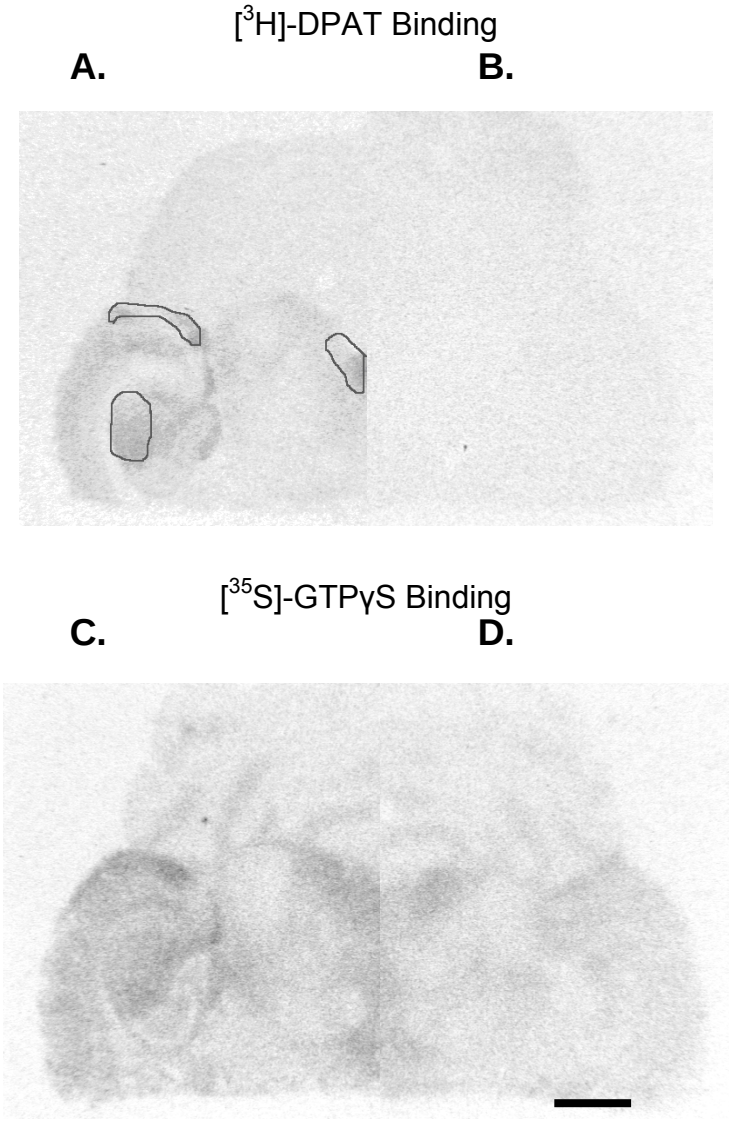

Figure 2.4 Autoradiograms of [^{3}H]-DPAT binding and DPAT-stimulated [^{35}S]-GTPγS binding in horizontal sections of brain from an untreated control mouse.

A: Total [^{3}H]-DPAT binding. B: Non-specific [^{3}H]-DPAT binding (binding in the presence of 20 μM unlabelled DPAT). C: [^{35}S]-GTPγS binding in the presence of 2 μM DPAT. D: Basal [^{35}S]-GTPγS binding (in the absence of added DPAT). Binding measurements were made in the ventral hippocampal CA1 *stratum*

radiatum, medial entorhinal cortex, (Layers 1-3) and the inferior colliculus. The horizontal bar at the bottom of panel D denotes a distance of 1 mm.

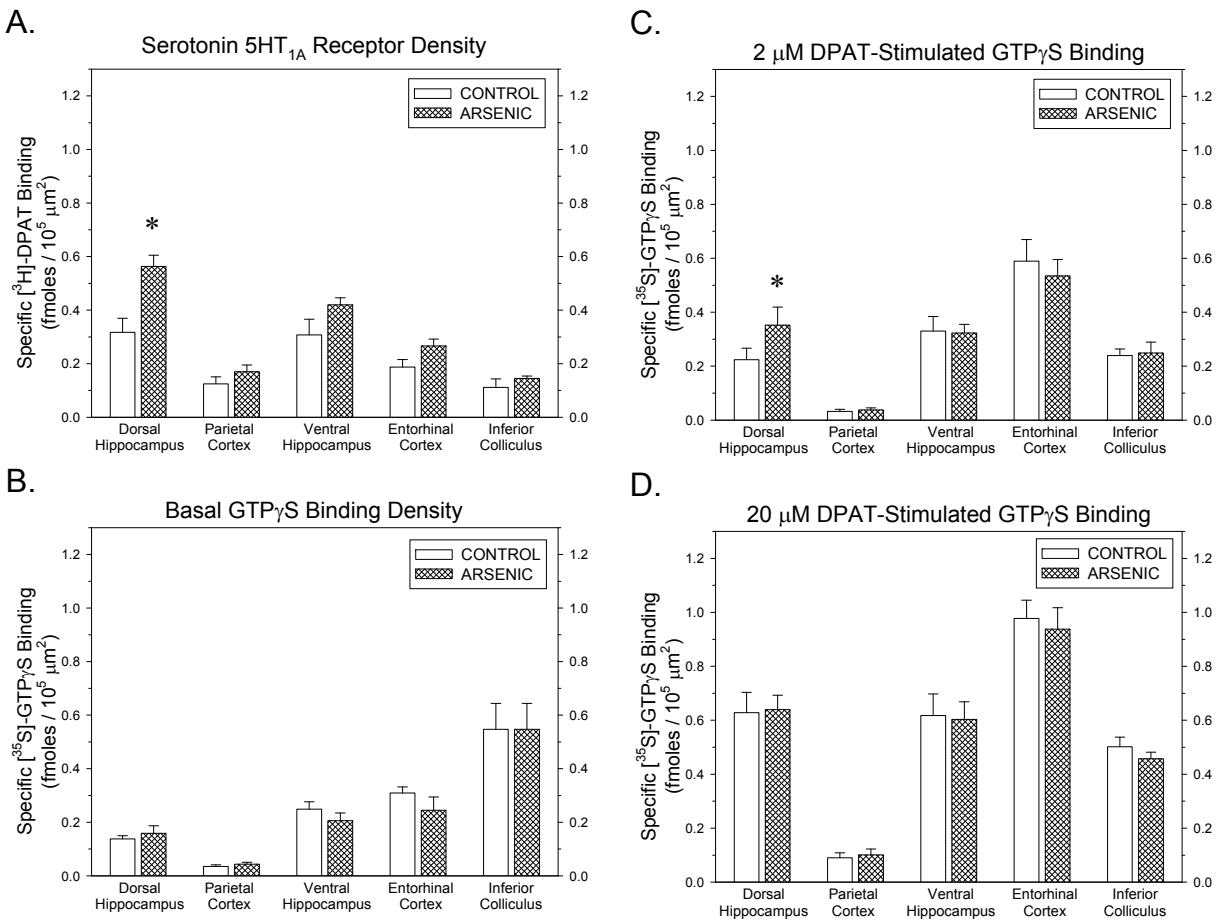

Figure 2.5 The effects of perinatal arsenic exposure on $5HT_{1A}$ receptor density and $5HT_{1A}$ receptor-effector coupling in five regions of adult mouse brain.

A: Specific [^{3}H]-DPAT binding. B: Basal [^{35}S]- GTPγS binding. C: 2 µM DPAT-stimulated [^{35}S]- GTPγS binding. D: 20 µM DPAT-stimulated [^{35}S]-GTPγS binding. Data bars represent the mean ± SEM of 5 to 11 mice in each group. Asterisks denote data significantly increased compared to the untreated control group (multivariate, one-way ANOVA $F(1,12)=9.8$ $p<0.001$ in Figure 5A; $F(1,10)=6.6$ $p<0.03$ in Figure 5C).

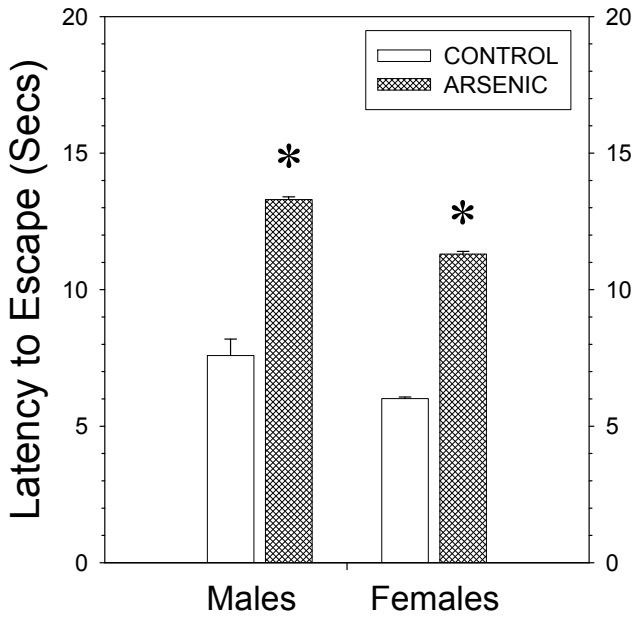

Figure 2.6 The effect of perinatal arsenic exposure on learned helplessness in adult male and female offspring.

Data bars represent the mean ± the SEM of five mice in each group. Asterisks denote significant differences between control and arsenic-exposed offspring. A two-way ANOVA was performed with a t-test as a post-hoc. There was a significant effect of learned helplessness training ($F(1,40) = 783$, $p<0.0001$), an effect of perinatal arsenic $F(1,40) = 194$ $p<0.0001$ and an interaction between perinatal treatment and learned helplessness training $F(1,40)= 63.6$ $p<0.001$.

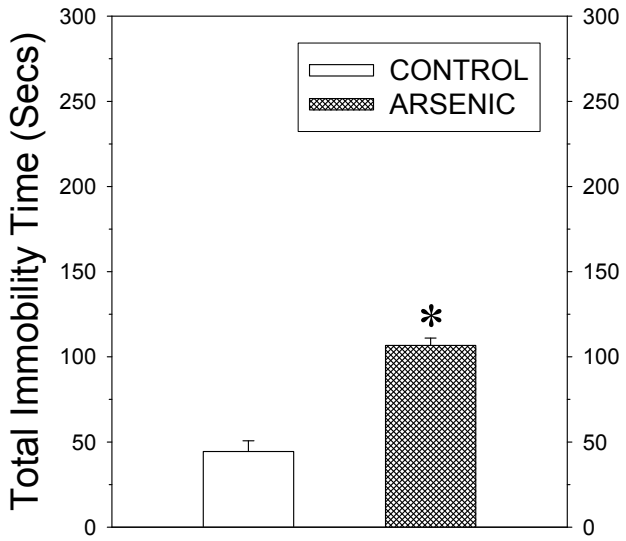

Figure 2.7 The effect of perinatal arsenic exposure on forced swim task in adult offspring.

Data bars represent the mean ± the SEM of eight mice in each group. Asterisks denote a significant elevation in time spent in immobile behaviors compared to the control water group (two-tailed t-test, $t(14) = 8.11$, $p < 0.001$).

Chapter 3. LEARNING DEFICITS IN C57BL/6 MICE FOLLOWING PERINATAL ARSENIC EXPOSURE: CONSEQUENCE OF LOWER CORTICOSTERONE RECEPTOR LEVELS?

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3.1 ABSTRACT

Most studies on arsenic as a drinking water contaminant have focused on its carcinogenic potential but a few suggest that arsenic can adversely affect cognitive development. One parameter of the hypothalamic-pituitary-adrenal axis, the corticosterone receptor (CR) has been shown to be altered by arsenic. These receptors are found throughout the central nervous system, particularly in the hippocampus, an area of the brain of central importance for learning and memory. We examined the impact of perinatal exposure to 50 parts per billion (ppb) sodium arsenate on CRs and learning and memory behavior in the C57BL6/J mouse. Measurements of CRs revealed that arsenic-exposed offspring have significantly lower levels of both glucocorticoid and mineralocorticoid receptors in the nuclear subcellular fractions than controls. Exposed offspring showed longer latency to approach a novel object than controls in an object recognition task. In the 8-Way Radial Arm Maze, arsenic offspring had a significant increase in the number of entry errors on the task compared to controls. Results suggest that moderate exposures to perinatal arsenic can significantly reduce CR levels in the hippocampus and can have adverse effects on learning and memory behavior. While these studies in themselves do not provide a direct link between the learning deficits and the alterations in the HPA axis, in context with previous work on stress and learning they suggest that damage to the HPA axis by perinatal arsenic could lead to learning deficits. Overall, this data suggests that moderate levels of perinatal arsenic can have a lasting impact on offspring.

KEY WORDS

Arsenic, Hippocampus, Glucocorticoid receptor, Mineralocorticoid receptor, Radial Arm Maze, Novel Object

3.2 INTRODUCTION

Arsenic is one of the most toxic naturally occurring contaminants found in the environment. A common source of human exposure to inorganic arsenic is through drinking water contamination. Exposure has been associated with skin, lung and bladder cancers, vascular diseases, hypertension, genotoxicity, cellular disruption and diabetes (ATSDR Arsenic, 2007). While most studies on arsenic have focused on its carcinogenic potential, studies in rodents pioneered by Rodriguez and colleagues suggest that arsenic can adversely affect cognitive development (Rodriguez et al., 2001, 2002), however, these effects on nervous system development and function have received less attention (Rodriguez et al., 2003). Literature describing the effects of arsenic in drinking water on human cognitive development is sparse, and has been best characterized through epidemiological studies, often based on research from countries with significantly higher arsenic concentrations than the United States (Calderon et al., 2001; von Ehrenstein et al., 2007; Wasserman et al., 2004). The majority of these studies have focused on children; and deficits in learning and memory, particularly in hippocampal-dependent tasks, following acute and chronic arsenic exposure have been reported. However, the cellular and molecular mechanisms involved in this process are still poorly understood.

Events that disrupt maturation and development of the hypothalamic-pituitary-adrenal (HPA) stress axis have been shown to permanently alter CR expression in the adult (de Kloet et al., 1998; Matthews et al., 2000; Welberg and Seckl, 2001). CRs are found in heterooligomeric complexes in the cytoplasm and upon binding corticosterone (CORT) are translocated to the nucleus where they influence diverse gene transcription. The glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR) are two forms of the CR which are activated by binding of CORT. Arsenic has been shown to perturb components of the HPA stress axis, like the GR (Bodwell et al., 2004) and alter gene expression of inducible genes (Hamilton et al., 1998). Perturbation of the HPA stress axis has been implicated in both cognitive damage and the promotion of carcinogenesis. Thus far, no studies of the effects of perinatal arsenic on GR and MR have been reported.

Neurons in the hippocampus contain both GRs and MRs. GRs are involved in consolidation of learned information (de Kloet et al., 1999) and MRs involved in interpretation of novel information (Berger et al., 2006), memory retrieval (Conrad et al., 1997), and visuospatial learning (Yau et al., 1999). Further, spatial learning and memory deficits have been shown in GR and MR knockout mice (Berger et al., 2006; Steckler et al., 1998) and sustained activation of GR by chronically elevated CORT impairs hippocampal function and memory processes (McEwen and Sapolsky, 1995). The connection between the hippocampus and learning and memory has been well established for decades. De Kloet and colleagues (1998) showed that both the presence of CORT and a functional GR are critical

to normal hippocampal function. Stress, through the actions of CORT and CRs, can either facilitate or impair learning and memory processes (Joels, 2006). Studies have shown that, at intermediate levels, CORT enhances learning and memory but low or high levels adversely affect cognition (Lupien and McEwen, 1997). We have previously shown that perinatal arsenic-exposed offspring have elevated levels of circulating CORT into adolescence and young adulthood (Martinez et al., 2008). Evidence from prenatal lead exposure studies suggest that excess fetal CORT levels can program pathologies in adult life (Cory-Slecta, 2004; Seckl and Meaney, 2004). High levels of circulating CORT have been frequently shown to be associated with a series of clinical diseases including neurodegenerative diseases, major depression and schizophrenia as well as aging (Alderson and Novack, 2002; Belanoff et al., 2001; Lupien et al., 1999).

Given our previous report of high basal CORT levels in perinatal arsenic-exposed offspring (Martinez *et al.,* 2008), we examined hippocampal GR and MR translocation to determine if this parameter was affected by the arsenic-induced changes in CORT level. We also examined spatial and non-spatial learning and memory behavior in our perinatal mouse model to determine if deficits were attributable to the role of glucocorticoids.

3.3 METHODS

Note: Arsenic is classified as a probable human carcinogen (ATSDR, 2007). All arsenicals were handled as potentially highly toxic compounds.

