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Prenatal Alcohol Exposure Effects on NMDA Receptor Subunit Composition and Function in the Mouse Dentate Gyrus

by

Megan Brady

B.S. Biochemistry, Marquette University, 2002

DISSERTATION

Submitted in Partial Fulfillment of the
Requirements for the Degree of

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PRENATAL ALCOHOL EXPOSURE EFFECTS ON NMDA RECEPTOR SUBUNIT COMPOSITION AND FUNCTION IN THE MOUSE DENTATE GYRUS

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ABSTRACT

Moderate prenatal alcohol exposure (PAE) in humans leads to learning and memory impairments later in life. The range of physical, behavioral, emotional, and social dysfunctions that are associated with PAE are collectively termed fetal alcohol spectrum disorder (FASD). It is estimated that approximately 12% of women consume alcohol at some time during their pregnancy and as many as 5% of children born in the United States are impacted by PAE. Extensive research has shown that even moderate maternal drinking (1-2 drinks per day) is, in some individuals, associated with cognitive and behavioral deficits.

Existing models for PAE have been useful in studying the effects of moderate exposure, including its effect on hippocampal learning and memory; however, these models often include factors, such as stress or malnutrition issues, that can confound results. To date, most studies on the behavioral consequences of PAE have focused on overall hippocampal-dependent behavioral deficits, withouth attempting to determine whether the impairment may be attributed to altered functioning of a subregion (e.g. the

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dentate gyrus) of the hippocampus. In contrast, biochemical and electrophysiological studies have identified dysfunctions in the hippocampal subregions. In the dentate gyrus, long term potentiation (LTP), which is considered to be a cellular mechanism of learning, is impaired in animals prenatally exposed to alcohol, as is *N*-methyl-D-aspartate (NMDA) receptor (NMDAR)-dependent activation of extracellular signal-regulated kinase 1/2 (ERK 1/2), which is important for LTP. This dissertation tested the hypothesis that PAE leads to impairments in dentate gyrus-dependent learning and memory, which is associated with NMDA receptor – dependent LTP deficits and NMDA receptor subunit composition alterations in the dentate gyrus.

A limited access PAE model in mice was developed, producing moderate ethanol intake in dams with no evident effect on litter size, pup weight, dam food and water intake, maternal weight gain, or maternal care. In addition, although PAE mice exhibited no differences in spontaneous locomotor activity compared to controls, they did demonstrate hippocampal-dependent fear conditioned learning deficits, similar to those seen previously in the literature. They also exhibited deficits in a delayed non-match to place (DNMP) task that was dentate gyrus-dependent, indicating that PAE does produce dentate gyrus impairments. Further studies using electrophysiological techniques found that PAE mice had deficits in NMDAR-dependent LTP in the dentate gyrus, as well as a reduced contribution of GluN2B-containing receptors to NMDAR-dependent field recordings. Biochemical subcellular fractionation and immunoblotting experiments found that, compared to controls, PAE mice had increased C2´-containing GluN1 and GluN3A subunits and decreased GluN2B subunit levels in the synaptic membrane fraction isolated from the dentate gyrus. These results indicate that PAE does indeed

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produce a dentate gyrus-dependent deficits in learning and memory, and this deficit is likely due to alterations in synaptic plasticity that result from changes in the NMDAR subunit composition at the synaptic membrane. These studies provide the basis for several future studies that aim to explore the impact of PAE on the control of NMDAR levels, trafficking, and function.

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1. Prenatal alcohol exposure and the Dentate Gyrus

1.1 Prenatal alcohol: incidence in human populations

In both humans and laboratory animal models, exposure to alcohol during development has been shown to cause a multitude of dose-dependent effects on the structure and function of the central nervous system, with the extent of damage being dependent on the pattern and chronicity of the exposure and the stage(s) of development during which it occurs (Queen et al., 1993; Miller, 1996; Goodlett and Johnson, 1997; Kelly and Richards, 1998; Sood et al., 2001; Savage et al., 2002; Mooney and Miller, 2009). Additionally, the effects of prenatal alcohol exposure (PAE) on the brain display regional variability (Guerri, 1998; Willford et al., 2004), with the hippocampus being particularly sensitive to the damaging effects of the exposure (Berman and Hannigan, 2000; Hamilton et al., 2003; Sakata-Haga et al., 2003; Sluyter et al., 2005). The range of physical, behavioral, emotional and social dysfunctions that are associated with PAE are collectively termed fetal alcohol spectrum disorder (FASD) (Kelly et al., 2000; Streissguth and O'Malley, 2000). It is estimated that use of any alcohol during pregnancy is about 12% (Floyd et al., 2009), with the incidence of newborns displaying prenatal alcohol-related damage as high as 5% in the United States (May et al., 2009). While heavy and binge (five or more drinks in a single episode) patterns of drinking during pregnancy are associated with the development of the most severe form of PAE, termed Fetal Alcohol Syndrome (FAS) (Jones and Smith, 1973; Clarren and Smith, 1978), extensive research shows that even moderate maternal drinking (1-2 drinks per day) is, in some individuals, associated with cognitive and behavioral deficits, often revealed only

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under stressful or challenging conditions (Streissguth et al., 1990; Streissguth et al., 1994; Willford et al., 2004).

1.1.1 Effects of PAE in humans

As mentioned above, the effect of PAE on humans depends on the amount and the timing of the exposure. Children diagnosed with FAS must have confirmed maternal alcohol exposure, evidence of a characteristic pattern of minor facial anomalies, evidence of prenatal and/or postnatal growth retardation, and evidence of deficient brain growth or abnormal morphogenesis (Hoyme et al., 2005). However, as implied by the term spectrum disorder, there is a range of deficits, and severity, for those with PAE, which are classified as partial FAS and alcohol-related neurodevelopmental disorder (ARND) (Mattson et al., 2011). These diagnoses, which typically involve moderate PAE (MPAE), often also exhibit neurodevelopmental abnormalities, although not as severe as those seen in FAS. Studies on children and adolescents have found moderate PAE may lead to decreased IQ scores, attention deficits, verbal and non-verbal learning and memory impairments, working memory deficits, and executive function impairments (Streissguth et al., 1994; Richardson et al., 2002b; Willford et al., 2004; Mattson et al., 2011). In addition, one study using MRI to study the corpus callosum in PAE adults and controls found higher variability in the size, volume, and shape in PAE adults (reviewed in (Chen et al., 2003)). These, and other studies, indicate that PAE can have long-lasting effects on brain structure and function in humans, even when exposure is considered moderate.

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1.1.2 Effects in animal models

1.1.2.1 Animal models of prenatal alcohol exposure

Animal models are useful tools to determine the mechanisms and outcomes of PAE, given that their physiological responses to alcohol are similar to those seen in humans (Hannigan and Abel, 1996). When designing an animal model of PAE, several factors need to be taken into consideration, including the age of exposure, the species used, the frequency and duration of exposure, and the method of administration (Thompson et al 2009). Existing rodent models for PAE employ various lengths and routes of administration, as well as a range of ethanol doses (Costa et al., 2000; Cudd, 2005). One of the more widely used PAE models uses ethanol-containing liquid diets, which limits food and water consumption (Berman and Hannigan, 2000). Ethanol is added to the diet, typically accounting for either 18% or 35% of the total caloric intake. Another commonly used method of administration is intragastric gavage, where the dams, or occasionally the pups, are intubated with ethanol solution (Berman and Hannigan, 2000). These methods commonly target the first and second trimester equivalent of human pregnancy in the rodents, although gavage can also target the third trimester equivalent. Although these methods often achieve moderate to high blood ethanol concentrations (BECs), they often elicit maternal stress and malnutrition, which may confound the results (Ward and Wainwright, 1989a; Slone and Redei, 2002). A newer model of PAE, which involves voluntary oral consumption of a sweetened ethanol solution, limits maternal stress and malnutrition issues (Allan et al., 2003).

Another important consideration when interpreting results from PAE studies are the BEC levels achieved. As mentioned above, intragastric gavage and liquid diet

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achieve moderate-to-high BECs, whereas voluntary oral consumption reaches low-to-moderate BECs. Studies have shown that the timing during gestation of PAE, the level of BECs reached, and the chronicity of exposure influences the severity the damage that is produced (Goodlett and Johnson, 1997; Guerri, 1998; Sood et al., 2001; Mooney and Miller, 2009). These factors must be considered when comparing results from different PAE paradigms.

1.1.2.2 Behavioral effects of PAE in animal models

Similar to the clinical presentation of PAE in humans, PAE causes a wide range of impairments in animal models, where PAE has been shown to be associated with increased learned helplessness, depression (Carneiro et al., 2005; Caldwell et al., 2008), increased seizure activity (Bonthius et al., 2001), increased ethanol consumption and preference (Barbier et al., 2009), and decreased sensitivity to the sedative effects of ethanol (Boehm et al., 2008; Barbier et al., 2009) in the offspring. In addition, PAE has been shown to produce spatial learning deficits in several tasks, including spatial alternation tasks (Incerti et al., 2010), Morris water maze (Richardson et al., 2002a; Christie et al., 2005; Savage et al., 2010), trace fear conditioning (Hunt et al., 2009), and contextual fear conditioning (Allan et al., 2003; Savage et al., 2010), all of which are hippocampal-dependent.

