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Consequences of Prenatal Ethanol Exposure and Maternal Stress on Offspring

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DISSERTATION

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Dedication

For Josh and Remie,

With love and gratitude,

Always

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Abstract

Extensive evidence in humans suggests that exposure to insults during gestation, such as ethanol or maternal stress, can negatively impact the developing fetus in manners which can last into adulthood. These deleterious effects can include various behavioral challenges including cognitive impairments and increases in anxiety. These findings have been replicated in animal models of prenatal ethanol exposure and prenatal stress exposure, but little work has been conducted on measuring the interactive effects of the two prenatal insults in combination. Currently, there is no animal model of combined prenatal ethanol and prenatal stress exposures which has been utilized to systematically evaluate the potential interactive effects of the two gestational insults on adult offspring behavior and expression of biochemical markers of activity-dependent synaptic plasticity. Based on previous literature, it is believed that maternal stress will potentiate the prenatal ethanol induced learning deficits on a hippocampally-sensitive task, as well as potentiate the reduction in expression of biochemical markers of activity-dependent dentate gyrus synaptic plasticity. The model developed here employed a previously described voluntary ethanol consumption model and a novel predator scent exposure paradigm, resulting in no significant deficits in maternal weight gain, maternal ethanol consumption, maternal stress reactivity, maternal care of litters, number of pups per litter, nor the amount of weight gained from birth to weaning in the pups. Exposure to prenatal ethanol resulted in a significant decrease in learning in a trace fear conditioning task, and prenatal stress exposure resulted in heightened anxiety as measured by

performance in the elevated plus maze. However, the two insults did not interact to worsen either outcome. Assessment of ARC protein expression, a marker of neuronal activity, and AMPA receptor subunit expression, indicated a significant elevation in basal expression of ARC protein and a significant reduction in GluA1 subunit expression in the dentate gyrus of animals prenatally exposed to one or both insults as compared to non-exposed animals, as well a significant elevation in GluA1 and 2 following training in the trace fear conditioning task in non-exposed animals, but absent in all other groups. It is possible that the basal elevations in ARC expression could be the result of a compensatory up-regulation of neuronal activity in prenatally exposed animals, which results in the endocytosis of AMPA receptors from the cellular membrane. The reduction of AMPA receptors could explain, in part, the significant deficits in hippocampal-sensitive learning in animals exposed to prenatal ethanol. Future directions following this work should include assessments of male offspring, electrophysiological characterization, and inquiry into the potential for amelioration of the learning deficits by ampakine administration.

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1. Prenatal Ethanol

1.1 Occurrence and Effects of Prenatal Ethanol Exposure in Humans

Exposure to alcohol in utero has long been known to cause deleterious effects to the developing fetus for centuries with literary references as far back in The Bible and Aristotle's *Problemata*. It has only been relatively recently, however, that the set of defects consistently seen in children born to mothers who consumed alcohol was given a name and a definition. In the 1970's, dysmorphologists in the United States and France observed distinct physical and behavioral characteristics in children born to alcoholic mothers (Jones et al 1973, Jones and Smith, 1973, and Lemoine et al 1968). These effects were termed Fetal Alcohol Syndrome (FAS), as it was believed that the facial dysmorphology, growth retardation, behavioral abnormalities, and cognitive deficits were due to exposure to alcohol during gestation. Since then, it has been noted that alcohol consumed during pregnancy has a wide ranging impact on the developing fetus, via alcohol's capacity to cross the placenta (Burd et al, 2007), and the severity of a child's impact due to exposure to alcohol in gestation is dependent on many factors including when during gestation the alcohol was consumed (Guerri et al, 2009), the quantity of consumption (Kelly et al., 2000 and Sood et al., 2001), and the pattern of consumption with binge-like consumption being worse than chronic exposures (Bailey et al, 2004).

Once it was discovered that varying types of alcohol exposure could result in varying impacts on exposed children, multiple levels of diagnoses were developed to more accurately describe the level of prenatal alcohol impact. This

includes FAS (characterized by the facial malformations, growth retardation, and mental retardation), partial FAS (PFAS, characterized by the same or similar level of mental retardation as FAS without the physical dysmorphology), alcohol related birth defects (ARBD, characterized by deficits in organ system functions), and alcohol related neurobehavioral defects (ARND, characterized by normal or close to normal growth and appearance, but significant nervous system damage and cognitive challenges). These groups are collectively termed Fetal Alcohol Spectrum Disorder (FASD) (Bertrand et al, 2004 and Hoyme et al, 2005).

The different levels of FASD have various cognitive challenges ranging from significant intelligence quotient (IQ) reductions in individuals with FAS, to deficits that only present in stressful or challenging situations. In 2009, Kodituwakku reviewed these assorted deficits, noting that exposure to gestational exposure to ethanol (EtOH) impairs higher-order cognitive functions with specific emphasis on executive control, IQ, attention, executive functioning, and learning and memory. In one specific example, children with a documented FASD demonstrated significant impairments on a human virtual Morris Water Maze (Hamilton et al, 2003), a task originally designed to measure hippocampal learning and memory in animals (Morris, 1981).

Since exposure to alcohol in utero is the number one preventable cause of mental retardation and developmental delays in the United States (Abel, 1998), a concerted effort is being made to educate women about the risks of fetal alcohol exposure. However, efforts to prevent pregnant women from consuming alcohol have not been entirely successful, with 7.6% of women reporting alcohol use

during pregnancy in a 2006-2010 study by the Centers for Disease Control (CDC, 2012). These rates of alcohol use among pregnant women have remained fairly consistent for an extended period of time (measured at 12.2% from 1991-2005, CDC, 2009), resulting in children continuing to be born with significant cognitive and behavioral deficits despite public health interventions and awareness campaigns.

In 2009, May et al estimated the rate of FAS and FASD occurrence in school age children in the United States to be as high as 0.2-0.5% and 2-5% respectively. These values, however, are only estimates as typically only the most severely affected children are diagnosed with a FASD, leaving many children undiagnosed. May and colleagues also noted that mothers in impoverished and low socioeconomic areas are more likely to give birth to a child with FAS than FASD, while the inverse is true for mothers in wealthy, high socioeconomic areas (May et al, 2009).

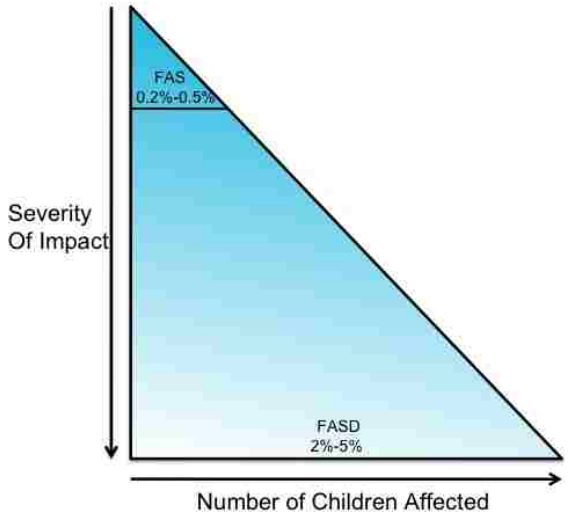

Figure 1.1. Illustration of FASD Distribution in US School Aged Children

As illustrated by this diagram, as the severity of prenatal alcohol exposure is reduced, the number of children included in the appropriate diagnosis increases. The number of children diagnosed with FAS constitutes a very small portion of the total number of children suspected to have a FASD.

The estimated cost of FAS in the United States, including but not limited to medical, social, educational, and judicial support services, was $5.4 billion in 2003 (Harwood, 2003) implying that individuals with this disorder place a significant strain on available resources. It is particularly striking how high this cost is, especially when this figure does not include the cost attributed to children with an FASD, which comprise the largest proportion of individuals suffering from the effects of prenatal EtOH exposure.

Multiple studies have examined various behavioral facets of the impact of prenatal ethanol exposure. Studies involving individuals diagnosed with an FASD have reported significantly reduced IQs (Streissguth et al, 1991, Mattson et al, 1997), impaired executive functioning (Rasmussen et al, 2007), and altered or

inappropriate social interactions (reviewed in Kully-Martens et al, 2012). Recent improvements in imaging technologies have provided evidence for reduced overall brain volume (including reduced hippocampal volume) (Norman et al, 2009). These behavioral deficits and physical abnormalities have been shown to persist into adulthood (Barr et al, 2006).

1.2 Effects of Prenatal Ethanol Exposure in Rodents

Taking into account the significant and long-lasting impact and cost of FASDs, it is evident that a greater understanding of how alcohol consumption is impacting the developing fetus is needed. Many of the effects of prenatal ethanol exposure observed in children and adults with an FASD can be mirrored in animal models, allowing for precise experimental control and assessment.

Similar to the relationship between the timing and quantity of alcohol consumption and the severity of impact seen in children, the magnitude of behavioral and cognitive deficits documented in rodents seems to be related to the timing (Sulik 2005) and quantity of alcohol exposure (Bonthius and West, 1988). Varying experimental models in rodents have been able to replicate many of the features of FASD such as the behavioral and cognitive deficits, and in some cases, the physical dysmorphology and growth retardation seen in more severe alcohol exposures (Sulik et al, 1986). This is discussed further in Chapter 3.

1.3 Summary

In this chapter, we have discussed the correlation between consumption of alcohol during gestation and the development of cognitive, behavioral, and physical challenges in the exposed offspring. The effects seen in humans can be replicated in animal models of prenatal ethanol consumption, and have aided in furthering our understanding of the mechanisms of alcohol induced damage. However, a better understanding of the underlying mechanisms resulting in cognitive deficits following moderate prenatal ethanol exposure is required.

2. Prenatal Stress

2.1 Occurrence and Effects of Prenatal Stress Exposure in Humans

While the effects of alcohol exposure during gestation can be fairly well summarized, the physical and behavioral changes in children prenatally exposed to high or persistent levels of stress are more equivocal. This is due to the nature of characterizing “stress” and the challenges in identifying pregnant women potentially exposed to elevated levels of stress. Biologically, stress is defined as any force that drives the individual from homeostasis. However, every individual’s personal definition and perception of stress is unique and dependent on prior experience, developmental environment, and genetic background. Stress, anxiety, and depression are some of the most widely reported health problems, particularly for women (Walters, 1993) and can serve as a compounding factor for pre-existing conditions. Calculating the percentage of children exposed to high levels of prenatal stress is complex due to the varying nature of the perception of stress by each individual. There is currently no explicit clinical test for stress during pregnancy. The most precise way to measure stress would be to take a biological sample (blood or saliva) and measure circulating stress hormones; however, this method of evaluation is often seen as impractical as stress hormone levels naturally fluctuate across the diurnal cycle in a circadian manner, which creates confounding factors in the experimental design. Pregnant women are often asked to identify potential stressors in their lives and quantify their level of stress, but self-reporting and personal perceptions of stress both serve to confound this methodology of gestational stress assessment.

Stress during pregnancy can arise from a wide variety of sources and be grouped in a number of categories (Figure 2.1). While pregnancy is in itself inherently biologically and emotionally stressful, additional stress can arise from personal relationships, employment situations, and personal or fetal health to name a few.

Figure 2.1. Potential Sources of Stress During Pregnancy

Adapted from Dunkel-Schetter, 2011. Sources of stress for pregnant women can be derived from individual, community, relationships, or sociocultural origins. Each woman will react differently to each stressor depending on her previous experience and background.

Stress can also be categorized as either acute (single or short lasting event) or chronic (enduring or on-going situation). Many sources of stress which involve living or working situations are considered sources of chronic stress, while examples of acute stress are singular events such as the terrorist attacks of September 11th, 2001, an earthquake, or the death of a loved one. However, the distinction between acute and chronic stress can be a bit vague as the ramifications of an acute stressor can have enduring effects.

Following an adverse experience, maternal stress hormones, including cortisol (corticosterone in rodents, CORT), are secreted by the maternal adrenal glands in high quantities into the maternal circulation. CORT can cross the placenta when present in sufficient quantities and can dysregulate the developing fetal stress axis (Figure 2.2). The majority of maternal CORT (mCORT) produced by the maternal adrenal cortex is converted to its inactive form by 11B-hydroxysteroid dehydrogenase 2 (11B-HSD2); however, mCORT can cross the placenta in its active form and has important roles in fetal organ development. When a pregnant mother is stressed, the total quantity of mCORT is elevated, and the efficacy of 11B-HSD2 to inactivate CORT is reduced (Vagnerova et al, 2008). This results in the fetus being exposed to excessive levels of mCORT. The placenta also reacts to the higher levels of mCORT by secreting corticotrophin releasing factor (CRF, pCRF when released from placenta), which is able to stimulate the fetal pituitary into releasing adrenocorticotrophic hormone ACTH, resulting in the release of fetal derived CORT (fCORT) from the fetal adrenal cortex. Both mCORT and fCORT can have down-regulating effects on the developing fetal HPA axis and reduce the quantity of mineralocorticoid (MR) and glucocorticoid receptors (GR) in the hippocampus, resulting in significant cognitive impairments (reviewed in Sandman et al, 2011).

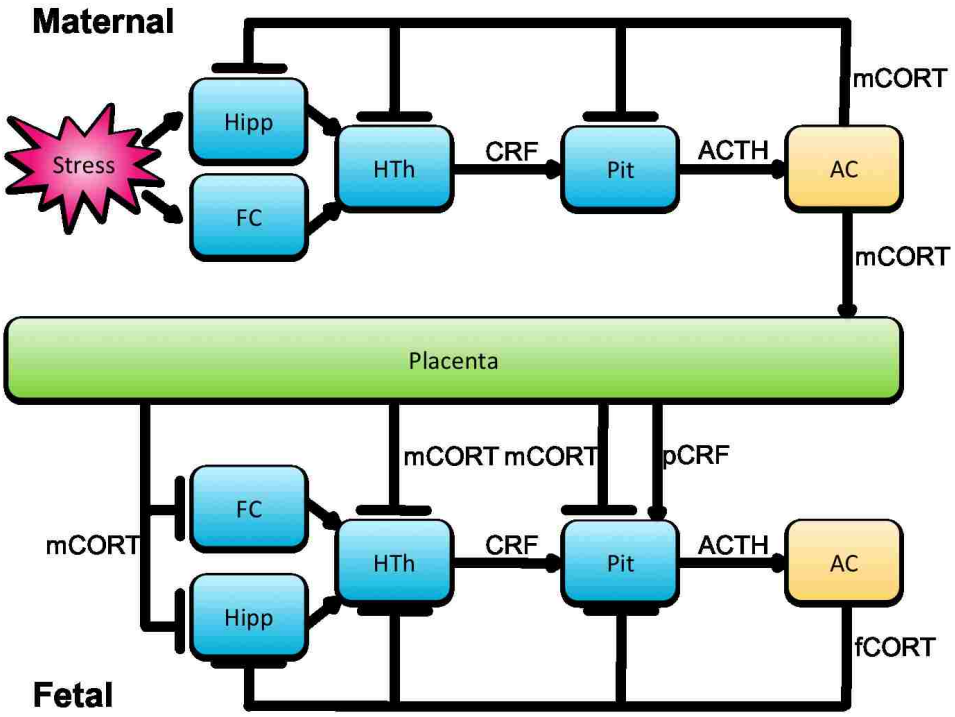

Figure 2.2. HPA Axis and the Maternal-Placental-Fetal Unit

Following exposure to a stressor, the maternal hypothalamus-pituitary-adrenal axis (HPA axis) is activated and maternal CORT (mCORT) is secreted into the circulation. MCORT can cross the placenta to dysregulate the developing HPA axis as well as down-regulate the expression of CORT receptors in the fetal hippocampus (Hipp), resulting in learning impairments.

While in utero, the developing fetus is able to adjust and respond to its surrounding environment. If the maternal levels of stress hormones are high, the developing hypothalamus-pituitary-adrenal axis (HPA axis) will be set to respond accordingly. This phenomenon is termed “fetal programming” and is derived from the “Barker Hypothesis” which claimed that the adaptations made in utero become a fixed state as the individual ages and develops (Hales and Barker, 1992). While this fetal plasticity is clearly of some evolutionary benefit by priming the fetus to the likely postnatal environment, in the case of maternal stress, it can

lead to a child that is poorly suited to the environment away from mom. Exposure to prenatal stress has been linked to low birth weight (Seckl, 2004, Bolten et al, 2011), reduced IQ (LeWinn et al, 2009), learning and memory deficits (O'Donnell et al, 2009, LeWinn et al, 2009), mood disorders (Kleinhaus et al, 2013), and attention disorders (Gutteling et al, 2006).

2.2 Effects of Prenatal Stress Exposure in Rodents

Since identifying children who were exposed to high levels of maternal stress can be challenging due to the disparities in maternal stress perceptions and reactivity, the vast majority of our knowledge about the effect of prenatal stress on cognition, behavior, and mechanisms of learning has been derived from animal models. Measuring the behavioral impact of prenatal stress is dependent on the time of day the behavior is measured (Kajer et al, 2011), the genetic background of the animal (Neeley et al, 2011), and the gender being evaluated (Weinstock, 2007). Studies vary widely in the outcomes described in adult offspring exposed to prenatal stress, but there are clear patterns of deleterious effects.

Experimental models examining the effects of maternal stress hormones on the developing fetus have also employed synthetic glucocorticoids (GCs) such as dexamethasone (DEX) and betamethasone (BETA). Administration of physiologically relevant doses of DEX to pregnant rat dams has been previously reported to impair learning in the Morris Water Maze, elongate the HPA axis response to a stressor, and reduced GR mRNA expression in the hippocampus

(Brabham et al, 2000). However, this same study demonstrated that cross-fostering the prenatally exposed offspring with non-exposed dams ameliorated these deficits, implying that maternal care can overcome these fetal programming challenges (Brabham et al, 2000).

However, these pharmacological agents only bind to the GR, while CORT is able to bind to both the MR and the GR. Therefore, in studies aimed at assessing the impact of maternal stress on the developing fetus, it is more relevant in animal models to elicit a systemic stress response rather than administering a synthetic GC. Similar to the alterations produced by prenatal ethanol exposure, offspring exposed to prenatal stress can display deficits in learning and memory (Lemaire et al, 2000, Yang et al, 2006, Zagron and Weinstock, 2006) and heightened anxiety (Takahashi et al, 1992, Vallee et al, 1997, Welberg et al, 2000). Unfortunately, these outcomes are not unequivocal, as other studies have shown no cognitive impairments (Bowman et al 2004) or no increase in anxiety-like behavior (Poltyrev et al, 2005). In addition to the experimental factors mentioned previously, these outcomes seem to be directly related to the method of stress administration and the gender of the offspring being evaluated (reviewed in Weinstock, 2008).

2.3 Summary

Here we have discussed the effects and mechanism of prenatal stress as well as the challenges in calculating the percentage of children born to mothers who experienced stress during pregnancy. Animal models have been helpful in

further understanding the explicit effects of prenatal stress, as human studies generally have large numbers of confounds which make directly linking the stress exposure to negative outcomes in children difficult. Stress during gestation has been demonstrated to result in hippocampal-sensitive cognitive deficits and heightened anxiety in adult offspring.

3. Models of Combined Exposure

3.1 Currently Existing Models

Assessing the direct effect of a single prenatal impact is exceptionally difficult when only evaluating human populations due to natural human variability and confounding factors. Genetics, maternal nutrition, maternal age, socioeconomic status, and the use of other drugs and pharmaceuticals, are just a few of the additional factors that can create problems when assessing human population data involving adverse fetal exposures during gestation. For this reason, animal models are often employed in scientific studies in order to assess the impact of a prenatal exposure on a physical or behavioral outcome in the offspring in order to minimize these confounding factors and provide a more explicit picture of the resulting developmental impact. However, choosing the appropriate model system is a challenging question as each has advantages and disadvantages when compared to humans.

Choosing the proper animal to serve as the model system is likely the most important decision when designing a novel model. Often non-human primates are used due to their neurological and behavioral similarities to humans, but they have a long gestational period and are cost prohibitive. Sheep are similarly used as a model of prenatal alcohol exposure due to their similarities to humans in gestational development, but are cost and time prohibitive. Rodents are the most frequently used animal model due to their relatively small size, large number of pups per litter, short gestational length, and short lifespan (rapid aging) (Cudd 2005).

Within the rodent order, the two most frequently utilized species are mice (Mus musculus) and rats (Rattus norvegicus). Mice have the experimental benefit of having their entire genome sequenced making them the best option for genomic studies. However, subtle learning and memory deficits are more difficult to assess since they require more extensive training in learning tasks. Rats are able to learn challenging tasks quickly, allowing for more discrimination between groups when the differences are subtle (Whishaw, 1995). There are also strain differences within each species to consider; three of the more common strains of experimental rat (Wistar, Sprauge-Dawley [SD], and Long-Evans [LE]) all demonstrate different behavioral characteristics that must be figured into the selection of a strain for use in any given model. LE rats have been shown to perform better in the Morris Water Maze than Wistars (Hort et al, 2000) and SDs (Harker and Whishaw, 2002), reduced anxiety behavior in the Elevated Plus Maze (EPM) and Open Field (OF) as compared to Wistars (Shaw et al, 2009), and increased reactivity to a stressor as compared to SDs (Bielajew et al, 2002). Another factor to consider is the usefulness of animals bred for a specific purpose. For example, “P Rats” are selectively bred, high alcohol consuming animals often used in models of alcoholism (Stewart et al, 1993). Similarly, rats have been previously reported as having been selectively bred for their high or low anxiety, useful in assessing predilections towards psychiatric disorders (Liebsch et al, 1998).

There are a vast number of both prenatal ethanol and prenatal stress models currently available, each with their own positive and negative features

(Summarized in Table 3.1 and 3.2). To date, there are only two models of combined prenatal ethanol and prenatal stress exposure. The Christie group has used a model featuring a 6% liquid diet ethanol administration and 3x daily restraint (gestational day [GD]12-20, 45minutes per session). However, their initial work using this model did not include assessments of behavioral outcomes, and much of their data is derived by comparing the ethanol consuming group to the ad libitum group, rather than the pair-fed group, which is the more appropriate control (Titterness and Christie, 2012). Similarly, there is a Russian model of combined exposure where the dams were exposed to forced ethanol (11%) consumption and placed in restraint tubes for 2 hours daily in the last week of gestation. Unfortunately, only the control (no ethanol, no stress) and combined exposure (ethanol and stress) groups are assessed, and only for sexual partner preference and effects of brain derived neurotrophic factor (BDNF) (Popova et al, 2011a, 2011b). Clearly, both the characteristics of the chosen ethanol administration and the validity of the analysis of the data derived in each of the models is of concern.

3.2 Our Model

The currently available rodent models of combined prenatal ethanol and prenatal stress exposures have significant drawbacks and limitations that prevent them from being the optimal paradigm for a dual gestational insult model. The Titterness and Popova models both utilize stressful ethanol administration models and prenatal stress exposures which the animals could potentially

habituate to. When developing this novel animal model of combined prenatal ethanol and prenatal stress exposure, many factors were taken into careful consideration, including mode of ethanol administration, stressor, and the timing of the two insults.

3.2.1 Ethanol Consumption

Method of Administration	Advantage	Disadvantage
Injection	Any BAC possible Exact dosing	Stressful Painful Potential infection at injection site May directly inject pups Not a natural route of administration
Oral Gavage	Any BAC possible Exact dosing Gastric absorption	Stressful Risk of injury to animal
Vapor Chamber	High BAC possible	Stressful Lung irritation BAC can vary widely Not a natural route of administration
Liquid Diet	Guaranteed consumption Gastric absorption	Stressful Malabsorption of nutrients in diet
Forced Consumption	Guaranteed consumption Gastric absorption	Stressful Possible dehydration
Voluntary Consumption	Gastric absorption Non-stressful delivery system	Low to moderate BAC only

Table 3.1. Currently Available Prenatal Ethanol Exposures

There are many possible routes of ethanol administration to animals. Nearly all of them, however, induce some level of stress during administration, and only some of them are administered orally.