3.3.1 Perinatal Arsenic Exposure Paradigm

The arsenic exposure paradigm and behavioral tasks employed in these studies were approved by the UNM Health Sciences Center Institutional Animal Care and Use Committee. All mice were bred and maintained on a reversed 12-h light/dark cycle (lights on from 17:00 to 09:00h) with food and water *ad libitum* in a temperature controlled (22ºC) room in the Animal Resource Facility. All dosing suspensions were prepared approximately weekly. Arsenic water (sodium arsenate, Sigma, St. Louis, MO) was prepared using standard tap water. Control mice were administered untreated tap water. Tap water at UNM contains approximately 5 ppb arsenic. Female C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) were assigned to either a control or 55 ppb arsenic water treatment group. After a 2-week acclimation period on the treated waters, male breeder mice were introduced into each female's cage. Three days later, the males were removed from the cages and nesting material was placed in the female's cage. Mouse dam water consumption was monitored throughout pregnancy and the dams continued to drink the treated waters until their offspring were weaned. Offspring were weaned at 23 days of age and maintained in same-sex, litter-mate housed cages with *ad libitum* access to untreated tap water and mouse chow (7004 Harlan Teklad S-2335, Denver, CO) until they were used in experimental procedures at 35-40 days of age. Mice (two per litter; 5-10 litters total per treatment group) were assigned to either one of the biochemical assay procedures or the behavioral testing methods. Offspring used in behavioral

studies were not used in biochemical studies. A sample size of five represents the total number of litters and not individual animals used.

3.3.2 Drinking Water Analysis and Data

Arsenic and control water bottles were monitored and recorded every other day for water consumption. Bottles from individual cages were weighed to the nearest tenth of a gram. Water samples (10mL) from each dosing suspension were sent in their liquid forms to the laboratory of Dr. Abdul-Mehdi Ali (UNM Department of Earth and Planetary Sciences) for determination of arsenic concentration according to US EPA method 200.8.

3.3.3 Offspring whole brain arsenic concentrations

To evaluate whole brain concentrations of arsenic, samples were analyzed using Inductively Coupled Plasma-Mass Spectrometry (ICP-MS, Perkin Elmer Elan DRC II, Waltham, MA) by Dr. Ali. The internal standard was Indium (In) at mass 115. The analysis was performed comparable to method US EPA 200.8. Exactly 10 mL of the standards, samples, and QC samples were spiked with 1 mL of 100 mg/L Indium (In) as internal standard to correct for drifts in the signal that may be caused by sample matrix, viscosity, and/or peristaltic pump (sample) pulsing. The system was calibrated using NIST traceable calibration standards (stock solution) and QC solutions (stock solution). The system is sensitive down to the parts per trillion level. Three mg brain samples were taken from one cerebellar lobe from arsenic-exposed and control mice. Samples were digested, using 2 mL nitric acid, at 90oC. After digestion was completed, digests were brought up to 10 mL

final volume and transferred into Inductively Coupled Plasma (ICP) plastic tubes. Results were then calculated using the starting weight and the final volume after digestion. Results were expressed as ug/g and then converted to ppb based on the standard curve. Samples were run in duplicate.

3.3.4 Western Immunoblotting

3.3.5 Tissue Preparation

Adolescent offspring, 35-40 days of age, were sacrificed by decapitation and whole hippocampal formation rapidly dissected. Subcellular fractions are prepared exactly as described by Buckley et al. (2004). Protein determinations were performed as described in Weeber et al. (2001) and were used to load equal amounts of protein in each well. To determine the lack of cytoplasmic contamination in the nuclear fraction, the membrane was probed between 68kDa and 21 kDa for anti-A-Raf (1:500; Santa Cruz) and anti-H-Ras (1:500; Santa Cruz) antibodies, respectively.

3.3.6 Glucocorticoid and Mineralocorticoid Receptors

Cytosolic and nuclear fractions from seven or eight hippocampi from arsenic-exposed and control animals were analyzed for GR and MR via western blot. The linear range of the total protein was calculated to ensure that the protein was not saturating the blot (data not shown). Control samples were run on the same blot as arsenic-exposed samples. The amount of total GR and MR of the sample was corrected for Beta-actin and data analyzed by t-test. Extracts were thawed on ice, diluted in 4X SDS-PAGE sample buffer (Invitrogen, Carlsbad, CA) and

heated at 70oC for 10 minutes. Samples (2ug protein per well (GR); 3ug protein per well (MR)) were separated using 4-12% NuPAGE Bis-Tris gels (Invitrogen) and transferred to 0.45-um-thick nitrocellulose membranes (Invitrogen). The GR and MR membranes were blocked with 0.25% I-block (Applied Biosystems, Foster City, CA) in TBS-T (25mM Tris-HCl pH 7.2, 150mM NaCl and 0.05% Tween-20) for one hour at room temperature. Blots were then cut and incubated with either a polyclonal primary antibody to GR (1:3 000; M-20, Santa Cruz Biotechnology, Santa Cruz, CA), MR (1:500; H-300 Santa Cruz) or B-actin (1:2 000; Cell Signaling, Boston, MA) overnight at 4oC. The reaction was stopped with four consecutive 5-minute washes in TBS-T. A goat anti-rabbit IgG (H+L):HRP (1:50 000, Thermo Fisher Scientific, Rockford, IL) was used for the secondary antibody incubation in 0.25% I-block one hour at room temperature. The reaction was stopped with four consecutive 5-minute washes in TBS-T. Membranes were then incubated in Supersignal West Pico Working Solution (Thermo Scientific) for five minutes and exposed to F-BX57 film (Phenix Research Products, Candler, NC). Film was developed in Kodak D-19 developer then washed and fixed in Kodak fixer. The developed film was scanned (Hewlett Packard Scan Jet 5P) and immunoreactivities quantified by measurements of optical densities using BioRad Quantity-One analysis software. Each protein sample was run in duplicate and the average optical density taken.

3.3.7 Novel Object Exploration

The novel object exploration task was conducted in a plexiglas open field apparatus (43 cm x 43 cm x 21 cm high, designed by Allan laboratory) using a

protocol described previously by Grailhe et al. (1999). The floor of the apparatus was black and divided into five areas: Four equal quadrants and one center area 14 cm. in diameter. The experiments were conducted in a dimly lit room with the aid of a video camera to minimize subject stress and anxiety. The floor and walls of the open field were wiped with 70% isopropanol before each test session. Each test consisted of two videotaped five-minute sessions. During the first session, mice were placed in the center area and allowed to explore and acclimate to the apparatus. A novel object, consisting of a pink and green striped gray cube (2.5 cm3) with an open side (designed by Allan laboratory), was placed in the center area with the open side facing the mouse. Response to the novel object during the second five minute session was videotaped. Subsequently, a trained observer blinded to experimental group identity analyzed the video tapes and measured latency to approach the object and total number of center line crosses in the presence of the novel object.

3.3.8 Eight-way Radial Arm Maze

Radial arm maze testing was conducted using the mouse runway system from Coulbourn Instruments (Whitehall, PA) using a protocol modified from Egashira et al. (2002). Plastic food cups containing sweetened condensed milk as a reinforcer, were placed approximately 2/3rds the way down in each arm. The maze was located in a room containing many extra-maze visual cues. Arm entry was recorded by an observer seated several feet away from the maze. All mice were habituated to the maze for 20 minutes on the day prior to testing. One week before testing, mice were placed on a caloric restriction diet where they received

3 grams of lab chow (7004 Harlan Teklad S-2335, Denver, CO) each day. Untreated tap water was available *ad libitum*. Testing was conducted over 3 consecutive days. For each 8 minute testing session, the mouse was placed in the center of the maze. For each testing session; the number of correct choices in the initial eight chosen arms and the number of errors which was defined as choosing arms which had already been visited were scored.

3.3.9 Statistical analysis

All data were analyzed by t-test comparing the perinatal arsenic to the control on each of the dependent measures. Unless otherwise noted, all behavioral data points were taken from two animals per litter and this average treated as a single result. A repeated measures ANOVA was used in the eight-way radial arm maze behavioral test. Significance was set at $p \leq 0.05$.

3.4 RESULTS

3.4.1 Arsenic Exposure Paradigm

No signs of overt toxicity (i.e. ataxia, redness, swelling, fetal malformations, death) were observed in the mice throughout the duration of the study. Absence of structural malformations is in accordance with what has been shown previously (Holson et al., 2000). The doses of arsenic used in present study were well tolerated and did not alter maternal body weight or body weights of the newborn pups (Martinez et al., 2008), consistent with what has been reported by others (Bardullas et al., 2009). Arsenic exposure did not change whole brain

weight (t<1, not significant) or hippocampal wet weight (t<1, not significant) of offspring at Day 35.

3.4.2 Drinking Water Analysis and Data

Samples from six arsenic treated and six control water samples from throughout the span of the study were sent to Earth and Planetary Sciences for analysis. Water arsenic concentrations, analyzed by ICP-MS, were ~56 ppb and untreated tap water (control group) concentrations were ~6 ppb. The average daily litter water consumption, monitored every other day, and averaged over the entire study period was 13.09 ± 0.93 grams/day for the control group and 15.98 ± 2.97 grams/day for the perinatal arsenic group. There were no significant differences in the amount of water consumed by the litters in either treatment condition (t<1, not significant). We did not measure the amount of arsenic present in mother's breast milk because this amount has been shown to be negligible according to several papers. One example comes from a study of Andean women exposed to high concentrations (about 200ppb) of inorganic arsenic in drinking water. The concentrations of arsenic in their breast milk ranged from about 0.0008 to 0.008ppm (Concha et al., 1998). Similarly, a World Health Organization study detected arsenic at concentrations of 0.00013-0.00082 ppm in human breast milk (Somogyi and Beck, 1993). Although these studies were not conducted in animals, extrapolation of the data would suggest that the concentration of arsenic that is present in the breast milk in our mice after exposure to arsenic in their drinking water is extremely low.

3.4.3 Brain Arsenic Concentrations

Total inorganic arsenic concentrations detected in brain tissue of offspring at 35days of age were measured. Control brain levels were 1.0 ± 0.24 ppb and perinatal arsenic offspring levels were 2.24 ± .02 ppb. Levels of arsenic in the perinatal exposure group significantly exceeded controls ($t(6) = 3.87$, $^{**}p < 0.008$). The source of arsenic in the brains of control animals may be either organic or inorganic given that untreated tap water at UNM has ~6ppb arsenic and approximate levels of arsenic within the chow are 0.16 ppm, based on analyses done at Harlan Labs (personal communication).

3.4.4 Glucocorticoid and Mineralocorticoid Receptors

Due to the high basal CORT levels in the perinatal-exposed mice that we previously reported (Martinez et al., 2008); we decided to examine the intracellular distribution of the GR and MR proteins. Intracellular distribution of the GR and MR is important for its function.

Figure 3.1, panels A-B and C-D, shows a representative western blot, together with results of the densitometric quantitation of the nuclear and cytosolic glucocorticoid receptor bands, respectively. The rabbit polyclonal antibody against the mouse glucocorticoid receptor recognized a prominent band at ~95kD, consistent with the molecular weight of the glucocorticoid alpha receptor. Perinatal arsenic exposure reduced levels of both nuclear and cytosolic GR receptors. Figure 3.1B shows the quantification of the nuclear compartment where there was significantly less GR in the perinatal mice than controls ($t(13) =$

3.81, **p < 0.002). The cytosolic fraction (Figure 3.1D) revealed a similar significance (t(13) = 2.11, *p < 0.05). This finding is curious as we would have predicted higher nuclear GR levels in the perinatal offspring due to their higher basal CORT levels.

Figure 3.2 shows a representative western blot, together with results of the densitometric quantitation of the mineralocorticoid receptor bands. The rabbit polyclonal antibody against the mouse mineralocorticoid receptor recognized a prominent band at ~102kD, consistent with the molecular weight of the mineralocorticoid receptor. In the perinatal arsenic exposed there was significantly less MR in the nuclear fraction compared to controls (Figure 3.2B) (t(13) = 5.95, ***p < 0.0001). Figure 3.2D shows the cytosolic fraction, where there is no difference in relative amount of MR (t<1, not significant).

3.4.5 Novel Object Exploration

Behavior is an informative end-point for assessing the neurobiological effects of arsenic exposure. Mineralocorticoid receptors have been shown to be associated with ability to learn in a novel situation. We used the novel object task as a measure of learning and memory. Figure 3.3, panels A and B, show the latency to novel object and entries to center in presence of novel object, respectively during a second five-minute period. Perinatal exposed mice took significantly longer to recognize the presence of the novel object, measured as latency to approach the novel object (t(12) = 13.79, ***p <0.0001). Compared to controls, perinatal mice made significantly fewer center entries in the presence of the

novel object ($t(12) = 4.61$, $^{***}p < 0.0006$). The increase in latency and center entries may be confounded by an anxiety response; therefore we conducted an additional behavioral test to rule out this variable.

3.4.6 Eight-way Radial Arm Maze

We used the 8-way radial arm maze to determine if the arsenic exposure was associated with decreased hippocampal-dependent spatial learning and memory. Because variations in maternal care within the same strain of animals can lead to differences in behavioral performance including spatial memory (Barha et al., 2007) we pulled offspring from different litters to preclude the litter effect. It has also been reported that males inherently perform better on spatial memory tasks than do females (Brandeis et al., 1989; Vorhees et al., 2004), thus only males were used on the behavioral tasks. All mice were acclimated to the testing field prior to test days. All control mice showed a progressive decline in number of entry errors over the three testing days. Arsenic offspring errors did not change significantly with test day (Figure 3.4). There was a significant effect of treatment ($F(1,18) = 50.79$, $^{***}p < 0.0001$) and these results indicate that perinatal arsenic exposure in mice might induce spatial-learning and memory impairment.

3.5 DISCUSSION

The persistence of arsenic in drinking water raises the importance of assessing the neurobiological consequences of exposure to low levels of arsenic. Our perinatal exposure model provides a unique opportunity to examine the effects of arsenic on the developing brain at an environmentally relevant level, magnitudes

lower than what has been previously studied. Arsenic has been shown to accumulate in target organs (Kenyon et al., 2008) and at 35 days of age, two weeks after weaning and absence of arsenic, our model revealed arsenic (total arsenicals) content in the brains of our adolescent offspring. We are currently seeking to determine which areas of the brain, if any, are preferentially affected. We previously showed elevated basal CORT levels in offspring perinatally exposed to 55ppb arsenic compared to their control counterparts (Martinez et al., 2008). These findings led us to evaluate the status of the receptors responsible for mediating changes in CORT levels. Results from the present experiments revealed lower abundance of hippocampal cytosolic GRs in perinatal arsenic-exposed offspring compared to controls but no difference in abundance of cytosolic MRs. Nuclear levels of both GR and MR were significantly lower than those of controls. In addition to this receptor deficit perinatal exposed mice exhibited behavioral underperformance in the learning and memory tests.

CORT binds and activates the cytosolic GR and MR hormone receptors initiating translocation of activated receptor to the nucleus. Although there is sufficient CORT to activate these receptors in our animals (Martinez et al., 2008), the amount of nuclear hippocampal GR and MR is significantly less in the arsenic-exposed offspring than in the controls (figs. 3.1AB-3AB). A plausible explanation for this is failure of the receptors to traffic to the nucleus. While other groups have investigated this possibility and found that the trafficking of these receptors to the nucleus is unaffected by arsenic (Kaltreider et al., 2001) these studies were conducted using a hepatoma cell line and the results may not be the same in the

brain, as is the case with many studies in other systems. In addition, the cell line fails to maintain the contribution of the intact HPA axis and does not account for genes that may have previously been affected. For example, high CORT levels have been shown to reduce dynein cytosplasmic intermediate chain 1 gene expression, reducing motor protein activity and axonal transport (Morsink et al., 2006), a result that would not have been taken into account in the hepatoma cell line. In accordance with our high CORT, the translocation of GR to the nucleus by dynein may be reduced. Lower levels of nuclear GR and MR would have far reaching consequences on gene transcription. It has been shown that, in particular for GR, nuclear translocation of the activated receptor and its ability to induce transcriptional activity are intimately correlated (de Kloet et al., 2009). Binding of GRs to promoter glucocorticoid response elements (GREs) regulates gene transcription both positively and negatively and approximately 20% of the genome is under this control. In support of this possibility, we previously showed increases in 5-HT_{1A} receptor density in the dorsal hippocampus of our perinatal arsenic-exposed mice (Martinez et al., 2008). The 5-HT_{1A} receptor is negatively regulated by corticosteroids through the GR (Chalmers et al., 1993; Datson et al., 2001; de Kloet et al., 1986; Meijer et al., 1997) and reduced GR binding to negative GREs would allow for transcription and ultimately upregulation of the 5-HT_{1A} gene and may be one example of the consequences of reduced nuclear GRs.

The finding of no difference in cytosolic MR protein levels compared to controls could be explained by the presence of two different promoters (P1 and P2) for

the MR gene. P1 and P2 can both be activated by glucocorticoids but P2 can also be activated by aldosterone with the same affinity (Viengchareun et al., 2007). Thus GR and MR although both activated by glucocorticoids are also differentially regulated. Activation of P2 by aldosterone, which is independent of the HPA axis, could be enough to keep MR levels constant in the cytoplasm. These differences in transcription regulation could help explain the differences in protein levels seen in our animals.