1.2 Prenatal alcohol exposure affects the dentate gyrus of the hippocampus

Although it was initially believed that *in utero* exposure to alcohol damaged all portions of the brain equally, later studies showed that certain regions of the brain, especially the hippocampal formation, are more vulnerable to the effects of prenatal

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alcohol(Guerri, 1998; Berman and Hannigan, 2000; Chen et al., 2003). Previous studies have shown that pre- and post-natal alcohol exposure can reduce cell numbers (Guerri, 1998), alter receptor levels and binding (Savage et al., 1991; Galindo et al., 2004; Samudio-Ruiz et al., 2010), affect neurotropic factors essential for normal brain development (Caldwell et al., 2008; Miki et al., 2008), and impair hippocampal-dependent learning and memory (see above).

The hippocampal formation, which is considered part of the limbic system, consists of the hippocampus, dentate gyrus, subiculum, presubiculum, parasubiculum, and entorhinal cortex (reference). The hippocampus is divided into regions that are classified as the cornu ammonis (CA) fields (CA1, CA2, CA3); many investigators also include the dentate gyrus as part of the hippocampus. The hippocampus is generally considered a trisynaptic circuit, with the major input received from the entorhinal cortex, synapsing onto the dentate gyrus via the perforant path. The dentate gyrus sends afferents to the CA3 region via the mossy fiber pathway, and the CA3 synapses onto the CA1 region via the Schaffer collaterals. The CA1, in turn, sends the main hippocampal output back to the subiculum and entorhinal cortex, thus forming a loop (Figure 1.1).

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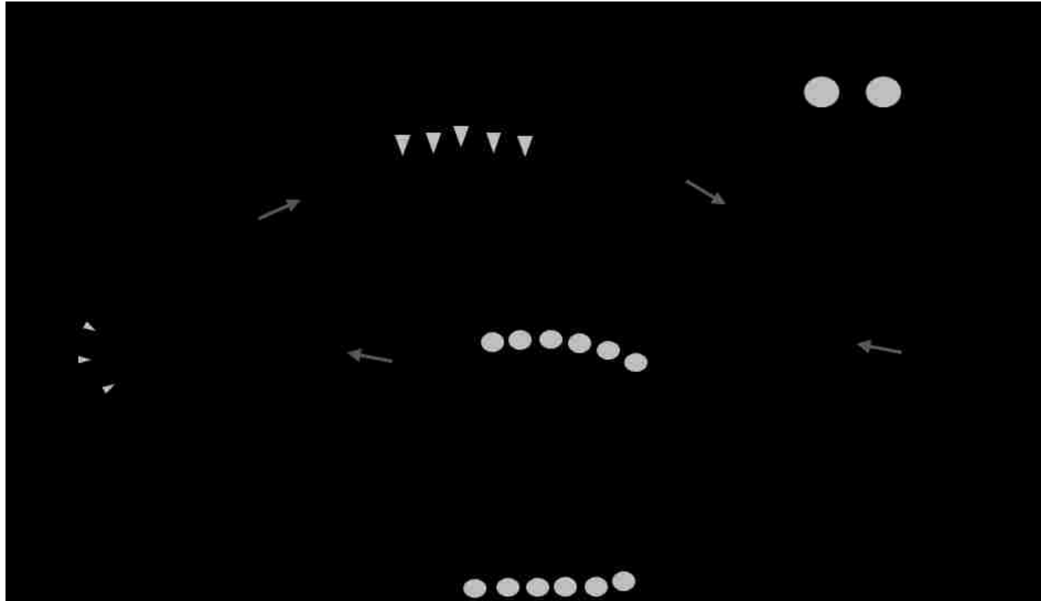

Figure 1.1 The hippocampus and associated pathways.

Although considerable attention has focused on the function of the Schaffer collaterals – CA1 pathway in learning, and the effects of PAE on this region of the hippocampus, less research has focused on the effect of PAE on the dentate gyrus, which plays an important role in hippocampal function.

1.2.1 The Dentate gyrus – Anatomy and function

The dentate gyrus is the site of efferent input into the hippocampus. It consists of three major layers – the molecular layer, the granule cell layer, and the hilus, or polymorphic layer (Figure 1.2). The hilus consists of several cell types, including the mossy cells, which are glutamatergic and form the commissural/associational connections (commissural projections not found in the primate brain), and the pyramidal basket cell, which are GABAergic interneurons that play a role in feedback inhibition. The granule cell layer primarily contains the dentate granule cells, which are the primary cells of the

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dentate gyrus. These cells have an elliptical cell body, and are densely packed into this layer (Amaral et al., 2007b). These cells form the blades of the dentate gyrus, the suprapyramidal and infrapyramidal blades, located above and below the pyramidal cell layer of the CA3, respectively (Amaral et al., 2007b).

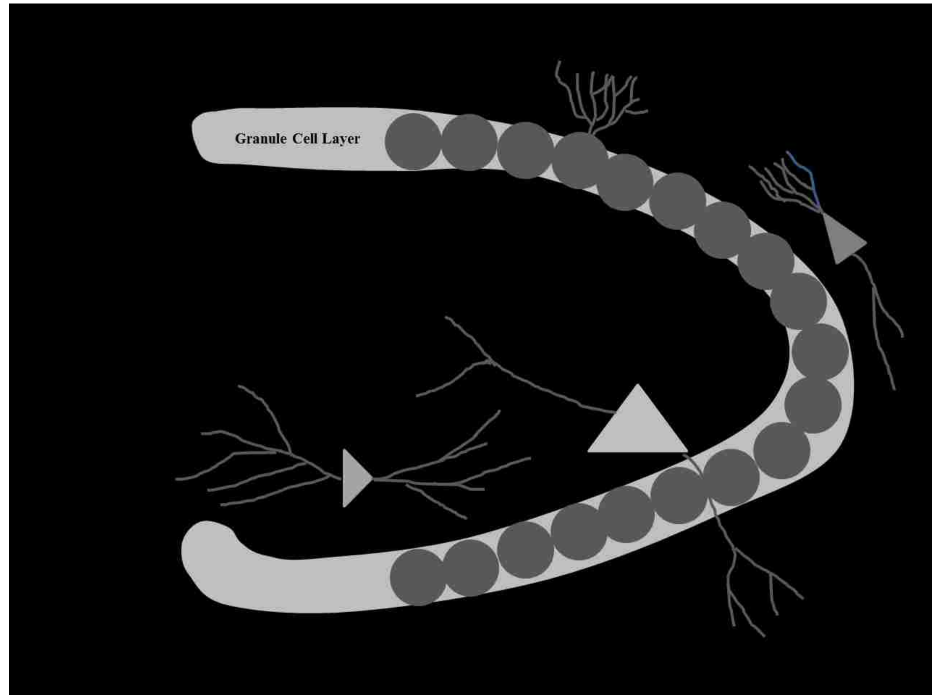

Figure 1.2 The Dentate Gyrus

The granule cells project their dendrites into the molecular layer, receiving the major excitatory input of the dentate gyrus, and the hippocampus, from the entorhinal cortex. The molecular layer consists of three subsections – the inner molecular layer, the middle molecular layer, and the outer molecular layer. The outer two molecular layers receive inputs primarily from layer II of the entorhinal cortex, with the medial entorhinal cortex extending to the middle molecular layer to form the medial perforant path (MPP), and the lateral entorhinal cortex extending to the outer molecular layer to form the lateral

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perforant path (LPP) (Witter, 2007). These pathways, while both generally considered glutamatergic, have other distinct receptor expression and electrophysiological properties, allowing them to signal differently to the granule cell layer (Witter, 2007). The inner third molecular layer receives its inputs from cells located in the hilus, primarily the mossy cell, which are immunoreactive for glutamate (Soriano and Frotscher, 1994). Although the molecular layer primarily consists of dendrites from the granule cells and hilus cells, as well as axon projections from the entorhinal cortex, the molecular layer also contains neurons, primarily the molecular layer perforant path-associated cell (MOPP), which are immunoreactive for GABAergic markers and likely provide inhibitory control of granule cells (Amaral et al., 2007a).

The dentate gyrus is believed to play several roles in the hippocampus, one of which is pattern separation, or the ability to produce separate representations of different, but similar, places (Kesner, 2007). The idea that the dentate gyrus plays a role in this function comes from its anatomy. Pattern separation may be facilitated by the sparse connections that the dentate gyrus granule cells make with the CA3 pyramidal cells via the mossy fibers. There is a low probability that any two CA3 neurons will receive mossy fiber inputs from a similar subset of dentate gyrus cells (Rolls, 1996). A critical role for the dentate gyrus in spatial learning has been demonstrated in studies showing that lesions of the dentate gyrus result in deficits similar to those produced by complete hippocampal lesions in spatial tasks (Walsh et al., 1986; Tilson et al., 1988). To determine the dentate gyrus' more specific role in pattern separation, a delayed non-match to sample task was designed to test discrimination between two similar spatial locations (Gilbert et al., 2001). Animals with dentate gyrus-specific lesions showed

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impairments in this task compared to controls, implicating the dentate gyrus in pattern separation. Another study found that minimal changes in the shape of an environment can alter correlated activity patterns among place-modulated granule cells in the dentate gyrus, implicating the role of the dentate gyrus in pattern separation (Leutgeb et al., 2007).