The route of ethanol administration should be as minimally stress inducing as possible. The most popular models of ethanol administration all share the feature of being highly stressful and thereby requiring a third control group to measure the impact of the stress of the chosen route of ethanol administration. These include intraperotoneal (IP) injection, oral gavage, and liquid diet. Each of

these routes of ethanol administration has been previously shown to increase stress in experimental animals; this confounding factor requires models utilizing these routes of administrations to employ a third control group that receives the same route of administration but no ethanol. This third group greatly increases the number of animals (and the resulting time and cost involved) required for each experiment. One of the most frequently used administration models, the ethanol-containing liquid diet, reduces the quantity of the diet consumed due to the rat's natural aversion to ethanol. This induces a nutritional deficit stress that must be controlled for with a pair-fed group that receives the same reduced quantity of diet as the ethanol exposed group consumed. Animals in the pair-fed group have significantly elevated levels of CORT, as well as prolonged elevations in CORT, as compared to controls (Weinberg and Gallo, 1982). This finding implies that the liquid diet is not a suitable model of ethanol exposure in isolation of other gestational impacts.

Voluntary ethanol administration, as described by Savage et al, 2010, allows the animal to choose to consume as much or as little of the sweetened ethanol solution as they like, with their house water and chow available ad libitum. Animals are restricted to 4 hours of access to the ethanol solution during their awake cycle. This method eliminates the stress of forced ethanol administration. This is also a more translationally relevant mode of ethanol consumption, as ethanol consumed by humans is gastricly digested (as opposed to directly administered to the circulation via injection) and voluntarily consumed (as opposed to forced consumption via oral gavage). This model also better

replicates the moderate levels of ethanol that the majority of children are exposed to in contrast to the high levels of ethanol used in other models. In the voluntary consumption model, the best measure for comparing prenatal ethanol exposures, the maternal blood alcohol content (BAC), reached a physiologically relevant dose of 80-100mg/dL, while other models can reach BACs of over 300mg/dL, levels often only observed in human cases of severe alcoholism.

3.2.2 Stress Exposure

Method of Administration	Advantage	Disadvantage
Restraint	Can be of varied length, Acute or Chronic	Animals can habituate
Crowding	Psychological stressor	Possible fighting Development of depression/defeat
Electric Shock	Fast Repeatable	Painful
Noise	Can be of varied intensity, tone, duration, pattern	Animals can habituate
Injections	Acute	Painful Risk of infection at injection site Animals can habituate Only delivers single drug/hormone
Predator Scent	No Habituation Non-invasive	Development of PTSD (chronic administration)

Table 3.2. Currently Available Prenatal Stress Exposures

There are many ways to induce a stress response in a rodent. A sampling is presented here. However, rodents are able to habituate to repeated exposures of many of them, and some only induce an elevation in a single stress hormone rather than eliciting a full systemic response.

An evolutionarily beneficial feature of most animals is their ability to habituate to stressors that are reoccurring. However, for the purposes of this model, it is imperative that the animals not habituate to the stressful experiences, and that the stress not interfere with drinking behavior or maternal care of litters.

The stressor should also be moderate in intensity to avoid growth retardation and litter effects, as these can both be confounds when assessing learning, memory, and anxiety behavior.

The most commonly employed form of prenatal stress, immobilization restraint, has the potential risk of habituation. Studies have shown that animals repeatedly restrained can adjust to the predictable, non-harmful stressor and cease producing a hormonal or behavioral stress reaction (Armario et al 1988, Hauger et al, 1990, Melia et al, 1994). To avoid this, some researchers employ a repeated variable stress paradigm where animals are exposed to a random assortment of stressors at random times of the day. While this approach eliminates the problem or habituation to a single stressor and predictability of scheduling, there is a concern of the timing of the stressors in relation to the ethanol exposure (discussed below).

The use of a biologically relevant predator scent as the stressor eliminates the concern surrounding habituation to the stress exposure. It is well documented that rodents will respond to the presentation of a predator scent (cat, fox, etc) with a behavioral and hormonal stress reaction (reviewed in Takahashi et al, 2005). Rodents can safely be repeatedly exposed to an extract of fox urine, trimethylthiazoline (TMT), and the use of a chemical compound allows for precise experimental control of the quantity of odor exposure (Wallace and Rosen, 2000).

3.2.3 Timing of Exposures

The timing of the ethanol and stress exposures is critically important, in relation to each other, in relation to the diurnal cycle, and in relation to gestation.

3.2.3.1 Daily Exposures

Rodents are nocturnal creatures, so it is important that the ethanol and stress exposures occur during a physiologically relevant time of day. Animals utilized in this model are maintained on a reverse light/dark cycle, and the ethanol and stress occur during the dark/awake phase of their daily cycle. For the ethanol exposure, this is the time of day that the animals would be likely consuming the most food and liquid.

For the stress exposure, the circulating CORT concentration will be higher during the awake cycle than the sleep cycle (Mohawk et al, 2007). Therefore, the stressor should be presented at a time during the awake cycle when the circulating CORT is lowest (but not necessarily the lowest CORT level of the 24-hour period) in order to evoke the greatest physiologically relevant elevation in the stress hormone. The stressor should also be presented at a temporally dissociated time from the presentation of the ethanol solution so as not to interfere with ethanol consumption.

3.2.3.2 During Gestation

As previously stated, the chronicity of ethanol and stress exposures during gestation can determine the type and severity of the impact on the offspring. Rodent gestation mimics the first two trimesters of human gestation. Ethanol can

impact the developing fetus at any point during gestation, but there is a specific window when stress will induce deleterious effects. Evidence suggests that maternal stress does not impart negative effects until the fetal HPA axis is semi-functional. In the rat, this occurs around the second week of gestation (Diaz et al, 1998).

3.3 Summary

In this chapter, we have discussed the various factors that must be considered when developing a new animal model for use in scientific study. These factors include the specific species and strain of animal used, the route of ethanol administration, the type of stressor, and the timing of the ethanol administration and stressor.

4. The Hippocampus and Dentate Gyrus

4.1 Structure

The hippocampus, named after the Latin word for seahorse, is part of the limbic system and plays a critical role in specific types of learning and memory. It is comprised of three primary regions, the dentate gyrus (DG), and the cornu Ammonis 1 and 3 (CA1 and CA3). Projections from the entorhinal cortex (EC) synapse first on the granule cells (GCs) of the DG, whose mossy fibers then synapse onto the pyramidal cells of the CA3. Projections from the CA3 pyramidal cells (the Schaffer Collaterals) synapse onto the CA1 pyramidal cells. The CA1 axons then project back out to the EC (Figure 4.1).

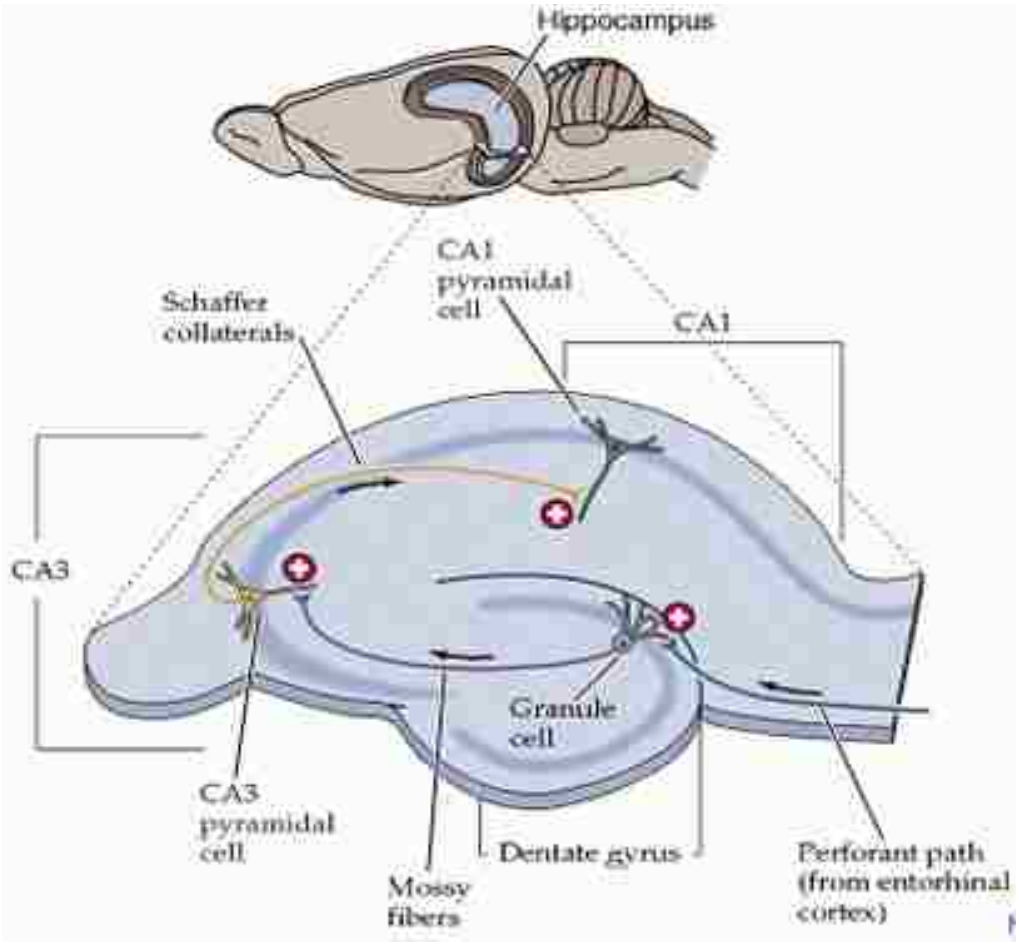

Figure 4.1. The Rodent Hippocampus

The hippocampus is comprised of the trisynaptic circuit: the dentate gyrus (DG), the cornu Ammonis (CA) 3, and CA1. Efferent fibers from the entorhinal cortex (EC) synapse on the granule cells (GCs) of the DG, which send mossy fiber projections to the CA3 pyramidal cells. The CA3 pyramidal cells then synapse on the CA1 pyramidal cells via the Schaffer collaterals. Finally, the CA1 send afferent projections back to the Perforant Path.

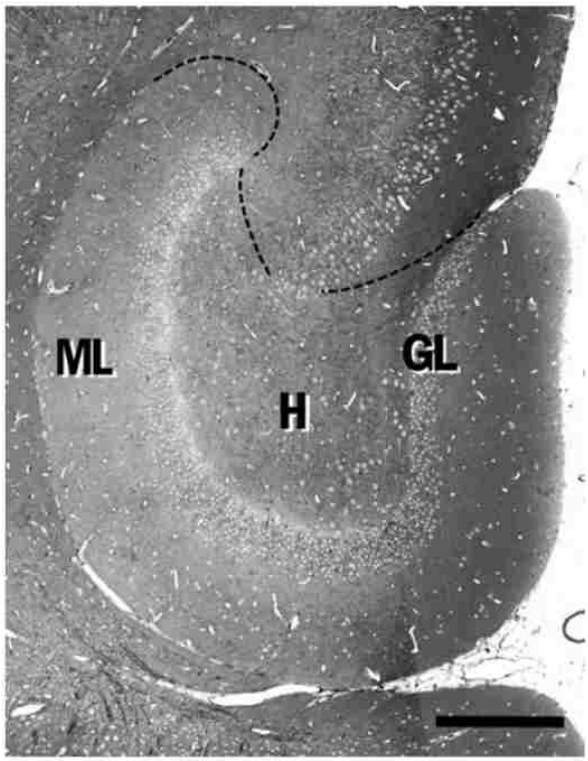

Figure 4.2. The Structure of the Dentate Gyrus

The DG is comprised of three primary layers: the molecular layer (ML) contains the granule cell dendrites, the cell bodies reside in the granule cell layer (GL) and the hillus (H) is comprised of the mossy fiber projections.

The DG (Figure 4.2) is of particular interest due to its sensitivity to ethanol (Miller, 1995) and stress (Hosseini-Sharifabad et al, 2012) exposure during development. It consists of three layers, the molecular layer, the granule cell layer, and the polymorphic layer (hilus). The molecular layer is primarily comprised of the GC dendrites and is the site of the major excitatory input for the DG and other regions of the hippocampus from the EC. The cell bodies of the GCs are the main component of the granule cell layer. The mossy fiber projections from the GCs are the primary component of the polymorphic layer or the hilus (Amaral et al, 2007), although there are GABAergic interneuron's which are thought to provide inhibitory feedback for the DG.

4.2 Dentate Gyrus Development

The granule cells in the DG begin to form 2 days prior to birth, with the majority of the DG structure formed in the early postnatal period. During late gestation, neuroblasts begin migrating from the ventricular zone to form the upper blade of the DG, while the lower blade is developed shortly following delivery (Piatti et al, 2006). Interestingly, the subgranular zone (SGZ) of the DG is one of only two sites of adult neurogenesis in the mammalian brain (the other being the subventricular zone [SVZ] of the olfactory bulb), with new GCs being produced into adulthood. This implies that the DG is a constantly changing and adapting structure, a critically important feature for a region of the brain involved with forming new learning and memories.

4.3 Dentate Gyrus Function

Evidence suggests that the DG is involved with specific types of learning and memory and its function is intimately associated with its neurogenic properties. There is extensive evidence that the DG is directly involved in the pattern separation portion of spatial memory (Clelland et al, 2009, Holden et al, 2012, Morris et al, 2012) but only limited work has investigated an additional role of the DG in the formation of temporal memories (Kesner 2013).

4.3.1 Alterations in the Dentate Gyrus due to Prenatal Ethanol Exposure

While the vast majority of the research measuring prenatal ethanol's impact has been performed on the CA regions or on the hippocampus as a whole, there has been some work detailing the effects seen in the DG. Prenatal

ethanol has been shown to have deleterious effects on the neurogenic properties of the DG, alter receptor expression, and impair performance in behavioral tasks.

While binge-like exposure in the early postnatal period of rodent development results in reductions in markers of neurogenesis as well as cell survival (Klintsova et al 2007), more moderate exposures of ethanol during gestation do not result in significant cell loss or deviations from normal progression of neurogenesis, altered receptor expression (Brady et al, 2013), and behavioral performance in DG specific tasks (Brady et al 2013).

4.3.2 Alterations in the Dentate Gyrus due to Prenatal Stress Exposure

Similar to prenatal ethanol, prenatal stress exposure imparts significant alterations to the dentate gyrus due in part to the excessive exposure to CORT. This effect is primarily derived through CORT's actions on the new neurons proliferating in the SGZ. The neurogenic cells in the SGZ are particularly sensitive to excessively high levels of CORT and proliferation has an inverse relationship with the level of circulating CORT. Not only are the neuronal precursors subjected to mCORT, the fetal programing of the developing HPA axis can result in constitutively high levels of fCORT once the offspring is born. This suppression of proliferation is thought to be responsible for the reduced performance on tasks of hippocampal dependent learning and memory.

4.4 Summary

The hippocampus is a region of the neocortex which is responsible for the intergration and formation of new memories. The DG is a subregion of the

hippocampus which receives efferent input from the EC. Both the hippocampus and the DG are involved in spatial and temporal memory formation, as well as being sensitive to prenatal ethanol and prenatal stress exposures, making the DG a prime target of investigation into the effects of developmental exposures to prenatal ethanol and prenatal stress.

5. Markers of Synaptic Plasticity

5.1 Synaptic Plasticity

A key feature of the adult brain is its capacity to be shaped by experience and strengthen or weaken synaptic connections in response. This phenomenon is known as synaptic plasticity and is exceptionally prevalent in the hippocampus. This results in long lasting changes in synaptic efficacy and firing (Hebb, 1949, Bliss and Lomo, 1973).

5.1.1 Activity Dependent Synaptic Plasticity

One specific type of synaptic plasticity is activity dependent synaptic plasticity. This phenomenon occurs when a synapse is strengthened or weakened following direct activation from a learning event and is the primary type of synaptic plasticity in the adult hippocampus. Forms of activity dependent synaptic plasticity include long-term potentiation (LTP), long-term depression (LTD), synaptic scaling, and metaplasticity (reviewed in Citri and Malenka, 2008). LTP is the enduring potentiation of a synapse following repeated presynaptic stimulation and postsynaptic firing (Martin and Shapiro, 2000), while LTD is the depression or reduction in synaptic strength following consistent stimulation (Dudek and Bear, 1992). Synaptic scaling is a phenomena wherein all the synapses of a neuron are inversely adjusted to continuous elevations (or in some cases reductions) in input activity by reducing the strength of the synapse to maintain homeostasis (Turrigiano and Nelson, 2004). The feature of metaplasticity is less well understood than the previously described forms of plasticity, but is centered on the study of the role of activity in the potential for

long-term plasticity of a synapse. Each of these forms of plasticity, particularly LTP, is permissible due to the neurons capacity for rapid molecular adjustment following stimulation.

There are multiple biochemical properties of synapses undergoing plasticity. Briefly, following electrical current stimulation, glutamatergic vesicles are transported to the presynaptic membrane and released into the synapse. Glutamate binds to receptors on the postsynaptic surface and cations, including in some cases calcium (Ca^{2+}) are able to enter the cell. The influx of Ca^{2+} triggers signaling cascades and activated various kinases in the postsynaptic cell, eventually resulting in the addition of more glutamate receptors in the postsynaptic membrane, as well as their phosphorylation or dephosphorylation. These modifications can alter the receptors location at the synapse and/or ionic conductivity (reviewed in Ho et al, 2011).

5.1.2 Impact of Prenatal Ethanol Exposure on Dentate Gyrus Synaptic Plasticity

The most commonly used measure of synaptic plasticity is LTP. There is a solid body of work investigating the impact of prenatal ethanol on DG specific synaptic plasticity and evidence from moderate prenatal ethanol exposure models demonstrate significant reductions in DG LTP in both mice and rodents (Sutherland et al, 1997, Christie et al, 2005, Samudio-Ruiz et al, 2009, Varaschin et al, 2010, Titterness and Christie, 2012, Brady et al, 2013). These deficits have been linked to directly and indirectly behavior (Savage et al, 2010, Brady et al,

2012) and are, in part, explained by reductions in, or altered subunit expression of receptors at the cell surface (Samudio-Ruiz et al, 2010, Brady et al, 2013).

5.1.3 Impact of Prenatal Stress Exposure on Dentate Gyrus Synaptic Plasticity

While there is a body of evidence that prenatal ethanol can impair synaptic plasticity in the DG, the influence of prenatal stress on synaptic plasticity in this specific region of the hippocampus has been scarcely evaluated. One report suggests that prenatal stress reduces hippocampal LTP in adult offspring (Son et al 2006), and there is only one study which reported a deficit in DG LTP as a result of prenatal stress (Titterness and Christie, 2012). Further work in this area is required to better understand how synaptic plasticity in the DG is altered due to prenatal stress.

5.2 Biochemical Markers of Synaptic Plasticity

5.2.1 Activity-Regulated Cytoskeletal Protein

Activity Regulated Cytoskeletal protein (ARC) is an immediate early gene (IEG) whose expression as both mRNA and protein is rapidly up-regulated following neuronal activation from both learning (Link et al, 1995, Lyford et al, 1995, Guzowski et al, 2001, Montag-Sallaz and Montag 2003), and electrical stimulation. Functionally, ARC is involved in supporting the expanding actin cytoskeleton and the physical expansion in the synaptic bouton following stimulation, as well as aiding in AMPA receptor endocytosis following neuronal activation. ARC knockouts are biologically viable, but LTP and learning are significantly diminished as a result, implicating a critical role for ARC in synaptic

plasticity and learning (Guzowski et al, 2000) and is an often utilized marker of neuronal activity.

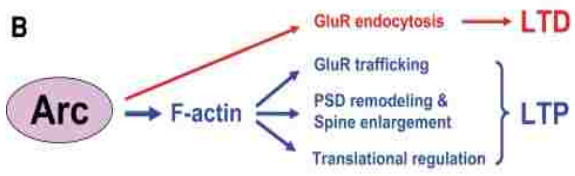

Figure 5.1. ARC Protein

ARC protein has many roles in the postsynaptic cytoplasm following stimulation. It has been implicated in the removal of glutamate receptors (GluRs), specifically AMPA-type GluRs, from the cytoplasmic membrane, as well as supporting the F-actin scaffold assembly. The assembly of F-actin results in increased GluR trafficking, enlargement of the postsynaptic density, and regulation of cytoplasmic translational activity. Adapted from Bramham et al, 2010.

5.2.1.1 Impact of Prenatal Ethanol Exposure

The existence and function of ARC is a relatively new finding in neuroscience, but researchers have rapidly embraced it as a marker of synaptic plasticity. However, there has been limited exploration of ARC expression in animal prenatally exposed to adverse gestational conditions. One study demonstrated that moderate prenatal ethanol exposure reduces the expression of ARC mRNA following social experience in the frontal cortex of rats (Hamilton et al, 2010a, 2010b), but this has been the only work to date using ARC as a marker for impaired synaptic plasticity. More research into ARC expression following a neuronal stimulation in prenatally ethanol exposed animals is needed to construct a more detailed model of the interaction between prenatal ethanol and activity dependent synaptic plasticity.

5.2.1.2 Impact of Prenatal Stress Exposure

There are no reports of the impact of prenatal stress on ARC expression following neuronal stimulation, but exposure to a direct stressor does induce a significant reduction in ARC expression in the frontal cortex of rats (Caffino et al, 2011). Contrastingly, stress exposure has been shown to increase ARC expression in the hippocampus following exposure to a novel environment or increase ARC expression in the frontal cortex following restraint stress (Mikkelsen and Larsen, 2006, Ons et al, 2004). These results, while providing contrasting results, imply a potential interaction of stress and ARC expression that should be further explored.

5.2.2 AMPA Receptor

The α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) is an ionotropic glutamate receptor found throughout the central nervous system (CNS), including the hippocampus and DG. It is comprised of four subunits, GluA1-GluA4, which are derived from four different genes, GRIA1-4. GluRA1-3 are expressed throughout life, while GluA4 is expressed predominately during development. The GluA2 subunit is subject to Q/R editing where a glutamine is substituted for an arginine, resulting in receptors containing the edited subunit being rendered Ca^{2+} impermeable. Nearly 99% of the GluA2 subunits produced contain this edit. Each subunit contains a “flip/flop” sequence, regulated by alternative splicing of the gene, near the ligand binding channel, which in the case of the GluA2 subunit, dictates the rate of conductance of the channel; “flop” GluA2 subunits are more quickly desensitized than the “flip”

subunit, but there seems to be no difference in conductance among the GluA1 subunit (reviewed in Shepherd and Huganir, 2007 and Keifer and Zheng, 2010).

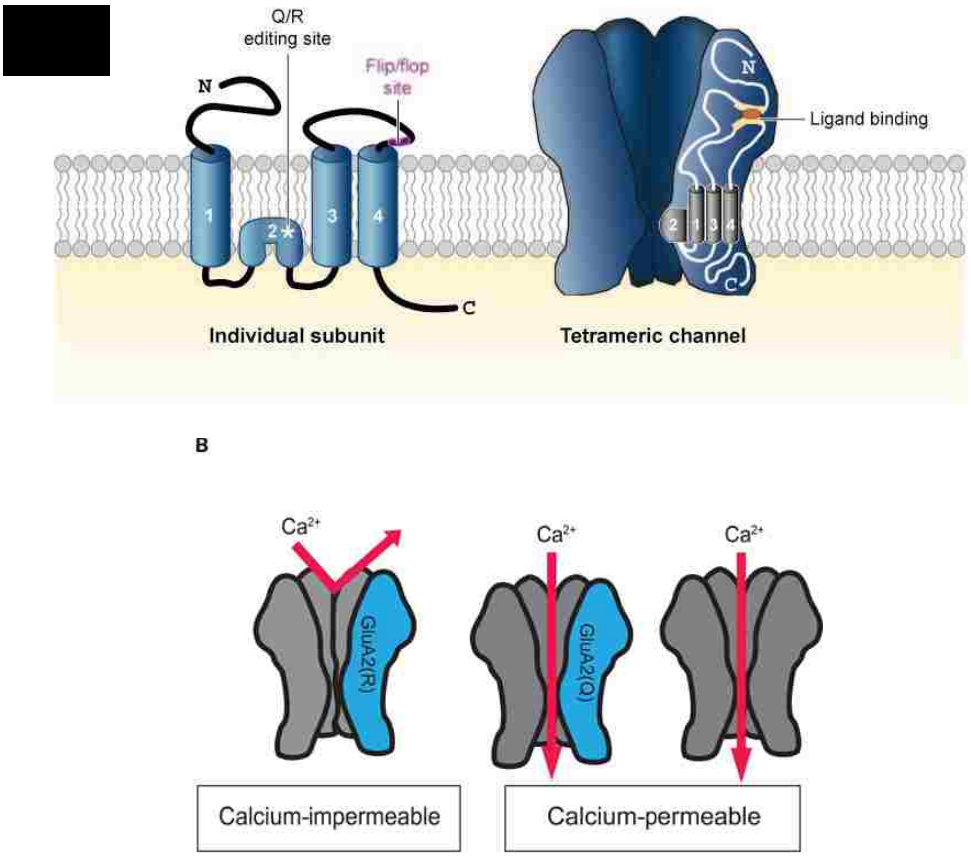

Figure 5.2. AMPA Receptor

A. The AMPA-type glutamate receptor is a Tetrameric channel typically comprised of dimers of dimers of the subunits GluA1-4. B. Depending on the editing of the GluA2 subunit, the channel of the AMPA receptor can be calcium permeable of impermeable. Adapted from A-Shepherd and Huganir, 2007 and B-Adapted from Keifer and Zheng, 2010.