The reductions in GR are important as studies using GR germline knockouts have shown that knocking down the receptor in all organs results in an inviable animal that dies approximately 1 week after birth due to atelectasis of the lungs (Erdmann et al., 2008). However, conditional knockouts and loss of the receptor in whole brain results in animals that are viable and exhibit many of the characteristics that we have seen in our animals. For example, GR knockout mice are reported to have elevated CORT levels, impaired spatial memory and reduced anxiety behavior (Erdmann et al., 2008). Our perinatal arsenic-exposed mice exhibit slight reductions in anxiety in the elevated plus maze (unpublished observations) and depression-like behavior in a learned helplessness task (Martinez et al., 2008). Dysregulation within the HPA axis has long been suggested to be part of depressive pathophysiology. The high CORT levels at the adolescent time point at which our measurements were taken suggests that there is dysregulation of the axis. We are currently investigating this possibility. GR expression is detectable at embryonic day 14.5 and HPA axis activity and regulation is assumed to be established around this time during development

(Michelsohn and Anderson, 1992). Our perinatal exposure period could be affecting the establishment of regulation of the axis, enabling sustained or cumulative damage with further high CORT levels resulting in an inability to return to homeostasis and prevent a maladaptive overshoot. GRs are important for terminating the stress response via negative-feedback control and MRs are thought to control the basal circadian rhythm of plasma CORT secretion (Sapolsky et al., 1986). A reduction in MRs and GRs, coupled with reduced nuclear transport, could affect the maintenance of homeostasis via the HPA axis and HPA response to stressful situations. Alterations in the HPA axis response to stress and changes in transcription mediated by GR are likely to play an important role in mechanisms underlying neural plasticity, providing a possible basis for linking neuroendocrine dysregulation and cognitive decline.

It is well established that chronically elevated levels of CORT, mediated by the binding to GRs and MRs, produce cognitive deficits (de Kloet et al., 2005, Joels et al., 2007; McEwen, 2007). Many epidemiological studies have focused on the issue of deficits in learning and memory associated with exposure to arsenic (Calderon et al., 2001; von Ehrenstein et al., 2007; Wasserman et al., 2004). Our perinatal arsenic-exposed offspring performed poorly on learning and memory tests. Results from the novel object exploration test revealed deficits in latency to the novel object and in number of center entries in perinatal-exposed animals suggesting that arsenic animals have learning and memory deficits (fig. 3.3). This impaired performance could be explained by loss of nuclear MR. Findings from mutant mice with inactivated MR resulted in impaired behavioral reactivity to

novelty (Berger et al., 2006). The 8-way Radial Arm Maze, an assessment of spatial working memory, illustrated that arsenic-exposed offspring have difficulties performing more arduous hippocampal-dependent learning tasks (fig. 3.4). Arsenic-exposed offspring failed to make improvements in the task as the days progressed. This is in agreement with other findings that long periods of stress or CORT treatment impair performance on a variety of spatial tasks (Brinks et al., 2007; Elizalde et al., 2008; Schwabe et al., 2008). For example, hypercorticism, after exposure to restraint stress for 6 hours per day for 3 weeks, was shown to result in impaired spatial memory in adult animals (Conrad et al., 1996; Luine et al., 1996). Furthermore, long-term high dose exogenous CORT treatment in Long-Evans rats was shown to produce disruptive effects in water-maze performance, a test for spatial memory similar to the 8-way radial arm maze (Bodnoff et al., 1995). MR appears to mediate the immediate effects of CORT on memory acquisition, while presence of a functional GR is responsible for modulation of spatial memory (Oitzl et al., 1997). While a direct link between the poor performance in these behavioral tasks and reduced GR levels is not established by these studies the findings are consistent with the interpretation that memory performance is affected by arsenic-induced decreases in GR in the hippocampus.

In conclusion, the present study indicates a relationship between decreased CR levels and cognition. While other studies have examined CR levels and subcellular localization in other target organs, to our knowledge ours is the first report of the brain receptor levels after perinatal arsenic exposure.

Changes in CR levels cannot fully explain the perinatal arsenic-induced impairments in cognitive performance. However, these lower CR levels are likely altering transcription of genes that are important for learning and memory. Overall this data suggests that moderate perinatal arsenic exposure can have long lasting effects on learning and memory well into adolescence, consistent with the Fetal Basis of Adult Disease hypothesis (White et al., 2007), which postulates that many adult diseases have a fetal origin. Further studies detailing the molecular effects of perinatal arsenic exposure on the central nervous system are essential to our understanding of arsenics production of learning and memory deficits.

3.6 FUNDING

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3.8 CONFLICTS OF INTEREST

The authors report no conflicting interests.

3.9 FIGURES

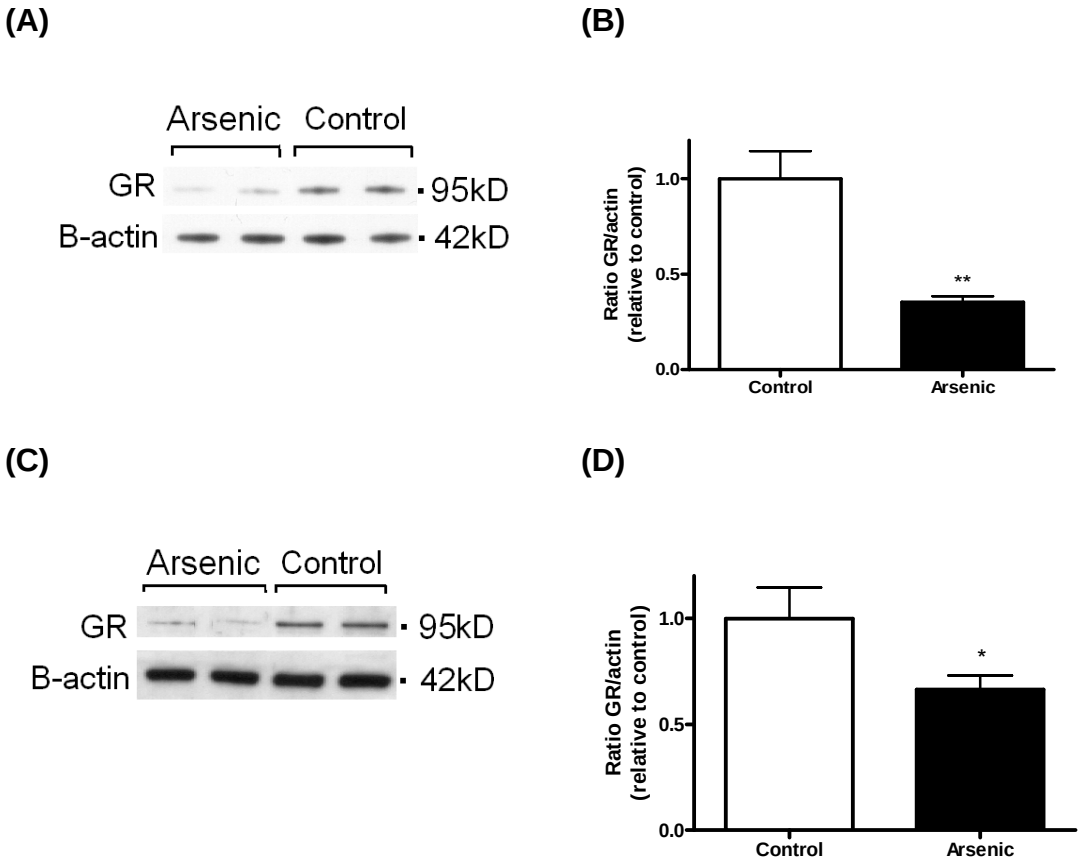

Figure 3.1 Nucleocytoplasmic trafficking of GRs and densitometric quantitation of GR bands.

(A) Representative western blot of GR in the hippocampal nuclear compartment of arsenic exposed mice. The anti-GR polyclonal antibody recognized a prominent band at ~95kD, representative of GRα. (B) Quantitation of GR bands in nuclear subcellular fraction of arsenic exposed mice was based on densitometric analysis (**$p < 0.002$ vs. control). (C) Representative western blot of GR in the hippocampal cytosolic compartment of arsenic exposed mice. (D) Quantitation of GR bands in cytosolic subcellular fraction of arsenic exposed mice was based on densitometric analysis (*$p < 0.05$ vs. control). Results are

expressed as a percentage of the GR signal to B-actin signal, relative to controls and are presented as mean ± SEM of seven-eight litters.

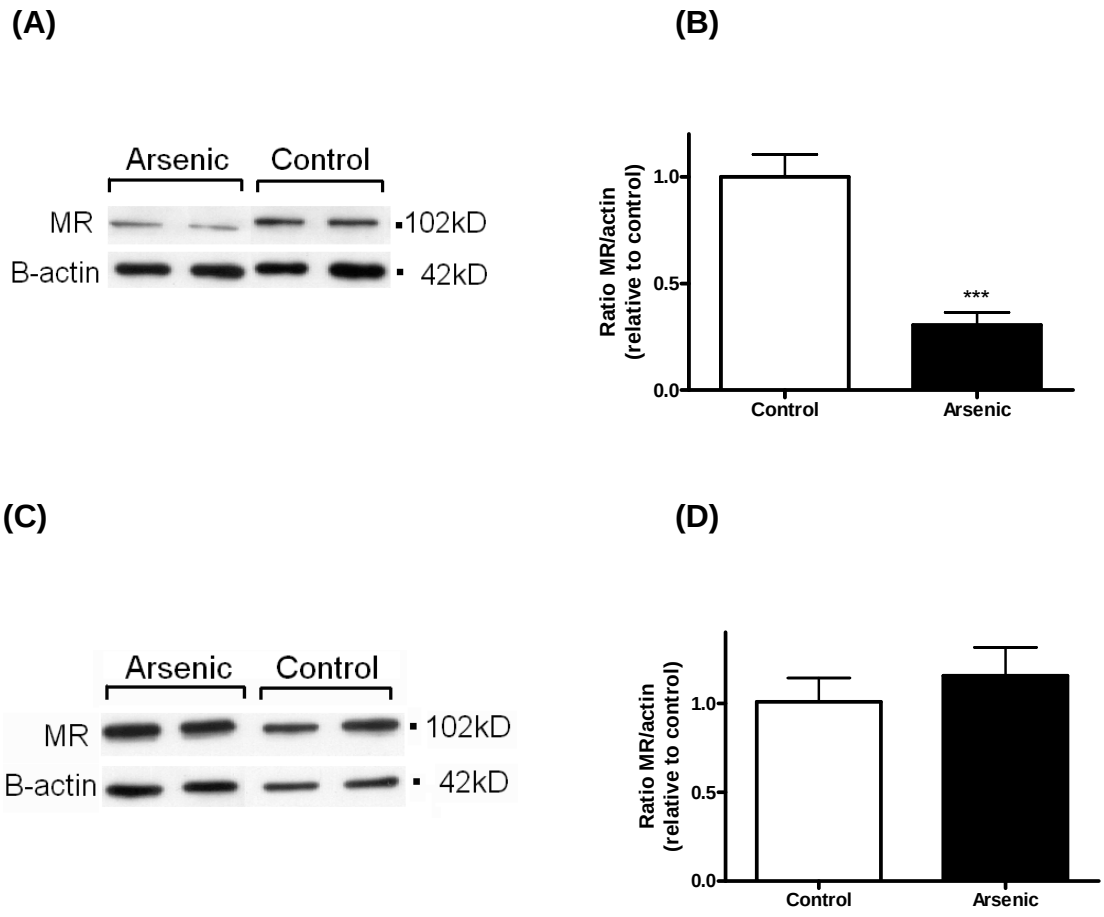

Figure 3.2 Nucleocytoplasmic trafficking of MRs and densitometric quantitation of MR bands.

(A) Representative western blot of MR in the nuclear compartment of arsenic exposed mice. The anti-MR polyclonal antibody recognized a prominent band at ~102kD. (B) Quantitation of MR bands in hippocampal nuclear subcellular fraction of arsenic exposed mice was based on densitometric analysis (***$p < 0.0001$ vs. control). (C) Representative western blot of MR in the cytosolic compartment of arsenic exposed mice. (D) Quantitation of MR bands in

hippocampal cytosolic subcellular fraction of arsenic exposed mice was based on densitometric analysis ($p > 0.05$ vs. control). Results are expressed as a percentage of the MR signal to B-actin signal, relative to controls, and are presented as mean ± SEM of six-eight litters.

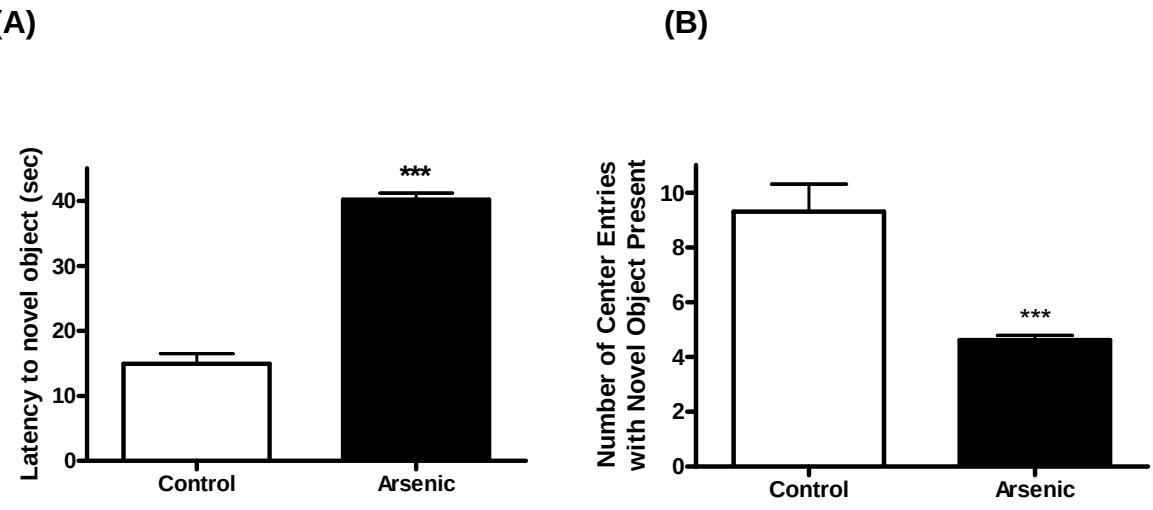

Figure 3.3 Effects of arsenic exposure to 50ppb arsenic on novel object exploration at D35.

(A) Latency to approach novel object after acclimation period (***$p < 0.0001$ vs. control). (B) Entries to center in the presence of the novel object (***$p < 0.0006$ vs. control). Data are presented as mean ± SEM of seven litters.

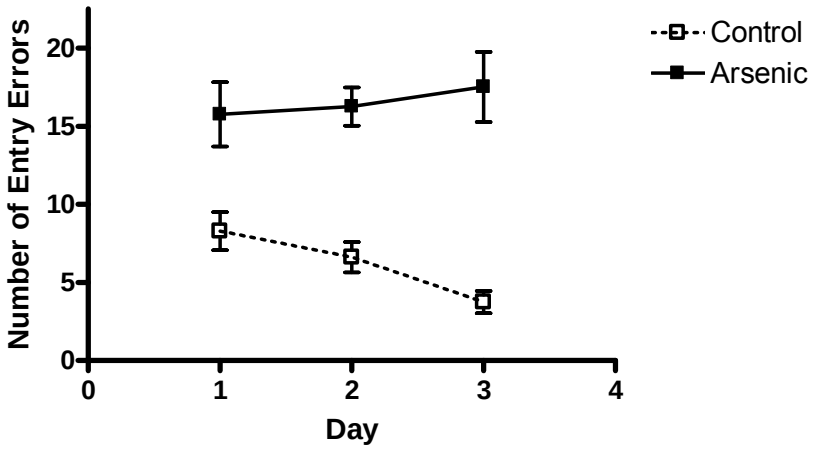

Figure 3.4 Effects of arsenic-exposure on number of entry errors in 8-way Radial Arm Maze at D35.

Day 1 represents first day after acclimation period. There was a significant effect of treatment (***p < 0.0001 vs. control). Data are presented as mean ± SEM of ten litters.

Chapter 4. ABERRANT GENE EXPRESSION OF MAPK/ERK GENES INDUCED BY GLUCOCORTICOID RECEPTOR DEFICITS IN PERINATAL ARSENIC-EXPOSED OFFSPRING

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[To be submitted]

4.1 ABSTRACT

Biosynthesis of members of the mitogen-activated protein kinase (MAPK) signaling pathway, shown to be involved in learning and memory, are modulated by glucocorticoid receptor (GR) binding glucocorticoid response elements (GREs) in their promoters. We have previously reported increased basal plasma levels of corticosterone, decreased hippocampal GR levels and deficits in behavioral learning and memory performance in perinatal arsenic-exposed mice. Two genes of the MAPK pathway, Ras and Raf, contain GREs which are activated upon binding of GRs. Due to the lower levels of GRs in our mice we evaluated the activity of GRs at Ras and Raf promoters via chromatin immunoprecipitation and real-time PCR. In the present study we report decreased binding of GR at these promoters which coincided with decreased mRNA levels of both of these genes in perinatal arsenic-exposed mice. We also investigated the role of extracellular-signal regulated kinase (ERK), a downstream target of Ras and Raf, whose transcriptional targets also play an important role in learning and memory. Nuclear activated ERK levels were decreased in the hippocampus of arsenic-exposed animals compared to controls. We performed electrophoretic mobility shift assay (EMSA) to assess the ability of hippocampal nuclear GRs to bind to a synthetic radiolabeled GRE oligonucleotide. Interestingly, nuclear cellular fractions were capable of binding to the GRE, although it is unclear whether or not GR is in this complex. GR-mediated transcriptional deficits in MAPK/ERK pathway could be an underlying

cause of the observed learning deficits and provide the link to arsenic-induced deficiencies in cognitive development.