The dentate gyrus is also one of two locations in the adult brain that exhibit adult neurogenesis, which occurs in the subgranular zone (SGZ), at the border of the hilus and dentate granule cell layer. This region contains stem cells that can proliferate and differentiate to give rise to new neurons. These neurons migrate into the granule cell layer and integrate into the existing circuitry, extending axons to the CA3 region (Stanfield and Trice, 1988) and initially having lower thresholds for long-term potentiation (LTP) and long-term depression (LTD) (Schmidt-Hieber et al., 2004), considered to be important for learning and memory. Neurogenesis is regulated by a number of factors, including hormones, neurotransmitters, age, and exercise (reviewed in Parent, 2007). Although the functions of adult neurogenesis in the dentate gyrus are unclear, several studies have shown that it plays a role in learning and memory. Disruption of neurogenesis via irradiation has been shown to disrupt learning in a dentate gyrus-specific delayed non-match to place radial arm maze pattern separation task (Clelland et al., 2009). In addition, stimulation of adult neurogenesis, either by environmental enrichment or by genetic manipulation, has been shown to improve learning (Bruel-Jungerman et al., 2005; Sahay et al., 2011)

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1.2.2 Development of the Dentate Gyrus

In humans, the dentate gyrus, similar to the rest of the hippocampus, begins development in the 13th-14th weeks of gestation, and continues after birth (Seress et al., 2001); in the rodent, development begins around embryonic day 13.5 (E13.5) and continues into the postnatal period (Li and Pleasure, 2007). At about E13.5, precursors migrate from the ventricular zone to the dentate notch, where they then form the suprapyramidal, then the infrapyramidal, blades of the dentate gyrus by about postnatal day 4 (PD4) (Frotscher et al., 2007; Li and Pleasure, 2007). Precursors also remain in a secondary proliferation zone which will later become the subgranular zone and the site of adult neurogenesis. Interestingly, afferents from the entorhinal cortex arrive in the region before the dentate granule cells form laminations, indicating that these afferents act independently of the target.

1.2.3 Effects of prenatal alcohol exposure on the dentate gyrus

As mentioned above, PAE has been shown to have severe consequences on the function of the hippocampus, with most studies having focused on either the hippocampus as a whole or the CA1 region of the hippocampus. However, some studies have examined the effects of prenatal alcohol on the dentate gyrus. Impairments in adult neurogenesis after pre- or post-natal alcohol exposure have been reported. Choi *et al.* (2005) reported that moderate PAE reduced the neurogenic response to an enriched environment, but diod not alter the initial number of proliferating progenitors. Klintsova et al. (2007) showed that an early postnatal binge alcohol exposure (PD 4-9) reduced the number of mature neurons in the adult dentate gyrus. Similarly, several, but not all, studies have reported pre- and postnatal alcohol exposure decreases in overall cell

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number in the dentate gyrus (reviewed in Gil-Mohapel et al., 2010). LTP has also been shown to be impaired in the dentate gyrus *in vivo* in rodents prenatally exposed to alcohol (Sutherland et al., 1997; Titterness and Christie, 2010; Varaschin et al., 2010). PAE has been associated with reductions in mGluR5 receptor number and function in adult rats (Galindo et al., 2004) and decreased extracellular signal-regulated kinase 1/2 (ERK 1/2) phosphorylation after *N*-methyl-D-aspartate (NMDA) receptor activation in adult mice (Samudio-Ruiz et al., 2009).

1.3 Summary

PAE has a relatively high incidence in the US population, with estimates as high as 5%, and can cause significant deficiencies in offspring, even at moderate exposure levels. The dentate gyrus, which is important for pattern separation learning and is the site of adult neurogenesis, is particularly vulnerable to PAE, with studies showing neurophysiological and neurochemical alterations in dentate gyrus after *in utero* ethanol exposure. The mechanism behind these alterations, however, has yet to be determined, and is the focus of study for this dissertation.

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2. NMDA Receptors and LTP in the Dentate Gyrus

2.1 Long term potentiation (LTP) is the cellular mechanism for learning and memory

LTP is the long-lasting, activity dependent increase in synaptic strength (Santos et al., 2009). The traditional form of LTP, or classical LTP, is *N*-methyl-D-aspartate (NMDA) receptor-dependent, occurs postsynaptically, and can be elicited throughout the brain, including in the dentate gyrus. LTP undergoes three main stages: LTP induction, LTP expression, and LTP maintenance (reviewed in Malenka and Bear, 2004). LTP induction occurs immediately after the stimulation protocol. Activation of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor by glutamate leads to postsynaptic depolarization, relieving the Mg^{2+} block of the NMDA receptor and allowing for the influx of Ca^{2+} into the cell. This Ca^{2+} acts as a second messenger,via regulation of the levls/activity of a number of downstream signaling molecules, such as protein kinase A (PKA), protein kinase C (PKC), cAMP, and calcium-calmodulin activated kinase II (CaMKII), which allows for the next stage of LTP, LTP expression. Signaling molecules generated during LTP induction lead to increased AMPA receptor trafficking to the surface, as well as phosphorylation of AMPA receptors already present; these events, in turn, lead to an increase in postsynaptic response to glutamate and, thus, potentiation. These changes would be short lived, however, if not for the third stage of LTP, LTP maintenance. During LTP maintenance, PKA, PKC, cAMP, and CaMKII go on to activate other signaling molecules, such as extracellular signal-regulated kinase 1/2 (ERK1/2) and cAMP response element binding protein (CREB), which, in turn, translocate into the nucleus to regulate gene transcription. In addition, these signaling molecules may regulate localized protein translation in the dendrite. New protein

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synthesis and gene transcription are required for LTP maintenance, and result in a number of long-lasting changes to the spine, including cytoskeletal reorganization (Malenka and Bear, 2004).

As mentioned, LTP is believed to be the cellular basis for learning and memory, and several recent studies have supported this notion. Studies have demonstrated that inhibition of the signaling cascades that are necessary for LTP result in learning impairment. For example, when a dominant-negative form of PKA is expressed in mouse hippocampus, LTP is impaired, as is memory consolidation in a one trial per day water maze task (Malleret et al., 2010). α-CaMKII autophosphorylation has been shown to be increased by learning paradigms (Rodrigues et al., 2004). Similarly, mutant mice that have α-CaMKII subunits that are unable to undergo autophosphorylation experience deficits in passive avoidance learning (Irvine et al., 2005). A role for PKC in LTP is supported by the demonstration that PKC is activated in hippocampal cells during associative learning (Olds et al., 1989), transfection of a constitutively active catalytic domain of PKC in the dentate gyrus of aged rats improved performance in the Morris water maze relative to controls (Zhang et al., 2009), and PKCγ knockout mice exhibit deficits in fear conditioning (Weeber et al., 2000). Further studies have tried to relate LTP directly to the process of learning. LTP induction in the dentate gyrus has been shown to disrupt acquisition of new spatial memories, but not of already-established memories (McNaughton et al., 1986; Moser et al., 1998); evoked potentials recorded *in vivo* from the dentate gyrus during an operant training protocol found a significant increase in population spikes over the course of training compared to non-training controls (Skelton et al., 1987); and *in vivo* recordings of field extracellular postsynaptic

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potentials (fEPSPs) in the CA1 demonstrated an increase in slope after inhibitory avoidance training but not in controls (Whitlock et al., 2006). These data indicate that synaptic plasticity, particularly LTP, is an important aspect of learning and memory; however, there is some evidence that spatial learning may occur without LTP (Saucier and Cain, 1995).

2.2 The NMDA Receptor

2.2.1 The NMDA receptor: general information

As mentioned above, activation of the NMDA receptor is important for classical LTP, at least in part due to its Ca^{2+}-permeability, which allows for activation of the downstream signaling molecules responsible for LTP. NMDA receptors are critical components of excitatory neurotransmission and plasticity in the mammalian central nervous system (Cull-Candy et al., 2001). NMDA receptors are cation channels, which in addition to being permeable to Ca^{2+} are permeable to Na^{+} and K^{+}; opening of the channel leads to membrane depolarization (Hardingham and Bading, 2003). Additional features of NMDA receptors include the presence of a voltage-sensitive Mg^{2+} block of the ion channel, the requirement of a co-agonist, glycine, their sensitivity to endogenous ligands and modulators (e.g., Zn^{2+} and polyamines), relatively slow activation/deactivation kinetics, and the ability to interact with various intracellular signaling molecules (Cull-Candy et al., 2001).