Under basal conditions, AMPARs are constitutively cycled from the cytosol to the cell membrane and back again. When the AMPAR subuinits bind glutamate, the receptor undergoes a conformational change and the ion channel opens, allowing for an influx of cations. This allows for a small depolarization of the synaptic membrane, resulting in the magnesium (Mg^{2+}) blockade of the N-

Methyl-D-aspartate receptor (NMDAR) to be ejected. The removal of the blockade, along with binding of glutamate and glycine to the NMDAR, open the channel and allow for an influx of Ca^{2+}. This Ca^{2+} current stimulates an intracellular signaling cascade, which results in a net increase in the number of AMPARs at the postsynaptic surface.

In the short term, the AMPARs delivered to the synaptic surface lack the GluRA2 subunit. This renders the open channel Ca^{2+} permeable and continues the potentiation between the pre- and post- synaptic cells. However, in order to prevent Ca^{2+} cytotoxicity in the long term, the GluA2-lacking receptors are eventually exchanged for GluA2-containing receptors. This allows for the safe maintenance of potentiation across the synapse.

There is some debate about whether the receptors are delivered the extra-synaptic membrane then shuttled to the synaptic membrane or are delivered there directly upon stimulation. However, the evidence clearly demonstrates that there is an increase in the total number of AMPARs at the membrane surface following synaptic strengthening. This implies that a measuring the change in AMPAR levels at the membrane surface can serve as a marker of activity dependent synaptic plasticity.

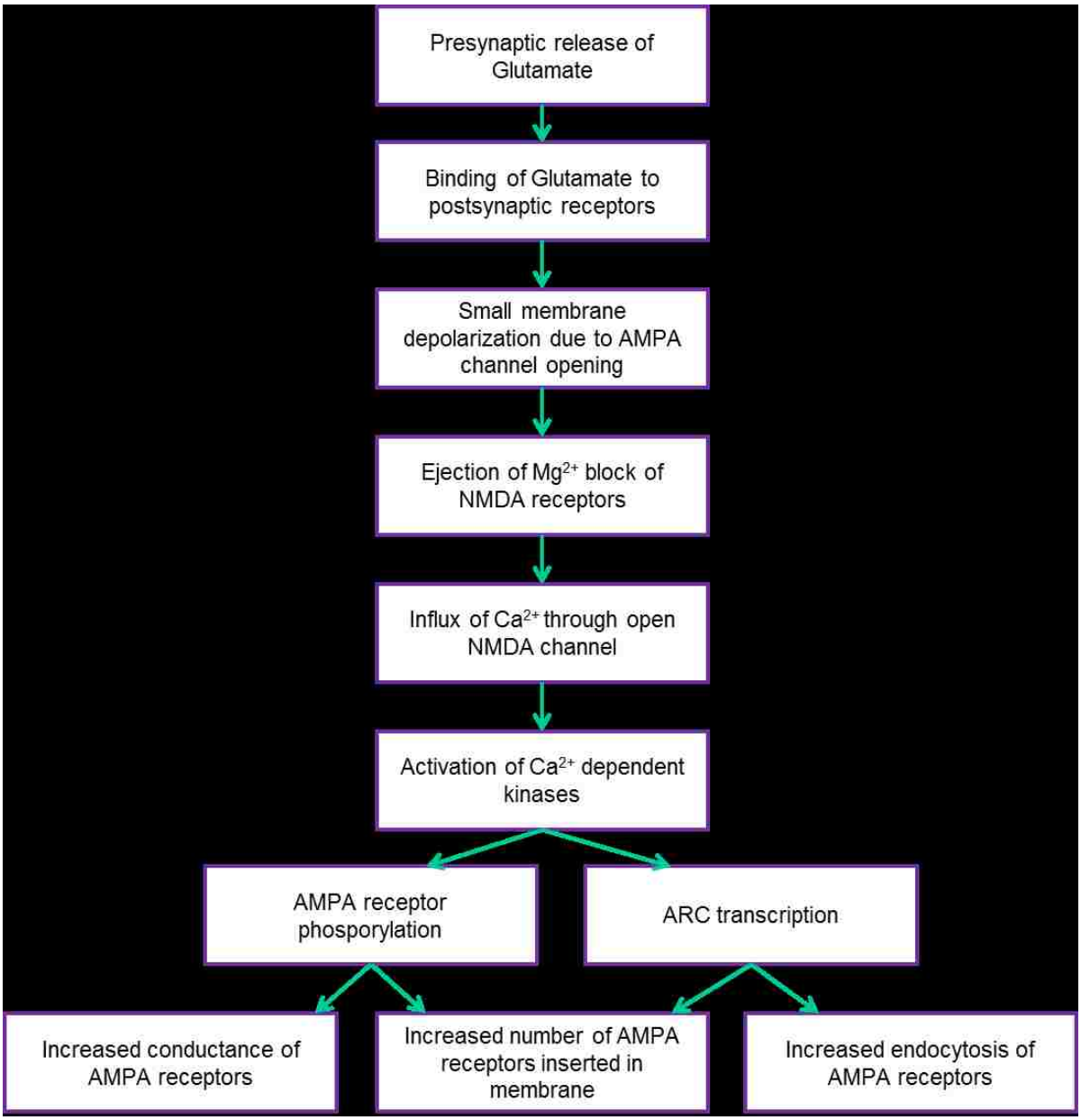

Figure 5.3. Events of Early LTP

The stimulation of LTP is a multistep process beginning with presynaptic glutamate release and resulting in an increased number of AMPA receptors inserted into the postsynaptic membrane.

An elevation in ARC protein following neuronal stimulation can have differential effects on the quantity of AMPARs expressed at the membrane depending on the elapsed time following stimulation that is being assessed. Following transcription, ARC is quickly phosphorylated and acts as a scaffolding

protein to assemble F-Actin polymers (Messaoudi et al, 2007). This serves to build a larger cytoskeletal network which vesicles containing AMPA that has been transcribed in the soma is then transported along for delivery to the cell synaptic (and extra-synaptic) membrane (Rust et al, 2010). In the hours and days that follow this initial potentiation, ARC interacts with the dynamin and endophilin endocytotic machinery to reduce the number of AMPA receptors available at the surface (Chowdhury et al, 2007).

5.2.2.1 Impact of Prenatal Ethanol Exposure

The effect of prenatal ethanol on AMPAR expression, and subunit composition are dependent on the quantity of ethanol and the model used in the research. Moderate prenatal ethanol exposure does not appear to alter the basal number of AMPARs in the hippocampus, but studies measuring a difference in evoked levels of AMPARs have not been conducted. Significantly high exposure (+300mg/dl) has demonstrated altered levels of AMPAR subunits in 3 day old pups (Bellinger et al, 2002). Ethanol during the gestational period of guinea pigs has been shown to increase the GluA2/3 subunits, with no effect of the GluA1 subunit. These results imply that exposure to ethanol during gestation could have negative impacts on the expression profile of the AMPA receptor in the hippocampus.

5.2.2.2 Impact of Prenatal Stress Exposure

The effect of prenatal stress on the number or functionality of AMPARs has not been well investigated. Acute stress has been demonstrated to alter the

phosphorylation state of AMPARs within the hippocampus (Caudal et al, 2010), while direct exposure to CORT results in an increase of the GluA2 subunit. These results imply that there may be an interaction of prenatal stress and AMPARs due to the developing fetus's exposure to elevated levels of CORT.

5.3 Summary

Activity dependent synaptic plasticity is the capacity for a synapse to strengthen or weaken in relation to behavioral activity. Changes in AMPAR and ARC expression can serve as a marker for activity dependent synaptic plasticity, as both have altered expression following behavioral activity. Similarly, there is evidence to suggest that exposure ethanol and stress during gestation can alter the quantities of AMPA subunit expression and ARC protein expression.

6. Research Rationale, Hypothesis, and Specific Aims

6.1 Research Rationale

Prenatal alcohol exposure impacts an estimated 2%-5% of school-aged children resulting in significant learning deficits that have been replicated in animal models of moderate alcohol exposure. Similarly, prenatal stress has been shown to result in learning deficits in children that have also been mirrored in animal models. While both prenatal insults result in similar behavioral outcomes, they have not been systematically evaluated together. Of particular interest is the impact the two prenatal insults have on learning and memory, anxiety, and markers of synaptic plasticity in the dentate gyrus.

6.2 Hypothesis

Based on the available information, I hypothesize that maternal stress during pregnancy will potentiate the fetal ethanol induced deficits in hippocampal sensitive learning and biochemical markers of activity dependent changes in dentate gyrus synaptic plasticity.

6.3 Specific Aims

In order to test this hypothesis, I have developed three specific aims with the goal to characterize the novel model, evaluate learning and anxiety in the offspring, and assess markers of activity dependent changes in the dentate gyrus.

6.3.1 Specific Aim 1

Specific Aim 1 is focused on the development and characterization of a novel dual prenatal exposure paradigm. This will require through characterization of both maternal and offspring characteristics and behavior.

6.3.1.1 Sub Aim 1A

Characterize the effect of maternal stress and ethanol consumption on maternal weight gain, ethanol consumption, stress reactivity, and care of litters.

6.3.1.2 Sub Aim 1B

Characterize the effect of maternal stress and ethanol consumption on number of pups delivered per litter, and the amount of weight gained by the pups from birth to weaning.

6.3.2 Specific Aim 2

Specific Aim 2 is focused on assessing changes in behavior due to prenatal ethanol and prenatal stress exposure.

6.3.2.1 Sub Aim 2A

Assess the impact of combined prenatal ethanol consumption and prenatal stress exposure on anxiety behavior.

6.3.2.2 Sub Aim 2B

Assess the impact of combined prenatal ethanol consumption and prenatal stress exposure on hippocampal sensitive learning.

6.3.3 Specific Aim 3

Specific Aim 3 is focused on assessing alterations in the levels of markers of activity dependent changes in the dentate gyrus of the hippocampus due to prenatal ethanol and prenatal stress exposure.

6.3.3.1 Sub Aim 3A

Evaluate the impact of combined prenatal ethanol consumption and prenatal stress exposure on ARC protein expression following learning in the dentate gyrus.

6.3.3.2 Sub Aim 3B

Evaluate the impact of combined prenatal ethanol consumption and prenatal stress exposure on AMPA receptor subunit expression following learning in the dentate gyrus.

7. Impact of Combined Prenatal Ethanol and Prenatal Stress Exposure on Anxiety and Hippocampal Sensitive Learning in Adult Offspring

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7.1 Abstract

7.1.1 Background.

Prenatal ethanol (EtOH) and prenatal stress have both been independently shown to induce learning deficits and anxiety behavior in adult offspring. However, the interactive effects of these two developmental teratogens on behavioral outcomes have not been systematically evaluated.

7.1.2 Methods.

We combined an established moderate prenatal EtOH consumption paradigm where Long-Evans rat dams voluntarily consume either a 0% or 5% EtOH solution in 0.066% saccharin water (resulting in a mean peak maternal serum EtOH concentration of 84 mg/dL) with a novel prenatal stress paradigm. Pregnant rats were exposed to 3% 2,3,5-trimethyl-3-thiazoline (TMT) for 20 minutes a day on Gestational Days 13, 15, 17, and 19. Adult female offspring were evaluated for anxiety-like behavior using an elevated plus maze and hippocampal-sensitive learning using a two-trial trace conditioning task.

7.1.3 Results.

TMT exposure produced a three-fold increase in maternal serum corticosterone compared to non-exposed, unhandled controls. Neither prenatal exposure paradigm, either alone or in combination, altered maternal weight gain, ethanol consumption, maternal care of litters, litter size, pup birth weight, or pup weight gain up to weaning. Offspring exposed to prenatal stress displayed significant increases in anxiety-like behavior in the elevated plus maze in terms

of open arm entries and time spent on the open arms, with no significant effect of prenatal EtOH exposure and no interaction of the two prenatal exposures. Performance in a two-trial trace conditioning task revealed a significant effect of prenatal ethanol exposure on freezing behavior on the testing day, with no significant effect of prenatal stress exposure and no interaction of the two prenatal exposures.

7.1.4 Conclusions.

While each prenatal exposure independently produced different behavioral outcomes, the results indicate that there is no significant interaction of prenatal ethanol and prenatal stress exposures on learning or anxiety at the exposure levels employed in this dual exposure paradigm. Subsequent studies will examine whether similar outcomes occur in male offspring and whether other measures of anxiety or learning are differentially impacted by these prenatal exposure paradigms.

7.2 Introduction

It is well established that exposure to ethanol during gestation is harmful to the developing fetus (Jones and Smith, 1973). The severity of the effects, which can range from intellectual and other behavioral deficits to physical dysmorphology, is related to the quantity of ethanol the mother consumed, how often during pregnancy she consumed ethanol, and when during her pregnancy the ethanol was consumed (Kelly et al., 2000 and Sood et al., 2001). These effects are termed Fetal Alcohol Spectrum Disorders (FASD), an umbrella phrase which collectively describes the varying ranges of possible effects due to maternal consumption of alcohol during pregnancy. While 12.2% of women report consuming ethanol at some point during their pregnancy (CDC 2002), the vast majority of the 2-5% of US children diagnosed with a FASD are on the less severe end of the spectrum (Willoughby et al., 2008, Green et al., 2009, Kodituwakku, 2009), meaning that their exposure to prenatal ethanol results in mild or subtle changes in intelligence which persist into adulthood in the absence of physical dysmorphology characteristic of Fetal Alcohol Syndrome. In addition to the well-characterized cognitive deficits, children prenatally exposed to alcohol have also been described as having non-cognitive deficits such as altered stress reactivity, heightened anxiety, hyperactivity and attention disorders (Famy et al, 1998, Steinhausen et al, 2003, and Kodituwakku, 2009). These behavioral changes can also persist well into adulthood and can exacerbate the cognitive challenges.

While alcohol is a known teratogen capable of causing a range of developmental defects, there are other pregnancy risk factors, such as maternal stress, that have been associated with adverse neurobehavioral outcomes in offspring. Due to the difficulty in defining and characterizing “maternal stress”, the precise epidemiology of prenatal stress is unknown, but the occurrence of anxiety during pregnancy is estimated at 8.5% (Ross and McLean, 2006). Similar to the effects of prenatal alcohol exposure, the effects seen in the offspring of mothers reporting stress or heightened anxiety during their pregnancy is dependent on the amount of stress incurred, the frequency of the stress, and the timing of the stress during their pregnancy (Mychasiuk et al., 2011, and O’Connor et al., 2003). In human studies of prenatal stress, altered emotionality, elevated levels of anxiety, attention disorders, and cognitive impairments are consistently reported (O’Connor et al., 2003, Laplante et al., 2004, Rodriguez and Bohlin, 2005, Martini et al., 2010). In one study, 17.6% of respondents self-reported high levels of anxiety and consumed high levels of alcohol (CDC 2002). Given the overlap in behavioral and cognitive consequences of prenatal ethanol and prenatal stress exposure, it is conceivable that when these two prenatal insults occur together, they may exacerbate the neurobehavioral consequences of each individual insult alone in affected offspring.

To date, there has been limited investigation on the putative synergistic effects of prenatal ethanol and prenatal stress exposure on behavioral outcomes. Using a non-human primate model, Schneider and colleagues have

demonstrated that moderate prenatal alcohol exposure in combination with chronic stress during mid to late gestation results in altered stress reactivity and reduced behavioral adaptation to a novel environment in both male and female offspring (Schneider et al., 2004). Additional evidence from this model shows that offspring from mothers who consumed ethanol and were exposed to chronic stress during gestation have an increased ratio of dopamine D2 receptors to dopamine synthesis and this observation correlated with reduced inhibition in a non-matching to sample task (Roberts et al., 2004).

While the non-human primate studies support the notion of synergistic interactions between prenatal ethanol and prenatal stress, it is not practical to pursue more detailed investigations of the underlying teratogenic mechanisms and the neurobiologic consequences of these interactions using this model. Thus, more detailed studies necessitated the development of a dual prenatal exposure model using rodents. However, the choice of prenatal ethanol and prenatal stress paradigms to employ in the characterization of a dual exposure paradigm warranted careful consideration from the onset. For example, many fetal alcohol researchers utilize methods of ethanol administration in their animal models which induce maternal stress, including intraperitoneal (IP) injection, oral gavage, and liquid diet ethanol administration (Norton and Kotkoskie, 1991), which each require the use of a third group (sham-injected, sham-gavage, or pair-fed, respectively) to control for the stress associated with the ethanol administration. However, the inherent additional stress involved with thesemodels could confound the interpretation of results in a dual exposure

paradigm as the dams exposed to both ethanol and stress would essentially be receiving two stressors. For example, Titterness and Christie (2011) recently reported that liquid diet administration produces a two-fold increase in circulating corticosterone level in rat dams compared to dams consuming rat chow ad libitum, which raises concern regarding the ability to systematically assess the effects of ethanol and stress in a dual exposure paradigm. Further, relative to repetitive maternal stress, it was important to utilize a stress procedure which the dams would not adapt to during the course of pregnancy, nor would the stressor adversely affect the level of ethanol consumption compared to non-stress controls.

In the present study, we employed a recently developed voluntary ethanol drinking paradigm (Savage et al., 2010), that minimizes the stress of ethanol administration and reduces the number of animals required for testing, in combination with a predator scent stressor paradigm, that minimizes the dam's habituation to the stressor, to examine the behavioral consequences of prenatal ethanol and prenatal stress exposure. Behavioral outcomes were measured in adult female offspring as they respond to forms of trace conditioning better than males (Dalla et al 2009) and females demonstrate increased anxiety as a result of prenatal stress compared to males (Matthews and Phillips, 2012 and Schulz et al, 2011).

7.3 Methods

Unless indicated in parenthetical text, all materials were obtained from Sigma Chemical Company, St. Louis, MO. All animal care and usage was in compliance with the University of New Mexico Health Sciences Center Institutional Animal Care and Use Committee.

7.3.1 Voluntary Drinking Paradigm.

As previously described in Savage et al (2010), four-month-old Long-Evan rat breeders (Harlan, Indianapolis, IN) were single-housed in plastic cages at 22o C and kept on a “reverse” 12-hour dark / 12-hour light schedule (lights on from 2100 to 0900 hours) with rat chow and water ad libitum. After acclimation to the animal facility, all female rats were provided 0.066% saccharin in tap water for four hours each day from 1000 to 1400 hours in addition to their ad libitum home cage food and water. On Days 1 & 2, the saccharin water contained 0% ethanol, on Days 3 & 4, the saccharin water contained 2.5% ethanol (v/v). On Day 5 and thereafter, the saccharin water contained 5% ethanol. Daily four-hour ethanol consumption was monitored for two weeks. At the end of two weeks of ethanol consumption, females that drank greater than one standard deviation below the mean of the entire group were removed from the study. The remainder of the females were assigned to either a saccharin (Sacc) control or 5% ethanol drinking (EtOH) group and matched such that the mean pre-pregnancy ethanol consumption by each group was similar. Subsequently, females were placed with proven male breeders until pregnant, as evidenced by the presence of a vaginal plug. Female rats did not consume ethanol during the breeding

procedure. Beginning on Gestational Day (GD) 1, rat dams were provided saccharin water containing either 0% or 5% ethanol for four hours a day in addition to their home cage food and water. The volume of 0% ethanol saccharin water provided to the controls was matched to the mean volume of saccharin water consumed by the ethanol drinking group. Daily four-hour ethanol consumption was recorded for each dam. Once a litter was delivered, the drinking tubes were no longer provided.

7.3.2 Predator Scent Stress Exposure Paradigm.

Prior to GD13, one-half of the dams in each of the two drinking groups were randomly assigned to either a control (No Stress) or predator scent exposure (Stress) group. Dams assigned to the No Stress group were left undisturbed in their home cages throughout the maternal stress procedures to minimize elevations in corticosterone levels. At 1800 hours on GDs 13, 15, 17, and 19, dams in the Stress group were transported from the breeding colony room to a nearby treatment room and placed separately in individual cages, which were connected via plastic tubing to an air pump. Three percent (v/v) 2,3,5-Trimethyl-3-thiazoline (TMT, Contech, Victoria, BC, Canada) was pipetted onto a small piece of filter paper and placed inline between the air pump and the cage. Dams were exposed to the scent gently blown into the cage for 20 minutes, and then returned to their home cage. Subsequently they were transported back to the breeding colony room where they were maintained in a separately vented cubicle to reduce scent contamination with the No Stress groups. On GD20, stressed females were returned to their original housing

location among the control females. A diagrammatic summary of the dual exposure paradigm is provided as Figure 7.1.

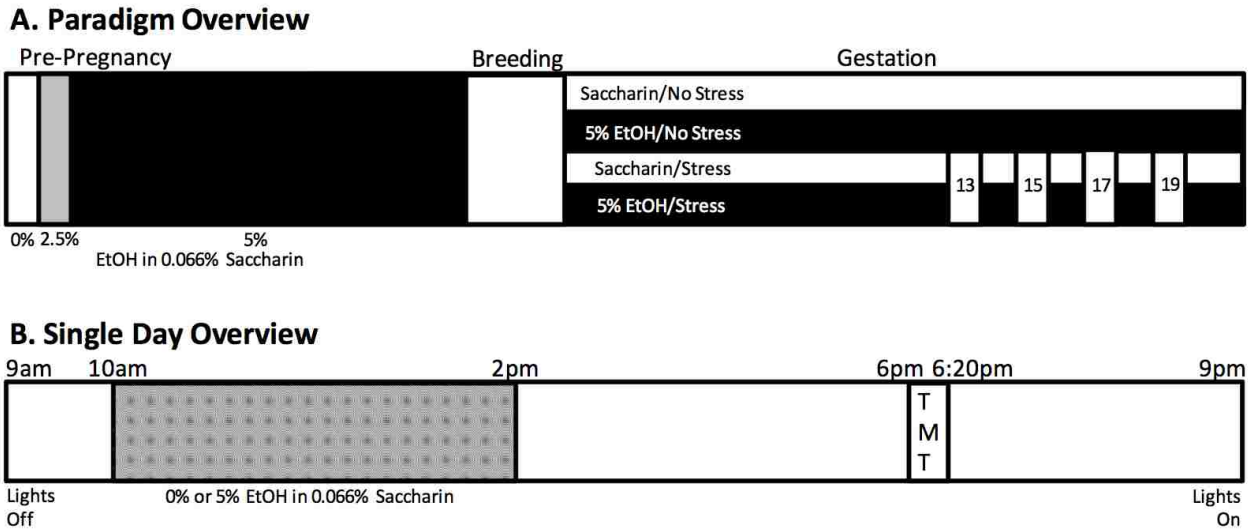

Figure 7.1. Schematic diagrams illustrating the design of the prenatal ethanol and prenatal stress exposure paradigms.

A: Disposition of female rats throughout the exposure paradigms. The concentration of ethanol (EtOH) is increased from 0% up to 5% (v/v) over the first five days and then 5% EtOH consumption measured for two weeks to establish pre-pregnancy drinking levels. After breeding, the dams are allocated into two groups. One group consumes saccharin water and the other consumes 5% EtOH. Beginning on GD13, each drinking group is subdivided with one-half subjected to maternal stress (3% TMT) and the other half left undisturbed in their home cage. MaterThe TMT exposure is repeated on GD15, GD17 and GD19. B: Diagram of events over a twenty-four hour cycle. Room lights go off at 0900 hours. Drinking tubes are introduced at 1000 hours and removed at 1400 hours. Maternal stress dams are placed in air-tight cages at 1800 hours, subjected to 3% TMT in air for 20 minutes and then returned to their home cage. Room lights go on at 2100 hours.