KEY WORDS

Arsenic, Glucocorticoid Receptor, Glucocorticoid Response Element, Extracellular-signal regulated kinase (ERK), Hippocampal formation, MAPK Signaling Pathway, Raf, Ras

4.2 INTRODUCTION

The persistence of arsenic in the environment beckons the need for continued research on arsenic and its health effects. Drinking water contamination is of particular concern as arsenic has been shown to be permissive to a variety of cancers, vascular diseases, and cellular disruption (Agency for Toxic Substances and Disease Registry Arsenic, 2007). In addition, several studies have identified arsenic as a potent neurotoxin and exposure poses a threat to cognitive development in both rodents (Rodriguez, 2001; Martinez et al., 2008; Martinez-Finley et al., 2009) and humans (Holson et al., 2000).

Moderate doses of arsenic have been shown to perturb glucocorticoid receptors (GRs) (Kaltreider et al., 2001; Bodwell et al., 2006). We have shown that GR and mineralocorticoid receptor (MR) protein levels in the hippocampus of brains of offspring perinatally exposed to arsenic are significantly decreased compared to age-matched controls at postnatal day 35 (Martinez-Finley et al., 2009). This is consistent with what others have observed: events that disrupt maturation of the

hypothalamic-pituitary-adrenal (HPA) axis, particularly during gestation, have been shown to permanently alter glucocorticoid receptor (GR) expression in the adult (Matthews, 2000; Welberg and Seckl, 2001). Increases and/or decreases in transcription of genes under the control of GRs have been described as one consequence of altered GR levels. In regards to arsenic, Kaltreider and colleagues have shown that nontoxic doses of arsenite can interact directly with GR complexes and selectively inhibit GR-mediated transcription (2001). At concentrations between 1-3uM, the effects of arsenic on GR-mediated gene activation are inhibitory (Bodwell et al., 2006). Hamilton and colleagues showed that a single, low, non-overtly toxic dose of arsenite significantly altered basal and inducible mRNA expression of the GRE-controlled phosphoenolpyruvate carboxykinase gene in cell culture and in a whole-animal model (1998). They also showed that arsenite had no effect on expression of GRE-non-inducible or constitutively expressed genes (Hamilton et al., 1998). Given that GR controls the transcription of a number of genes, the present work investigated the effects of decreased levels of GR in the hippocampus on two genes shown to be transcriptionally activated by GR.

Ras and Raf-1, two important members of the mitogen-activated protein kinase (MAPK) signaling pathway are transcriptional targets of GR (Revest et al., 2005). Ras and Raf-1 are core elements of the MAPK signaling pathway and their activation ultimately leads to phosphorylation, activation and nuclear localization of extracellular signal-regulated kinase (ERK1/2). The MAPK/ERK pathway plays a crucial role in learning and memory (Sweatt, 2001; Sweatt, 2004). At the

biochemical level, the MAPK pathway is linked to synaptic plasticity, which contributes to memory formation and is important in information processing (reviewed in Thomas and Huganir, 2004). Induction of long-term potentiation (LTP), the best studied form of cellular learning and memory, relies on the regulation of protein phosphorylation (reviewed in Thomas and Huganir, 2004) and many forms of LTP have been shown to be ERK-dependent (English and Sweatt, 1996; Atkins et al., 1998; Ohno et al., 2001). In addition to the decreased levels of GR in the hippocampus, we have reported learning and memory deficits in our perinatal arsenic-exposed offspring (Martinez-Finley et al., 2009). Because the MAPK pathway has been implicated in learning and memory and GR has been shown to transcriptionally regulate its members we evaluated this pathway as a unifying factor for the effects that we have seen.

The present study focused on the connection between perinatal arsenic exposure, decreased hippocampal GR and genes of the MAPK signaling pathway in brains of exposed offspring as alterations in these cascades could underlie the learning and memory deficits observed in these animals. We investigated the binding association of nuclear GR with promoters in the Ras and Raf-1 genes in hippocampal cells from our perinatal arsenic and control animals, as well as GR association with a synthetic GRE. In addition, we assessed the effects of decreased binding at these promoter sites by measuring mRNA levels and activation of the downstream protein ERK.

4.3 MATERIALS AND METHODS

Arsenic is classified as a probable human carcinogen and all arsenicals were handled with extreme caution (ATSDR, 2007).

4.3.1 Perinatal Arsenic Exposure Paradigm

The arsenic exposure paradigm employed in these studies was approved by the UNM Health Sciences Center Institutional Animal Care and Use Committee. All mice were bred and maintained on a reversed 12-h light/dark cycle (lights on from 21:00 to 09:00h) with food and water *ad libitum* in a temperature controlled (22^0C) room in the Animal Resource Facility. All dosing suspensions were prepared approximately weekly. Arsenic water (sodium arsenate, Sigma) was prepared using standard tap water. Control mice were administered untreated tap water. Tap water at UNM contains approximately 5 ppb arsenic. Mice were bred according to our established protocol (Martinez et al., 2008; Martinez-Finley et al., 2009). Offspring were weaned at 21 days of age and maintained in same-sex, litter-mate housed cages with *ad libitum* access to untreated tap water and standard mouse chow until they were used in experimental procedures at 35-40 days of age. A sample size of six (n = 6) represents the total number of litters and not individual animals used.

4.3.2 Nuclear Extracts

Adolescent offspring, 35-40 days of age, were sacrificed by decapitation and whole hippocampal formation rapidly dissected. Preparation of nuclear extracts

and protein determinations were performed as described in Martinez-Finley et al (2009).

4.3.3 Chromatin Immunoprecipitation (ChIP) Real-time PCR Assays

The protocol followed was a modified Upstate Biotechnology ChIP protocol as described. Hippocampal tissue was collected and homogenized on ice in 1% cross-linking solution (10% formaldehyde, 0.1M NaCl, 1 mM EDTA, 0.5 mM EGTA, 50 mM Hepes pH 8.0 and double distilled H_2O) for 15 minutes. Cells were centrifuged at 1,000 x *g* at 4ºC for six minutes and washed five times with 1.5mL PBS containing 1µL/mL protease inhibitor cocktail (Sigma, P8340). Cell pellets were resuspended in cell lysis buffer (5mM PIPES pH8.0, 85mM KCl, 0.5% 2-[2-(4-nonylphenoxy)ethoxy]ethanol(IGEPAL-CA-630)) and allowed to sit on ice for 5 minutes. The cells were then pelleted (1,000 *x g* at 4ºC for 6 minutes), cells were resuspended in cell lysis buffer and repelleted. Cells were then resuspended in nuclei lysis buffer (50mM Tris pH 8.1, 10mM EDTA, 1% SDS). At this point samples were frozen in the -80ºC overnight. The following day, crosslinked DNA was sheared via sonication (Kontes. Micro Ultrasonic Cell Disrupter; setting = 25%, four times 10 seconds, waiting a couple minutes between rounds) to approximately 500-600 base pairs. To check the size of chromatin, 10µl of sample was treated with 2µl of 10mg/ml proteinase K for 20 minutes at 50ºC to reverse the cross-links. Sample was run on 1.5% (w/v) agarose gel. After brief sonication to shear genomic DNA the samples were centrifuged (minimum of 10,000 *x g* but not exceeding 15,000 *x g* at 4ºC for 10 minutes). To prepare the beads, swell protein G agarose (Sigma, P7700) in PBS (approximately 80µl

settled beads per sample) and spin (1,000 *x g* at 4ºC for 1 minute) and resuspend in blocking buffer (25µl 10mg/ml sonicated salmon sperm DNA, 100µL 1mg/ml BSA, q.s. to 1ml with H2O). Block 15 minutes at 4ºC rotating. The mixture was then washed 2 times in 1.5 ml PBS and resuspended in 50/50 slurry with ChIP dilution buffer. To preclean the cell lysate, 150µl of the supernatant of cell lysate was diluted with 900µl ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2mM EDTA, 16.7mM Tris pH 8.1, 167mM NaCl, 1µL/mL protease inhibitor cocktail) and 80µl protein G slurry to eliminate nonspecific binding. Samples were rotated at 4ºC for 1 hour, followed by pelleting of protein G (1,000 *x g* for 1 minute). Protein G was then discarded. A small fraction of the lysate (1%) was then saved as "input DNA" (positive control). Input DNA was treated in parallel with immunoprecipitated DNA during reverse crosslinking and purification steps. After discarding the protein G, cell lysates were incubated with 5µg anti-glucocorticoid receptor antibody (GR, M-20 Santa Cruz, sc-1004) or normal rabbit IgG (Santa Cruz, sc-2027) as a negative control and rotated overnight at 4ºC. The following day 60µl of the 50/50 protein G agarose slurry was added and samples were left to rotate for 2 hours at 4ºC. Protein G was then pelleted (1,000 *x g* for 1 min.), supernatant was discarded and sample was washed with 1.5ml of ChIP Dilution buffer and rotated for 5 minutes at 4ºC. Sample was pelleted (1,000 *x g* for 1 min.). Wash step with ChIP dilution buffer was repeated. Next sample was washed with 1.5ml TSE-500 (0.1% SDS, 1% Triton X-100, 2mM EDTA, 500mM NaCl) for 5 minutes and pelleted (1,000 *x g* for 1 min) and this wash was repeated. Next the pellet was washed with 1.5ml LiCl/Detergent (100mM Tris pH

8.1, 500mM LiCl, 1% IGEPAL, 1% deoxycholic acid) for 5 minutes and pelleted (1,000 *x g* for 1 min) and this wash was repeated. Finally, the pellet was washed in 1.5ml 1X TE, for 5 minutes and pelleted (1,000 *x g* for 1 min) and this wash was repeated. The DNA was eluted from the beads with 250µl IP Elution Buffer (50mM $NaHCO_3$, 1% SDS). Samples were vortexed and left to rotate 15 minutes at room temperature. Protein G was pelleted (3,000 *x g* for 5 min.) and supernatant was saved. Elution step was repeated and elute was pooled. Crosslinks were reversed overnight at 65ºC (20µl 5M NaCl). Saved input sample received 460µl TE, 20µl 5M NaCl overnight at 65ºC. The following day samples were incubated for 1 hour at 55-65ºC in 32µl (0.5M EDTA, 1M Tris pH6.5, 10mg/mL proteinase K) solution. DNA was isolated using Qiaquick Spin Columns (Qiagen, 27104) according to manufacturers protocol and quantified by NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). The ChampionCHIP One-Day Kit (SABiosciences) was also used according to manufacturer's instructions to confirm results. Purified DNA was used in PCR amplification for 40 cycles using the Power SYBR green master mix (Applied Biosystems, 4367659). The primers used for ChIP were based on sequences published in Revest et al. (2005). Non-immune rabbit IgG was used to determine non-specific antibody interactions and was subtracted from specific GR interactions. Results are presented as relative promoter binding, relative to controls, after subtracting for non-immune rabbit IgG interactions. Data are represented as the result of four-five samples per condition, run in five independent experiments. PCR products were run out on 1.5% (w/v) agarose gel

and results were visualized using a Fluor-S Multiimager (BioRad) and Quantity One Software (BioRad).

4.3.4 Electromobility shift assays (EMSA)

EMSAs included 10pmol GR consensus oligonucleotide (Santa Cruz Biotechnology, sc-2545) with 20pmol γ^{32}P-ATP at 6000Ci/mmol (Perkin Elmer, NEG002Z) along with T4 Polynucleotide Kinase (PNK) Buffer (10X) and T4 PNK (USB, Cleveland, 70031Y) and water for a total of 50µL. Contents were mixed well and centrifuged briefly. The reaction was incubated at 37ºC for 30 minutes followed by termination of the reaction by heating at 65ºC for 10 minutes. The 5'-end-labeled oligo was separated from precursor ATP using a QIAquick Nucleotide Removal Kit (Qiagen, cat# 28304). Radioactivity was quantified using a liquid scintillation fluid counter. The following 5X binding buffer was prepared on ice for each sample, 50mM Tris pH 7.5, 20% glycerol, 2.5mM EDTA, 250mM NaCl, 2.5mM DTT and 10µg poly (di-dC). The 5X binding buffer was then added to 10µg of nuclear extract, and 1µl labeled probe and water to 20µl and incubated at room temperature for 20 minutes. Following the incubation either anti-GR antibody (1µg, Santa Cruz, sc-1002X) or cold competitor probe was added to the mixture and was incubated for an additional 20 minutes at room temperature. Complexes were separated by 5% polyacrylamide gel electrophoresis in 1X TBE buffer, 105V, 45 minutes. Gels were dried via gel dryer (BioRad Model 583) for approximately 45 minutes and visualized using a Personal Molecular Imager FX (BioRad) and Quantity One Software (BioRad) at 50µm resolution.

4.3.5 mRNA isolation

mRNA was isolated using the Oligotex Direct mRNA Mini Kit (Qiagen, Valencia, CA) per manufacturer's protocol. The mRNA concentration was determined (OD 260 nm) using a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Purified mRNA was stored at -80^{o}C.

4.3.6 cDNA synthesis

Reverse transcription reactions were performed in 20 µL following the manufacturer's protocol (Invitrogen). A 10µL reaction mixture containing 20ng mRNA, 500ng oligo dT, 1mM of each dNTP was heated at 65^{o}C for 5 minutes and quick chilled on ice for 5 minutes. The contents was centrifuged briefly and 1µL RNaseOUT (40units/µL), 2 µL DTT (0.1M) and sterile RNase free water was added to 19µL. The contents was mixed gently and incubated at 37^{o}C for 2 minutes followed by addition of 1µL (200units) M-MLV reverse transcriptase. The reaction was then incubated at 37^{o}C for 50 minutes followed by inactivation by heating at 70^{o}C for 15 minutes. Synthesized cDNA was stored at -20^{o}C until used. The suitability of the cDNA for PCR was determined using 2 µL of cDNA added to a 40 µL PCR. A control reaction containing 2µl of purified mRNA was run without reverse transcription to test for presence of DNA contamination in the purified mRNA samples. The PCR products were run out on a 1.5% (w/v) agarose gel and visualized using Fluor-S Multiimager (BioRad) and Quantity One Software (BioRad).

4.3.7 Primers

Oligonucleotide sequences for H-Ras and Raf-1 were based on sequences reported by Revest et al. (2005) and β-actin as reported by Caldwell et al. (2008). The specificity of each primer pair was confirmed by the identification of a single PCR product of predicted size on 1.5% (w/v) agarose gels.

4.3.8 Semi-quantitative determination of transcript levels by Real-time PCR

Real-time PCR was conducted in a Gene Amp 7300 sequence detection system (Applied Biosystems) using a 96-well plate (MicroAmp™ Fast). The relative quantification of the Ras and Raf genes in different tissue samples was determined using the $2^{-\Delta\Delta Ct}$ method described by Livak and Schmittgen (2001); Initially, the ΔCt value, the difference between the average of the triplicate Ct values for the H-Ras and Raf-1 and the internal control β-actin, for each sample was calculated ($\Delta Ct_{sample} = \Delta Ct_{Ras} - \Delta Ct_{B\text{-}actin}$). Next, the average of the ΔCt values for the control animals ($\Delta Ct_{Con\ av}$) was determined and subtracted from each sample ($\Delta Ct_{sample} - \Delta Ct_{\ Con\ Av}$), giving a ΔCt value for each. This was used to calculate the $2^{-\Delta\Delta Ct}$ value for each. Because 2^0 is one, the mean $2^{-\Delta\Delta Ct}$ value for each control sample was approximately one, depending on the variability in the samples, and the mean $2^{-\Delta\Delta Ct}$ value for the arsenic samples was expressed relative to one.

4.3.9 Activated ERK Protein

Nuclear fractions from 8 hippocampi from arsenic-exposed and 8 control animals were analyzed for phosphorylated ERK via western blot according to Buckley et

al. (2004) and our established protocols. Briefly, extracts were thawed on ice, diluted in 4X SDS-PAGE sample buffer (Invitrogen, Carlsbad, CA) and heated at 70oC for 10 minutes. Samples (3µg protein per well) were separated using 12% NuPAGE Bis-Tris gels (Invitrogen) and transferred to 0.45-um-thick nitrocellulose membranes (Invitrogen). The membranes were blocked with 0.25% I-block (Applied Biosystems, Foster City, CA) in TBS-T (25mM Tris-HCl pH 7.2, 150mM NaCl and 0.05% Tween-20) for one hour at room temperature. Blots were then incubated with a polyclonal primary antibody to anti-phospho-ERK1/2 (1:3,000; Cell Signaling, 9101) overnight at 4oC. The reaction was stopped with four consecutive 5-minute washes in TBS-T. A goat anti-rabbit IgG (H+L):HRP (1:50 000, Thermo Fisher Scientific) was used for the secondary antibody incubation in 0.25% I-block one hour at room temperature. The reaction was stopped with four consecutive 5-minute washes in TBS-T. Membranes were then incubated in Supersignal West Pico Working Solution (Thermo Scientific) for five minutes and exposed to F-BX57 film (Phenix Research Products, Candler, NC). Film was developed in Kodak D-19 developer then washed and fixed in Kodak fixer. The blots were then stripped (45 minutes in 10ml Restore Western Blot Stripping Buffer, Thermo Scientific, 21059) and reprobed for total ERK (1:50,000; Cell Signaling, 9102) and stripped again and probed for β-actin (loading control)(Cell Signaling, 4967L). Blotting for ERK 1 and 2 follow the protocol described by Buckley et al. (2004). The linear range of the phosphorylated and total protein was calculated to ensure that the protein was not saturating the blot. Control animals were run on the same blot as arsenic-exposed animals. Gels were run

and analyzed in duplicate. The developed film was scanned (Hewlett Packard Scan Jet 5P) and immunoreactivities quantified by measurements of optical densities using BioRad Quantity-One analysis software. The amount of p-ERK were compared to the total ERK of the sample and also compared to β-actin and data were analyzed by t-test.