Functional NMDA receptors are heteromeric tetramers composed of two obligatory GluN1 subunits along with modulatory GluN2 (A-D) or GluN3 (A-B) subunits (Cull-Candy et al., 2001; Ron, 2004; Paoletti and Neyton, 2007). Each subunit consists of a large extracellular N-terminus; three hydrophobic transmembrane domains (TM1,

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TM3, and TM4) and a transmembrane segment (TM2) making a loop within the membrane and which contribute to the formation of the channel pore; and an intracellular C-terminal tail, which plays a role in intracellular signaling (Cull-Candy et al., 2001). Eight isoforms of the GluN1 subunit have been identified, arising from alternative splicing of a single gene. These splice variants arise from three different splice sites, producing three different cassette inserts: the N1 cassette, located in the N-terminus, and the C1 and C2/C2' cassettes, located in the C-terminal tail (Al-Hallaq et al., 2001). In contrast, GluN2 and GluN3 subunits are encoded by six separate genes (Paoletti and Neyton, 2007). Although NMDA receptors are conventionally believed to form diheterotetramers (e.g., GluN1/GluN2A), there is evidence that NMDA receptors can exist as triheterotetramers incorporating two types of NR2 subunits- i.e., GluN1/GluN2A/GluN2B (Thomas et al., 2006; Paoletti and Neyton, 2007). Initially, it was estimated that approximately two-thirds of the GluN2A-containing and GluN2B-containing NMDA receptors on the surface of hippocampal cells are associated as GluN1/GluN2A or GluN1/GluN2B diheterotetrameric complexes and the remaining one-third are associated as triheterotetrameric complexes of GluN1/GluN2A/GluN2B (Al-Hallaq et al., 2007); however, a recent study, using electrophysiological techniques, determined that triheterotetramers may constitute the primary NMDAR subtype at adult hippocampal synapses (Rauner and Kohr, 2011). This difference in diheterotetrameric vs. triheterotetrameric may play a large role in determining the functionality of the receptor at the synapse.

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2.2.2 NMDA receptor expression during development

The expressions of the different NMDAR subunits vary throughout development, and have been primarily characterized in rodents. GluN1 subunit expression begins around E14, peaking in the third postnatal week and then decreases to adult levels (Laurie and Seeburg, 1994; Paupard et al., 1997). The expression of the different splice variants, although exhibiting variations across different brain regions, generally does not change through development. The GluN1-1a (corresponding to the splice variant containing the N1, C1, and C2 cassettes), GluN1-2a (corresponding to the splice variant containing the N1 and C2 cassettes), and GluN1-4a (corresponding to the splice variant containing the N1 and C2' cassettes) are the most abundantly expressed GluN1 splice variants in the hippocampus (Paupard et al., 1997).

The GluN2 subunits exhibit differential expression in the brain throughout development (Ewald and Cline, 2009). At about E14, GluN2B and GluN2D expression begins at low levels, peaking at around PD7, with GluN2B expressed at higher levels than GluN2D in the hippocampus. GluN2D expression eventually decreases to very low levels in the adult hippocampus. Around the first postnatal week, expression of GluN2A in the hippocampus begins, peaking in the third postnatal week and gradually declining to adult levels. GluN2C is primarily expressed in the cerebellum, peaking at PD12 and is expressed at high levels throughout adulthood.

Electrophysiological recordings have also confirmed developmental changes to GluN2 subunit levels, focusing primarily on the developmental switch between GluN2B and GluN2A subunits. The GluN2A subunit confers lower glutamate sensitivity, faster decay times, and lower open probabilities to the NMDA receptor compared to the

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GluN2B subunit, while GluN2B has been shown to have a longer current duration and contribute more to the Ca^{2+} load (Lau and Zukin, 2007). During development, the expression of primarily GluN2B-containing NMDARs leads to longer decay kinetics in electrophysiological recordings, which gradually shorten with age due to the increased expression of GluN2A (Cull-Candy et al., 2001; Ewald and Cline, 2009).

GluN3 subunit levels change dramatically over development. GluN3A is expressed at low levels in the embryo, peaking early in the postnatal period and decreasing into adulthood, while GluN3B levels are low postnatally and increase into adulthood (Henson et al., 2010). These expression profiles are seen in many regions of the brain, including the hippocampus. Interestingly, the GluN3A subunit has been shown to form heterotrimers with GluN1 and GluN2 subunits, leading to reduced Mg^{2+} sensitivity, reduced Ca^{2+} permeability, and alterations in open probabilities and decay kinetics (Henson et al., 2010), which may alter the function of NMDARs during development.

2.2.3 Localization and trafficking of the NMDA receptor

The NMDA receptor is trafficked to the surface in several different manners, typically dependent on the subunit composition of the receptor. Initially, it was believed that each GluN1 splice variant expresses an endoplasmic reticulum (ER) retention (ERR) signal, keeping it in the ER until it combines with a GluN2 or GluN3 subunit, which covers the ERR and allows for forward trafficking (Hall and Soderling, 1997). However, several papers have demonstrated that only GluN1-1 is primarily retained intracellularly, while other GluN1 splice variants have been shown to reach the surface when expressed

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in heterologous systems (Okabe et al., 1999; Standley et al., 2000). Furthermore, Horak and Wenthold (2009) showed that there are two independent ERR motifs present in the C1 cassette, and C2 has an additional inhibitory effect on forward trafficking, while the C0 (the portion of the GluN1 C-terminus found in all splice variants) and C2' cassettes enhance trafficking to the surface. Thus, the specific expression of GluN1 splice variants can alter the trafficking of the NMDA receptor. ERR signals have been identified in both GluN2A (Qiu et al., 2009) and GluN2B (Horak et al., 2008), although these signals are masked when the subunit is complexed with GluN1. GluN2/GluN3 heterodimers are also retained in the ER until combining with GluN1 (Perez-Otano et al., 2001).

The NMDA receptor is trafficked to the surface in endosomes from the trans-Golgi network (TGN) via kinesin motor proteins on polarized microtubules and exocytosed to the cell surface via the SNARE machinery (reviewed in Petralia et al., 2009). Typically, the receptor is accompanied by a chaperone protein, typically a PDZ domain-containing protein that binds primarily to the GluN2 subunit. SAP97 and CASK, two PDZ-containing proteins, have been shown act together to use an alternative secretory pathway to traffic the NMDA receptor to the surface quicker than the traditional secretory pathway (Jeyifous et al., 2009). SAP102, another PDZ domain-containing protein, has also been shown to play a role in NMDA receptor trafficking (Sans et al., 2003; Sans et al., 2005).

Once at the surface, the NMDA receptor can be localized either within or outside of the synapse. The receptors are primarily inserted into the extrasynaptic membrane, and can then travel laterally within the cell membrane into the synapse (Lau and Zukin, 2007), although there is some evidence that they can directly enter the post-synaptic

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density (PSD) or perisynaptic membrane (Petralia et al., 2009). The localization of the NMDAR is important because it may determine the downstream signaling to which the receptor can couple, and thus affect the role that the receptor plays. Synaptic NMDA receptors have been associated with ERK1/2 and CREB activation (Hardingham et al., 2002; Ivanov et al., 2006; Mulholland et al., 2008), which are associated both with cell survival and LTP. Conversely, extrasynaptic NMDA receptors have been associated with ERK1/2 and CREB deactivation, and may result in excitotoxicity (Hardingham et al., 2002; Ivanov et al., 2006; Mulholland et al., 2008). It has been shown that a number of factors dictate whether the NMDA receptor remains in the extrasynaptic membrane or translocates to the synaptic membrane. Some studies have suggested that GluN2B-containing receptors are more likely to be retained in the extrasynaptic membrane, while GluN2A-containing receptors are more likely to move to the synaptic membrane (Tovar and Westbrook, 1999); however, recent evidence indicates that levels of both subunits are equally distributed in both fractions (Harris and Pettit, 2007). In addition, the extracellular matrix protein Reelin (Groc et al., 2007) and the adhesion protein EphB2 (Nolt et al., 2011), among others, have been shown to affect NMDA receptor subunit mobility, thereby controlling the localization.

Once the NMDA receptor is localized to the synaptic fraction, it is incorporated into a large, macromolecular signaling complex composed of synaptic scaffolding proteins and adaptor proteins, which help to link the receptor to downstream signaling molecules and maintain the receptor in the synapse (Lau and Zukin, 2007). The scaffolding proteins primarily consist of the PDZ domain-containing proteins PSD-95, PSD-93, and SAP102, which anchor the receptors to the synaptic membrane. PSD-95

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has been shown to target GluN2B subunits to the synaptic membrane (Sans et al., 2003; Prybylowski et al., 2005). It has been shown, however, that GluN1 splice variants containing the C2' cassette also contain a PDZ binding domain, although whether it plays a role in trafficking and localization has yet to be determined (Standley et al., 2000).

Endocytosis of the NMDA receptor primarily occurs for receptors residing in the extrasynaptic membrane, via clathrin-coated pits. Endocytosis occurs both through the constitutive and the regulated pathway. Regulated endocytosis can be activated either by agonist binding to the NMDA receptor or indirectly via metabotropic glutatmate receptor (mGluR) activation (Snyder et al., 2001a; Nong et al., 2003). The internalization of the GluN2 subunits appears to occur by different pathways (reviewed in Petralia et al., 2009). Generally, internalization of the GluN2B subunit occurs by the AP-2 adaptor protein binding to a specific internalization motif on the C-terminus. Although the GluN2A C-terminus has a similar internalization motif, it does not appear to interact with AP-2 . This leads to more robust internalization of GluN2B subunits compared to GluN2A subunits. The different GluN2 subunits also follow different endocytic pathways. While both enter early endosomes, GluN2A subunits are processed through late endosomes and are degraded, while GluN2B subunits are recycled back to the surface .