Rat dams from all four prenatal exposure groups were weighed on GDs 1, 7, 14, and 20. Prior to weighing on postnatal day (PN) 3, a single pup was removed from the nest and place on the opposite side of the cage. The latency for the dam to approach the displaced pup was recorded. Litters were weighed on PNs 3, 7, 14, and 21. The number of pups per litter was recorded on PN3. Litters were culled to 10 pups (five males and five females optimally) on PN3 and litters with less than 5 pups were not used in the study. Observed neonatal

mortality in litters in each of the exposure conditions was less than 0.01% (data not shown). Dams were videotaped for 30 minutes PN7, 8, or 9 in their home cage in the home room between 1000 hours and 1200 hours. Time spent on their nest was recorded. All litters were weaned on PN25-27 into sex- and exposure group-specific pair housing (cages contained 2-3 adult females). All offspring were maintained in group housing conditions until behavioral testing.

7.3.3 Blood Collection, Preparation and Corticosterone Assay.

In a separate set of dams at 1830 hours on GDs 13 and 19, pregnant dams were briefly anesthetized with isoflurane. A small nick was made at the base of the tail and 900 µL of blood collected in gel collection tubes (Ram Scientific, Yonkers, New York). Samples were kept on ice until the collections were complete, then centrifuged at 3500 x g for 5 minutes using a MiniSpin tabletop centrifuge (Eppendorf, Hamburg, Germany). Following centrifugation, plasma was collected and stored at -80o C until analysis. Plasma samples were thawed and maintained at 4oC prior to analysis. Samples were processed using a 125I-Corticosterone Radioimmunoassay Kit (RIA, Siemens, Los Angeles, CA) according to the manufacturer's instructions. Samples were measured in a gamma counter (Auto-Gamma 5000, Packard Instrument Company, Meriden, CT).

7.3.4 Elevated Plus Maze.

Anxiety-like behavior was assessed using an elevated plus maze (EPM) apparatus similar to that originally described by Pellow et al., (1985). Adult

female offspring (12-14 weeks of age) were exposed to used male bedding in their home cage to synchronize their estrus cycles into the diestrous phase 24 hours prior to behavioral testing. Synchronicity was confirmed via vaginal cytology in a separate set of female offspring similarly exposed to male bedding to avoid additional stress effects in behavioral testing. Animals were the same mean age and no difference in behavioral performance was noted between 12 week and 14 week old offspring. Animals were transferred to a testing room beginning at 0900 hours and immediately placed in the maze. The maze consisted of two open arms (50.8 cm long) and two closed arms (50.8 cm long). The open arms had a ledge 1 cm high to prevent the animals from slipping off the edge. The closed arms were surrounded by walls 30.5 cm tall and were covered to provide an enclosed environment. The plus-maze platform was elevated 50.8 cm off above the floor. Animals were allowed to freely move in the maze for five minutes. Their activity was video recorded and open arm entries, closed arm entries, and time spent in the open arms were recorded by an investigator blinded to the experimental condition of the subject.

7.3.5 Two Trial Trace Conditioning (TTTC).

Adult female offspring (12-14 weeks of age) were exposed to used male bedding in their home cage to synchronize their estrus cycles into the diestrous phase 24 hours prior to behavioral testing. Synchronicity was confirmed via vaginal cytology in a separate set of female offspring similarly exposed to male bedding to avoid additional stress effects in behavioral testing. Animals were the same mean age and no difference in behavioral performance was noted between

12 week and 14 week old offspring. On the conditioning day, animals were transferred from their home room to the conditioning room one at a time starting at 0900 hours. The animal was placed in the conditioning chamber, which consisted of a Habitest System (Coulbourn Instruments, Allentown, PA) equipped with a Precision Regulated Shocker and a 12" W x 10" D x 12" H test cage having a metal grid floor and allowed to acclimate to the novel surroundings for three minutes. As illustrated in Figure 7.2, immediately following the acclimation period, a constant, 30-second, 90 dB tone (the conditioned stimulus, CS) was played. After a 60-second trace interval where no stimulus was present, a two-second, one mA foot shock (the unconditioned stimulus, US) was administered. Ninety seconds later, the tone-trace-foot shock pairing was repeated. One minute following the second foot shock, the animal was moved back to their home cage and home room. The testing phase of the task began 24 hours after the conditioning session. The animals were moved individually from their home cage to a novel room and placed in a clean, plexiglass arena with a video monitoring camera positioned directly above it. The animal was placed in the novel environment for three minutes before the same 30-second, 90 dB tone from the conditioning session was played. Following the tone, the animal remained in the testing cage for three minutes and was then removed back to their home cage and home room. The entire six and a one-half minute testing sessions were recorded and freezing behavior was continuously scored across the session.

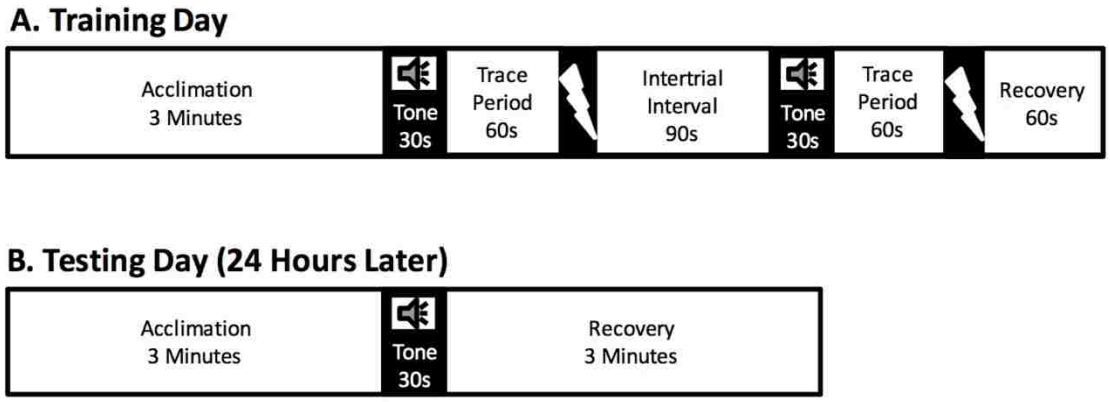

Figure 7.2. Diagram illustrating the experimental design of the two-trial trace conditioning paradigm.

A: Training phase. B: The testing phase, which occurred 24 hours following training. Freezing behavior was measured during the three-minute recovery period after the tone.

7.3.6 Statistical Analysis.

Statistical analysis was conducted using SPSS (two-way Repeated Measures ANOVA, maternal ethanol consumption [Figure 3] and maternal stress reactivity [Figure 4]) and SigmaPlot 11.0 (2-way ANOVA, all other analysis). All graphs were assembled in Graphpad Prism 5.

7.4 Results

7.4.1 Maternal Ethanol Consumption.

Daily four-hour ethanol consumption was measured throughout pregnancy. The weekly consumption data for both of the ethanol-consuming No Stress and Stress groups is presented in Figure 7.3. A repeated measures two-way ANOVA analysis (stress X week of pregnancy) revealed a main effect of pregnancy week [$F(2,216)=5.63$, $p=0.004$] with no significant difference in ethanol consumption between the No Stress and the Stress exposed dams. Both groups drank significantly more during the second week of gestation compared to the first and third weeks. Overall, the dams in the No Stress group consumed a mean of 2.44 ± 0.09 g EtOH/kg/day while the TMT-exposed consumed 2.39 ± 0.09 g EtOH/kg/day. There was no statistical difference in the blood ethanol concentrations at the end of the four-hour drinking period between the dams exposed to TMT and No Stress dams (data not shown).

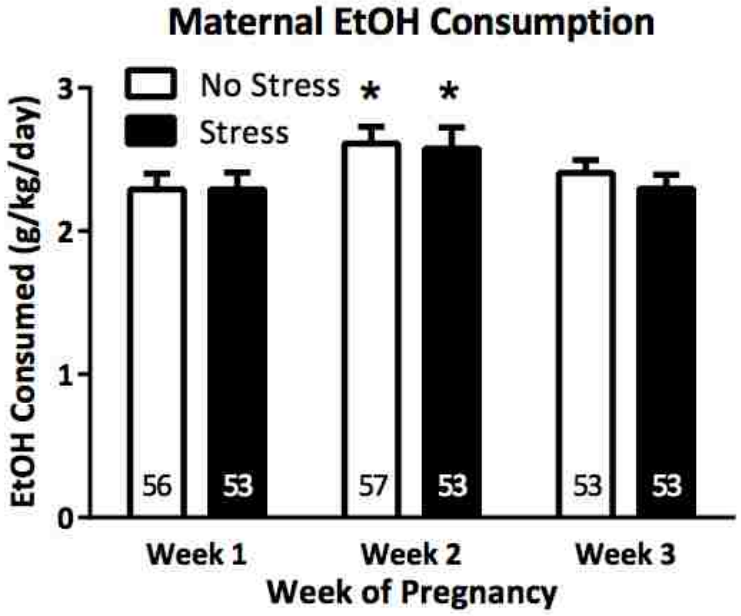

Figure 7.3. Weekly ethanol consumption during pregnancy and the impact of maternal stress on ethanol consumption.

Data bars represent the mean ± S.E.M. ethanol consumed each week in the No Stress group (open bars) and the Stress group during the third week of gestation (filled bars). Asterisk denotes a main effect of gestational week on ethanol consumption (2-way ANOVA; *-$p < 0.05$).

7.4.2 Maternal Stress Exposure.

In order to monitor the pregnant dams' response to the predator scent, blood samples were analyzed for plasma corticosterone (CORT), a hormone produced in response to stressful stimuli. Samples were measured 30 minutes following the onset of the scent presentation or time-matched for non-stressed controls, following the first presentation of scent on GD 13 and last presentation on GD 19. A repeated measure (with missing measures) two-way ANOVA (Ethanol x Stress) revealed a significant main effect of Stress on CORT levels [$F(1,26)=134.75$, $p<0.01$], and a significant interaction of Stress and Ethanol [$F(1,26)=6.23$, $p=0.02$]. Post-hoc comparisons revealed a significant difference

between the Sacc/Stress group and the EtOH/Stress group on GD 13 (p=0.02) with no effect between the two groups on GD 19 (p> 0.05). There was no significant difference between the CORT levels on GD 13 and those measured on GD 19 for any of the four exposure groups.

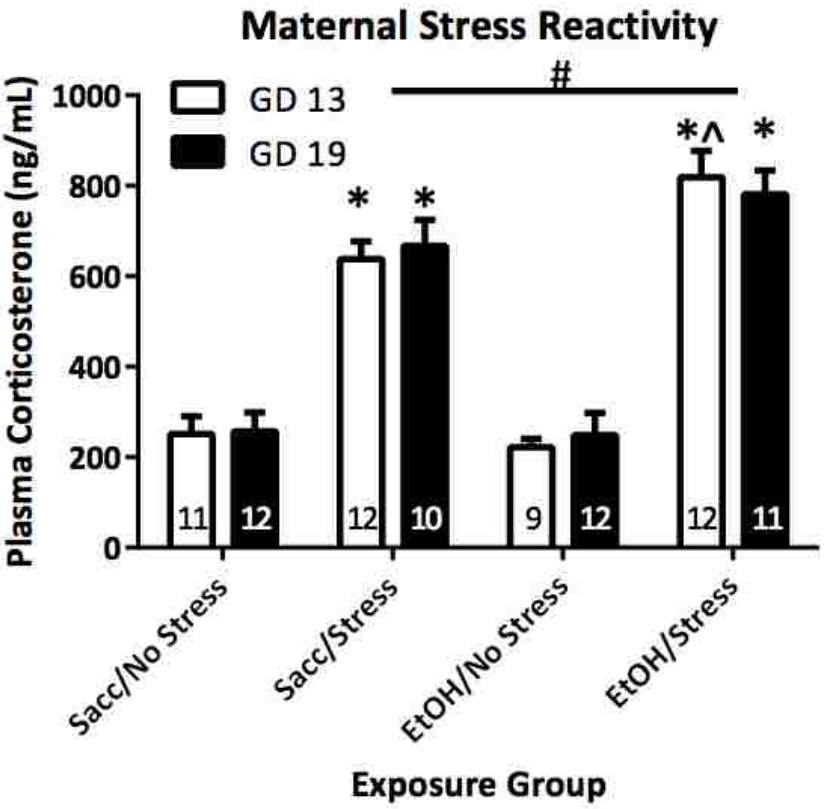

Figure 7.4. Impact of 3% TMT exposure on maternal serum corticosterone levels on Gestational Days 13 and 19.

Data bars represent the mean ± S.E.M. corticosterone on GD13 (open bars) and GD19 (filled bars) in rat dams. Asterisks denote a statistically significant main effect of Stress (repeated measure two-way ANOVA; $p < 0.05$). Hatch symbol denotes a significant interaction of Stress and Ethanol (repeated measure two-way ANOVA; $p < 0.05$) and arrow denotes a significant increase in CORT levels in EtOH/Stress as compared to Sacc/Stress on GD 13 only (post-hoc comparison; $p < 0.05$).

7.4.3 Other Exposure Paradigm Data.

Table 7.1 summarizes other data collected on the rat dams and their offspring. Maternal weight gain during pregnancy was not affected by either prenatal exposure paradigm, either alone or in combination. Likewise, the number of live pups born and their body weights on PN 3 and body weight at weaning were not different among the four prenatal treatment groups. Further, the quality of maternal care was not affected, based on measures of the latency to retrieve a displaced pup and the percent of time a dam spent on her nest.

	Sacc/No Stress	Sacc/Stress	EtOH/No Stress	EtOH/Stress
Maternal weight gain during pregnancy (g)	81.2 ± 45.4[a] (11)[b]	76.5 ± 29.3 (13)	94.6 ± 26.0 (22)	94.8 ± 1.6 (16)
Number of Pups on PN 3	10.6 ± 1.9 (10)	10.5 ± 1.9 (10)	11.3 ± 1.5 (18)	9.6 ± 1.8 (13)
Pup retrieval time PN 3 (s)	12.7 ± 10.8 (10)	8.7 ± 5.9 (12)	9.9 ± 7.4 (20)	7.9 ± 6.0 (14)
Time spent on nest PN 7-PN 9 (%)	49.1 ± 4.2 (11)	58.1 ± 7.6 (9)	49.4 ± 4.4 (16)	60.7 ± 6.3 (9)
Pup growth to weaning PN 25-27 (g)	40.9 ± 8.47 (190)	39.7 ± 9.7 (12)	39.8 ± 10.3 (20)	39.5 ± 7.1 (15)

Table 7.1. Paradigm Characteristics

Summary of the effects of prenatal exposure to ethanol and/or stress on rat dams and their offspring. [a]Mean ± SEM, [b]Sample Size

7.4.4 Elevated Plus Maze.

A two-way ANOVA analysis (Ethanol X Stress) of behavior on the elevated plus maze using adult females generated from the current model of combined exposure demonstrated a main effect of stress resulting in a reduction

of percent of time spent on the open arms [$F(1, 36)=4.39$, $p=0.04$] and the number of open arm entries [$F(1, 36)=4.11$, $p=0.05$], with no effect of ethanol exposure and no interactive effect of the dual exposure. While there was a 47% difference between the Sacc and EtOH groups in the No Stress group in time spent in the open arms, this difference was not statistically significant (unpaired two-tailed t-test, $t=1.214$, $df=17$, $p=0.24$).

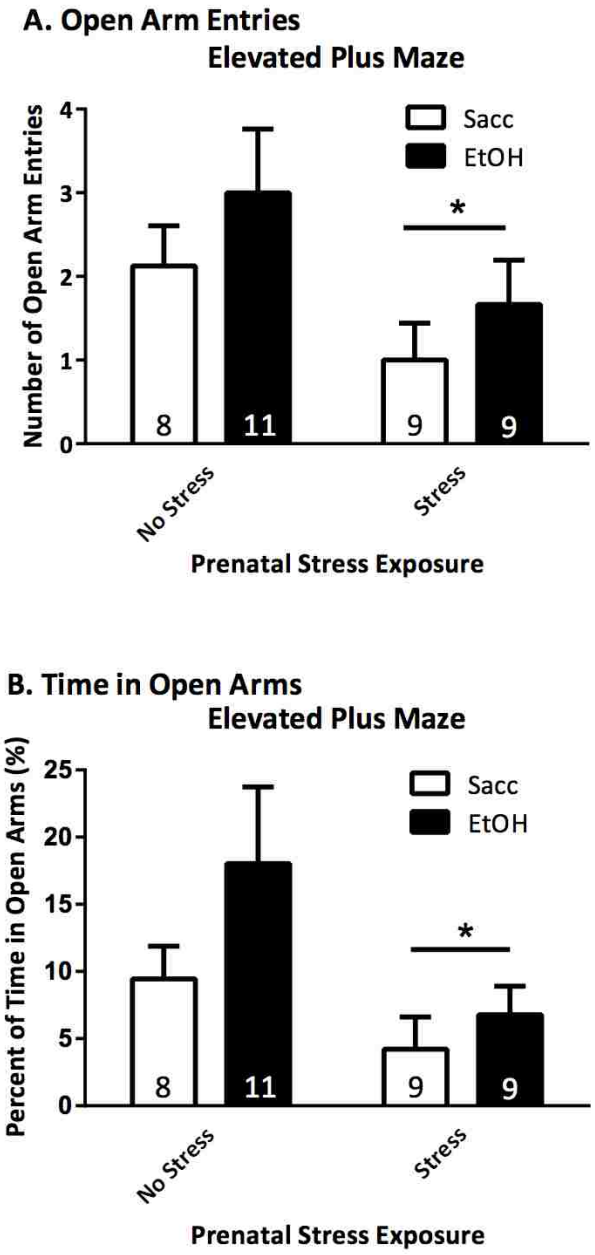

Figure 7.5. Impact of prenatal stress and/or prenatal ethanol exposure on female adult offspring behavior in the elevated plus maze.

A: Number of open arm entries. Data bars represent the mean ± S.E.M. B: Percent time spent in the open arms. Data bars represent the mean ± S.E.M. Asterisks denote a significant main effect of Stress on exploratory behavior in the elevated plus maze (two-way ANOVA; $p \leq 0.05$.)

7.4.5 Two-Trial Trace Conditioning.

We assessed behavioral performance in a hippocampal-sensitive learning task (Raybuck and Lattal, 2011), the Two-Trial Trace Conditioning task (TTTC). A two-way ANOVA (Ethanol X Stress) analysis of the freezing behavior during the three minutes following the tone on the testing day indicated a main effect of Ethanol resulting in a reduction of freezing behavior [$F(1, 44)=4.88$, $p=0.03$]. There was no significant effect of Stress, nor an interaction of the dual exposure.

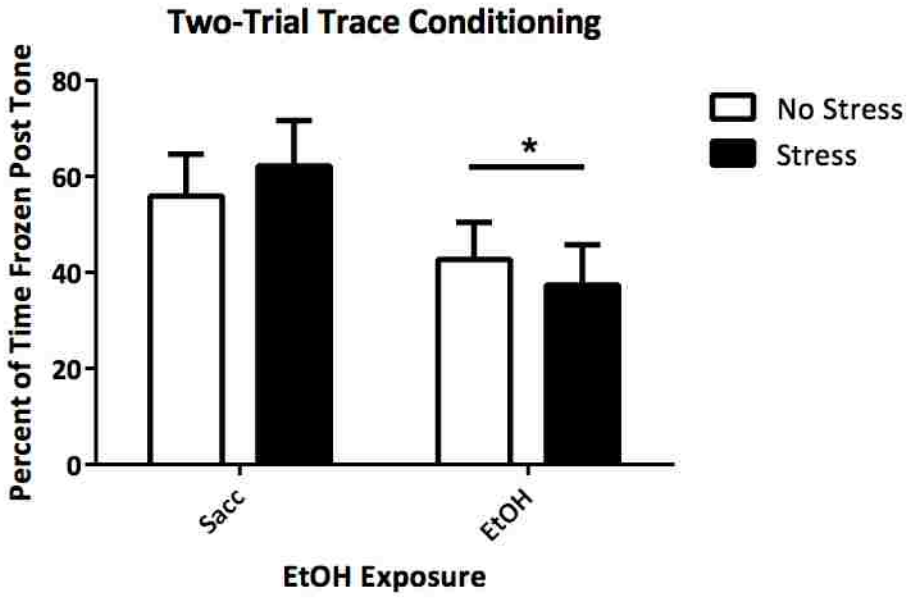

Figure 7.6. Impact of prenatal stress and/or prenatal ethanol exposure on hippocampal-sensitive learning in the Two-Trial Trace Conditioning task.

Data bars represent the mean ± S.E.M of twelve adult female offspring in each experimental group. Adult females prenatally exposed to ethanol showed reduced freezing on the testing day compared to their Sacc-exposed counterparts, with no impact of Stress. (Ethanol main effect; $p<0.05$).

7.5 Discussion

The primary objective of this study was to establish a dual exposure prenatal ethanol and prenatal stress paradigm that would allow a systematic examination of the impact of each of these two pregnancy risk factors on behavioral outcomes in the offspring. Operationally, the goal was to overlay a relatively novel predator scent stress paradigm with a previously established moderate prenatal ethanol exposure paradigm (Savage et al., 2010) where each exposure was delivered during the rodent awake cycle, yet in a manner that would minimize potential confounds of the dual exposure paradigm. The predator scent exposure time was scheduled near the awake-phase trough of the diurnal serum corticosterone (CORT) cycle both to maximize its impact on maternal CORT levels as well as to minimize potential alterations in voluntary ethanol consumption. As illustrated in Figure 7.2, TMT exposure during the third week of gestation did not alter ethanol consumption compared to the No Stress group (Figure 7.2).

Exposure to TMT elicited a roughly three-fold increase in maternal plasma CORT levels compared to No Stress controls. The elevations in maternal CORT corticosterone reported here are similar to the levels to those reported by Patin et al., (2002) as a result of acute and repeated exposure to cat predator scent on GD 10 or GD 14, as well as the levels reported by Barbazanges et al., (1996) as a result of a 45-minute restraint stress session during the last week of gestation. A small, but statistically significant, increase in the TMT exposure-induced elevation in maternal CORT was observed in the dams also consuming ethanol

(Figure 7.3). However, this interactive effect was not associated with any synergistic effects on the various outcome measures examined in the course of this study.

Other potential confounds which could arise as a result of this dual exposure paradigm and thus create a challenge in interpreting the behavioral outcome data in adult offspring included altered maternal nutritional intake (reviewed in Alamy and Bengelloun, 2012), reduced care of litters (Liu et al., 2000), and the possibility that both prenatal ethanol and prenatal stress exposures can result in reduced litter size (Baker et al., 2008, Patin et al., 2002), or pup growth retardation (Lopez-Tejer et al., 1986, Levitt et al., 1996, and Drago et al., 1999). However, no significant differences were observed among the four exposure groups relative to maternal weight gain during pregnancy, maternal care, number of pups per litter or pup weight gain to weaning (Table 7.1). Further, the lack of differences in the level of maternal care and the amount of weight gained by the pups until weaning mitigated the need to cross-foster the pups in order to avoid these possible confounds. Taken as a whole, this data also implies that each of the these prenatal exposure procedures as conducted in this study, either alone or in combination, are relatively moderate in nature as they did not cause some of the gross deviations reported using more severe prenatal ethanol or prenatal stress exposure conditions.

Moderately severe prenatal stress exposure, elicited by the application of eighty 0.5 mA foot-shocks over a two-hour period to the dams every other day throughout gestation, has been shown to induce anxiety-like behavior in the adult

female offspring (Estanislau and Morato, 2006). In the present study, we demonstrate that a more moderate prenatal stress procedure can also elicit increased anxiety-like behavior using the elevated plus maze (Figure 7.5). Further, this difference cannot be attributed to differences in either locomotor or exploratory activity as these measures were not different among the four prenatal exposure groups (data not shown).