4.3.10 Statistical analysis

All data were analyzed by t-test comparing the perinatal arsenic to the control. Significance was set at $p \leq 0.05$. Data were analyzed and graphs were generated using GraphPad Prism (v4.03).

4.4 RESULTS

4.4.1 Arsenic Exposure Paradigm

The dose of arsenic used in present study did not significantly alter maternal water consumption, maternal body weight, body weights of the newborn pups, brain wet weights or hippocampal wet weights (Martinez et al., 2008; Martinez-Finley et al., 2009).

4.4.2 Chromatin Immunoprecipitation

To determine if GR is able to bind to GREs found in promoters of genes of the MAPK pathway we used ChIP to assess the *in vivo* association of GR with Ras and Raf-1 promoters. Compared to controls, perinatal arsenic animals had lower GR binding to GREs in the promoters of Ras genes (fig. 4.1A) ($t(8) = 2.38$;*$p = 0.04$). Figures 4.1B and 4.1D are representative of the PCR products of each

real-time run. Compared to controls, perinatal arsenic animals had lower GR binding to GREs in the Raf-1 promoter (fig. 4.1C) ($t(7) = 2.15$; $p = 0.06$), although not statistically significant there is an obvious reduction in binding. Figures are the result of four to five samples per condition run in five independent experiments.

4.4.3 Supershift EMSA

To determine if the decreased binding seen in the ChIP experiments was an artifact of the reduced GR levels we performed an electrophoretic mobility shift assay (EMSA). Nuclear hippocampal extracts from perinatal arsenic exposed and control animals were incubated with a radiolabeled consensus GRE (fig. 4.2). Control and arsenic-exposed samples were incubated with or without anti-GR antibody to reveal the presence of GR in the nuclear protein-radiolabeled GRE complex. The specificity of the EMSA was confirmed by competitition experiments using unlabeled oligonucleotide sequences in 5-fold molar excess. The results are representative of four assays. We did not deem it necessary to stimulate with dexamethasone prior to extracting the nuclear fraction of the cells as others have shown a modest increase in intensity after stimulation but presence of the band without stimulation (Tirumurugaan et al., 2008; Barr et al., 2009). For our purposes, we were solely interested in whether or not binding was apparent in our treatment group compared to our controls. Results showed that nuclear extracts from both control and arsenic-exposed animals were able to bind to a consensus GRE (fig. 4.2). We are unsure whether or not GR is in this complex as all attempts to probe for GR have turned out unsuccessful. While this

is important to know, it is not the end of the story as GRs ability to activate transcription is not always dependent on direct binding of GR to GREs (Reichardt et al., 1998).

4.4.4 Ras and Raf-1 mRNA levels

To determine if the decreased binding in our ChIP experiment was associated with a significant reduction in mRNA, we examined Ras and Raf-1 mRNA levels. In the perinatal arsenic-exposed offspring Ras and Raf-1 mRNA expression was inhibited as determined by real-time reverse transcriptase-polymerase chain reaction. Figure 3A Ras mRNA levels ($t(16) = 4.35$, ***$p = 0.0005$). Figure 3B Raf-1 mRNA levels ($t(15) = 3.55$, **$p = 0.003$). The Cycle threshold (Ct) values were determined in triplicate for both groups. The average Ct. values for β-actin, which was used as a housekeeping gene were not different between the treatment and control groups. Data are presented as mean ± SEM of eight-ten litters.

4.4.5 Activated ERK

To assess the effect of lower mRNA levels on activation of the MAPK pathway we assessed the activation of the downstream target ERK1/2 (fig. 4). ERK1/2 has been implicated in learning and memory, thus it seemed a logical target to assess. We examined phosphorylated and total ERK1/2 expression levels in both treatment groups. Treatment with arsenic resulted in decreased phosphorylation of ERK1/2 (p42/44), as quantified by densitometery and normalized to total levels

(t(9) = 4.05, **p < 0.003). There was no difference in total ERK1/2 (data not shown).

4.5 DISCUSSION

We have previously reported decreases in GR and MR levels in hippocampus of moderate (50ppb) perinatal arsenic-exposed animals leading us to examine the status of genes that are under the control of the GR. Many components of the MAPK pathway have been shown to be under this type of control. The MAPK pathway has been associated with learning and memory and depression, two behavioral responses that were altered in our animals (Martinez-Finley et al., 2009; Martinez et al., 2008). In this work we provide evidence for decreased GR/GRE binding leading to a decrease in transcripts of genes that are under the control of GR.

Our ChIP-real-time PCR data indicates decreased GR binding at Ras and Raf-1 promoter sites (fig. 4.1A, 4.1C) and lower Ras and Raf-1 mRNA levels (fig. 4.3). Because many promoters contain GREs, genes other than the ones studied here could be affected. In support of this possibility, we previously showed increases in 5-HT_{1A} receptor density in the dorsal hippocampus of our perinatal arsenic-exposed mice (Martinez et al., 2008). The 5-HT_{1A} receptor is negatively regulated by corticosteroids through the GR (Chalmers et al., 1993; Datson et al., 2001; de Kloet et al., 1986; Meijer et al., 1997). Reduced GR binding to negative GREs would allow for transcription and ultimately upregulation of the 5-HT_{1A} gene and may be one example of the consequences of reduced nuclear GRs or reduced

binding ability. The data from the EMSA experiments show that proteins in the nuclear fraction of perinatal arsenic-exposed offspring are able to bind to the synthetic GREs, even though we are unsure as to whether or not GR is in this complex (fig. 4.2). This is somewhat perplexing but can be explained by the fact that transcriptional regulation by GR does not require direct association with GREs, but instead may affect transcription through the presence of other transcription factors. One caveat of these experiments is that transcriptional activity of steroid receptors may be dependent not only on specific DNA-binding elements but also on combinatorial control generated by interactions with other receptors and additional DNA-binding and non-DNA binding proteins. It is well recognized that the specificity of the response depends on the tissue and cell type, as well as on the characteristics of the promoter. At the PND 35 time point when we are testing, arsenic is no longer detectable in the hippocampal region in our animals (unpublished results) and therefore cannot be regulating transcription via a steric hindrance. Our data suggest that there is a deficit in GR binding to promoter regions in vivo but EMSA results suggest that binding ability is present when assessed with synthetic GREs. Thus, we suspect that the inhibition is occurring at the promoter regions. Hypermethylation at these regions could be a possible mechanism of inhibition that is initiated by arsenic during the perinatal period. We are currently investigating this theory.

In our studies using gene expression as a tool for toxicity assessment we detected mRNA effects after moderate (50ppb) perinatal arsenic exposure (fig. 4.3). These lower levels mRNA levels are thought to be mediated by the GR, as

transcription of Ras and Raf-1 are under the control of GR (Revest et al., 2005). Rapid actions of GRs on cellular signaling pathways, such as an increase in the expression levels of components of the MAPK pathway but with a later increase in the phosphorylated forms of these proteins, have been described (Revest et al., 2005). Even though we did not measure protein levels of Ras and Raf-1, we did assess the activation of ERK, which is under control of Ras and Raf-1 (B-raf is also implicated in brain MAPK signaling but we did not measure these levels and therefore cannot comment on them here) (reviewed in Avruch, 2007). It is not always the case that mRNA levels are correlated to protein levels or coincide with effects on cell function but it has been shown that mRNA levels are more predictive of protein levels than total RNA levels are (Pradet-Balade et al., 2001;Greenbaum et al., 2003). Revest and colleagues have shown that the GR-mediated activation of the MAPK pathway that culminates in the phosphorylation of ERK seems to be mediated by an increase in the steady-state levels of upstream proteins (Revest et al., 2005). If the decrease in activated ERK levels in our arsenic offspring (fig. 4.4) are indicative of lower protein activity, then it is possible that this is the result of decreased levels of Ras and Raf-1 proteins.

The MAPK signaling cascade is involved in various forms of synaptic and behavioural plasticity (Sweatt, 2001) but most importantly for this study, couples GR activation to ERK phosphorylation. ERK2 phosphorylation was dramatically decreased in our animals, in the absence of change in total ERK levels (fig. 4.4). If the amount of Ras and Raf-1 protein is reflected by the degree of phosphorylation of ERK and the lower levels of Ras and Raf-1 transcripts, the

data further supports the hypothesis that the MAPK pathway is involved in mediating the learning and memory deficits in response to GR. The discovery that ERKs are widely expressed in post-mitotic neurons in the mammalian nervous system and are activated in response to excitatory glutamatergic signaling led to the identification of an important role for ERK in synaptic plasticity and learning and memory (English and Sweatt, 1996; Sweatt, 2001; Thomas and Huganir, 2004). We have reported deficits in learning and memory tasks in our perinatal arsenic-exposed mice (Martinez-Finley et al., 2009). Intrahippocampal infusions of MAPK pathway inhibitors, prior to training, impair memory retention after training in the Morris Water Maze (Blum et al., 1999; Selcher et al., 1999). There is new evidence that suggests that the ERK2 isoform is especially important in learning and memory. A mouse ERK2 knockout study reported deficits in associative learning in a fear conditioning assay (Samuels et al., 2008). Satoh and colleagues reported impairment in fear conditioning and deficits in two spatial memory tasks in ERK2 hypomorphic mice that show a 30% reduction in ERK2 levels and an overtly normal brain (Satoh et al., 2007).

Arsenic has been shown to disrupt gene transcription in various models (Hamilton et al., 1998; Kaltreider et al., 2001; Bodwell et al., 2006). Because of differences in the exposure periods and the in vitro vs. in vivo systems it is difficult to interpret work presented by others with regard to arsenic and GR-mediated transcription. Our perinatal model is a unique view of arsenic disruption of gene regulation. Prolonged exposure to low concentrations of persistent neurotoxic environmental pollutants can lead to neuronal dysfunction, particularly

during development, when the brain is most sensitive. It is difficult to predict the consequences of long-term dysregulation of GR-mediated transcription, as we have not followed our mice for longer than 35 days. By analyzing changes in the gene expression profile of cells exposed to toxicants, it will be possible to find genes/gene clusters that are commonly or specifically challenged in response to damage associated with developmental neurotoxicants (Sunol, 2010). Overall, these data suggest that moderate perinatal arsenic can have long-term effects on disruption of steroid hormone-regulated gene transcription well into adolescence, long after the arsenic has been metabolized.

4.6 FUNDING

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4.7 ACKNOWLEDGEMENTS

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4.8 FIGURES

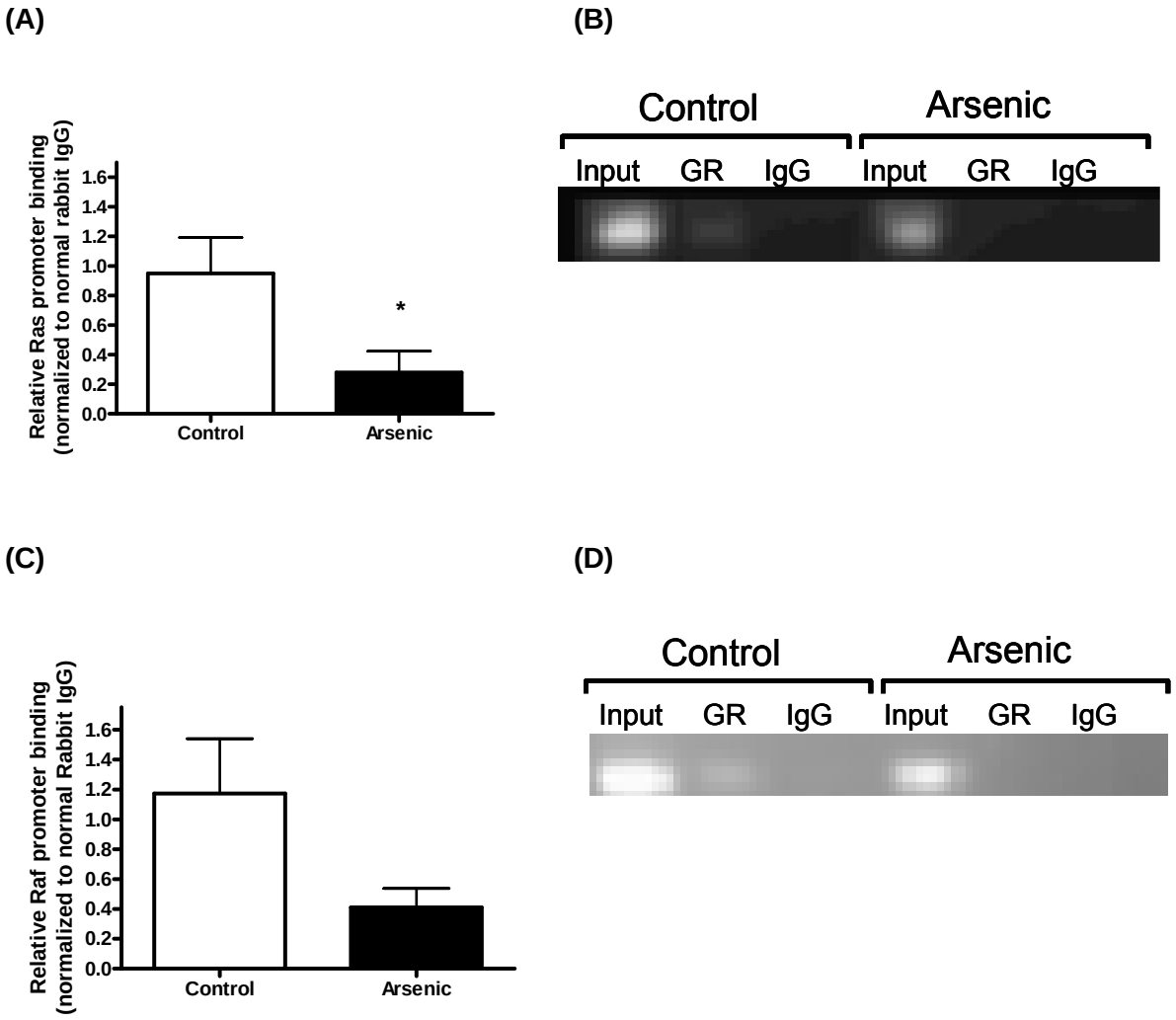


Figure 4.1 Perinatal arsenic exposure reduces glucocorticoid receptor (GR) binding to Ras and Raf-1 promoters. (A) Results of chromatin immunoprecipitation with anti-GR antibody and nuclear tissue extracts and real-time PCR for Ras (*p = 0.04 vs. control). (B) Gel showing real-time PCR product bands for Input (positive control), GR and IgG (negative control). (C) Results of chromatin immunoprecipitation with anti-GR antibody and nuclear tissue extracts and real-time PCR for Raf-1 (p = 0.06 vs. control). (D) Gel showing real-time

PCR bands for Input (positive control), GR and IgG (negative control). Results are presented as relative promoter binding, relative to controls, after subtracting for non-immune rabbit IgG interactions. Results are representative of five separate experiments and are presented as mean ± SEM of four-five litters. GR: Glucocorticoid Receptor; IgG: normal rabbit IgG