Phosphorylation also plays a role in NMDA receptor trafficking and localization. Casein kinase II (CK2) has been shown to phosphorylate GluN2B at Ser1480 on the C-terminus, which disrupts the association of GluN2B with PSD-95, leading to decreased surface expression (Chung et al., 2004). Conversely, phosphorylation of Tyr1472 by Fyn, a Src family kinase (Prybylowski et al., 2005), on the GluN2B subunit promotes surface expression and prevents endocytosis (Goebel-Goody et al., 2009).

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Dephosphorylation, via protein tyrosine phosphatases (PTPs), also plays a role in NMDAR assembly. The phosphatase striatal–enriched tyrosine phosphatase (STEP) can directly dephosphorylate the Tyr1472 residue on GluN2B (Kurup et al., 2010), or dephosphorylate Fyn (Nguyen et al., 2002), also resulting in decreased phosphorylation at Tyr1472 on GluN2B , both leading to increased endocytosis. Surprisingly, inhibition of PTPs led to disassembly of GluN1, GluN2A, and GluN2B subunits of the NMDA receptor complex (Ferrani-Kile and Leslie, 2005), indicating that a balance of phosphorylation and desphosphorylation is required to maintain the NMDAR complex at the surface.

Phosphorylation has also been shown to play a role in NMDA receptor function (reviewed in Salter et al., 2009). PKC phosphorylates the GluN1 (Ser890 and Ser896), the GluN2A (Ser1416), and GluN2B (Ser 1303 and Ser1323) subunits, all of which may lead to enhancement of NMDAR currents, possibly by potentiating channel activity. PKA has also been shown to indirectly potentiate NMDAR currents by stimulation of G-protein coupled receptors and directly via phosphorylation of GluN1, 2A, and 2B. Phosphorylation of NMDAR subunits by PKA and PKC has also been shown to inhibit the ERR signal retention, regulating forward trafficking. In addition, Src family kinases (SFKs) have been shown to play a large role in phosphorylation of NMDARs leading to potentiation. Application of exogenous Src or Fyn, or peptides that activate Src or Fyn, increases both NMDA-evoked currents and NMDA receptor-dependent excitatory postsynaptic currents (EPSCs) by directly phosphorylating GluN2A and/or GluN2B. SFKs are believed to be activated downstream of mGluR activation and PKC signaling

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(Heidinger et al., 2002), and have been found to be necessary for LTP induction (Lu et al., 1998).

2.3 NMDAR, learning and memory, and LTP

2.3.1 NMDAR and learning

Several studies have shown that NMDARs are critical for learning and memory (reviewed in Riedel et al., 2003). Most studies have focused on the role of the NMDAR in spatial memory. Blockage of NMDAR function by the antagonist MK-801 has been shown to impair contextual fear conditioning in rats when infused either into the dorsal or ventral hippocampus (Zhang et al., 2001; Bast et al., 2003). In addition, contextual fear conditioning was impaired in mice with an inducible CA1-specific NR1 knockout (Shimizu et al., 2000). Conversely, transgenic mice with overexpression of the NR2B subunit show an improvement in contextual fear conditioning as compared to wild type (Tang et al., 1999). Spatial memory has been shown to be impaired in the Morris water maze and an eight-arm radial arm maze task upon application of an NMDA antagonist, (Morris et al., 1986; Bischoff and Tiedtke, 1992), as well as in a spatial delayed alternation task (Watson et al., 2009). Some studies have shown NMDAR involvement in non-spatial learning tasks. NMDA infusion into the dorsal or ventral hippocampus also blocks auditory fear conditioning in addition to contextual fear conditioning (Zhang et al., 2001; Bast et al., 2003). Ro 25-6981, an NMDAR antagonist specific to those receptors containing the NR2B subunit, has been shown to impair learning in a trace conditioning paradigm (Valenzuela-Harrington et al., 2007), a procedure requiring temporal processing. Similarly, an inducible CA1-specific NR1 knockout in mice is associated with an impairment in trace fear conditioning (Huerta et al., 2000) and in a

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nonspatial transverse patterning task (Rondi-Reig et al., 2001). Knockdown of the NR1 subunit using siRNA results in impaired performance in a passive avoidance task, whereas overexpression of the subunit results in better performance in the same task as compared to wild type (Kalev-Zylinska et al., 2009).

Many studies involving NMDARs' role in learning and memory have tried to determine at which time point during learning and memory formation NMDARs have a role, with conflicting results. It is generally agreed that there are three major stages of memory – acquisition or encoding, consolidation, and retrieval. Mice with the inducible CA1-specific GluN1 knockout have shown an inability to learn in several different tasks, implicating NMDAR's role in acquisition (Tsien et al., 1996; Shimizu et al., 2000; Rondi-Reig et al., 2001). Supporting this, application of NMDAR antagonists during the Morris water maze at time points just prior to either acquisition learning or retrieval demonstrated only acquisition impairments (Morris et al., 1986; Woodside et al., 2004). However, some studies implicate its role in both acquisition and retrieval (Ahlander et al., 1999; Shimizu et al., 2000; Yoshihara and Ichitani, 2004), while a recent study implicates it in retrieval, but not acquisition, in weanling rats (Watson and Stanton, 2009). Although some studies have shown no role for the NMDAR in consolidation (Yoshihara and Ichitani, 2004), one study has shown that NMDAR reactiviation is necessary for consolidation (Shimizu et al., 2000), although the uncertain time period for consolidation may limit the interpretations of these results. The reason for these differences in results is unclear. The timing of the pharmacological intervention may play a role – if not administered at the appropriate time, the window of opportunity to interfere with a certain aspect of memory may be missed. In addition, there may be differences in the role of

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NMDARs in the timing of these stages in different types of learning; spatial memory may require the involvement of NMDARs at one specific stage, whereas non-spatial learning may require its involvement at another. In addition, NMDARs' interactions with other receptors, such as the AMPA receptor and mGluRs, may influence the extent of NMDAR's role in these processes. Further research is needed to determine NMDAR's precise role.

2.3.2 NMDAR and LTP

Given the role of NMDA receptors in some types of learning, it is not surprising that the NMDA receptor plays an important role in LTP. As mentioned previously, classical LTP, which has primarily been characterized at the Schaffer collaterals-CA1 pathway but is also present at the perforant path-dentate gyrus molecular layer pathway, is dependent on the NMDA receptor. Early studies showed that NMDA receptor antagonists, such as 2-amino-5-phosphonopentanoate (AP5) or MK-801, prevent the induction of LTP in the CA1 region *in vitro* and the dentate gyrus *in vivo* in rodents (Collingridge et al., 1983; Morris et al., 1986; Coan et al., 1987; Davis et al., 1992). In addition, mice lacking the GluN1 subunit exhibit reduced hippocampal LTP compared to control littermates (Sakimura et al., 1995). Given that the NMDA receptor requires both agonist activation, via presynaptic glutamate release, and release of the Mg^{2+} block, via postsynaptic depolarization, it functions well as a coincidence detector (Bliss and Collingridge, 1993).

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2.3.2.1 Downstream signaling of the NMDA receptor important for LTP

One of the primary reasons that NMDA receptors are important for LTP is the receptor's permeability to Ca^{2+}. An important initial study showed that LTP could be prevented by postsynaptic intracellular injection of EGTA, a Ca^{2+} chelator (Lynch et al., 1983). Tetanic stimulation has also been shown to increase Ca^{2+} levels in dendritic spines (Muller and Connor, 1991), and a rise in Ca^{2+} lasting less than 3 seconds has been shown to be sufficient for LTP induction (Malenka et al., 1992). A study done by the Clapham lab showed that mimicking pre- and postsynaptic activation by pairing extracellular glutamate uncaging and postsynaptic depolarization resulted in a large residual Ca^{2+} influx that was not blocked by Cd^{2+}, a blocker of voltage-gated calcium channels (VGCCs), but was blocked by AP5, implicating NMDA receptors as a primary source of Ca^{2+} during LTP (Schiller et al., 1998). However, it is quite possible that other sources of Ca^{2+}, such as from intracellular stores or VGCCs, play a role in NMDA receptor-dependent LTP, since NMDA receptor activation can lead to mobilization of Ca^{2+} from these sources (Schiller et al., 1998; Emptage et al., 1999).