The effects of prenatal ethanol exposure on anxiety have mainly been reported in male offspring. Prenatal exposure to relatively high ethanol levels (~350 mg/dL peak maternal serum ethanol concentrations) resulted in a significant reduction in the time spent on the open arms of the elevated plus maze (Dursen et al., 2006). However, this effect was not observed in adult female offspring using a more moderate prenatal ethanol exposure paradigm that produced a 135 mg/dL peak maternal serum ethanol concentration (Osborn et al., 1998). Similarly, our voluntary maternal ethanol consumption paradigm which produces a peak of 80 mg/dL did not affect the time spent on the open arms of the elevated plus maze. Further, the combination of prenatal ethanol exposure with prenatal stress did not increase anxiety-like behavior compared to prenatal stress alone suggesting the level of prenatal ethanol exposure achieved with our voluntary drinking paradigm was possibly too low to exacerbate the effect of moderate prenatal stress on the elevated plus maze.

Previous work utilizing the prenatal ethanol arm of the model presented here demonstrated reduced learning in a contextual delay conditioning task in prenatal ethanol-exposed female offspring (Savage et al., 2010). We replicated

this finding using a hippocampal-sensitive two-trial trace conditioning task (Raybuck and Lattal, 2011), where prenatal ethanol-exposed offspring demonstrated significantly less freezing behavior in response to the acoustic stimulus on the testing day (Figure 7.6). In contrast, prenatal stress exposure did not impair learning in this task. This lack of an effect in female offspring from prenatal stress was also observed by Zagron and Weinstock (2006) who subjected dams to a single 30 minute session of restraint stress each day during the last week of gestation and Salomon et al (2011) who used a variable stress paradigm during the last week of gestation. These observations are in contrast to findings that more severe levels of prenatal stress do cause learning deficits (Lemaire et al., 2000, Lordi et al., 2000, and Wu et al., 2007) suggesting that the impact of maternal stress on learning may, in part, be dependent on the duration, frequency and/or intensity of the stressor events, gender being evaluated, and type of learning being assessed. However, direct comparisons between different prenatal stress studies is difficult because the stress paradigms employed vary widely by stressor type, the duration, frequency and intensity of stressor events, and by the fact that quantifiable markers, such as maternal corticosterone levels following stress exposure, are often not reported. In addition to not affecting two-trace conditioning alone, combining prenatal stress with prenatal ethanol exposure did not exacerbate the effect of prenatal ethanol exposure alone on learning, again suggesting a lack of interaction between these two prenatal insults on a specific behavioral response using the moderate exposure conditions employed in this study.

In summary, the work presented here represents a first effort to systematically characterize the neurobehavioral consequences of a dual prenatal ethanol and prenatal stress exposure paradigm. We observed differential behavioral effects in that moderate prenatal stress produced anxiety-like behavior in offspring but did not affect hippocampal-sensitive learning, whereas moderate prenatal ethanol exposure produced an opposite effect on these two measures. No synergistic interactions were observed at the levels of prenatal exposure employed here, suggesting that separate teratologic mechanisms and brain regions may be involved in mediating prenatal stress versus prenatal ethanol effects. A similar lack of synergistic interaction was recently reported relative to the effects of maternal stress on prenatal ethanol-induced alterations in synaptic plasticity (Titterness and Christie, 2012). Given that higher or more severe levels of prenatal exposure to either ethanol or stress affect both anxiety and learning behavior (Dursen et al., 2006, Lemaire et al., 2000, Lordi et al., 2000, and Wu et al., 2007), one alternative explanation for our results is that different behavioral outcomes have differential sensitivities or "exposure thresholds" for producing adverse neurobehavioral outcomes in response to different prenatal insults. Thus, variations in a "behavioral phenotype" may likely be attributable to varied degrees of exposure to variable combinations of prenatal insults.

A number of different types of follow-up studies employing this dual exposure paradigm are contemplated for future study. For example, one question is whether these behavioral responses to prenatal ethanol and/or

prenatal ethanol are affected by the estrus cycle phase during which the behavioral testing is conducted. The diestrous phase was selected for initial study here as hormone levels remain relatively stable for the duration of the two-day phase, minimizing the potential impact of hormonal variations during the two-day trace conditioning task. Re-assessing these behaviors across different phases of estrous would be of interest since these variations can play a major role in learning and memory performance, as well as anxiety levels in female rats (Warren and Juraska, 1997, Marcondes et al., 2001). Another question is the impact of the dual exposure paradigm on male offspring. Females were employed in this initial study due to the effects of the voluntary maternal drinking paradigm on female offspring performance in a delay fear conditioning task (Savage et al., 2010) as well as a heightened susceptibility of female offspring to anxiety compared to controls (Weinstock 2011). It would be important to evaluate male offspring to determine whether the dual exposure paradigm produces sex differences in behavioral responses. It is also possible that the male offspring from the combined exposure group would demonstrate interactive effects of prenatal ethanol and prenatal stress, as males have been reported to exhibit learning and memory impairments as a result of prenatal stress, while females are more often subject to increased anxiety behavior (reviewed in Weinstock 2011). Beyond putative estrus phase- and gender-based differences in behavioral outcomes, it would be of interest to conduct a more systematic investigation of the impact of this dual exposure paradigm on different types of learning as well as other kinds of behavioral responses.

Another avenue for subsequent investigation would be to examine the extent to which manipulations in the design of the dual exposure paradigm would affect behavioral outcomes. For example, it is conceivable that a more severe prenatal stress paradigm may have produced deficits in two-trial trace conditioning and/or a synergistic interaction with the effects of moderate prenatal ethanol exposure on learning. Conversely, higher levels of prenatal ethanol exposure may have produced anxiety-like behavior and/or exacerbated the effects of prenatal stress on anxiety responses. While the voluntary ethanol consumption paradigm employed in the current studies minimizes the potential confound of stress due to the method of ethanol administration, it is limited in that it is not possible to produce markedly higher maternal serum ethanol concentrations. Higher maternal ethanol levels can be achieved with other ethanol administration paradigms, such as oral gavage or ethanol vapor inhalation. However, these administration paradigms are more stressful, thus increasing a potential confound for interpreting ethanol-stress interactions. Another experimental design manipulation to consider would be reversing the order of presentation of the prenatal exposures, having the maternal stress precede or even overlap with the ethanol consumption period, a pattern with perhaps more clinical relevancy. It is possible that the temporal separation of the prenatal exposures employed here contributed to the lack of interactive effects of the dual exposure paradigm on offspring. Again however, this approach would need to be carefully examined to determine whether concurrent exposures affects either ethanol consumption or

stress response in a manner that could limit the ability to assess the interactive effects of each factor on offspring outcome measures.

8. Impact of Combined Prenatal Ethanol and Prenatal Stress Exposures on Markers of Activity Dependent Synaptic Plasticity in the Rat Dentate Gyrus

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8.1 Abstract

Prenatal ethanol exposure and prenatal stress can each cause long-lasting deficits in hippocampal-sensitive learning. These prenatal insults have also been shown to alter hippocampal synaptic plasticity. To date, the impact of either prenatal ethanol or prenatal stress on the neurobiological substrates that subserve synaptic plasticity following a learning event have not been investigated. We examined the effects of prenatal ethanol exposure and prenatal stress exposure, either alone or in combination, on the cytosolic expression of Activity-regulated Cytoskeletal (ARC) protein and the synaptosomal expression of AMPA receptor subunits in dentate gyrus of female adult offspring under baseline conditions and after two-trial trace conditioning (TTTC). Surprisingly, baseline cytoplasmic ARC expression was significantly elevated in both prenatal treatment groups. In contrast, synaptosomal GluA1 receptor subunit expression was decreased in both prenatal treatment groups. Similar effects were not observed for synaptosomal GluA2 subunit expression. TTTC did not alter ARC levels compared to an unpaired behavioral control (UPC) group in any of the four prenatal treatment groups. In contrast, TTTC significantly elevated both synaptosomal GluA1 and GluA2 subunit expression relative to the UPC group in control offspring, an effect that was not observed in any of the other three prenatal treatment groups. Given ARC's role in regulating synaptosomal AMPA receptors, these results suggest that prenatal ethanol- or prenatal stress exposure-induced increases in baseline ARC levels could contribute to reductions in both baseline and activity-dependent changes in AMPA receptors in

a manner that diminishes the role of AMPA receptors in dentate gyrus synaptic plasticity and hippocampal-sensitive learning.

Keywords: Fetal Alcohol spectrum Disorder, Prenatal Stress, AMPA Receptor, ARC, Western Blot

8.3 Introduction

Prenatal exposure to ethanol results in long-lasting deleterious effects on IQ, behavior, attention and, in case of more severe exposures, physical dysmorphology. The severity of ethanol's impact on the developing fetus has been shown to be related to the quantity of ethanol consumed, the pattern of ethanol consumption (Bailey et al, 2004), and the gestational timing of exposure (Kelly et al, 2000; Sood et al, 2001). The resulting range of outcomes has been termed Fetal Alcohol Spectrum Disorder (FASD). In the US, an estimated 2-5% of school-aged children suffer from FASD (May et al, 2009), the majority of which have no physical characteristics, but demonstrate significant reductions in IQ and cognitive deficits (Willoughby et al, 2008, Green et al, 2009, Kodituwakku, 2009). These reductions in cognition have been shown to last into adulthood (Barr et al, 2006), signifying a persistent role of ethanol exposure during development on learning and memory.

Various animal models of prenatal ethanol exposure have been employed to elucidate teratogenic mechanisms of ethanol in the developing fetus (Cudd, 2005). These models allow for precise experimental control, as well as providing for potential cellular and molecular analysis of animals demonstrating behavioral deficits which parallel those observed in humans. Studies of adult animals exposed to moderate amounts of ethanol during gestation have reported reductions in hippocampus-sensitive learning and memory tasks (Berman and Hannigan, 2000; Sutherland et al., 2000; Weeber et al., 2001; Savage et al, 2002; Savage et al, 2010; Brady et al, 2012), as well as deficits in measures of

hippocampal synaptic plasticity (Sutherland et al., 1997; Savage et al, 2002, Varaschin et al, 2010, Titterness and Christie, 2012; Brady et al, 2013), a major functional aspect of learning.

Similar to ethanol, maternal stress during pregnancy can also cause adverse neurodevelopment impact on the fetus. In human populations, children born to women who have survived acute trauma (severe weather events, terrorist attacks, etc) or have adverse living circumstances (chronic stress) have been reported to suffer from significant cognitive impairments (O'Connor et al, 2003; Laplante et al, 2004; Rodriguez and Bohlin, 2005; Martini et al, 2010). However, identifying and characterizing stress in human populations is challenging due to the high number of variables associated with individual perceptions of stress. Thus, most knowledge regarding the effects of prenatal stress on offspring behavior has come from animal model studies. Animals born to mothers who were exposed to stressors during gestation are typically smaller (Rice et al, 2010), demonstrate heightened anxiety and stress reactivity (Vallee et al, 1997), and display cognitive deficits, particularly on hippocampal-sensitive tasks as well as long-term potentiation (Yang et al, 2006). The severity of these characteristics are related to the type and duration of the stress the mother was subjected to during her pregnancy (O'Connor et al, 2003, Mychasiuk et al, 2011), as well as her predisposition to stress reactivity.

While a great deal is known regarding the neurochemical changes associated with synaptic strengthening in long-term potentiation (see review by Shepherd and Huganir, 2007), the neurochemical mechanisms underlying the

deficits in activity-dependent synaptic plasticity as a consequence of either prenatal ethanol exposure or prenatal stress are not well understood. The quantity, activity and/or translocation of a number of proteins are critical to the expression or maintenance of long-term potentiation (LTP), thought to be a prevailing cellular mechanism underlying learning (see review by Citri and Malenka, 2008). In the present study, we elected to examine the impact of either prenatal ethanol exposure and/or prenatal stress exposure on the expression of two proteins involved in activity-dependent changes in synaptic plasticity, namely, Activity-Regulated Cytosolic protein (ARC) and two AMPA-glutamate receptor subunits (GluA1 and GluR2). ARC protein has been shown to be up-regulated within four hours following maximal electroconvulsive shock (MECS) (Lyford et al, 1995), immediately following classical eyeblink conditioning (Mokin et al, 2005), one hour following auditory cued fear conditioning (Longeran et al, 2010), and one hour following the final training in a lever-press task (Kelly and Deadwyler, 2003). ARC has direct roles in the expanding cytoskeletal network within the postsynaptic cell (Huang et al, 2007). Likewise, previous work has demonstrated activity-dependent increases in AMPA receptors following LTP induction (Shi et al, 2001, and Takahashi et al 2003), with specific increases observed in GluA1 and GluA2 expression following various forms of fear conditioned learning (Yeh et al, 2006, Matsuo et al, 2008). Interestingly, the interaction of ARC protein expression and the AMPA receptor has implications of various forms of synaptic plasticity. While ARC is known to play a role is supporting the expanding actin cytoskeleton, it also has been demonstrated to

play a role in AMPA receptor endocytosis from the synaptic membrane (Chowdhury et al, 2006), implicating a role for long-term depression (LTD) (Waung et al, 2008).

As described previously, both prenatal ethanol and prenatal stress individually induce deficits in learning and memory performance and LTP. ARC expression and AMPA receptor expression have been demonstrated to be required for proficient performance on learning tasks, with recent evidence demonstrating an elevation in ARC expression following various models of trace fear conditioning (Chau et al, 2013, Chia and Otto, 2013) as well as sufficient LTP expression (Plat et al, 2006). In models of prenatal ethanol exposure, basal quantities of AMPA receptor subunits are differentially expressed as compared to non-exposed controls (Dettmer et al, 2003), and ARC mRNA expression is reduced as compared to controls following a novel social experience (Hamilton et al, 2010). However, basal ARC protein expression has not been evaluated in animals prenatally exposed to ethanol, stress, or the combination. Similarly, changes in ARC protein nor AMPA receptor subunit expression has not been assessed following behavioral activation in animals prenatally exposed to ethanol, stress, or the combination.

We recently reported that in a model of combined prenatal ethanol and prenatal stress exposure, the moderate prenatal ethanol exposure decreased learning of a behaviorally challenging two-trial trace conditioning (TTTC) paradigm (Staples et al., 2013). Employing the training portion of TTTC as a behavioral activation paradigm, we tested the hypothesis that prenatal ethanol

exposure and/or prenatal stress exposure would diminish TTTC-induced elevations in ARC and AMPA receptor subunit expression.

8.4 Materials and Methods

All chemicals were from Sigma-Aldrich (St. Louis, MI) unless otherwise noted. All animal care and usage was in compliance with the University of New Mexico Health Sciences Center Institutional Animal Care and Use Committee.

8.4.1 Animals

Prenatal ethanol- and prenatal stress-exposed Long-Evans rat offspring were generated as previously described by Staples *et al* (2013). Briefly, females and breeder males were single-housed and maintained on a reverse light/dark cycle. Prior to mating, females were gradually acclimated to voluntarily drinking 5% ethanol in 0.066% saccharin (Sacc) for four hours during the awake phase of the light-dark cycle. Tap water and rat chow were available continuously. Following two weeks of 5% ethanol consumption, females drinking within one standard deviation of the group mean were paired with a proven breeder male until pregnancy was confirmed via the presence of a vaginal plug. Ethanol was not available during breeding. Pregnant rat dams were then returned to their single housing conditions and voluntarily consumed either 0% or 5% ethanol in 0.066% saccharin until delivery. Dams assigned to the prenatal stress group were exposed to 3% 2,3,5-Trimethyl-3-thiazoline (TMT, Contech, Victoria, BC, Canada) for 20 minutes four hours following the removal of the ethanol or saccharin tube on gestational days (GD) 13, 15, 17, and 19 (dates selected based on preliminary data not published). Following delivery, dams and litters were left undisturbed until weaning on postnatal day 25 when the offspring were moved into sex-matched group housing with littermates. Except for routine cage

bedding changes, offspring were left undisturbed until experimental use. This is visually summarized in Figure 8.1.

Figure 8.1. Schematic diagram illustrating the combined prenatal ethanol and prenatal stress exposure paradigms.

Pregnant dams were exposed to either 0% or 5% ethanol in 0.066% saccharin for the duration of their pregnancy. Half of each group was exposed to 3% predator scent (TMT) on gestational days (GD) 13, 15, 17, and 19. Litters were weaned into sex and exposure matched cages on postnatal day (PN) 25. Behavioral training and tissue collection occurred on PN 90.

Only female offspring at three months of age were used in the experiments described in this work due to their propensity to learn trace conditioning (Dalla et al, 2009). One week prior to experimentation, eighteen subjects from each of the four prenatal treatment groups were randomly separated into three experimental groups (six rats per prenatal treatment / experimental condition group) and maintained in single-housed cages. One group, designated as the non-handled control for assessing baseline biochemical measures, was not further disturbed until 24 hours prior to sacrifice for tissue processing, when females from all experimental groups were exposed to used male bedding to synchronize their estrous cycle, in order to reduce estrus cycle variations in estrogen, which can create confounds in analyzing data derived

from female subjects. Synchronicity of the estrous cycle was confirmed in a separate set of females similarly exposed to the used male bedding.

8.4.2 Two-Trial Trace Conditioning Paradigm

The other two experimental groups of female rat offspring were subjected to behavioral training 24 hours after exposure to used male bedding. One group of subjects was exposed to the training portion of a Two-Trial Trace Conditioning (TTTC) paradigm, described by Staples *et al*, (2013). Briefly, each rat was placed in the conditioning chamber, which consisted of a Habitest System (Coulbourn Instruments, Allentown, PA) equipped with a Precision Regulated Shocker and a 12" W x 10" D x 12" H test cage having a metal grid floor and allowed to acclimate to the novel surroundings for three minutes. Immediately following the acclimation period, a constant, 30-second, 90 dB tone was played. After a 60-second trace interval where no stimulus was present, a two-second, one mA foot shock was administered. Ninety seconds later, the tone-trace-foot shock pairing was repeated. One minute following the second foot shock, the animal was moved back to their home cage and home room. The rat was sacrificed one hour following the termination of training. This behavioral training paradigm was selected due to it's hippocampal sensitivity, and limited number of training trials.

The remaining experimental group of subjects served as a behavioral control for TTTC. These rats were housed for the week prior to experimentation in plastic cages that had been modified with metal grid rods across cage floor

approximately 0.5 cm above the bottom of the cage. On the testing day, these rats were subjected to all three of the relevant stimuli (context, tone, and foot shock,) but at temporally and contextually dissociated points in time so as to prevent the formation of association among the three stimuli. These so-called "Unpaired Control" (UPC) group of rats were allowed to explore the context chamber for 8.5 minutes and then were returned to their grid floor home cages. One hour later, the UPC animals were given two presentations of the tone while remaining in their grid floor cages. Subsequently, the rat was moved back to the home housing room. One hour later, the UPC rat was shocked twice in their grid floor cage, in the home room, and then sacrificed one hour following the foot shock.

8.4.3 Tissue Collection and Preparation

Preliminary time-course studies using Saccharin / No stress control offspring established that 60 minutes post TTTC training was an optimal time to measure both ARC and AMPA receptor subunit expression. Rats were rapidly decapitated and the cortex was quickly removed from the skull. The DG portion of the hippocampus was micro-dissected from the surrounding cortical tissue, frozen in liquid nitrogen and stored at -80^{o}C until further processing. The two DG from a single animal were pooled to serve as a single sample and were frozen, stored, and processed together.

Similar to the sample preparation described in Samudio-Ruiz et al (2010), samples were thawed on ice, then homogenized in a glass Dounce homogenizer

in 300 µL of homogenization buffer (320 mM Sucrose, 10 mM Tris HCl, 1 mM $NaVO_4$, 5 mM NaF, 1 mM EDTA, 1 mM EGTA, and protease inhibitor [Roche, Indianapolis, IN]) with six up and down strokes each of a “loose-fitting” and then a tight-fitting pestle. The resulting homogenate was centrifuged at 1000xg for six minutes at 4 ^{o}C. The supernatant (S1) was then centrifuged at 10,000xg for 15 minutes at 4 ^{o}C resulting in a crude synaptosomal fraction (P2) and a crude cytosolic fraction (S2). One percent sodium dodecyl sulfate (SDS) was added to each sample and the protein concentration was determined by BCA protein assay (Thermo Scientific, Rockford, IL) according to the manufacturer’s instructions. Homogenized samples were frozen in liquid nitrogen and stored at -80 ^{o}C until western blot analysis. This is visually summarized in Figure 8.2.

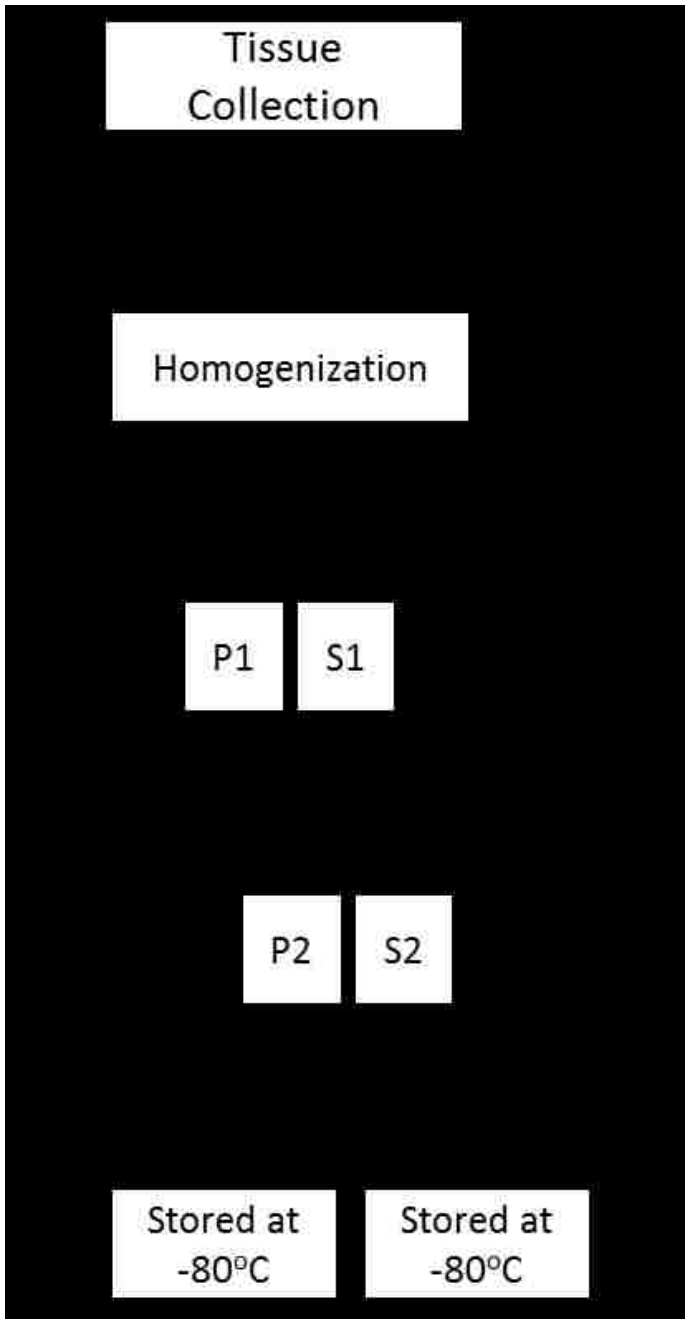

Figure 8.2. Schematic diagram illustrating the crude synaptosomal and crude cytosolic sample preparation.

Following rapid decapitation, the brain was removed from the skull, and the dentate gyrus from both hemispheres was separated from the surrounding hippocampal tissue and homogenized in a glass Dounce homogenizer in homogenizing buffer. The resulting homogenate was then centrifuged at 1000xg for 6 minutes at 4°C. The supernatant (S1) was then centrifuged again at 10000xg for 15 minutes at 4°C. The resulting pellet (P2) was the crude synaptosomal fraction and the resulting supernatant (S2) was the crude cytosolic fraction. Both were stored at -80°C until use.