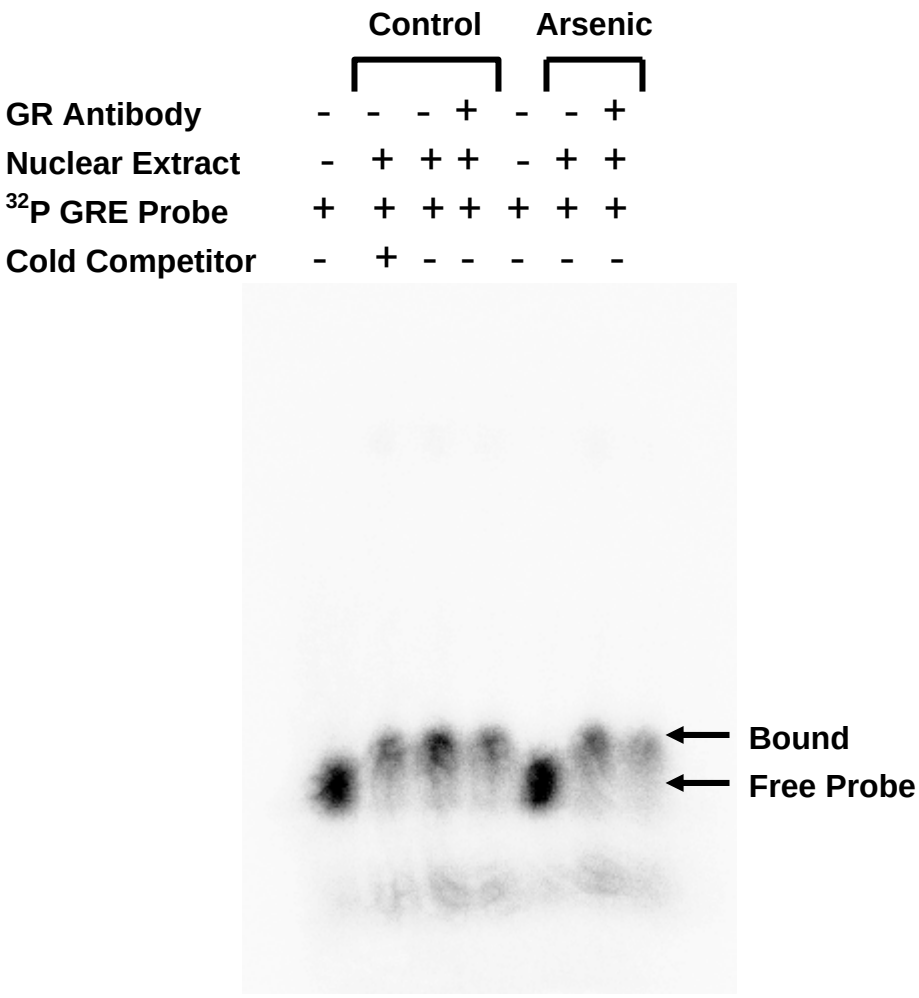
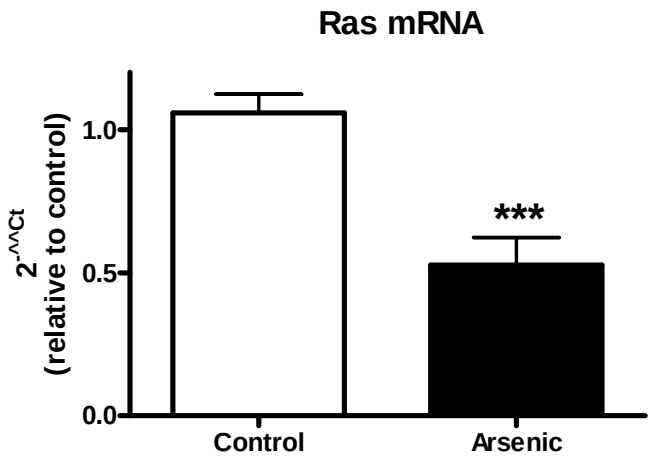


Figure 4.2 Perinatal arsenic exposure does not affect GRE binding in nuclear extracts. Bands labeled as 'bound' are the result of binding of [^{32}P]-labeled consensus GRE with nuclear proteins from arsenic-exposed and control hippocampal tissue resulting in a 'shift' of the complex on the gel versus the free probe. Free probe represents [^{32}P]-labeled consensus GRE that was run in the absence of nuclear extract. Specificity was determined using [^{32}P]-labeled consensus GRE along with 5-fold molar excess cold oligonucleotide and labeled as Cold Competitor (CC). In this figure the CC was incubated along with the first control sample. Result is representative of eight independent experiments.

(A)



(B)

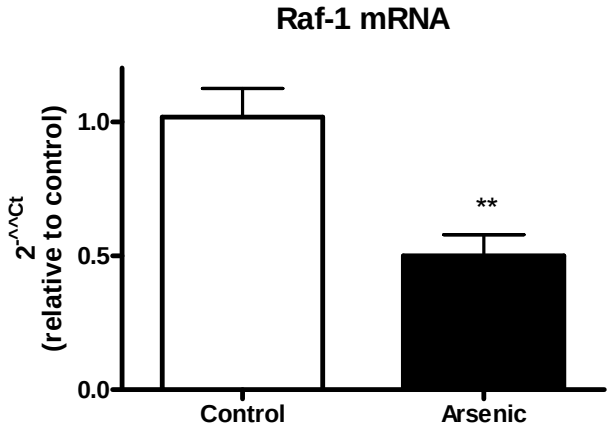
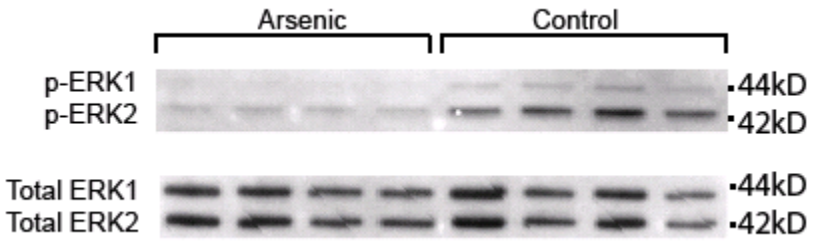


Figure 4.3 Reduced MAPK pathway mRNA levels in perinatal arsenic-exposed offspring compared to controls. (A) Ras mRNA levels in arsenic-exposed offspring and controls (***p = 0.0005 vs. control). (B) Raf-1 mRNA levels in arsenic-exposed offspring and controls (**p = 0.003 vs. control). Results are expressed as a percentage of the Ras or Raf signal to the β-actin signal, relative to control animals, and are presented as mean ± SEM of eight-ten litters.

(A)



(B)

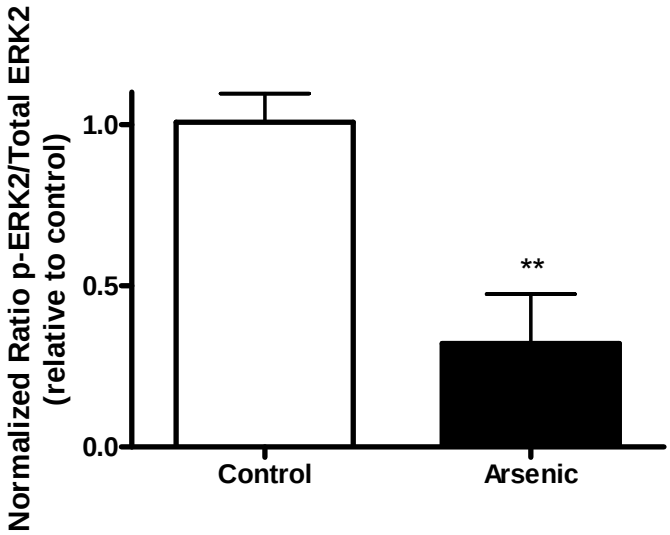


Figure 4.4 Activated ERK in hippocampal nuclear subcellular fraction. (A) Representative western blot of p-ERK1/2, total ERK1/2 and β-actin in the nuclear compartment of perinatal exposed mice. (B) Quantitation of p-ERK, total ERK, and B-actin bands in hippocampal nuclear subcellular fraction of arsenic exposed mice was based on densitometric analysis (**p = 0.003 vs. control). Results are expressed as a percentage of the p-ERK2 signal to total ERK2 signal, relative to control animals, and are presented as mean ± SEM of five-six litters.

Chapter 5. CONCLUSIONS

5.1 OVERALL FINDINGS

At the onset of this dissertation project it was known that arsenic is capable of inducing toxicological effects in multiple body systems, while less was known about its neurotoxicity. Further, prior to our studies there was limited information on arsenic as a prenatal insult and an incomplete description of the mechanistic damage. The overall goal of our research was threefold. The first goal was to reveal whether or not arsenic exposure, at levels commonly seen in drinking water in the US, was detrimental to learning. The second was to give some indication of exposure time periods (gestational vs. postweaning) when cognitive function would be particularly susceptible to damage by arsenic. And the third was to explore a mechanism for arsenic damage which could explain the cognitive deficits reported in human populations.

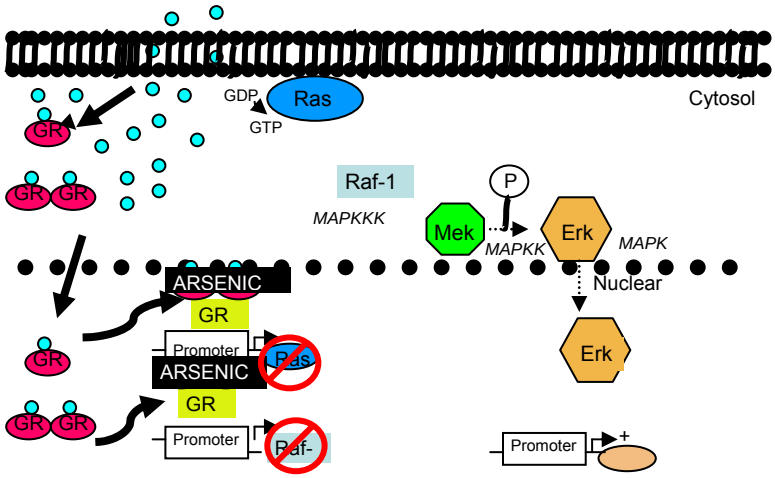


Figure 5.1 Diagram of arsenic-induced changes in perinatal-exposed offspring

Learning and memory impairments after exposure to arsenic were first described in a Mexican study in 2001 (Calderon et al., 2001). That report was soon followed by others (from different countries) that correlated learning and memory deficits in adolescents to arsenic exposure (Wasserman et al., 2004; von Ehrenstein et al., 2007). The arsenic-exposure model developed in the present studies displayed measurable deficits in learning and memory (figs. 3.3-3.4) and depressive-like behavior (figs. 2.6-2.7) similar to those reported in human studies. Results from the novel object exploration test revealed deficits in latency to the novel object and in number of center entries in perinatal-exposed animals indicating that arsenic-exposed animals have learning and memory deficits (fig. 3.3). The 8-way Radial Arm Maze illustrated that arsenic-exposed offspring have difficulties performing more arduous hippocampal-sensitive learning tasks (fig. 3.4). Arsenic-exposed offspring failed to make improvements in the task as the days progressed. While unable to definitively link these behavioral alterations to a specific neurochemical change, our data strongly implicate deficits in glucocorticoid regulation of gene expression. In support of this conclusion was the finding that arsenic induced lower GR levels (figs. 3.1 and 5.1) and produced deficits in GR-mediated transcription of genes within the MAPK pathway (figs. 4.1, 4.3 and 5.1) critical for learning and memory. These findings add to the overall picture of arsenic neurotoxicity by describing the learning deficit in a rodent model after moderate (50ppb) exposure, identifying the perinatal period as a susceptible time period for arsenic-induced damage and by providing a possible mechanism of arsenic damage in the brain.

5.2 LIMITATIONS

In our mouse model, moderate perinatal arsenic exposure produced deficits in learning and memory. However there are several limitations to our current set of studies. First, it is difficult to directly link a behavioral deficit, like learning and memory, to an environmental insult or to a particular neurochemical mechanism of that insult. For example, arsenic is likely to cause a number of cellular changes, including disruption of zinc-associated proteins (reviewed in Beyersmann and Hartwig, 2008), inhibit DNA repair (Qin et al., 2008; reviewed in Gentry et al., 2010), induce chromosomal aberrations (reviewed in Salnikow and Zhitkovich, 2008), alter apoptosis (Pi et al., 2005), induce oxidative stress through hydroxyl radical formation (Shi et al., 2004) as well as other mechanisms. To accomplish a direct causal connection between perinatal arsenic and the subsequent adolescent behavior, we would need to be able to reverse the effect at a molecular level and restore normal learning in the animal. At this point, the best that we can do is correlate the presence of the behavior to the disruptions in the MAPK pathway, as several studies support a requirement for the MAPK/ERK pathway genes in hippocampal-sensitive associative and spatial learning (Sweatt, 2004).

While MAPK gene expression is necessary for learning, there are other biochemical pathways which, if damaged, will also lead to failed learning performance. The impaired performance found in our studies could be explained by loss of nuclear MR, which was seen in the arsenic-exposed mice. Findings from mutant mice with inactivated MR showed impaired learning of the water-

maze task and deficits in measures of working memory on the radial arm maze, as well as hyperreactivity toward a novel object, but normal anxiety-like behavior (Berger et al., 2006). Alternatively, anxiety differences between the treatment groups could have been misinterpreted as performance deficits. Habituation to the novel object open field prior to testing should have ameliorated this concern. The 8-way Radial Arm Maze illustrated that arsenic-exposed offspring have difficulties performing more arduous hippocampal-sensitive learning tasks (fig. 3.4). Arsenic-exposed offspring failed to make improvements in the task as the days progressed. If the arsenic mice had a reduced anxiety response, the mice would have displayed a greater willingness to enter the maze arms and this was not seen in our study.

A reported effect following oral exposure to inorganic arsenic is the development of peripheral neuropathy (ATSDR Arsenic, 2007). It is not possible that the learning and memory behaviors that we have seen are due to decreased ambulation as a result of peripheral neuropathy. We ran measured quadrant crossing in both the novel object and radial arm maze as well as measured startle response in these animals and found that the perinatal arsenic animals ambulated just as much as controls (data not shown).

Another limitation to the studies described in this dissertation is that the effects of arsenic were assessed in adolescents long after the perinatal exposure. The temporal separation between the exposure and the measurements makes it more difficult to correlate the two events because of the possibility that

experiences occurring in the intervening period could have contributed to the altered phenotype. There is an added level of difficulty in determining if the effects are a direct result of the toxin itself or a secondary, indirect effect of the exposure on the mother. The delivery of arsenic was to the pregnant dam and not to the developing fetus, thus, the maternal response to the toxin can produce its own insult. An example of this is the high plasma corticosterone levels that we have reported in our perinatally exposed animals, which was assessed under baseline, non-stressed circumstances (fig. 2.1). It is unclear whether those levels are the direct result of arsenic or the result of the mother's response to the arsenic during gestation. The absence of the elevated basal CORT in the postnatal exposure group (fig. D1) suggests that the high levels seen in our perinatal animals may be a result of the mother's response to the arsenic. While this may be the case, it doesn't make it any less of a problem in our perinatal animals. More research needs to be done to parse out what the effect of high CORT is versus the effect of the arsenic exposure. Even if high CORT is the result of the maternal response to arsenic exposure producing a programming change in the fetal HPA axis and not a direct fetal response to the presence of arsenic, our conclusions in these studies would remain unchanged.

Initial analysis of the high corticosterone suggested that the HPA axis was overactive in the perinatal arsenic-exposed offspring. A dexamethasone-stimulated corticosterone response was measured to determine the functionality of the negative feedback pathway at the level of the adrenals of the HPA axis. Although we had a small sample size, our results indicate that the HPA axis has

a higher set point in perinatal arsenic-exposed animals but can elevate corticosterone levels in response to a stressor (saline injection) and inhibit corticosterone secretion in response to dexamethasone in accordance with negative feedback regulation (fig. A.1). These results indicate that, at the level of the adrenals, the HPA axis responds 'normally' (fig. A.1). However, we did not measure CRF or ACTH secretion and thus cannot remark on the status of the receptors at these levels nor can we conclusively say that the regulation at either the hypothalamus or the pituitary is 'normal.' We can conclude from this test that the amount of regulation present is enough to dampen the amount of corticosterone secreted after DEX administration, an outcome that is present in a 'normal,' intact HPA axis. To better characterize regulation of the axis, a CRF-stimulated ACTH/CORT response should be a focus of future studies. This type of test would give us more information about the functioning of the negative feedback arm of the HPA axis at the level of the pituitary.

Results from the present experiments revealed lower abundance of hippocampal cytosolic GRs in perinatal arsenic-exposed offspring compared to controls but no difference in the abundance of cytosolic MRs. Nuclear levels of both GR and MR were significantly lower than those of controls (Martinez-Finley et al., 2009). A plausible explanation for lower nuclear GR and MR levels is failure of the receptors to traffic to the nucleus. While other groups have investigated this possibility and found that the trafficking of these receptors to the nucleus is unaffected by arsenic (Kaltreider et al., 2001), these studies were conducted using a hepatoma cell line and the results may not be the same in the brain.

Differential programming of the GR in different tissues has been shown and is attributed to the presence of multiple tissue-specific alternate first exons/promoters of the GR gene (Seckl and Meaney, 2004). In addition, the cell line fails to maintain the contribution of the intact HPA axis and does not account for genes that may have previously been affected. For example, high CORT levels have been shown to reduce dynein cytosplasmic intermediate chain 1 gene expression, reducing motor protein activity and axonal transport (Morsink et al., 2006), a result that would not have been taken into account in the hepatoma cell line. In accordance with our high CORT, the translocation of GR to the nucleus by dynein may be reduced. Lower levels of nuclear GR and MR would have far-reaching consequences on gene transcription. It has been shown that, in particular for GR, nuclear translocation of the activated receptor and its ability to induce transcriptional activity are intimately correlated (de Kloet et al., 2009).