As mentioned earlier, Ca^{2+} influx is important because of its role as a second messenger, activating such downstream molecules such as CaMKII, PKA, and PKC. The NMDA receptor has also been linked to the activation of these molecules, likely though its role in Ca^{2+} influx. NMDA receptor-dependent activation of these molecules are important for the expression of LTP, with CaMKII and PKC implicated primarily with LTP induction, and PKA implicated in LTP maintenance (Malinow et al., 1989; Klann et al., 1991; Frey et al., 1993; Fukunaga et al., 1993; Matthies and Reymann, 1993; Ahmed and Frey, 2005). CaMKII has been shown to translocate to the PSD and incorporate

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itself into the NMDA receptor signaling complex, where it can stay active even after Ca^{2+}/calmodulin disassociation and can interact with other proteins located in the PSD (reviewed in Lisman et al., 2002). It has been shown to phosphorylate Ser891 on the GluR1 subunit of the AMPA receptor, leading to increased channel conductance (Benke et al., 1998; Lee et al., 2000). It may also be involved in AMPA receptor trafficking to the surface, although the mechanism has not been elucidated (Lisman et al., 2002). In addition, CaMKII may lead to ERK1/2 activation, which is important for LTP expression (Sweatt, 2001; Thomas and Huganir, 2004). PKC activation can also activate ERK1/2 via the small G-protein Ras-Raf-MEK pathway (Sweatt et al., 1998; Impey et al., 1999; Levenson et al., 2004), and PKC-dependent AMPA receptor phosphorylation at Ser818 may lead to increased expression at the surface (Boehm et al., 2006). In addition, a particular atypical isozyme of PKC, protein kinase M zeta (PKMξ), has been shown to be important in LTP maintenance, possibly by directing AMPA receptor trafficking (Sacktor et al., 1993; Yao et al., 2008). PKA, which is activated via cAMP, is implicated in the activation of several downstream substrates, including CREB and the AMPA receptor (reviewed in Nguyen and Woo, 2003); however, there is evidence that PKA activation may inhibit the Ras/Raf pathway, thus inhibiting ERK1/2 activity (Sweatt, 2001). The activation of ERK1/2 and CREB are important because of their roles in activation of immediate early genes (IEGs), which are important for LTP maintenance (reviewed in Bozon et al., 2003). ERK1/2 can directly activate Elk-1 and indirectly activate CREB, two transcription factors that can induce transcription of *zip268*, which has been shown to be necessary in LTP maintenance. In addition, CREB has been shown to lead to increases in brain-derived neurotropic factor (BDNF), *cfos*, and *arc* expression, which

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have also been shown to be important in LTP maintenance (Czerniawski et al., 2011; Peng et al., 2011).

2.3.2.2 Other factors involved in NMDA receptor-dependent LTP

Although classical LTP is dependent on NMDA receptor activation, there are modulators that may affect the extent of LTP expressed. Group I mGluRs, which consist of mGluR1 and mGluR5 and are coupled to the G_q pathway, have been shown to potentiate NMDA receptor currents by activating PKC, which can then phosphorylate the NMDA receptor leading to current potentiation (Skeberdis et al., 2001; Heidinger et al., 2002). In addition, co-activation of mGluR5 and NMDA receptors has been shown to synergistically activate ERK1/2, which may also affect LTP (Yang et al., 2004). Acute nicotine has been shown to enhance LTP *in vitro* in the dentate gyrus via α7 nicotinic acetylcholine receptors (nAChRs). Naloxone, a μ-opioid receptor antagonist, and norbinaltorphimine (NBNI), a κ-opioid receptor antagonist, enhanced LTP in the dentate gyrus in rodents (Terman et al., 1994; Xie and Lewis, 1995), indicating that endogenous opioids play an inhibitory role in NMDA receptor-dependent LTP. Agonists of D1 receptors have been shown to upregulate NMDA receptor-mediated LTP in a CaMKII-dependent manner in hippocampal primary cultures (Nai et al., 2010); lead to increased phosphorylation of GluN1 Ser897 and GluN2B Ser1303 in hippocampal slices, which may lead to increased expression of the subunits at the surface; and synergistically activate the ERK1/2 pathway (Sarantis et al., 2009). Histamine may interact directly with the NMDA receptor to potentiate NMDA receptor currents (Burban et al., 2010). In addition, presynaptic histamine H_3 receptors may inhibit glutamate release in the dentate gyrus *in vitro* (Brown and Reymann, 1996), thereby affecting the activation of AMPA

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and NMDA receptors. Besides neurotransmitters, cell adhesion and repulsion molecules have been shown to affect LTP, and NMDA receptor interactions with the extracellular matrix protein Reelin and the receptor tyrosine kinase EphB has been shown to alter NMDA receptor levels as the surface, having an effect on NMDA receptor function (Groc et al., 2007; Nolt et al., 2011).

2.3.2.3 NMDA receptor subunit composition may alter the NMDA receptor's role in LTP

Although the NMDA receptor has been shown to be necessary for classical LTP, the importance of the individual subunits present is much less clear. Given that LTP is easier to elicit early in development, when the GluN2B receptor is highly expressed, and becomes more difficult to induce later into adulthood when the GluN2A subunit is predominant, implicates the GluN2B subunit as important for LTP expression (Flint et al., 1997). Several lines of work have supported this theory. Pharmacological blockade of GluN2B-containing receptors with Ro 25-6981 prevented increases in the slope of fEPSPs recorded *in vivo* in the perforant path-dentate gyrus pathway seen during conditioning in a trace conditioning paradigm (Valenzuela-Harrington et al., 2007). GluN2B has also been shown to interact with CaMKII, and this interaction has been shown to be necessary for LTP induction (Barria and Malinow, 2005; Zhou et al., 2007). GluN2B has also been shown to couple directly to RasGRF1, which activates the Ras/Raf/ERK1/2 signaling pathway, necessary for LTP and learning (Krapivinsky et al., 2003). In addition, disruption of GluN2B levels at the synapse, either with genetic (Brigman et al., 2010) or molecular (Gardoni et al., 2009) techniques, also resulted in reduction of LTP in the hippocampus.

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However, other studies have supported a primary role of GluN2A, rather than GluN2B, in LTP expression. Electrophysiological studies in rodent hippocampal or perirhinal cortex slices using NVP-AAM077 to pharmacologically block GluN2A-containing NMDA receptors found that LTP was reduced compared to controls (Liu et al., 2004; Massey et al., 2004). Interestingly, both studies found no inhibition of LTP using GluN2B blockers. NVP-AAM077 has also been shown to block dendritic translation, necessary for LTP maintenance, in primary hippocampal neuron cultures using GFP as a reporter (Tran et al., 2007). However, the specificity of NVP-AAM077 in rodents has recently come under question (Neyton and Paoletti, 2006). It has also been shown that mutant mice expressing C-terminally truncated GluN2A subunits exhibit reductions in LTP expression (Kohr et al., 2003), which would support a role of GluN2A in LTP. Furthermore, some studies have shown either no inherent specificity for the subunit composition for LTP (Berberich et al., 2005) or involvement of both subunits, but with different roles in LTP (Foster et al., 2010). In addition, although most LTP research has focused on the different roles of the GluN2 subunits, overexpression of the GluN3A subunit in adulthood in the hippocampus has been shown to impair LTP (Roberts et al., 2009), further complicating the precise role of the NMDA receptor in LTP.

2.4 The Effects of PAE on LTP and the NMDA Receptor

2.4.1 The Effects of PAE on the NMDA Receptor

Several studies have attempted to evaluate the effect of PAE on NMDA receptor subunit expression. Real-time polymerase chain reaction (RT-PCR) analysis of adult whole brain mouse tissue after a 25% ethanol solution i.p. injection on gestational day 8 (GD8) found a significant decrease in GluN2B mRNA expression and a significant

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increase in GluN2A mRNA expression compared to controls (Toso et al., 2005). In rat pups from mothers offered a 10% ethanol solution as their sole drinking source during pregnancy, the developmental expression profile of the GluN1 splice variants GluN1a and GluN1-1b, GluN2C, and GluN2D mRNAs in the hippocampus are altered by ethanol exposure (Naassila and Daoust, 2002). In a pair-fed liquid diet paradigm, no difference in GluN2A, GluN2B, or GluN2C mRNA levels were found in forebrain samples from PD1 (Hughes et al., 2001). The same study found no alteration in PSD-95 and GluN2A or GluN2B association as measure by immunoprecipitation, although they did find an alteration in GluN1 association with calnexin, a chaperone protein in the ER.

Studies have also examined protein levels of NMDA receptor subunits after pre- and postnatal alcohol exposure. In the cerebral cortex, GluN2B levels have been shown to be decreased at PD61 in an oral administration model of PAE in guinea pigs (Dettmer et al., 2003). Another study found decreased association of PSD-95 with GluN2A and GluN1 splice variants containing the C2' cassette at PD21 in the cortex after a pair-fed liquid diet PAE paradigm (Honse et al., 2003). In the hippocampus, the same pair-fed liquid diet paradigm produced decreased GluN2B subunit levels at PD7(Hughes et al., 1998). Prenatal exposure via pair-fed gavage in combination with postnatal exposure via artificial rearing resulted in an increase in GluN2A subunit levels at PD 10 in the hippocampus (Nixon et al., 2004), while the same postnatal exposure resulted in an increase in GluN2A levels in the cortex (Nixon et al., 2002). Prenatal ethanol exposure by a voluntary two-bottle choice paradigm found a decrease in PSD-95 association with GluN2B in the hippocampus (Samudio-Ruiz et al., 2010).

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Analysis and comparison of these studies become difficult for several reasons. First, the differences in PAE administration could alter the effects of ethanol. As mentioned in the previous chapter, paradigms involving gavage or liquid diet may elicit a stress response in the mother, which may have adverse effects on the offspring independent of, and perhaps interacting with, the alcohol exposure. In addition, the timing of the exposure also is important, with prenatal and postnatal exposure, as well as selective daily exposure during gestation, possibly targeting different developmental time periods and therefore may have differential effects. The BECs reached in these models also vary greatly, ranging from 80 mg/dL to as high as 330 mg/dL, with some BECs referencing the mother's levels and some referencing the offspring's levels. Differences in BECs will also greatly affect the interpretation of the outcomes. Finally, many of these studies are done early in development, often before PD21, while many studies of behavioral deficits occur in older animals, making it difficult to relate neurochemical alterations with behavioral alterations.