8.4.4 Western Blot Analysis

A total of 10 µg of each sample was loaded onto 8% SDS polyacrylamide gel and electrophoresed at 150 V for one hour then transferred to a PVDF membrane at 100 V for one hour or at 30 V overnight at 4 ^{o}C. Membranes were then washed in TBS for five minutes then blocked in iBlock (Life Technologies, Grand Island, NY) for one hour at room temperature. Subsequently, membranes were incubated in primary antibody (GluA1-1:600, Millipore #AB1504; GluR2-1:500, Abcam #AB20673; or ARC-1:150, Santa Cruz Biotechnology #17839) and 0.1% Tween in iBlock for one hour at room temperature or overnight at 4 ^{o}C. Membranes were washed for 3x5 minutes in TBST and then incubated in the appropriate secondary fluorescent antibody (1:10000 anti-rabbit or 3:10000 anti-mouse, Li-Cor Biosystems, Lincoln, NE) in iBlock with 0.1% Tween and 0.01% SDS for 45 minutes at room temperature. Finally, the membranes were washed 3x5 minutes in TBST and 2x5 minutes in TBS. Membranes were scanned using an infrared imaging system (Li-Cor Odyssey, Li-Cor Biosciences, Lincoln, NE) in the 800 nm wavelength.

Blots were then incubated with 0.4% Coomassie stain (Coomassie Brilliant Blue R-250, Bio-Rad, Hercules, CA) in 50% methanol, 10% acetic acid, and 40% ddH_2O for 30 minutes at room temperature. The membranes were destained using 50% ddH_2O, 43% methanol, and 7% acetic acid. Destaining solution was exchanged for fresh solution until protein bands were clearly resolved. The blot was then dried and scanned on a flatbed scanner (Canon, Melville NY). Optical densities of proteins of interest and Coomassie staining were measured using

Image Studio Lite (Li-Cor Biosciences, Lincoln, NE). The optical density of each band was first normalized to the Coomassie staining of the lane to control for variation in total protein loaded (Welinder and Ekblad, 2011), then controlled to a hippocampal homogenate loading control which was present on all blots to control for differences in incubations between blots.

8.4.5 Statistical Analysis

Statistical analysis was conducted using SigmaPlot 11.0 (San Jose, CA). Graphs were generated using GraphPad 6.0 (La Jolla, CA).

8.5 Results

8.5.1 Impact of the Dual Prenatal Exposure Paradigm on Maternal Status and Offspring

Previous work characterizing the dual exposure model used here reported no significant reduction in maternal ethanol consumption as a result of stress exposure or maternal stress response as a result of ethanol consumption, the quantity of weight gained by dams during gestation, offspring birth weight, measures of maternal care, litter size, or pup growth to weaning (Staples et al, 2013). Pregnant dams in this model consumed an average of 2.4 ± 0.09 g EtOH/kg body weight/gestational day. The adult female offspring generated from this paradigm demonstrated significant impairments on the TTTC task, a hippocampal-sensitive test of associative learning and memory, as a result of prenatal ethanol exposure, whereas prenatal stress exposure alone did not significantly impair learning (Staples et al., 2013). Adult female animals prenatally exposed to ethanol demonstrated 33% less freezing than their non-exposed counterparts, regardless of prenatal stress exposure (Staples et al, 2013).

8.5.2 Baseline ARC and GluA Receptor Subunit Levels

8.5.2.1 Cytosolic ARC Expression.

A two-way ANOVA revealed an interactive effect of prenatal ethanol and prenatal stress exposures ($F[1,19]=9.83$, $p=0.005$) on baseline cytoplasmic ARC levels (Figure 8.3). Post-hoc comparisons revealed a significant elevation in basal ARC expression in rats exposed to either prenatal stress or prenatal ethanol as compared to their Sacc/No Stress control group ($p=0.013$, $p=0.003$

respectively) and a nearly significant elevation due to the combine prenatal exposure as compared to the Sacc/No Stress controls (p=0.07).

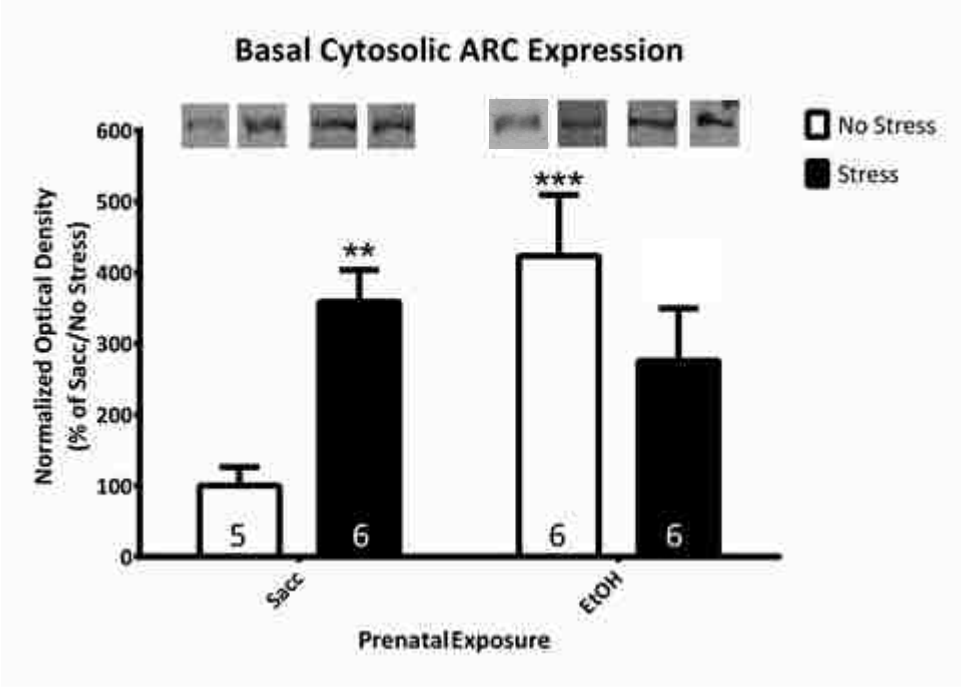

Figure 8.3. Effect of prenatal ethanol and/or prenatal stress exposure on baseline cytoplasmic ARC protein levels.

Representative bands from separate western blots are presented above each corresponding data bar. Open bars- No Stress, Filled bars- Stress. Data bars represent mean ± S.E.M. optical density normalized to the Sacc/No Stress control group. Asterisks denote significant differences between the Sacc/No Stress control group and all other prenatal exposure groups, *p<0.05, **p<0.01, ***p<0.005.

8.5.2.2 Synaptosomal GluA1 Expression.

A two-way ANOVA analysis revealed a main effect of prenatal ethanol exposure ($F[1,19]=7.68$, $p=0.01$), and a trend toward a significant main effect of prenatal stress ($F[1,19]=3.37$, $p=0.08$) (Figure 8.4A). Post-hoc comparisons revealed a significant reduction in baseline GluA1 expression in the synaptosomal fraction in animals exposed to dual prenatal ethanol and prenatal

stress exposure as compared to animals not exposed to ethanol or stress (p= 0.005) or prenatal stress (p=0.038).

8.5.2.3 Synaptosomal GluA2 Expression.

A two-way ANOVA analysis revealed a main effect of prenatal stress exposure ($F[1,20]=8.31$, $p=0.009$) (Figure 8.4B). Post-hoc comparisons revealed a significant elevation in basal GluA2 expression in the synaptosomal fraction in animals exposed to only prenatal stress as compared to Sacc/No Stress animals ($p=0.035$).

In addition, the sum total of GluA1 and GluA2 expression in the synaptosomal fraction for the four exposure groups was determined by adjusting the optical density of each group normalized to the corresponding Sacc/No Stress optical density. A three-way ANOVA analysis (ethanol x stress x subunit) resulted in significant main effects of prenatal ethanol exposure ($F[1,40]=10.5$, $p=0.002$) and subunit ($F[1,40]=13.0$, $p<0.001$), and a significant interactive effect of prenatal stress exposure and subunit ($F[1,40]=12.6$, $p=0.001$) (Figure 8.4C). Post hoc comparisons revealed a significant reduction in the total quantity of GluA1 and GluA2 in the synaptosomal fraction of the EtOH/No Stress and EtOH/Stress exposure groups as compared to the Sacc/No Stress group ($p<0.05$ for both comparisons) as well as a significant reduction in the quantity of GluA1 in the synaptosomal fraction in EtOH/Stress when compared to Sacc/No Stress group ($p<0.05$).

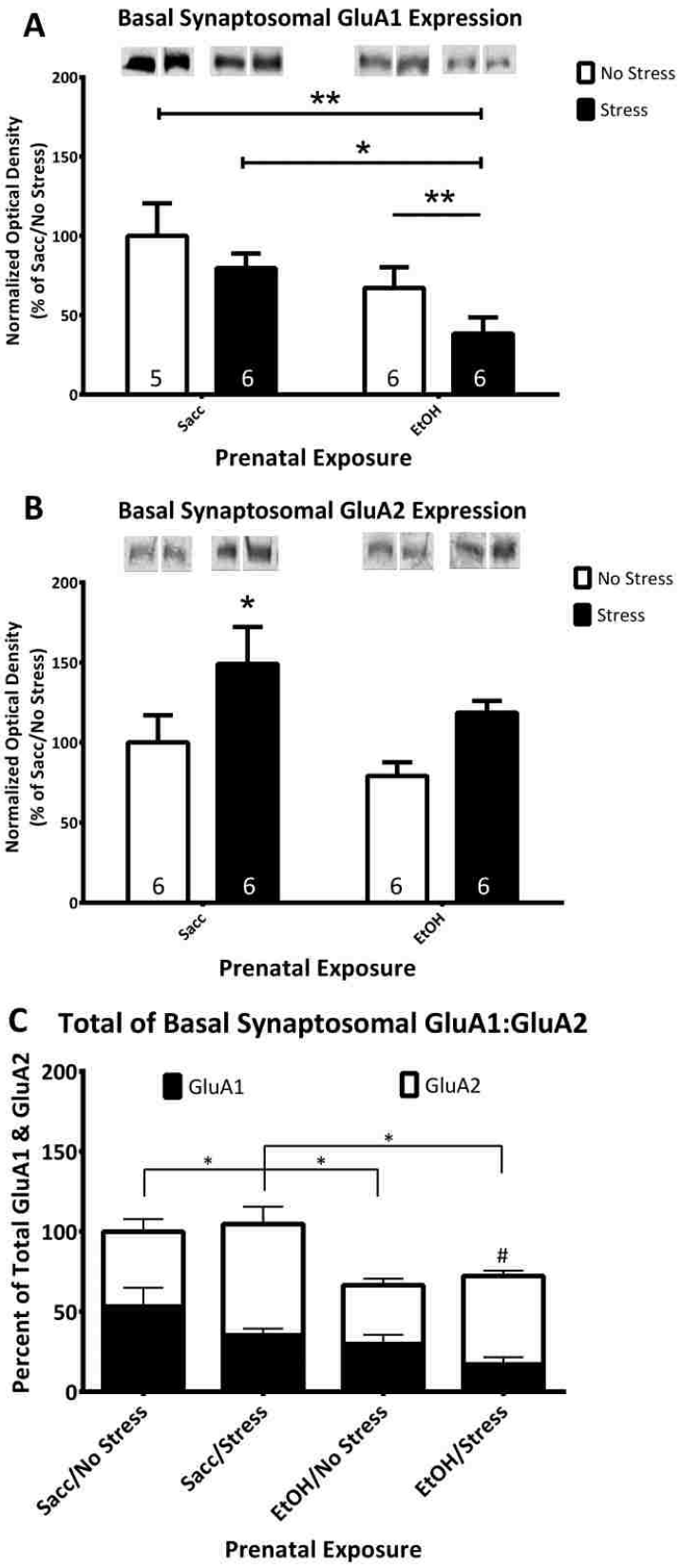

Figure 8.4. Effect of prenatal ethanol and/or prenatal stress exposure on baseline levels of GluRA receptor subunits.

Representative bands from separate western blots are presented above each corresponding data bar. **8.4A**: Basal synaptosomal GluA1 subunit expression. Open bars- No Stress, filled bars- Stress. Data bars represent mean ± S.E.M. optical density normalized to Sacc/No Stress. Asterisks denote significant main effect of prenatal ethanol exposure and significant differences between EtOH/Stress and Sacc/No Stress and Sacc/Stress, *p<0.05, **p<0.01. **8.4B**: Basal synaptosomal GluA2 subunit expression. Asterisk denotes significant difference between Sacc/No Stress and Sacc/Stress, *p<0.05. **8.4C**: Sum total of synaptosomal GluA1:GluA2 expression under basal conditions. Filled bars- quantity of GluA1 localized in synaptosomal fraction, open bars- quantity of GluA2 localized in synaptosomal fraction. Data bars represent mean ± S.E.M. optical density normalized to Sacc/No Stress. Asterisks denote a significant difference in the total quantity of GluA1 and GluA2 between the Saccharin and EtOH groups within the prenatal stress exposure groups. Hatch denotes a significant reduction in the quantity of GluA1 in the synaptosomal fraction in EtOH/Stress when compared to Sacc/No Stress group *p<0.05, #p<0.05.

8.5.3 Impact of TTTC on ARC and GluA Receptor Subunit Expression

8.5.3.1 Cytosolic ARC Expression after TTTC.

ARC levels in each of the four prenatal treatment groups were similar in the UPC control rats (Figure 8.5) in comparison to the naïve unhandled baseline control rats reported in Figure 8.3. A three-way ANOVA analysis (ethanol x stress x training) revealed a main effect of prenatal ethanol ($F[1,37]=7.56$, $p=0.009$), and a main interaction of ethanol and stress ($F[1,37]=47.2$, $p<0.001$) (Figure 8.5). Post-hoc comparisons revealed significant elevations in ARC protein as a result of each prenatal exposure condition as compared to its Sacc/No Stress behavioral counterpart ($p<0.05$ for all measures). However, there were no significant elevations in ARC protein expression as a result of TTTC compared to the UPC control group for any prenatal exposure group, including the Sacc/ No Stress group (Figure 8.5).

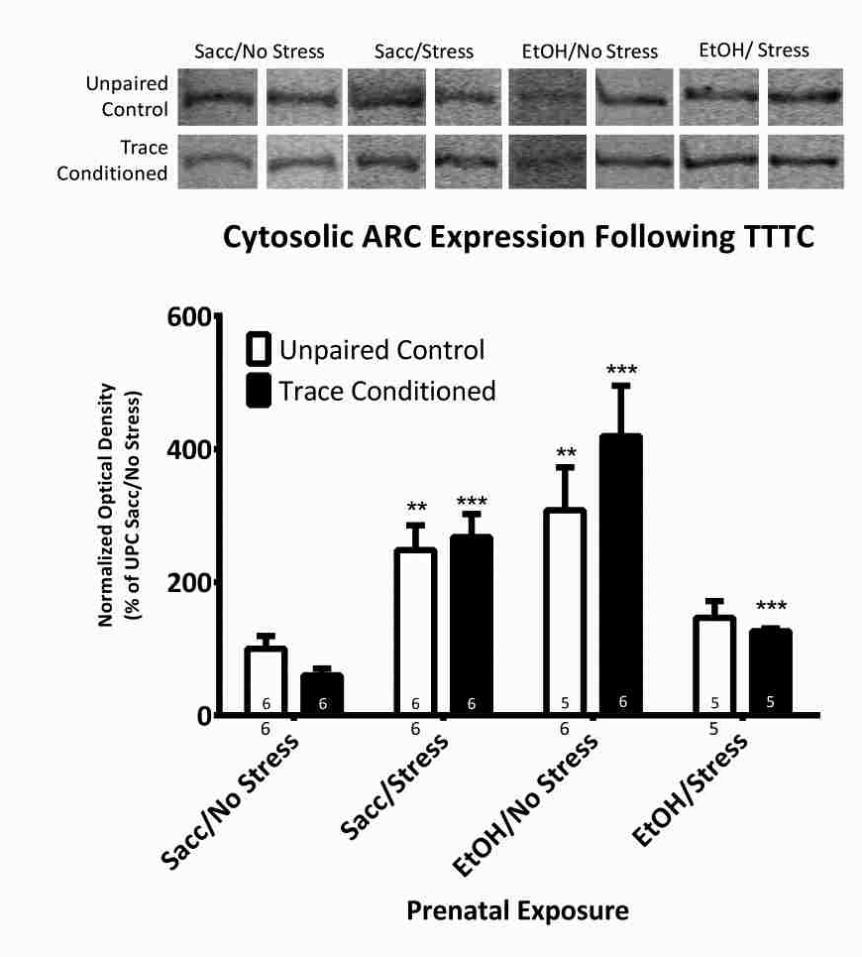

Figure 8.5. Effect of prenatal ethanol and/or prenatal stress exposure on TTTC-induced changes in cytoplasmic ARC protein levels.

Representative bands from separate western blots are presented above each corresponding data bar. Open bars- Unpaired Control (UPC), filled bars- Two-trial Trace Conditioned (TTTC). Data bars represent mean ± S.E.M. optical density normalized to Sacc/No Stress. **p<0.01, ***p<0.005.

8.5.3.2 Synaptosomal GluA1 Expression after TTTC.

A three-way ANOVA analysis (ethanol x stress x training) revealed a main effect of prenatal ethanol exposure ($F=[1,40]=9.52$, $p=0.004$), a main interaction of ethanol and stress ($F[1,40]=10.8$, $p=0.002$), and a nearly significant main effect of training ($F[1,40]=3.07$, $p=0.08$) (Figure 8.6A). Post-hoc comparisons revealed a significant elevation as a result of TTTC in GluA1 expression in the

Sacc/No Stress animals (p=0.04), an effect that was not observed in any other prenatal exposure group following TTTC.

8.5.3.3 Synaptosomal GluA2 Expression after TTTC.

A three-way ANOVA analysis (ethanol x stress x training) revealed a main effect of prenatal stress exposure (F=[1,38]=5.17, p=0.029) and a main interaction effect of ethanol x stress x training (F[1,38]=4.76, p= 0.035) (Figure 6B). Post-hoc comparisons revealed a significant elevation in GluA2 expression in the Sacc/No Stress animals (p=0.05) that was not observed in any other prenatal exposure group.

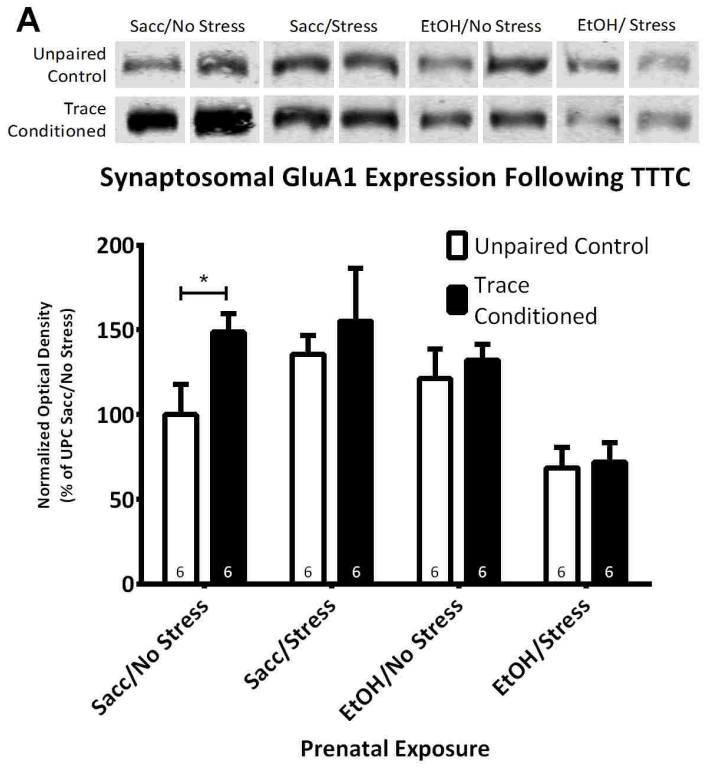

Figure 8.6. Effect of prenatal ethanol and/or prenatal stress exposure on TTTC-induced changes in AMPA receptor subunit expression.

Representative bands from separate western blots are presented above each corresponding data bar. **8.6A**: Synaptosomal GluA1 expression after TTTC. Open bars- Unpaired Control (UPC), filled bars- Two-trial Trace Conditioned (TTTC). Data bars represent the mean ± S.E.M. optical density normalized to Sacc/No Stress. Asterisks denote a significant difference between the UPC and TTTC conditions of the Sacc/No Stress group *p<0.05. **8.6B**: Synaptosomal GluA2 expression after TTTC. Open bars- UPC, filled bars- TTTC. Data bars represent mean ± S.E.M. optical density normalized to Sacc/No Stress. Asterisks denote significant differences between the UPC and TTTC conditions of the Sacc/No Stress group *p<0.05.

8.7 DISCUSSION

There are three salient observations based on the work presented in this report, two of which were in contrast to our predictions. Prenatal ethanol and/or prenatal stress: 1) significantly increased basal levels of ARC in the dentate gyrus, 2) interactively reduced basal synaptosomal GluA1 levels in patterns not consistently observed for GluA2 and, 3) abolished activity-dependent increases in both synaptosomal GluA1 and GluA2 in dentate gyrus.

At the onset of this project, we had predicted that the basal measures of ARC and GluA subunits would not be affected by either prenatal ethanol or prenatal stress exposure, and any observable differences would more likely occur as a function of activity-dependent conditions. These predictions were based on prior observations that moderate prenatal ethanol exposure did not affect the density of specific [^{3}H]-AMPA receptors (Savage et al., 1992) and dentate gyrus baseline input/output curves, as a measure of AMPA receptor responsiveness, are not altered in prenatal ethanol-exposed offspring (Sutherland et al., 1997; Varaschin et al., 2010).

In contrast to our prediction, both prenatal ethanol and prenatal stress exposure resulted in three-fold increases in basal cytosolic ARC protein levels (Figure 8.3). No further increase in basal ARC expression was observed in the dual exposure group. Currently, we are unable to find any evidence in the literature to suggest a mechanistic basis for increased basal ARC expression in these prenatal treatment groups. However, given that both prenatal ethanol (Weinberg, 1988, Kelly et al, 1991, Lan et al, 2006) and prenatal stress (Vallee et

al, 1997, Maccari et al, 2003) have been shown to elevate HPA axis responsiveness to stressors, and single housing and social isolation have been shown to increase stress hormones (Weiss et al, 2004, Stranahan et al, 2006), it is possible that maintenance in the single-housed cage environment during the last week prior to sacrifice caused sufficient stress to increase neural activity in a manner that differentially elevated ARC levels in otherwise naïve unhandled control conditions. Future work should include an assessment of the impact of single-housing stress on learning in the TTTC task, as well as a comparison of the expression of ARC protein and AMPA receptor subunit expression in animals group or pair housed compared to isolated animals. Similarly, future work should also include electrophysiological investigations into the capacity of these animals for LTD, as ARC protein is critical for LTD, and has been shown to be involved in the removal of AMPA receptors from the membrane during LTD stimulation. Either scenario, or potentially both, could assist in explaining the findings detailed in this work, and their relation to hippocampal-sensitive learning.

The second unexpected observation from this study was that both prenatal ethanol exposure and prenatal stress reduced baseline levels of synaptosomal GluA1 receptor subunit expression (Figure 8.4A). In this case, the dual exposure paradigm produced an interactive effect. Curiously, prenatal stress exposure alone elevated GluA2 level, an effect that was not observed with prenatal ethanol exposure alone or in the dual exposure paradigm (Figure 8.4B). However, prenatal ethanol exposure significantly reduced the sum total of synaptosomal GluA1 and GluA2 (Figure 8.4C) whereas the sum was not different

in the prenatal stress group. Our results in prenatal ethanol-exposed offspring are in contrast to a report by Bellinger et al (2002) where early postnatal ethanol exposure resulting in peak serum ethanol concentrations of 330 mg/dL reduced both GluA1 and GluA2 subunit levels in cortex. The differences in outcomes may be attributable to the timing of ethanol exposure during development, the difference in peak ethanol concentrations, and/or the brain region and age of analysis in the two studies.

A third unexpected outcome in these studies was the lack of an effect of TTTC on ARC expression levels in the dentate gyrus (Figure 8.5). This outcome is in contrast to previous other studies demonstrating significant increases in ARC levels following various behavioral challenges. For example, Kelly and Deadwyler (2003) observed significant elevations in cerebral cortical ARC levels following multiple days of training in a lever press. Mokin et al (2005) reported significant elevations in ARC protein in turtle brain stem following classical eye-blink conditioning. Lonergan et al (2010) found significant elevations in ARC protein in dorsal hippocampus of males sampled one hour following four trials of auditory cued fear conditioning. Thus, differences in the nature, duration or intensity of a behavioral challenge, the brain regions sampled and other factors such as gender may produce differential ARC responses. Relative to our results, it is possible that the TTTC paradigm employed in the present study was not sufficient behavioral challenge to induce an activity-dependent ARC response in the dentate gyrus.