We tested GR transcriptional activity of two genes of the MAPK pathway and found reduced binding of GR to their promoters (fig. 4.1) and reduced mRNA levels in our perinatal arsenic-exposed animals compared to controls (fig. 4.3). Arsenic's inhibition of GR binding at Ras and Raf-1 promoters (fig. 4.1) is not likely to be due to steric hinderance affecting the binding of GR to the GRE because arsenic levels were undetectable in hippocampal tissue at the 35 day time point, when our tests were conducted (data not shown). Although we are not certain what the exact mechanism might be, based on our ChIP data, we suspect that there is a decrease in GR-GRE binding ability (fig. 4.1). A co-immunoprecipitation study targeting the transcription cofactors that interact with

GRE-bound GR may show a decreased association with GR in pre-exposed animals. An alternative explanation for the reduced levels of Ras and Raf expression is that arsenic does not inhibit GR binding to GRE; rather it inhibits recruitment of the transcription machinery. However, our EMSA data, which shows binding of nuclear protein with synthetic GREs, suggest that nuclear proteins are able to be recruited and bind to a GRE, although we are unsure whether or not GR is in the complex. Given these observations, it is possible that arsenic could induce changes in methylation at promoter sites which would reduce expression of Ras and Raf and would explain the lack of an arsenic effect in the EMSA data (fig. 4.2). Methylation appears to influence gene expression by affecting the interactions with DNA of both chromatin proteins and specific transcription factors (Razin and Cedar, 1991). Our EMSA data (fig. 4.2) show that nuclear proteins from both arsenic-exposed and control animals are able to interact with synthetic GREs, suggesting that the binding problem in our ChIP data (fig. 4.1) may be the result of changes in the *in vivo* promoters. There can be multiple methylation sites in promoters, which will change how tightly the chromatin is bound and what types of interactions can occur. If there were hypermethylation in our arsenic-exposed animals, then we would expect to see reduced binding of GR in *in vivo* promoters but not in synthetic sequences, which is what our ChIP and EMSA data report.

The perinatal period, defined in our model from gestational day 1 through PND 23, encompasses the key developmental periods in most species; the brain is particularly vulnerable during development and the damage induced by

neurotoxic agents is likely to be long-lasting. This period is critical because it influences how the animals will respond to their external environment throughout their lives. Prenatal glucocorticoid excess or stress might link fetal maturation and adult pathophysiology, as prenatal glucocorticoid exposure permanently increases basal plasma corticosterone levels and reduces GRs and MRs in the hippocampus in adult rats (Seckl and Meaney, 2004). Our perinatal exposure period could be affecting the establishment of regulation of the HPA axis, enabling sustained or cumulative damage with further high CORT levels resulting in an inability to return to homeostasis and prevent a maladaptive overshoot. It is well established that chronically elevated levels of CORT, mediated by the binding to GRs and MRs, produce cognitive deficits (de Kloet et al., 2005, Joels et al., 2007; McEwen, 2007). A drawback of these studies is that we have not followed the perinatal-exposed animals past the 35 day time point to determine if deficits are persistent or can be recovered after a significant amount of time. In addition, we have reported that the hippocampal formation is one of the brain regions most susceptible to the consequences of perinatal arsenic exposure. Our studies have focused on the hippocampus, although other areas of the brain may also be affected. Also, learning and memory, although tied to the hippocampus, is also dependent on other areas of the brain, which we have yet to examine. It is reasonable to consider these other areas as arsenic is not preferentially sequestered in the hippocampus (Sanchez-Pena et al., 2010).

5.3 FUTURE DIRECTIONS

Further studies detailing the molecular effects of perinatal arsenic exposure on the central nervous system are essential to our understanding of arsenic's production of learning and memory deficits. Potential leads to follow include epigenetic effects, mainly changes in DNA methylation, as this has been shown to be important in long-term fetal programming and could be the mediator of the changes that we have shown. A second study would involve the assessment of other brain regions to determine the amount of damage associated with those areas and their contribution to the observed behavioral deficits. Future directions could also include monitoring the animals after a significant amount of time unexposed to see if the damage is persistent into later adulthood.

5.4 CONCLUSIONS

While a direct link between the poor performance in these behavioral tasks and reduced GR levels is not established by these studies, the findings are consistent with the interpretation that memory performance is affected by arsenic-induced decreases in GR and decreases in GR-mediated transcription of the MAPK pathway in the hippocampus. Overall the data suggest that moderate perinatal arsenic exposure can have effects on learning and memory and HPA axis function well into adolescence, consistent with the Fetal Basis of Adult Disease hypothesis (White et al., 2007), which postulates that many adult diseases have a fetal origin.

APENDICES

APPENDIX A: PERINATAL ARSENIC-EXPOSED ANIMALS HAVE NORMAL HPA NEGATIVE FEEDBACK REGULATION

Rationale: Due to the elevated CORT levels and lower levels of GRs in our perinatal exposed animals we chose to evaluate whether or not the arsenic-exposed offspring could produce a 'normal' stress response. A DEX-stimulated CORT response was measured to determine the functionality of the negative feedback arm of the HPA axis. Dexamethasone, a synthetic corticosteroid acting through GRs, when administered two hours prior to CORT measurement should have the effect of suppressing the secretion of CORT in accordance with negative feedback regulation. We did not measure CRF or ACTH secretion and thus cannot remark on the status of the receptors at these levels nor can we conclusively say that the regulation at either of these levels is 'normal.' We can conclude from this test that the amount of regulation present is enough to dampen the CORT response after DEX administration, an outcome that is present in a 'normal,' intact HPA axis.

Experimental Design and Data Analysis: Mice were given an intraperitoneal injection of dexamethasone (0.1mg/kg) or saline (0.1mg/kg) at 0900. Dexamethasone (Sigma-Aldrich cat #46165) was reconstituted in saline (0.91% w/v). The mice were then sacrificed by rapid decapitation 2 hours post injection and trunk blood was collected in EDTA-coated tubes. Plasma was separated and corticosterone levels were evaluated using a ^{125}I-RIA Corticosterone Kit for Rats/Mice (MP Biomedicals). Cort levels were analyzed by t-test comparing the

perinatal arsenic animals to the controls. Statistical significance was set at $p \leq 0.05$.

Results:

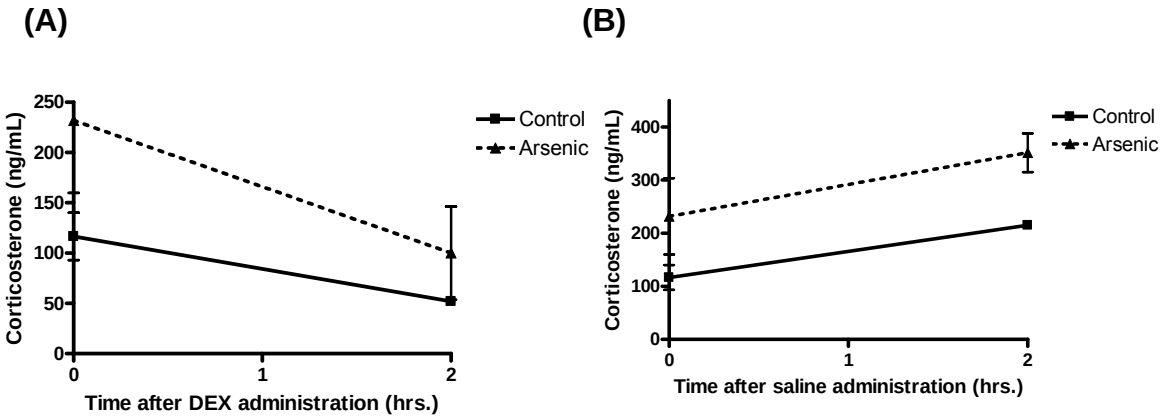


Figure A.1 The effect of DEX or saline administration on corticosterone negative feedback. Data bars represent the mean ± SEM nanograms corticosterone per mL plasma from one or three mice per treatment group.

Conclusions: The perinatal arsenic-exposed offspring appear to have higher basal CORT levels, confirming what we have previously published (Martinez et al., 2008). The higher baseline corticosterone seen in the perinatal-arsenic exposed animals does not appear to have an effect on their negative feedback response as they are able to lower the amount of CORT secreted in response to Dexamethasone administration. The arsenic-exposed animals are also able to mount a response as seen in the saline test, thus they have not reached a maximum level of CORT secretion. This data suggests that the perinatal exposed mice do not have a dysregulated HPA axis because the results suggest that the negative feedback regulation is intact.

APPENDIX B: INTRODUCTION TO POSTNATAL EXPOSURE

B.1 Rationale Behind Postnatal Exposure Model

Many of the changes that we have reported (Martinez et al., 2008; Martinez-Finley et al., 2009) in the perinatal arsenic-exposed mice could be the result of the sensitivity of the time of exposure as the developmental time period is a particularly sensitive time. One question that has not been addressed is whether or not the time of exposure to arsenic matters; both perinatal and postnatal arsenic exposure could potentially produce the deficits that we have reported. Thus far, epidemiological studies have been unable to separate the perinatal and postnatal exposure periods. Consequently, it is uncertain as to whether or not exposure to arsenic during development is more damaging than postnatal exposure. Postnatal acute arsenic exposure studies were run alongside the perinatal arsenic exposure studies and compared to control groups. The goal of this postnatal model was to discern if the deficits seen in arsenic exposed populations are due to developmental changes or due to a direct effect of the metal on nervous system functioning. This discernment becomes especially important when considering the moderate levels of arsenic which we are testing. The postnatal acute exposure paradigm was a short-term 50ppb exposure period occurring after weaning and lasting until day 35, when the animals were tested. We began the studies by looking for the learning and memory deficits. The results of those tests increased the importance of testing the acute postnatal group in other parameters to distinguish the developmentally linked with the acute arsenic effects. Postnatal arsenic exposure was well tolerated, as seen in

drinking water consumption, and did not result in overt toxicity, as seen in body, whole brain and hippocampal weights and litter sizes. Total inorganic arsenic concentrations detected in brain tissue of offspring at 35days of age were measured. Control brain levels were 1.0 ± 0.24 ppb and postnatal arsenic offspring levels were 3.18 ± 1.27 ppb. Levels of arsenic in the postnatal exposure group exceeded controls but was not significant due to the small sample size ($t(5) = 1.98$, $p \geq 0.05$).

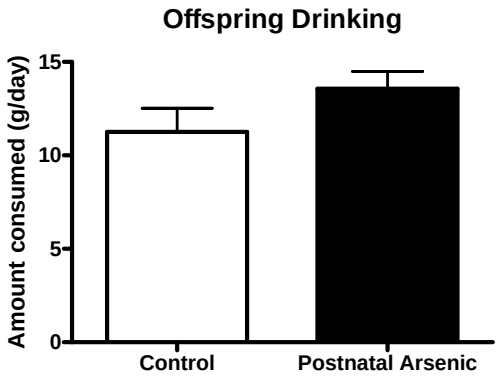


Figure B.1 Presence of arsenic in drinking water had no effect on amount consumed.

The presence of arsenic in the drinking water had no effect on the amount consumed ($p \geq 0.05$, not significant).

Treatment	Litter Size	Whole body weight at PND35	Whole brain weight at PND35	Hippocampal weight at PND35
Control	5.333 ± 0.802	16.94 ± 0.158	0.485 ± 0.011	0.036 ± 0.003
Postnatal As	7.714 ± 0.918	18.25 ± 0.133*	0.481 ± 0.008	0.040 ± 0.005

Figure B.2 Effect of postnatal arsenic exposure on litter size, whole body, whole brain and hippocampal weights.

There was no effect of arsenic on litter size, whole brain or hippocampal weights D35 ($p \geq 0.05$, not significant). There was an effect on whole body weight at D35, with arsenic-exposed animals weighing slightly more than controls (*$p \leq 0.05$). PND = Postnatal Day

APPENDIX C: IMPACT OF MODERATE POSTNATAL ARSENIC EXPOSURE ON SPATIAL AND NON-SPATIAL LEARNING AND MEMORY BEHAVIOR

Rationale: Epidemiological analysis has shown learning deficits in adolescents exposed to high concentrations of arsenic in drinking water (Calderone et al., 2001; Wasserman et al., 2004; von Ehrenstein et al., 2007). We examined the impact of postnatal arsenic exposure on two types of hippocampal learning tasks, nonspatial and spatial. The radial arm maze is a hippocampal-dependent task used to measure spatial learning and memory. The novel object recognition task is a nonspatial task which tests hippocampal and frontal cortical function (Baker and Kim, 2002). The 8-way radial arm maze was selected because it should be a sensitive measure of hippocampal spatial memory and the number of trials can be altered to either increase group differences or increase group reliability.

Experimental Design and Data Analysis: Radial Arm Maze: Radial arm maze testing was conducted using the mouse runway system from Coulbourn Instruments (Whitehall, PA) using a protocol modified from Egashira et al. (2002). Plastic food cups containing sweetened condensed milk as a reinforcer, were placed approximately 2/3rds the way down in each arm. The maze was located in a room containing many extra-maze visual cues. Arm entry was recorded by an observer seated several feet away from the maze. All mice were habituated to the maze for 20 minutes on the day prior to testing. One week before testing, mice were placed on a caloric restriction diet where they received 3 grams of lab chow (7004 Harlan Teklad S-2335, Denver, CO) each day. Untreated tap water was available *ad libitum*. Testing was conducted over 3 consecutive days. For

each 8 minute testing session, the mouse was placed in the center of the maze. For each testing session; the number of correct choices in the initial eight chosen arms and the number of errors which was defined as choosing arms which had already been visited were scored. **Novel Object Exploration:** The novel object exploration task was conducted in a plexiglas open field apparatus (43 cm x 43 cm x 21 cm high, designed by Allan laboratory) using a protocol described previously by Grailhe et al. (1999). The floor of the apparatus was black and divided into five areas: Four equal quadrants and one center area 14 cm. in diameter. The experiments were conducted in a dimly lit room with the aid of a video camera to minimize subject stress and anxiety. The floor and walls of the open field were wiped with 70% isopropanol before each test session. Each test consisted of two videotaped five-minute sessions. During the first session, mice were placed in the center area and allowed to explore and acclimate to the apparatus. A novel object, consisting of a pink and green striped gray cube (2.5 cm3) with an open side (designed by Allan laboratory), was placed in the center area with the open side facing the mouse. Response to the novel object during the second five minute session was videotaped. Subsequently, a trained observer blinded to experimental group identity analyzed the video tapes and measured latency to approach the object and total number of center line crosses in the presence of the novel object. Data were analyzed via t-test using GraphPad Prism (V.5)

Results:

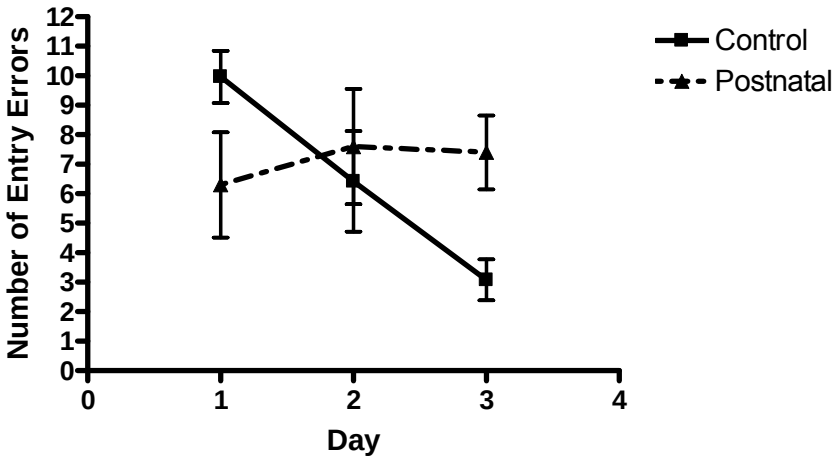


Figure C.1 Effects of postnatal arsenic-exposure on number of entry errors in 8-way Radial Arm Maze at D35.

Day 1 represents first day after acclimation period. Data are presented as mean ± SEM of five litters.

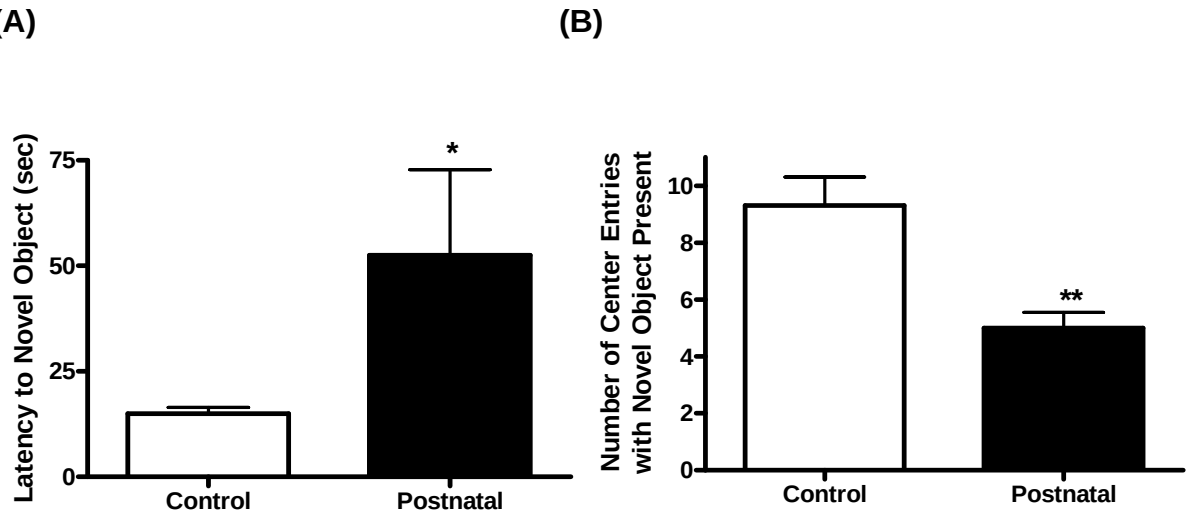


Figure C.2 Effects of postnatal arsenic exposure to 50ppb arsenic on novel object exploration at D35.

(A) Latency to approach novel object after acclimation period (*p < 0.03 vs. control). (B) Entries to center in the presence of the novel object (**p < 0.007 vs. control). Data are presented as mean ± SEM of four-seven litters.