2.4.2 Effects of PAE on LTP

Although the effect of PAE on learning has been studied in great detail, the effect of PAE on LTP has not been as thoroughly explored. An initial study examined the effects of two i.p. injections of 2.9 g/kg ethanol on GD8 and found that rats prenatally exposed to ethanol showed lower high frequency stimulation (HFS) threshold for LTP in the granule cell layer of the dentate gyrus (Gomez et al., 1992). However, later studies found deficits in LTP in the dentate gyrus measured *in vivo* in adult rats (Sutherland et al., 1997; Varaschin et al., 2010) using either a pair-fed liquid diet or a limited access paradigm. In the adult guinea pig, LTP deficits were also observed in the CA1 after oral

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administration of 4g/kg ethanol throughout gestation (Richardson et al., 2002a). It is unknown why the initial study found an LTP enhancement after prenatal alcohol compared to the later studies, although the difference in exposure (one day of exposure vs. exposure throughout the entire gestation) may account for the difference. The agreement of the later studies on LTP deficits, despite the differences in ethanol administration and species differences, is intriguing, as LTP deficits may account for learning deficits seen after PAE.

2.5 Summary

LTP, considered by many to be the cellular mechanism of learning, is dependent on the NMDA receptor in many regions of the brain, including the dentate gyrus. Regulation of the NMDA receptor, including the subunit composition and trafficking of the receptor, is important in maintaining proper cellular function, including LTP. Although both the NMDA receptor and LTP have been shown to be altered by prenatal alcohol exposure, no studies have determined whether alterations in NMDA receptor expression is associated with LTP deficits seen in the dentate gyrus, despite the importance of the NMDA receptor in LTP induction and maintenance.

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3. Research rationale, hypothesis, specific aims

In both humans and laboratory animal models, exposure to alcohol during development has been shown to cause a multitude of dose-dependent effects on the structure and function of the central nervous system, with the extent of damage being dependent on the pattern and chronicity of the exposure and the stage(s) of development during which it occurs (Queen et al., 1993; Goodlett and Johnson, 1997; Kelly et al.,2000; Soodet al., 2001; Savage et al., 2002; Mooney and Miller, 2009). The incidence of FASD has been estimated to be 0.2-5% in the United States (Sampson et al., 1997; May et al., 2009). Several studies have found that 10-20% of women report drinking low-to-moderate amounts of alcohol during pregnancy, with the prevalence of drinking decreasing as the pregnancy progresses (Flynn et al., 2003; Caetano et al., 2006). In animal models, this type of voluntary, intermittent drinking has been modeled in rhesus monkeys (Schneider et al., 2009), whereas, in rodent models, it is typically accomplished through injection or gavage (Bonthius et al., 2001; Carneiro et al., 2005). However, these routes of administration may induce stress in the mother, confounding the effects of ethanol, and may not be appropriately modeling human behavior.

The hippocampal formation, including the dentate gyrus, is one of the brain regions most vulnerable to the damaging effects of PAE (Gil-Mohapel et al., 2010). Effects of PAE on the dentate gyrus may have far-reaching consequences since this structure serves as a major cortical input pathway for the hippocampus (O'Reilly and McClelland, 1994; Leutgeb et al., 2007; Witter, 2007). Adult mice that were exposed to moderate levels of alcohol throughout gestation display deficits in hippocampal-dependent (Allan et al., 2003) learning and memory tasks, as well as impaired survival of

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newly generated dentate granule neurons in response to an enriched environment in adult mice (Choi et al., 2005). In addition, PAE is associated with decreased NMDA receptor-dependent activation of mitogen-activated protein kinase/extracellular signal regulated kinase 1/2 (ERK1/2) in the adult mouse dentate gyrus (Samudio-Ruiz et al., 2009). Numerous other effects of PAE on hippocampal functioning have been reported, including deficits in NMDA-dependent long-term potentiation (LTP) in the dentate gyrus (Sutherland et al., 1997; Varaschin et al., 2010).

Studies thus far have not investigated the effect of PAE on behavior that is dependent on the functioning of the dentate gyrus, nor have they fully evaluated the reason for the LTP deficits in the dentate gyrus. Although the work done in our lab previously implicates the NMDA receptor, the association between LTP deficits and NMDA receptor alterations after PAE has not been explored. The goal of this dissertation was, first, to design a more appropriate PAE animal model that produced learning deficits in a paradigm that had been shown to be dependent on the dentate gyrus; second, to determine whether the model displayed alterations in synaptic transmission in the dentate gyrus; and third, to determine if there were corresponding changes in NMDAR subunit composition and/or localization in the dentate gyrus.

3.1 Hypothesis

The hypothesis tested in this dissertation was that a limited access prenatal alcohol exposure model produces dentate gyrus-dependent learning impairments that are associated with NMDAR-dependent synaptic plasticity deficits and altered NMDAR subunit composition.

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Specific Aims

Specific Aim 1 – Development and characterization of a limited access, voluntary consumption model of PAE in mice

- Characterize the effect of maternal ethanol consumption on dam behavior and pregnancy outcomes
- Characterize the effect of PAE in hippocampal-dependent learning tasks, including contextual fear conditioning and trace fear conditioning
- Characterize the effect of PAE in a dentate gyrus-dependent pattern separation task

Specific Aim 2 – Evaluate alterations in NMDA receptor-dependent functions in the dentate gyrus of PAE animals

- Determine if basal glutamatergic synaptic transmission is altered in PAE animals
- Determine if NMDAR-dependent LTP is altered in PAE animals
- Determine if functional GluN2B-containing NMDA receptors are affected in PAE animals

Specific Aim 3 – Evaluate alterations in NMDA receptor subunit number and composition in the dentate gyrus in PAE animals

- Determine if PAE alters synaptic membrane NMDA receptor subunit populations

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- Determine if PAE alters extrasynaptic membrane NMDA receptor subunit populations

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4. A Limited Access Mouse Model of Prenatal Alcohol Exposure that Produces Long-lasting Deficits in Hippocampal-dependent Learning and Memory

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ABSTRACT

Background: It is estimated that approximately 12% of women consume alcohol at some time during their pregnancy and as many as 5% of children born in the United States are impacted by prenatal alcohol exposure (PAE). The range of physical, behavioral, emotional and social dysfunctions that are associated with PAE are collectively termed fetal alcohol spectrum disorder (FASD).

Methods: Using a saccharin-sweetened ethanol solution, we developed a limited access model of PAE. C57BL/6J mice were provided access to a 10% (w/v) ethanol in 0.066% (w/v) saccharin solution for 4 hours per day, with 0.066% (w/v) saccharin solution serving as control. After establishing consistent drinking, mice were mated, and continued drinking during gestation. Following parturition, ethanol solutions were decreased to 0% in a step-wise fashion over a period of 6 days. Characterization of the model included measurements of maternal consumption patterns, blood ethanol levels, litter size, pup weight, maternal care, and the effects of PAE on fear-conditioned and spatial learning, and locomotor activity.

Results: The mothers had mean daily ethanol intake of 7.17 ± 0.17 g ethanol/kg body weight/day, with average blood ethanol concentrations (BECs) of 68.5 ± 9.2 mg/dL after two hours of drinking and 88.3 ± 11.5 mg/dL after 4 hrs of drinking. There was no difference in food or water consumption, maternal weight gain, litter size, pup weight, pup retrieval times, or time on nest, between the alcohol-exposed animals and the control dams. The animals that were exposed to ethanol prenatally displayed no difference from control offspring in spontaneous locomotor activity but demonstrated learning deficits

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compared to controls in three hippocampal-dependent tasks: delay fear conditioning, trace fear conditioning, and the delay non-match to place radial arm maze task.

Conclusions: These results indicate that this model appropriately mimics the human condition of PAE, and will be a useful tool in studying the learning deficits seen in FASD.

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4.1 Introduction

In both humans and laboratory animal models, exposure to alcohol during development causes a multitude of dose-dependent effects on the structure and function of the central nervous system, with the extent of damage being dependent on the pattern and chronicity of the exposure and the stage(s) of development during which it occurs (Riley and McGee, 2005). Additionally, the effects of prenatal alcohol exposure (PAE) on the brain display regional variability (Guerri, 1998), with the hippocampus being particularly sensitive to the damaging effects of the exposure (Berman and Hannigan, 2000). The dysfunctions that are associated with PAE are collectively termed fetal alcohol spectrum disorder (FASD) (Streissguth and O'Malley, 2000). It is estimated that use of any alcohol during pregnancy is about 12% (Floyd et al., 2009), with the incidence of newborns displaying prenatal alcohol-related damage as high as 5% in the United States (May et al., 2009). While heavy and binge (five or more drinks in a single episode) patterns of drinking during pregnancy are associated with the development of Fetal Alcohol Syndrome (FAS) (Jones and Smith, 1973; Clarren and Smith, 1978), extensive research shows that even moderate maternal drinking (1-2 drinks per day) is, in some individuals, associated with cognitive and behavioral deficits, often revealed only under stressful or challenging conditions (Streissguth et al., 1999).