In contrast to the effects of TTTC on ARC expression levels, both synaptosomal GluA1 (Figure 8.6A) and GluA2 (Figure 8.6B) were significantly elevated in Saccharin / No stress controls after TTTC in comparison to their respective UPC control groups. This effect was consistent with prior electrophysiological (Shi et al, 1999) and behavioral stimulation (Schiapparelli et al, 2006); Shi et al (1999) tetaniclly stimulated hippocampal primary neurons in culture and observed that GFP-tagged GluA1 was delivered to the dendritic spine within 30 minutes of stimulation. Schiapparelli et al (2006) measured an elevation in GluA1 in the membrane fraction of the hippocampus in animals one hour after training in a passive avoidance task. As predicted, and in contrast to the Saccharin / No Stress control prenatal treatment group, TTTC did not increase either GluA1 or GluA2 levels in any of the other three prenatal treatment conditions. These results contrast to a prior report where using restraint stress three times a day for 45 minutes in each session during the last week of gestation did not affect activity-dependent changes in GluA1 or GluA2 receptor subunit expression in the prefrontal corticies of rats (Fumagalli et al, 2009).

Taken together, these major findings imply that exposure to prenatal ethanol and prenatal stress alter the biochemical machinery underlying learning in a hippocampal-sensitive task. At the present time, there is a paucity of information in the literature to help interpret how the results of these experiments shed light on putative underlying mechanisms contributing to the synaptic plasticity deficits in dentate gyrus of prenatal ethanol or prenatal stress-exposed offspring. Given that ARC has been associated with the endocytosis of AMPA

receptors from the cellular membrane (Chowdhury et al, 2006), one parsimonious explanation that incorporates the salient observations in this study is the notion that the three-fold increase in basal cytoplasmic ARC levels (Figure 8.3) disrupted the cycling of AMPA receptor subunits in a manner that reduced basal levels of synaptosomal GluA1 (Figure 8.4A) and interfered with behavioral activation-dependent increases in both synaptosomal GluA1 and GluA2 in the dentate gyrus. As GluA1 is required for the initiation of LTP (Sanderson et al., 2008) this putative mechanism provides one explanation for the deficits in dentate gyrus LTP observed in prenatal ethanol- (Sutherland et al, 1997, Samudio-Ruiz et al, 2009, Varaschin et al, 2010, Titterness and Christie, 2012, Brady et al, 2013) and prenatal stressed (Titterness and Christie, 2012) offspring. Further, reductions in GluA subunit expression have been also associated with reduced performance on object recognition and passive avoidance tests (Schiapparelli et al, 2006) and animals lacking the GluA1 subunit have reduced performance on hippocampal-sensitive tasks such as radial arm tasks and non-matching to place T-maze, two measures of working memory (Sanderson et al, 2008).

Clearly, a great deal of additional work will be required to better elucidate the mechanistic basis for prenatal ethanol-induced and prenatal stress-induced deficits in synaptic plasticity and learning. For example, one important factor in AMPA receptor-mediated synaptic plasticity is the phosphorylation status of the AMPA receptor protein. Phosphorylation of S845 of the GluA1 subunit results in increased conductivity and inward rectification of current (Banke et al, 2000),

while phosphorylation of the S831 site results in increased membrane localization (Hayashi et al, 2000). Thus, future work assessing synaptic plasticity in these models should measure changes in the levels of phosphorylation at these two sites under activity-dependent conditions. One potential drawback of this work is that only the presence of AMPA receptor subunits was measured, while the functionality of such receptors would be of critical importance. We also have no way of assessing if the reduction in AMPA receptor subunits reported here is due to internalization of the receptors or restricted delivery of the receptors to the membrane by using western blotting techniques. Alternative experiments would be required to further elucidate the exact localization and function of the receptors described here.

Beyond AMPA receptor function, baseline and activity-dependent assessment of other glutamate receptor proteins, such as NMDA and mGluR receptors, along with the multiple cellular signaling systems triggered by these receptor subtypes (see review by Medina, 2011) is warranted to gain a clearer insight on how these prenatal insults cause long-lasting changes in the ability of neuronal populations to respond to their environment. For example, there is substantial evidence to show that both prenatal ethanol and prenatal stress can alter the expression, subunit composition, and synaptic membrane localization of NMDA receptors (Savage et al, 1991, Kinnunen et al, 2003, Barros et al, 2004, Brady et al, 2013). Further, prenatal ethanol exposure has been shown to alter the levels of several phospholipases (Allan et al., 1997; Weeber et al, 2001), protein kinase C activity, (Perrone-Bizzozero et al., 1998; Tanner et al., 2004;

Galindo et al., 2004) and ERK kinase (Samudio-Ruiz et al., 2010). More comprehensive knowledge of how prenatal insults alter these systems, particularly under activity-dependent conditions, is critical for a clearer understanding of the neurobiological bases of synaptic plasticity deficits, which could subsequently lead to the establishment of more rational therapeutic approaches for ameliorating the synaptic plasticity and learning deficits associated with these neurodevelopmental insults.

9. General Discussion

9.1 Summary of Relevant Findings

The work presented in this thesis was focused on testing the hypothesis that maternal stress exposure would potentiate the prenatal ethanol (EtOH) induced deficits on hippocampal-sensitive learning and dentate gyrus (DG) activity-dependent synaptic plasticity. The three aims established to test this hypothesis were to 1) develop a model of combined prenatal EtOH and prenatal stress wherein the two exposures are relatively moderate and do not impact each other, 2) assess the long-lasting behavioral impact of the combined prenatal exposures, and 3) identify changes in markers of activity-dependent synaptic plasticity in the DG of animals exposed to both prenatal EtOH and maternal stress in adult offspring.

The development of any novel animal model requires careful consideration of the appropriate animal, EtOH and stressor paradigms, and timing of exposures to employ. The voluntary, two-bottle choice ethanol administration paradigm was the optimal method of prenatal ethanol exposure as it is minimally stressful to the pregnant rat dams. In this model, the animals were given a bottle of EtOH in sweetened solution in addition to their ad libitum cage water and food; since their normal food and water was available during the EtOH exposure, the animals could elect to not to drink the ethanol solution, eliminating the stress induced with forced administration models (ie: injection, oral gavage, or liquid diet). Similar care was used when selecting a prenatal stressor; the animals should have a physiological response to the stressor (which is absent in

models directly delivering corticosterone (CORT) or synthetic glucocorticoids via injection or subcutaneous pellet implant), and the animals should not be able to habituate to the stressor as it was to be presented multiple times during a single gestational period. The use of a vaporized predator scent satisfies all these criteria. Pregnant dams exposed to ethanol, stress, or both drank similar quantities of the 5% EtOH solution (Figure 7.3), gained similar amounts of weight, and cared for their litters in a similar fashion. Litters were of similar pup number and weight at birth and gained a similar amount of weight from birth to weaning (Table 7.1). Tracking these features was important in order to eliminate potential confounds due to stress-induced binging or abstinence of EtOH, maternal starvation, abandonment or neglect, or low birth weight. The predator scent did elicit a significant elevation in circulating CORT concentrations in the exposed dams, and this effect was compounded by prenatal EtOH exposure measured on gestational day (GD) 13 (Figure 7.4).

The second goal of this work was to assess behavioral alterations in the offspring exposed to both prenatal EtOH and prenatal stress. Of particular interest was the impact of the two prenatal insults on hippocampal-sensitive learning and anxiety-like behavior. To measure hippocampal-sensitive learning, adult female offspring were trained and tested in a novel Two-Trial Trace fear conditioning (TTTC) task where the animals were exposed to two pairings of a tone which is temporally dissociated from a foot shock (Figure 7.2). Animals prenatally exposed to EtOH displayed significant deficits in freezing behavior during the testing phase of this task (Figure 7.6), confirming the reduction in

performance in hippocampal-sensitive tasks described previously utilizing this EtOH exposure model (Savage et al 2010). Prenatal stress exposure in addition to prenatal ethanol exposure did not exacerbate the learning deficits induced by prenatal ethanol alone. Anxiety-like behavior was assessed in the Elevated Plus Maze (EPM); prenatal stress significantly increased anxiety-like behavior (Figure 7.5), with no additional impact of EtOH. These outcomes seem to demonstrate that even with the additive effect of ethanol on stress reactivity in the pregnant dams, the individual exposures were too moderate to elicit combinatorial alterations in behavior.

The final goal of this dissertation was to assess alterations in biochemical markers of activity-dependent synaptic plasticity in the DG of the hippocampal structure. Utilizing western blotting techniques, significant differential alterations in basal and evoked expression of AMPA receptor subunits and ARC protein in the cytosolic and synaptosomal fractions of animals in all four prenatal exposure groups were detected. At basal levels, western blot analysis revealed significant increases in cytosolic ARC protein expression in all prenatal exposure groups as compared to Sacc/No Stress controls (Figure 8.2), elevated synaptosomal GluA2 subunit expression in Sacc/Stress animals as compared to Sacc/No Stress controls (Figure 8.1), and reduced GluA1 subunit expression in the EtOH/Stress animals as compared to the Sacc/No Stress group (Figure 8.1). Following training in the TTTC task, the animals in the Sacc/No Stress group had significantly elevated quantities of GluA1 and 2 which were not present in any of the other three prenatal exposure condition groups (Figure 8.3). The

Sacc/Stress, EtOH/No Stress, and EtOH/Stress groups had significantly more GluA2 in the synaptosomal fraction (Figure 8.3), as well as more ARC protein in the cytosolic fraction (Figure 8.4), as compared to the Sacc/No Stress group. These findings demonstrate prenatal EtOH and prenatal stress-induced alterations in the biochemical markers of activity-dependent synaptic plasticity, and imply altered synaptic scaling, that, in the case of the prenatal EtOH exposure group, corresponds to reductions in hippocampal-sensitive learning (Figure 7.6, Savage et al, 2010).

9.2 Interpretation and Significance of Findings

9.2.1 Significance of Model

The work described in this dissertation is the first to combine a moderate voluntary EtOH exposure paradigm with a moderate predator scent exposure paradigm in order to assess in detail the impact of the two insults in combination on behavior and biochemical markers of plasticity. While combined exposure models have been developed previously, this is the first that used rodents as the animal model, utilizes an EtOH exposure paradigm that is minimally stressful, a stress exposure which animals did not habituate to, measured learning and anxiety behavior, and described the findings in relation to the individual prenatal exposures by employing a four-group experimental design.

9.2.2 Significance and Interpretation of Paradigm Characteristics

9.2.2.1 Maternal and Litter Demographics

It was critical when designing the combined exposure model that the two exposures not interact to cause confounds which could make interpreting the data difficult. These could have included consuming a significantly greater or less quantity of EtOH, maternal malnutrition, deviations in maternal care, litter number, and pup size. Each of these factors has been previously reported as altered by either prenatal exposure, and was closely monitored in this combined model to avoid the development of potential confounds.

Since the goal was to combine a moderate prenatal exposure with a previously described prenatal EtOH exposure (Savage et al, 2010), it was important that the stress exposure not result in a significant increase or reduction in the quantity of EtOH consumed. The deleterious effects of high quantity and binge-like EtOH consumption has been well-documented in the literature, both in humans (Jacobson et al, 1998, Nulman et al, 2004) and in rodent models (Bonthius and West 1990, Maier et al, 1995). However, the number of heavy drinking mothers (and the resulting FAS diagnosis for their children) is a very small percentage of the total number of women who consume alcohol during pregnancy (Whitehead and Lipscomb, 2003). Of more clinical relevance is an understanding of the effects neurologic underpinnings of moderate EtOH exposure. Both human (Holahan et al, 2001, Jacobsen et al, 2001) and animal studies (Nash and Maickel, 1985, Hilakivi-Clarke and Lister, 1992) have reported significant increases in EtOH consumption following a stressful experience, but in

our model, there was no observable difference in the quantity of EtOH consumed between the dams exposed to stress and those not exposed to stress, indicating that the temporal dissociation between the availability of the EtOH solution and the presentation of the predator scent prevented a change in consumption patterns.

Maternal nutrition during gestation plays a significant role in development of healthy offspring both in humans (Kretchmer et al 1996, Yajnik and Deshmukh, 2008) and animal models (Huber et al, 2013). There is evidence that maternal diets deficient in certain nutrients can result in smaller cortices, impaired cognition, and negative outcomes in the offspring (Chase and Martin, 1970, Gage et al, 2013), and that maternal exposure to both EtOH (Wainwright et al, 1990, Vavrousek-Jakuba et al, 1991) and stress (Kinsley and Svare, 1986) during pregnancy can reduce the amount of weight gained. However, this was not a factor in this model, as all dams gained similar quantities of weight across gestation in all four exposure groups. Since the provided diet was specifically formulated for pregnant and lactating animals, the relative quantity of weight gained serves as an indicator of sufficient nutritional intake.

The most likely confound to occur in this model was significant deviations in maternal care of litters due to the predator scent exposure, the EtOH intake, or the combination. The amount of time a mother spends caring for her offspring has been directly related to behavioral outcomes in the offspring both in humans (Canetti et al, 1997) and in animal models (Caldji et al, 1998), and has been reported in models of both maternal stress (Champagne and Meaney, 2006) and

maternal EtOH consumption (Ponce et al, 2011). Licking-grooming behavior and arched back nursing by the dam in the first two weeks following delivery of the litter have been well characterized as indicators of the level of maternal care (Caldji et al, 2000) and the correlated behavioral outcomes in the offspring (Caldji et al, 1998). Maternal care has also been implicated in the development and function of the hippocampus; offspring from low maternal care dams had increased latencies to find the hidden platform in the Morris Water Maze and reduced expression of synaptic markers (Liu et al, 2000). Due to the reduction in visibility due to the elimination of light in the animal housing rooms, analysis of licking/grooming behavior and position of nursing was not possible. Instead, the total time a dam spent on her nest was calculated, with no differences found between prenatal exposure groups. Similarly the length of time a dam took to retrieve a displaced pup from the nest has been used as an index of maternal care (Hess et al, 2002, Champagne et al, 2007) but there was no difference in this measure as a result of prenatal EtOH, prenatal stress, or the combination.

Taken together, these findings indicate that the addition of a novel prenatal predator scent stressor in the last week of gestation to a previously established voluntary prenatal EtOH consumption paradigm is able to produce adult animals that were exposed to both prenatal insults without significantly altering maternal and litter characteristics which could create potential confounds in further analysis of behavior and synaptic plasticity.

9.2.3 Significance and Interpretation of Behavioral Outcomes

9.2.3.1 Elevated Plus Maze

Both prenatal EtOH and prenatal stress have been reported to produce heightened anxiety in the adult offspring (Martini et al, 2007, Davis and Sandman, 2012). Anxiety in rodent models can be measured in a number of ways including the elevated plus maze (EPM), open field, light/dark exploration, and social interaction (reviewed in Bailey and Crawley, 2009). These tests rely on measuring the contrast between a rodent's natural drive to explore novelty and their preference for dark, enclosed spaces over open, bright environments (termed thigmotaxis). The maze consists of an elevated plus-shaped structure where two of the arms are open and brightly illuminated, and the other two are enclosed and darkened (Pellow et al, 1985). The measure of the time spent in the open arms (as compared to total time in the maze) and the number of entries onto the open arms of the maze (as compared to the total number of arm entries) have been validated as quantitative measures of anxious behaviors in rodents exploring the EPM (Pellow et al, 1985).

For this dissertation, animals exposed to both prenatal EtOH and prenatal stress were assessed for anxiety-like behavior in the EPM as it was the optimum test for various reasons. First, the EPM has been widely characterized and is considered to be the "gold standard" of anxiety testing (Carobrez and Bertoglio, 2005). Second, assessments such as the social interaction task have aspects which have been shown to be altered in ways that are not representative of anxiety behavior (Hamilton et al, 2010a and b). Third, common experimental

paradigms of tasks such as the open field and light/dark exploration require lengthy trials per animal (30 minutes in the open field as compared to 5 minutes in the EPM, Bailey and Crawley, 2009) which could mask subtle differences expected as the result of a moderate prenatal insult. Results from the EPM in adult animals prenatally exposed the EtOH, stress, or both identified a significant increase in anxiety-like behavior as a result of prenatal stress, with no effect of EtOH alone and no compounding effect of the two prenatal exposures (Figure 7.5). As described in Chapter 7, this singular effect is likely due to the moderate nature of the prenatal EtOH exposure as heightened anxiety has only been observed in offspring exposed to very high blood alcohol concentrations (BACs) (Dursun et al, 2006). In addition to ethanol not independently producing an increase in anxiety-like behavior, it also did not potentiate the heightened anxiety displayed by animals prenatally exposed to stress.

9.2.3.2 Two Trial Trace Fear Conditioning

Moderate prenatal EtOH exposure typically results in mild to moderate deficits in learning and memory which can be difficult to detect in both humans and rodents. There are many well-characterized assessments for hippocampal-sensitive learning which can be utilized when testing rodents, however, most require multiple trials and only reveal gross deviations from behaviors observed in control animals (Gerlai 2001). In order to measure the deleterious effects of prenatal EtOH and prenatal stress on hippocampal-sensitive learning and memory, as well as utilize this learning task as the behavioral activation for measuring activity-dependent changes in synaptic plasticity (Chapter 8), the

learning task needed to be a single learning event rather than multiple trials of acquisition, as well as be sufficiently challenging to detect subtle deficits in learning.

The test that satisfied these two requirements was a novel Two-Trial Trace Conditioning Task (TTTC), a specific type of classical or Pavlovian conditioning that measures an animal's capacity for associative learning. The primary attribute of classical conditioning involves the learned association of a conditioned stimulus (CS) and an unconditioned response (UR) (Pavlov, 1927). In fear conditioning, the CS is typically a loud auditory tone of specific decibel to which the rodent has no innate reaction to, and the UR is typically a freezing response. In order to teach the animal the association, the training trials include an unconditioned stimuli (US, typically a mild foot shock) which evokes the innate UR. When the stimuli are presented in the correct order (CS followed by US), an intact animal can form the associative memory that the CS is predictive of the US. If the animal has created the correct connection between the CS and US, the animal will respond to a subsequent presentation of the CS in the absence of the US with the UR, now termed the conditioned response (CR) as it was displayed by the animal following the CS not the US (described in more detail in Curzon et al 2009).

An intact hippocampus is required for the associative leaning of fear conditioning under select experimental conditions. When the CS is the context where the training took place, termed contextual fear conditioning (CFC), the hippocampus is required to form a spatial memory of the chamber (Phillips and

LeDoux, 1999). Similarly, when the CS and the US are temporally dissociated, termed trace conditioning (TC), the hippocampus is required to build a memory bridging the separation between the two stimuli (Kim and Fanselow, 1992, Wallenstein et al, 1998, McEchron et al, 1998, McEchron and Disterhoft, 1999, Anagnostaras et al, 2001, Quinn et al, 2002). TC is more challenging (Beylin et al, 2001) and is more sensitive to hippocampal damage than other forms of fear conditioning, such as delay conditioning (DC) where the CS and US co-terminate, which can involve other regions of the brain, including the amygdala (Beylin et al, 2001). The longer the duration of the trace period between the two stimuli on the training day, along with fewer numbers of CS and US pairings, the more challenging the task (Beylin et al 2001, Shors, 2004).

For the reasons described above, TTTC, where the CS and US were presented twice during the single training session, was the optimal choice for measuring learning in the dual exposure animals. Animals tested 24 hours following training demonstrated significant reductions in learning-associated freezing behavior as a result of prenatal EtOH exposure. Animals exposed to prenatal stress did not display learning deficits, and the combination of the two prenatal exposures did not potentiate the deficits observed as a result of prenatal EtOH alone (Figure 7.6). Similar to the threshold effect of moderate EtOH exposure on anxiety-like behavior, it is likely that hippocampal-learning deficits are only apparent in animals exposed to more severe forms of prenatal stress (Lemaire et al., 2000, Lordi et al., 2000, and Wu et al., 2007).

These findings, taken together, indicate that moderate prenatal EtOH and prenatal stress do not interact to exacerbate the independent prenatal exposure impacts on anxiety-like behavior and hippocampal-sensitive learning. This is different from the hypothesized outcome, and is possibly due to the moderate nature of both prenatal exposures.

9.2.4 Significance and Interpretation of Biochemical Findings

As described in Chapters 4 and 5, the hippocampus is an area of significant plasticity, particularly following neuronal stimulation. While this feature can be measured many ways, one way that detects changes due to activation as well as implying functional relevance is assessing biochemical markers of synaptic plasticity. To this end, the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) and activity-regulated cytoskeletal (ARC) protein expression are impacted differentially by neuronal activity and have significant implications on synaptic plasticity. For this reason, AMPAR subunit expression and ARC protein were selected as the biochemical markers of activity-dependent synaptic plasticity.

9.2.4.1 ARC Protein Expression

As described in Chapter 5, ARC protein has been demonstrated to be increased following synaptic activity (Link et al, 1995, Lyford et al, 1995, Guzowski et al, 2001). ARC protein expression has not been assessed in animals prenatally exposed to ethanol, stress, or the combinations, either at baseline nor following learning. Both basal and evoked ARC expression were

elevated in the prenatally exposed groups (Figure 8.1 and 8.3), possibly implying a hyperactivity of the DG, but there were no training induced elevations for any exposure group, including the Sacc/No Stress group, as would have been expected based on the previous literature (Monti et al, 2006, Lonergan et al, 2010, and Kelley and Deadwyler, 2003).

9.2.4.2 AMPA Receptor Expression

As described in Chapter 5, AMPAR subunits are differentially expressed depending on the activity level of the synapse and the quantity of AMPARs is significantly up-regulated following sustained potentiation (Shi et al, 1999, Schiapparelli et al, 2006). The quantity and composition of these receptors is tightly regulated due to their roles in synaptic plasticity and potential to increase the level of intracellular Ca^{2+}. Immediately following stimulation, there is an increase in the quantity of synaptosomal GluA1; over the course of sustained potentiation, a significant proportion of the GluA1 subunits are exchanged for GluA2, rendering the receptors Ca^{2+} impermeable and preventing Ca^{2+} induced cytotoxicity (reviewed in Shepherd and Huganir, 2007).

Previous work in animals exposed to prenatal EtOH has demonstrated an increase in basal levels of GluA2/3 but no change in GluA1 (Dettmer et al, 2003) and prenatal stress has been reported to reduce the quantity of the GluA1 subunit (Yaka et al, 2007). Western blot analysis of subcellular quantitation of basal GluA1 and GluA2 demonstrated a shift in the localization of GluA1 due to prenatal EtOH exposure with a significant reduction in the synaptosomal expression and a significant increase in the cytosolic expression (Figure 8.2A-C).

There was no difference in GluA1 expression as a result of prenatal stress, but there was an increase in GluA2 in the synaptosomal fraction. Taken together, these findings could potentially provide a mechanistic explanation for the significant reductions in learning observed in the prenatal EtOH exposed animals in the TTTC task but not in prenatal stressed animals; the reduction in the total number of AMPA receptors would limit the postsynaptic initiation of synaptic potentiation.

Quantitation of evoked GluA1 and GluA2 subunits have not been measured in animals prenatally exposed to either EtOH or stress. Following training in the TTTC task, there were significant elevations in the quantity of both GluA1 and GluA2 that were not observable in any other prenatal exposure group, but the expression level of the GluA1 and GluA2 were significantly elevated in the prenatally exposed groups in the unpaired conditioning group (UPC), potentially reaching a ceiling effect and preventing further increase in subunit expression following behavioral training. However, this is speculative, and further assessment into the mechanisms of the synaptic plasticity impairments would be required to further elucidate a causational relationship.

9.3 Overall Significance

When considered in its entirety, the work presented in this thesis demonstrates that prenatal ethanol exposure induces significant learning deficits, which may be explained, in part, by the significant reduction of GluA1 available at the membrane in the dentate gyrus following prenatal ethanol exposure. This

reduction in GluA1 expression, as well as the lack of an evoked increase in GluA1 and 2 in animals prenatally exposed to ethanol, stress, and the combination could be due to the substantial elevations in ARC protein expression, which has been documented to be involved in AMPAR endocytosis. No interactive effects were detected in many of the assessments conducted with animals prenatally exposed to both gestational insults, possibly implying that both prenatal exposures were too moderate in nature to elicit interactive effects.