Conclusions: Novel object test revealed a significant increase in latency to novel object in arsenic-exposed offspring compared to controls. Arsenic mice did not improve in number of entry errors in the 8-way radial arm maze as days progressed, similar to the perinatal exposed mice but unlike the controls. These data suggest that postnatal-arsenic exposed mice take longer to process novel information and fail to learn when challenged with hippocampal-dependent, spatial learning and non-spatial memory tasks.

APPENDIX D: IDENTIFYING POSTNATAL ARSENIC-INDUCED CHANGES WITHIN THE HYPOTHALAMIC-PITUITARY-ADRNEAL (HPA) STRESS AXIS

Rationale: The HPA axis findings in our perinatal arsenic exposed animals led us to question the integrity of components of the HPA axis in our postnatal arsenic-exposure model. As a result we evaluated corticosterone levels and nuclear and cytosolic GR levels in our postnatal arsenic animals. A DEX-stimulated CORT response was measured in addition to the basal CORT levels. Analysis of the levels of HPA hormones and receptors gave insight into the status of the feedback regulation in the postnatal exposure model.

Experimental Design and Data Analysis: CORT Levels: Litter mates of the mice tested in the behavioral tests were used in these studies. Five to six mice per treatment group were used. Radioimmunoassay (RIA) kits were used, according to manufacture instructions for CORT using manufacturer established protocols. Taking into account circadian rhythms, blood was taken between the hours of 0800 and 1000 to minimize variance. Plasma concentrations of CORT were determined through standard curve analysis. When we failed to get enough plasma from one animal, blood from males from the same litter were pooled and used for the analysis Comparisons between the arsenic treatment group and the corresponding control group was via t-test. **Dexamethasone Suppression Test:** Mice were given an intraperitoneal injection of dexamethasone (0.1mg/kg) or saline (0.1mg/kg) at 0900. Dexamethasone (Sigma-Aldrich cat #46165) was reconstituted in saline (0.91% w/v). The mice were then sacrificed by rapid decapitation 2 hours post injection and trunk blood was collected in EDTA-coated

tubes. Plasma was separated and corticosterone levels were evaluated using a ^{125}I-RIA Corticosterone Kit for Rats/Mice (MP Biomedicals). **GR Receptor:** Adolescent offspring, 35-40 days of age, were sacrificed by decapitation and whole hippocampal formation rapidly dissected. Subcellular fractions are prepared exactly as described by Buckley et al. (2004). Protein determinations were performed as described in Weeber et al. (2001) and were used to load equal amounts of protein in each well. To determine the lack of cytoplasmic contamination in the nuclear fraction, the membrane was probed between 68kDa and 21 kDa for anti-A-Raf (1:500; Santa Cruz) and anti-H-Ras (1:500; Santa Cruz) antibodies, respectively. Cytosolic and nuclear fractions from seven or eight hippocampi from arsenic-exposed and control animals were analyzed for GR via western blot. The linear range of the total protein was calculated to ensure that the protein was not saturating the blot (data not shown). Control samples were run on the same blot as arsenic-exposed samples. The amount of total GR of the sample was corrected for Beta-actin and data analyzed by t-test. Extracts were thawed on ice, diluted in 4X SDS-PAGE sample buffer (Invitrogen, Carlsbad, CA) and heated at 70°C for 10 minutes. Samples (2ug protein per well GR) were separated using 4-12% NuPAGE Bis-Tris gels (Invitrogen) and transferred to 0.45-um-thick nitrocellulose membranes (Invitrogen). The GR membranes were blocked with 0.25% I-block (Applied Biosystems, Foster City, CA) in TBS-T (25mM Tris-HCl pH 7.2, 150mM NaCl and 0.05% Tween-20) for one hour at room temperature. Blots were then cut and incubated with either a polyclonal primary antibody to GR (1:3,000; M-20, Santa Cruz Biotechnology,

Santa Cruz, CA) or B-actin (1:2,000; Cell Signaling, Boston, MA) overnight at 4°C. The reaction was stopped with four consecutive 5-minute washes in TBS-T. A goat anti-rabbit IgG (H+L):HRP (1:50,000, Thermo Fisher Scientific, Rockford, IL) was used for the secondary antibody incubation in 0.25% I-block one hour at room temperature. The reaction was stopped with four consecutive 5-minute washes in TBS-T. Membranes were then incubated in Supersignal West Pico Working Solution (Thermo Scientific) for five minutes and exposed to F-BX57 film (Phenix Research Products, Candler, NC). Film was developed in Kodak D-19 developer then washed and fixed in Kodak fixer. The developed film was scanned (Hewlett Packard Scan Jet 5P) and immunoreactivities quantified by measurements of optical densities using BioRad Quantity-One analysis software. Each protein sample was run in duplicate and the average optical density taken. All data were analyzed by t-test comparing the perinatal arsenic to the control on each of the dependent measures. Statistical significance was set at $p \leq 0.05$.

Results:

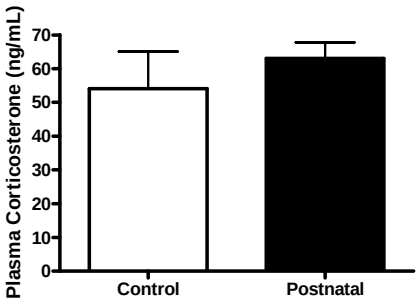


Figure D.1 The effect of moderate postnatal arsenic exposure on plasma corticosterone levels.

Data bars represent the mean ± SEM nanograms corticosterone per mL plasma from five or six mice per treatment group. There was not a significant difference in plasma corticosterone levels compared to the control water group ($p \geq 0.05$; not significant).

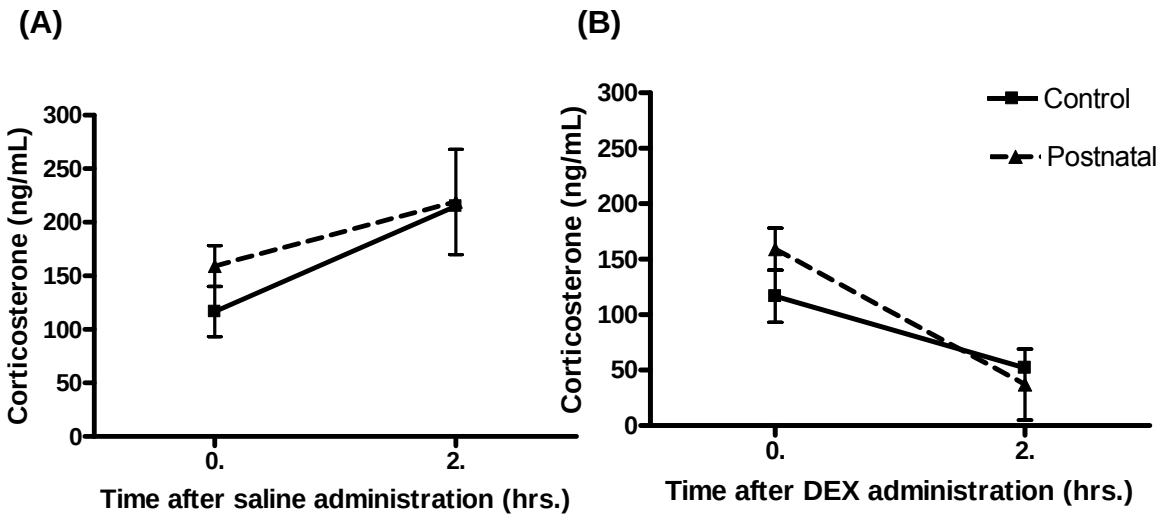


Figure D.2 The effect of saline or DEX administration on corticosterone negative feedback.

Data bars represent the mean ± SEM nanograms corticosterone per mL plasma from one or three mice per treatment group. There was not a significant difference in plasma corticosterone levels compared to the control group ($p \geq 0.05$; not significant).

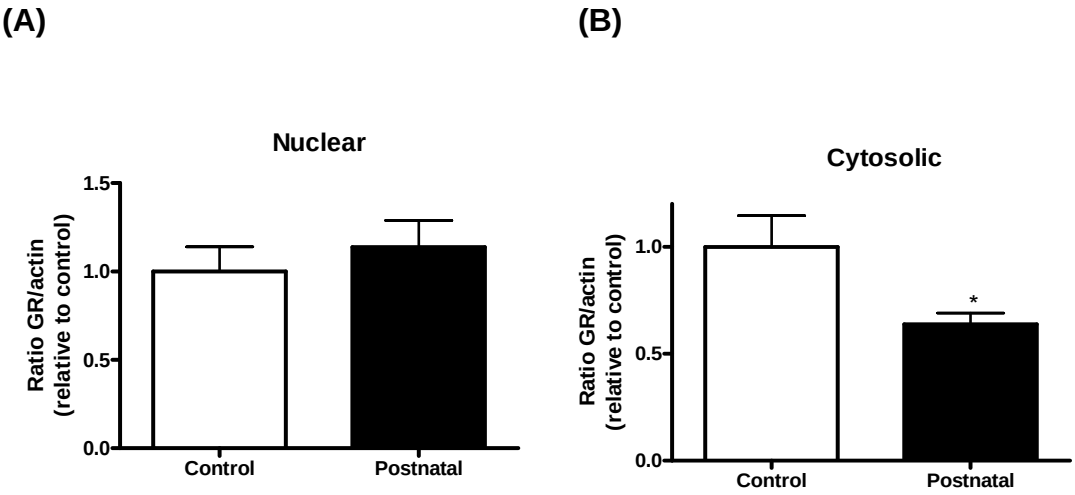


Figure D.3 Nucleocytoplasmic trafficking of GRs and densitometric quantitation of GR bands.

(A) Quantitation of GR bands in cytosolic subcellular fraction of arsenic exposed mice was based on densitometric analysis ($p > 0.05$ vs. control). (B) Quantitation of GR bands in nuclear subcellular fraction of arsenic exposed mice was based on densitometric analysis ($*p < 0.05$ vs. control). The anti-GR polyclnal antibody recognized a prominent band at ~95kD, representative of GRα. Results are expressed as a percentage of the GR signal to B-actin signal, relative to controls and are presented as mean ± SEM of seven-eight litters.

Conclusions: The postnatal exposure model revealed basal corticosterone levels similar to those seen in the controls. Dex-stimulated cort response was also similar to that of controls, indicating that both the setpoint and reponse to stimulation of the axis was not altered by presence of arsenic. Nuclear levels of GR were not different than controls; however there was a slight decrease in amount of cytosolic receptor relative to controls but not as robust a decrease as in the perinatal animals. Given these findings it is likely that the deficits reported in our perinatal arsenic animals are due to the sensitive perinatal time period. Although we cannot completely rule out the significance of having arsenic on-board because it may be that the exposure period in our postnatal animals is not long enough to elicit deficits in HPA axis parameters.

APPENDIX E: POSTNATAL ERK DATA

Rationale: Extracellular-signal regulated kinase (ERK) has been shown to be required in many different forms of learning in many species. Activation of the MAPK pathway leads to ERK phosphorylation (and ultimately its activation). In most cases, the effects on p-MAPK levels are not accompanied by changes in total MAPK levels. It is postulated that GR-activated transcription can ultimately lead to ERK phosphorylation (Revest et al., 2005). Therefore, arsenic inhibition of GR-induced transcription will ultimately lower the level of ERK phosphorylation. P-ERK has been shown to be important in molecular mechanisms for learning and memory.

Experimental Design and Data Analysis: Nuclear fractions from 5-8 hippocampi from arsenic-exposed and control animals were analyzed for phosphorylated ERK via western blot according to Buckley et al. (2004) and our established protocols (Martinez et al., 2008; Martinez-Finley et al., 2009). The blots were stripped and reprobed for total ERK and stripped again and probed for B-actin (loading control). Control animals were run on the same blot as arsenic-exposed animals. Gels were run and analyzed in duplicate. The amount of p-ERK was compared to the total ERK of the sample and also compared to B-actin and data were analyzed by t-test.

Results:

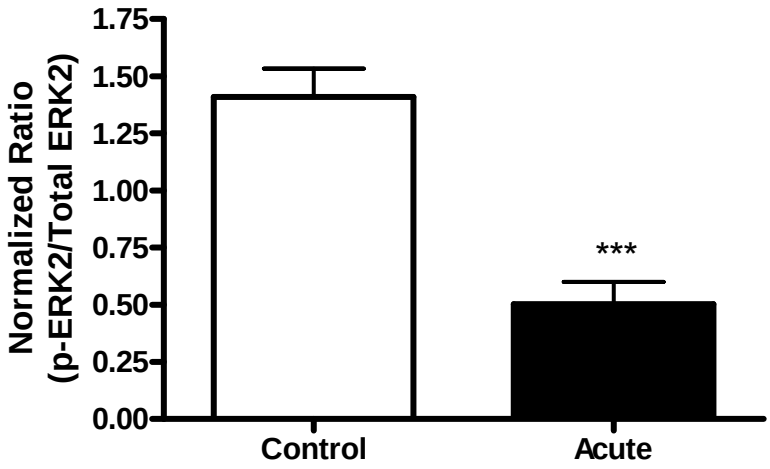


Figure E.1 Activated ERK in hippocampal nuclear subcellular fraction.

Quantitation of p-ERK, total ERK, and B-actin bands in hippocampal nuclear subcellular fraction of postnatal exposed mice was based on densitometric analysis (***$p < 0.0001$ vs. control). Results are expressed as a percentage of the p-ERK2 signal to total ERK2 signal, relative to controls, and are presented as mean ± SEM of nine-ten litters.

Conclusions: There was a decrease in activated ERK2 levels compared to controls. Lower levels of p-ERK could provide a mechanistic rationalization for the deficits in learning and memory observed in the arsenic-exposed mice.

APPENDIX F: OVERALL CONCLUSIONS FROM POSTNATAL DATA

To determine if the changes that we reported (Martinez et al., 2008; Martinez-Finley et al., 2009) in the perinatal arsenic-exposed mice were the result of the sensitivity of the time of exposure, we ran a subset of tests using an acute postweaning model. Results from the postnatal exposure model helped tease out which effects reported in the perinatal group were the direct result of arsenic on-board and which were the result of insults during the sensitive perinatal period. Novel object test revealed a significant increase in latency to approach the novel object in arsenic-exposed animals compared to controls (fig. C.2) along with a decrease in number of center entries in the presence of the novel object (fig. C2). These findings are consistent with the perinatal data. Postnatal arsenic-exposed mice did not improve in number of entry errors in the 8-way radial arm maze as days progressed (fig. C.1), similar to the perinatal exposed mice and unlike the controls (fig. 3.4). Collectively, these data suggest that postnatal-arsenic exposed mice take longer than controls to process novel information and fail to make the proper adjustments when challenged with hippocampal-dependent, spatial learning and non-spatial working memory tasks. The fact that we were able to elicit a learning and memory deficit in the postnatal exposure model (figs. C1-C2) indicates that the perinatal exposure period is not the only time-period that is sensitive to arsenic.

In the molecular studies, postnatal arsenic exposure decreased activated ERK2 levels compared to controls (fig. E.1) similar to those seen in the perinatal studies (fig. 4.4). Lower levels of p-ERK could provide a mechanistic rationalization for

the deficits in learning and memory observed in the arsenic-exposed mice. The learning and memory tests along with the ERK data suggest that arsenic is working through a similar mechanism in both exposure paradigms. We do not have enough data on the postnatal model to implicate the MAPK pathway in the learning and memory deficit but the ERK data suggests that the MAPK pathway may be involved, as ERK is one of the major MAP kinases.

The postnatal exposure model produced basal corticosterone levels similar to those seen in the controls (fig. D.1). However, when arsenic exposure occurs during the early developmental period as in the perinatal exposure model, basal corticosterone levels are significantly higher than controls (fig. 2.1), suggesting that the HPA axis is sensitive to arsenic damage during critical periods of the axis development. In support of this conclusion, the dexamethasone (DEX)-stimulated corticosterone response in the postnatal arsenic-exposure group was also similar to that of controls (fig. D.2), indicating that both the set point and response to stimulation of the axis were not altered by presence of arsenic when given at this later time point during development. Given that the DEX-stimulated corticosterone response was also intact in the perinatal group it appears likely that the perinatal animals have a higher set point but are able to both suppress and mount a stress response (fig. A.1).

There were some specific changes in the postnatal arsenic-exposure mice in the subcellular levels of glucocorticoid receptor. While postnatal nuclear levels of GR were not different than controls; there was a slight decrease in amount of

cytosolic receptor relative to controls. This decrease in the cytosolic GR fraction was not as robust a decrease as in the perinatal animals (figs. D.3, 3.1 and 3.2) suggesting that the degree of arsenics impact on GR distribution levels is dependent upon the timing of arsenic exposure.

The pattern of HPA axis damage as a result of either prenatal or postnatal arsenic exposure is variable with some measures not affected (fig. D1-D3) while others are affected to a differing degree (fig. D2). These data indicate that arsenic-induced damage to the HPA axis may be dependent upon the developmental stage of the axis. The overall mechanism of arsenic damage may be different in the two exposure paradigms. A careful assessment of pre- and postnatal exposures could be helpful in identifying the sensitive time points and the aspects of the HPA axis that are affected during the critical periods.

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