9.4 Limitations

When developing a novel animal model such as the one described in this thesis, many important features and outcomes are disregarded in order to form an initial characterization of the paradigm. In this case of this model, that unfortunately includes measures of more severe prenatal EtOH and stress, the timing of the stress exposure with respect to the EtOH administration, the use of only a single gender, the use of only a single behavioral outcome for learning and anxiety, and focusing on only two potential markers of synaptic plasticity. Each of these shortcomings could be assessed in greater detail in future work using this model in order to form a more complete picture of the interactive effect of the two prenatal insults.

9.5 Future Directions

While the combined exposure model has answered the few specific questions posed in the specific aims of this project, there are numerous questions yet to be proposed and tested. The following is a brief description of

prominent and important features to explore in future experiments which are not proposed and described elsewhere in this dissertation (Chapter 7 discussion, Chapter 8 discussion).

9.5.1 Dentate Gyrus-Sensitive Behavioral Assessment

The learning task used in these studies was a trace fear-conditioning task that was hippocampal-sensitive. While there is some evidence that implicates the DG in temporal memories, the primary function associated with the DG is the formation of memories specific to the separation of patterns (Kesner, 2013). To this effect, it would be of interest to measure performance in a task specifically sensitive to DG damage (Clelland et al, 2009). In the pattern separation variation of the Radial Arm Maze (RAM), animals are tasked with the challenging task to identify correctly baited arms that are in near proximity to each other. This is a spatial-memory task that requires multiple trials to acquire, so it would not be appropriate as a learning activation task. However, it may be of interest to measure biochemical markers of synaptic plasticity once each individual animal had reached a predetermined threshold of successful baited arm identifications or once the control animals had reached the success threshold.

However, while the pattern separation task would provide a more detailed measurement of DG-sensitive learning, this task requires a level of dietary restriction to motivate the animals to seek out the food bait at the end of the arms. This restriction to access of food could confound the results of the task in the prenatally stress exposed animals who have already been shown to have

heightened levels of anxiety. It would be difficult to separate the impact of the heightened anxiety in the test and a reduction in learning if the animals have significantly more errors in the task.

9.5.2 Adult Neurogenesis

The subgranular zone DG of the hippocampus is one site of two in the adult brain with lifelong neurogenesis (Curtis et al, 2012). Various reports have demonstrated differential effect of prenatal EtOH exposure, which appears to be dependent on the administration model used (Gil-Mohapel et al, 2010). Prenatal stress exposure could be linked to disrupted neurogenesis as excessive quantities of the stress hormone corticosterone (CORT) have been shown to inhibit the various stages of neurogenesis (Brummelte and Galea, 2010), and many models of prenatal stress exposure have been shown to result in heightened CORT concentrations and prolonged CORT elevations following a stressful event (Vallee et al, 1997). Since the DG has been shown to be particularly sensitive to prenatal EtOH and prenatal stress, it would be of interest to assess the neurogenic properties of the DG granule cells in animals exposed to both prenatal insults.

9.5.3 Effects of Cognition Enhancing Pharmaceutical Agents

One potential therapeutic intervention for individuals suffering from cognitive challenges is the family of aniracetam derivatives, called ampakines. These are drugs which serve as allosteric modulators of the AMPA receptor (Arai and Kessler, 2007), and have been shown to dose-dependently improve behavioral

performance in hippocampal-sensitive tasks (Davis et al, 1997). This improvement is thought to be the result of the enhanced AMPA channel currents (Arai et al, 1994). Since the work presented in Chapter 8 demonstrated a significant reduction in the basal quantity of select AMPAR subunits, it would be of interest to determine if the administration of an ampakine can ameliorate the cognitive deficits observed in animals exposed to prenatal ethanol simply by improving the AMPAR channel conductance. Based on previous experimental evidence in similar fear conditioning paradigms (Smith and Wehner, 2002, Lu and Wehner, 1997) and prenatal ethanol exposure models (Vaglenova et al, 2001, Vaglenova et al, 2008, Wijayawardhane et al, 2008), I would predict that animals given the potential cognition enhancer which were subjected to prenatal ethanol would show marked cognitive improvements. A study of this nature would provide more direct evidence that an underlying mechanism of learning deficts in animals prenatally exposed to ethanol is a reduction in the available AMPAR, as well as provide evidence for a potential theraputic for individuals with cognitive deficits due to prenatal ethanol exposure.

Appendix A: Supplemental Western Blot Data

A.1. Antibody Optimization Curves

In order to analyze the optical density (OD) of a protein of interest (POI), it must be first confirmed that the quantity of protein being loaded on the gel will fall in the linear range of expression for the concentration of primary antibody being used for incubation. Multiple quantities (1, 2.5, 5, 10, and 20ug) of total protein were loaded onto 8% Polyacrylamide gels and processed as described in chapter 8. The following are the resulting OD curves.

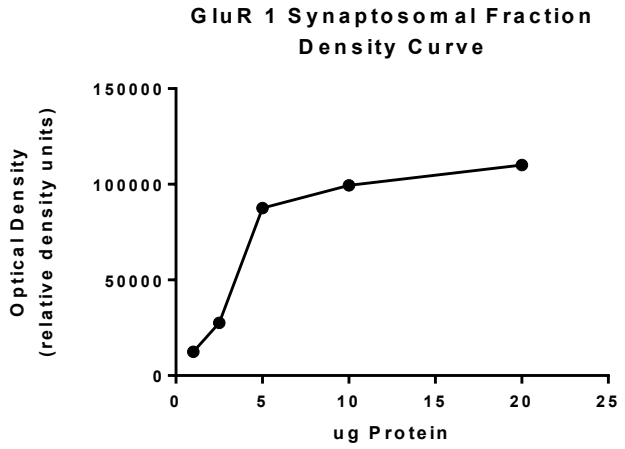

Figure A.1 Synaptosomal GluA1 Density Curve

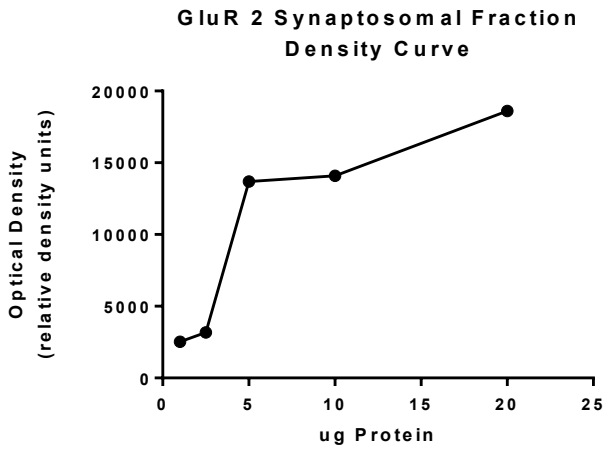

Figure A.2 Synaptosomal GluA2 Density Curve

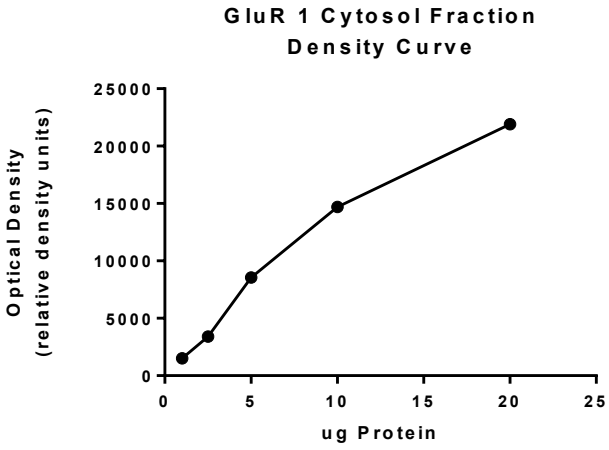

Figure A.3 Cytosolic GluA1 Density Curve

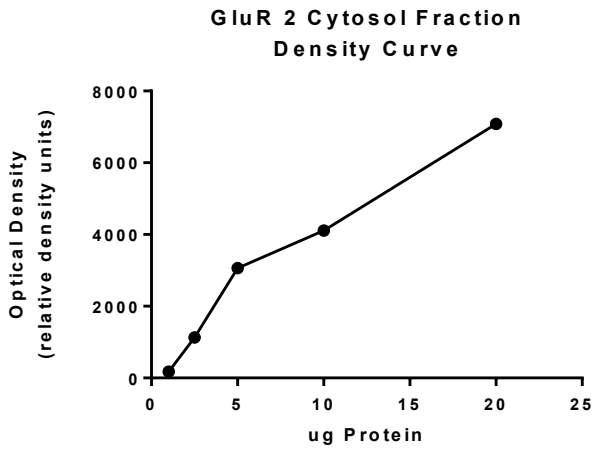

Figure A.4 Cytosolic GluA2 Density Curve

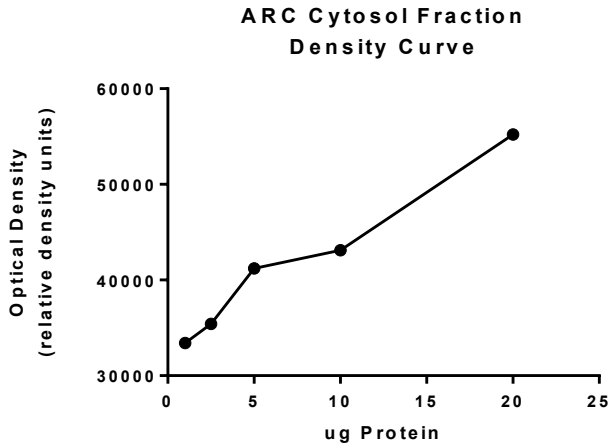

Figure A.5 Cytosolic ARC Density Curve

A.2. Trace Conditioning Time Course

In order to determine the optimal time to sacrifice the animals following UPC or TC, a small time-course study was conducted where animals were trained in the TTTC task, then sacrificed at o, 30, 60 or 90 minutes following the end of the

training session. The following graphs are the resulting protein expression from this experiment.

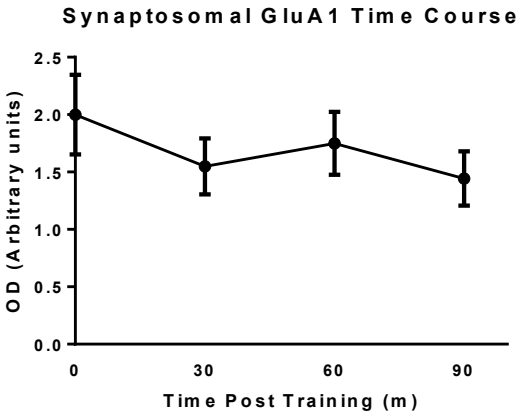

Figure A.6 Synaptosomal GluA1 Time Course

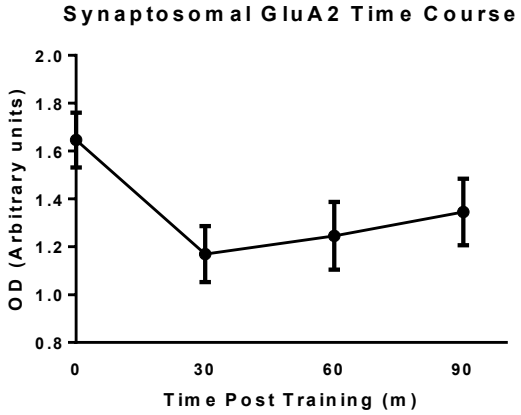

Figure A.7 Synaptosomal GluA2 Time Course

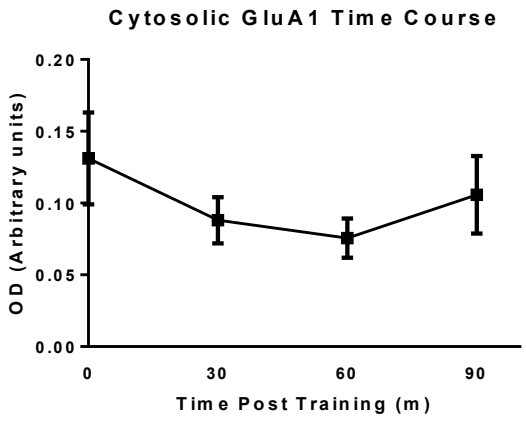

Figure A.8 Cytosolic GluA1 Time Course

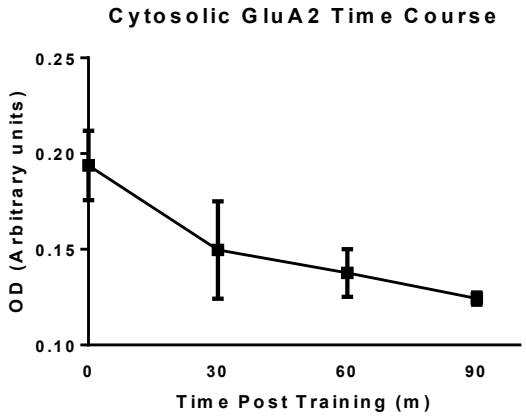

Figure A.9 Cytosolic GluA2 Time Course

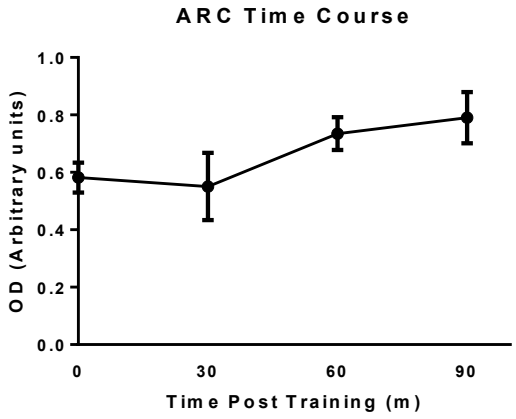

Figure A.10 Cytosolic ARC Time Course

A.3. Sample Coomassie Staining

In order to verify that the reductions or elevations in the expression of the protein of interest (POI) on a western blot has not been falsified by loading the gel with a reduced or increased quantity of total protein, the optical density (OD) of a POI is normalized to the OD of a “loading control”, or a protein present in the sample whose expression should not change with the conditions of the experiment. Actin is most frequently used as the loading control in western blot analysis, but there are issues surrounding the use of this protein in this manner. Actin is a cytoskeletal protein present in abundance in the cytoplasm of all cells; this high level of expression makes it a poor choice of a loading control for proteins of low abundance (Dittmer and Dittmer, 2006). Also, its restriction to the cytoplasm prevents it from serving as a loading control for proteins expressed in membrane fractions. Further complicating the use of Actin in these studies is the variation of Actin expression following cell stimulation and growth (Ruan and Lai,

2007). Therefore, in order to confirm that equivalent quantities of protein were loaded for each sample on each gel, the PVDF membrane was stained with coomassie stain following OD imaging, as described in Welinder and Ekblad, 2011. Since coomassie stain binds to every available protein on the membrane, this is a valid method of controlling for protein loading. OD of each sample was normalized to it's respective coomassie staining.

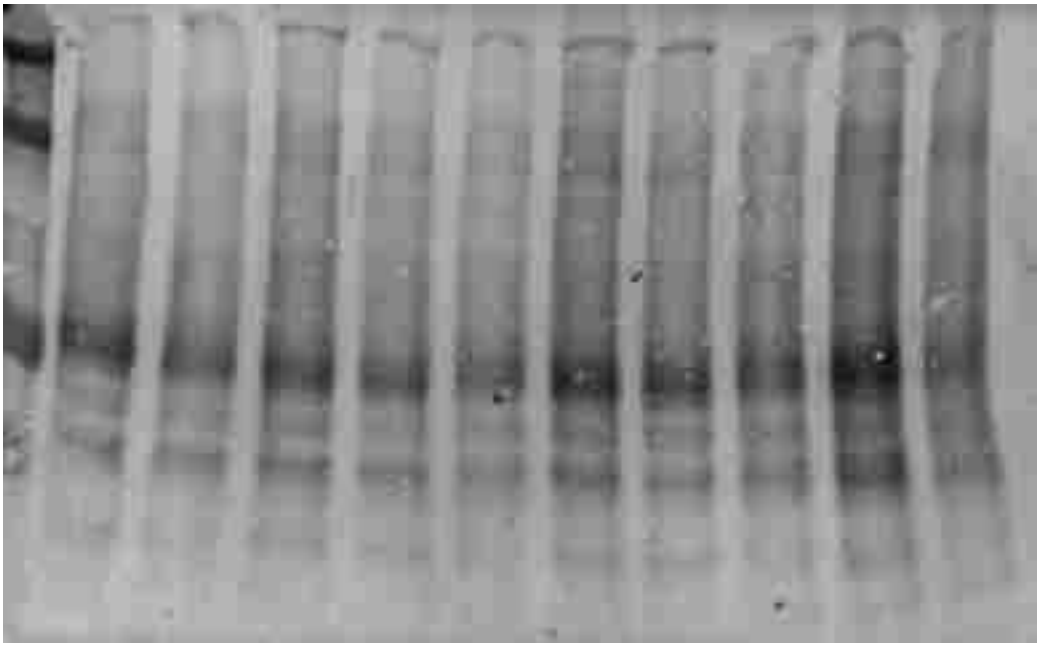

Figure A.11 Sample of Coomassie Staining Following Secondary Antibody Incubation

Appendix B: Recipes and Notes

B.1. Solutions

Animals

0.066% Saccharin Solution

Final Volume of Solution (L)	Saccharin (g)	Tap Water (L)
1	0.66	1
2	1.32	2
3	1.98	3
4	2.64	4

Table B.1 0.066% Saccharin Solution

5% EtOH Solution

Final Volume of Solution (L)	95% EtOH (mL)	Saccharin Solution (L)
1	53	1
2	106	2
3	159	3
4	212	4

Table B.2 5% EtOH Solution

3% TMT (v/v) for Stress Exposure

300uL TMT (Straight from vial)
1mL 10% EtOH
9mL DI Water

Dilute TMT in 10% EtOH, then in DI water. Pipette 250uL onto a small piece of filter paper. Roll filter paper and insert in to in-line tubing. Store unused 3%TMT in fridge desicator.

Western Blots

5x Loading Buffer (Blue Juice), 100mL

62.5mM Tris Base pH 6.8 (6.25mL of 1M Stock)
12.5mM EDTA (2.5mL of 0.5M stock)
2% SDS (20mL of 10% stock)
10% glycerol (20mL of 50% stock)
0.1% (w/v) Bromophenol Blue (BPB)

NOTE: Aliquot 2-3mL and add 125uL B-Mercapto ethanol per 1 mL. This is the final solution to use when prepping samples for gels.

4x Separating Buffer (500mL)

0.75M Tris Base (90.96g)
4% SDS (20mL of 10% stock)
DI Water to 500mL

Autoclave

4x Stacking Buffer (500mL)

0.25M Tris Base (30.45g)
4% SDS (20mL of 10% stock)
DI Water to 500mL

Autoclave

10% APS

0.1g APS per 1mL DI water. Store at room temperature.

Gel Recipes (for 2 gels)

	8%		10%		12.5%		15%		20%	
	Separating	Stacking	Separating	Stacking	Separating	Stacking	Separating	Stacking	Separating	Stacking
40% Polyacrylamide (mL)	3.6	2.25	4.5	2.25	5.63	2.25	6.75	2.25	9	2.25
4x Separating Buffer (mL)	4.5	0	4.5	0	4.5	0	4.5	0	4.5	0
4x Stacking Buffer (mL)	0	2.5	0	2.5	0	2.5	0	2.5	0	2.5
DI Water (mL)	9.81	5.16	8.91	5.16	7.785	5.16	6.66	5.16	4.41	5.16
10% APS (uL)	80	80	80	80	80	80	80	80	80	80
TEMED (uL)	10	10	10	10	10	10	10	10	10	10
Total Volume (mL)	18	10	18	10	18	10	18	10	18	10

Table B.3 Recipes for Polyacrylamide Gels

10x Running Buffer Stock(1L)

250mM Tris Base (30.28g)
1.9M Glycine (144.2g)
10g SDS
DI Water to 1L

Autoclave

Use 1X Running Buffer to run Gels (100mL stock to 900mL DI Water). Prep in a glass bottle or bucket to mix thoroughly.

10x Transfer Buffer Stock (1L)

250mM Tris Base (30.28g)
1.9M Glycine (144.2g)
DI Water to 1L

Autoclave

10x TBS (1L)

0.15M Tris HCl (24.24g)
30mM Trizma Acetate (5.56g)
1.4M NaCl (80g)
pH to 7.6
DI Water to 1L

Autoclave

NOTE: Use 1x TBS (100mL of stock to 900mL of DI H2O) for washes.TBS-T

iBlock (per manufacturer's instructions) (100mL)

0.2% iBlock (0.2g)
100mL 1x TBS

Stir and heat (DO NOT BOIL!) until dissolved. Solution will be cloudy. Cool to room temp, aliquot and store at -20^{o}C.

Li-Cor Stripping Buffer (1L)

25mM Glycine (1.88g)
DI Water to 1L
pH to 2
1% SDS (10g)

Coomassie Stain (100mL)

50mL Methanol
7mL Acetic Acid
43mL DI Water
0.1% Coomassie Brilliant Blue (.1g)

Destain Solution (1L)

400mL Methanol
70mL Acetic Acid
530mL DI Water

Abbreviations Used

(In Order of First Appearance)

FAS	Fetal Alcohol Syndrome
PFAS	Partial Fetal Alcohol Syndrome
ARBD	Alcohol Related Birth Defects
ARND	Alcohol Related Neurobehavioral Defects
FASD	Fetal Alcohol Spectrum Disorder
IQ	Intelligence Quotient
EtOH	Ethanol
CORT	Cortisol (humans), Corticosterone (rodents)
mCORT	Maternal Cortisol or Corticosterone
11B-HSD2	11B- hydroxysteroid dehydrogenase 2
CRF	Corticotrophin Releasing Factor
pCRF	Placental Corticotrophin Releasing Factor
ACTH	Adrenocorticotropic Hormone
fCORT	Fetal Cortisol or Corticosterone
MR	Mineralocorticoid Receptor
GR	Glucocorticoid Receptor
HPA axis	Hypothalamic-Pituitary-Adrenal axis
Hipp	Hippocampus
DEX	Dexamethasone
BETA	Betamethasone
SD	Sprauge-Dawley rat strain
LE	Long-Evans rat strain

EPM	Elevated Plus Maze
OF	Open Field
GD	Gestational Day
BDNF	Brain Derived Neurotrophic Factor
IP	Intraperotoneal Injection
BAC	Blood Alcohol Content
TMT	Trimethylthiazoline
DG	Dentate Gyrus
CA1	cornu Ammonis 1
CA3	cornu Ammonis 3
EC	Entorhinal Cortex
GC	Granule Cells
SGZ	Subgranular Zone
SVZ	Subventricular Zone
LTP	Long-term Potentiation
LTD	Long-term Depression
Ca^{2+}	Calcium
ARC	Activity-Regulated Cytoskeletal Protein
IEG	Immediate-Early Gene
GluR1	Glutamate Receptor
AMPA	α-Amino-3-Hydroxy-5-Methyl-4-Isoxazolepropionic Acid
AMPAR	α-Amino-3-Hydroxy-5-Methyl-4-Isoxazolepropionic Acid Receptor
CNS	Central Nervous System
Mg^{2+}	Magnesium
NMDA	N-Methyl-D-aspartate

NMDAR	N-Methyl-D-aspartate Receptor
Sacc	Saccharin
PN	Postnatal Day
RIA	Radioimmunoassay
TTTC	Two-Trial Trace Conditioning
CS	Conditioned Stimulus
US	Unconditioned Stimulus
ANOVA	Analysis of Variance
BEC	Blood Ethanol Concentration
mEPSCs	Miniature Excitatory Postsynaptic Currents
NHC	Non-Handled Control
TC	Trace Conditionied
UPC	Unpaired Control
UR	Unconditioned Response
CR	Conditioned Response
CFC	Contextual Fear Conditioning
DC	Delay Conditioning
RAM	Radial Arm Maze
OD	Optical Density
POI	Protein of Interest

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