Existing rodent models for PAE employ various lengths and routes of administration, as well as various concentrations of ethanol (Costa et al., 2000; Cudd, 2005). Each model has its advantages and disadvantages. For example, although liquid diets and intragastric intubation are capable of producing moderate-to-high blood ethanol

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concentrations (BECs), they often induce maternal stress and malnutrition, which may confound the results (Ward and Wainwright, 1989b; Slone and Redei, 2002). Voluntary oral consumption can reduce stress and malnutrition issues, but it may be difficult to reach moderate-to-high BECs with this paradigm (Finn et al., 2005). However, limiting access to ethanol in voluntary consumption paradigms has been shown to increase the amount of self-administration (Finn et al., 2005; Rhodes et al., 2005; Rhodes et al., 2007). A recent study (Boehm et al., 2008) employed this method to develop a PAE model using C57BL/6J mice, in which pregnant dams were given access to 20% ethanol for two hours during the dark cycle. These mice reached BECs ranging from 100-200 mg/dL, and the offspring exhibited motor deficits at postnatal day (PD) 33.

We sought to expand upon the model originally developed by Boehm et al. (2008) to determine its impact on hippocampal-dependent learning tasks. Significant deficits in both delay- and trace-conditioned learning and a delayed non-matching to place radial arm maze (RAM) task in adult PAE mice were observed. The model will likely be an important tool in the study of molecular mechanisms underlying cognitive dysfunction associated with PAE.

4.2 Methods and Materials

4.2.1 Animals

All of the procedures used in the current studies were approved by the University of New Mexico Health Sciences Center Institutional Animal Care and Use Committee.

Sixty day-old C57BL/6J male and female mice (Jackson Laboratory, Bar Harbor, ME) were acclimated for one week in the vivarium [kept at 22°C on a reverse 12 hr dark /

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12 hr light schedule (lights on at 2000)] in group-housed cages. After this acclimation period, animals were then individually housed for one week before the start of the alcohol (or control) drinking procedure. Standard chow was available *ad libitum* in all cages. Water was available at all times except during the ethanol exposure period for female mice. Offspring were group-housed in same-sex, littermate cages with free access to tap water and chow and maintained on a reverse light-dark cycle, as described above. All behavioral measurements were conducted during the dark period between 0900 and 1400 in a behavioral room lit with red lighting.

4.2.2 Prenatal alcohol exposure procedure

Prenatal exposure of mice to ethanol was performed using a modification of our previously published method employing saccharin-sweetened solutions (Allan et al., 2003). Two hours into the dark cycle, female mice were offered access to either 0.066% (w/v) saccharin (Sigma-Aldrich, St. Louis, MO) or an ethanol (DLI, King of Prussia, PA) solution sweetened with 0.066% (w/v) saccharin for four hours (from 1000 to 1400 hr.). Research has shown that saccharin may increase ethanol intake without affecting blood alcohol concentrations (Czachowski et al., 1999; Roberts et al., 1999). The concentration (w/v) of ethanol was increased in two, 2-day steps from 0% to 5%, then from 5% to 10%. After one week of drinking 10% ethanol, or the saccharin control solution, individual females were placed into the cage of singly housed males for two hours immediately after the drinking period (from 1400 to 1600 hr.). Water and food were available *ad libitum* in the males' cages. Females continued to consume ethanol and saccharin solutions throughout the five-day mating period, with consumption amounts measured daily. Five days was chosen for the length of the mating period based on the average estrous cycle

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length of 4-5 days in C57BL/6J mice (Jemiolo et al., 1986; Walf et al., 2009). Pregnancy was positively determined by monitoring weight gain. The length of gestation in C57BL/6J mice is approximately 19.5 days and is not affected by maternal consumption of alcohol (Murray et al., 2010; Kleiber et al., 2011). Thus, we identified gestational day 1 as 20-days prior to the day of parturition. Food and water consumptions were determined both before mating and during the last week of pregnancy. Weight determinations were made every 3-4 days during pregnancy. Within one day of birth, the alcohol and saccharin concentrations were halved every two days, until the animals were drinking only water.

4.2.3 Blood ethanol concentration measurements

Maternal BECs were determined in the last week of pregnancy, based on a method described previously (Smolen et al., 1987). Briefly, blood samples (40 μL) were collected from the submandibular vein at both the two hour (1200 hr) and four hour (1400 hr) time points, with only a single sample being collected from an animal. The samples were treated with 2 mL of 3.5% (v/v; 0.58 M) perchloric acid (Mallinckrodt Baker, Inc., Phillipsburg, NJ) and centrifuged (300 x *g*) for 10 min at 4 °C to obtain serum. Eighty microliters of serum, or known ethanol standard ranging from 0-400 mg/dL, were incubated in 2 mL of reaction buffer [10 units alcohol dehydrogenase (Sigma Aldrich, St. Louis, MO), 2.0 mM NAD (Sigma Aldrich), 0.5 M Tris-HCl, pH 8.8] for 15 minutes at 30 °C. The optical density of the sample was measured at 340 nm using a Beckman DU 380 Spectrophotometer. Sample blood ethanol values were determined by regression analysis. Blood alcohol was determined for three separate breeding rounds of

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PAE animals. The offspring of the dams used for determination of BECs were not used in the present studies.

4.2.4 Litter size and offspring weight

Litters were left undisturbed for seven days (except for those used for pup retrieval test) at which time litter size was determined. Litters were not culled. Offspring were weighed on PD 7, 14, and at weaning (approximately PD 23). Weights of the entire litter were collected, and average pup weight was determined by dividing the total weight of the litter by the number of pups present in each litter.

4.2.5 Characterization of maternal care

The percentage of time spent on nest in a one hour period was evaluated at PD 5 or 6 between 1000 and 1400 hr. Pup retrieval time was performed in the home cage on PD 8. A pup was removed from the nest and placed in the farthest corner away from the nest (approximately 30 cm away), and latency to retrieve the pup was measured.

4.2.6 Locomotor activity

Male offspring 90-150-days old were tested for their spontaneous locomotor activity, as described previously (Caldwell et al., 2008). Briefly, mice were placed in a dim red light-illuminated open field apparatus (102.5 x 102.5 x 47.5 cm; Opto-Varimex, Columbus Instruments, Columbus, OH, USA) for 15 min on two consecutive days. The first day examined locomotor activity in a novel environment, and the second day examined locomotor activity in a familiar environment. Open field locomotor (horizontal) activity was measured as the number of photo beam interruptions recorded

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on both days. All equipment was cleaned thoroughly with 70% (v/v) isopropanol between uses.

4.2.7 Delay fear conditioning

Delay fear conditioning was conducted with 90-150-days old male offspring using a procedure previously described (Allan et al., 2003). Briefly, animals were placed into a Coulbourn Instruments (Allentown, PA) Habitest® System for 90 sec of habituation. Subsequently, they received the conditioned stimulus (CS), an 80dB, 6Hz clicker, for 30 sec. The unconditioned stimulus (US), an electric foot shock (0.7mA), was delivered during the last 2 sec of the CS. This sequence was repeated one time, providing a total of two CS-US pairings. Thirty seconds after co-termination of the second CS/US pairing, the animal was removed from the conditioning context and returned to its home cage. Approximately 24 hr later, the mouse was placed back into the training context, and the animal's behavior was videotaped. Freezing (the absence of movements other than those necessary for respiration) during the first two minutes in the context was measured, and expressed as a percentage of the total time. Neither the CS nor the US was delivered during the testing period. The subject's behavior was scored by two individuals, one of whom was blind to the prenatal exposure history of the animal.

4.2.8 Trace fear conditioning

Trace conditioning was conducted with 90-150-days old male offspring in the same apparatus used for delay conditioning. The trace conditioning procedure was a modification of a procedure described previously (Huerta et al., 2000). Briefly, after 90 seconds of habituation in the chamber, male offspring experienced seven trials each

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consisting of the CS (10 sec 80 dB 6 Hz clicker), a 30 second trace, the US (1 sec 0.8 mA scrambled foot shock), and a 210 second intertrial interval; the animal experienced this sequence 7 times total, and was removed from the chamber 60 seconds following the delivery of the seventh US. The equipment was cleaned thoroughly with 70% (v/v) isopropanol between uses. A separate "untrained" group of mice was exposed to all of the same conditions except for delivery of the US.

Approximately 24 hours after completion of the training session, freezing to the CS delivered while the subject was in the novel context (a standard, clean mouse cage with minimal bedding) was assessed. Five minutes after being placed in the novel environment, the 10-sec CS was sounded; four minutes later another 10-sec CS was sounded. The test subject remained in the cage for 3 minutes after the second CS and then was returned to its home cage. The animal's behavior was recorded using a camera and video recorder, and the amount of time spent freezing during the tone and 30 sec after the tone (40 sec total duration) was scored for each CS. The average for the two measures for each mouse was calculated, and expressed as a percentage of time spent freezing. Untrained animals (cage mates from the same litters) also experienced the CS in the novel context, and the percentage of time spent freezing was similarly determined.

4.2.9 Delay non-match to place spatial memory

A delay non-match to place (DNMP) spatial memory test was performed with 90-150-days old male offspring based on a published method with modification (Clelland et al., 2009) using a radial arm maze purchased from Coulbourn Instruments. The maze consisted of an octagonal-shaped center arena (28 cm diameter x 25.5 cm height) with 8

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arms (68.5 x 9.5 x 12.1 cm, l x w x h) radiating out from the center arena. The arms were made of clear Plexiglas with a wire grid floor and a clear Plexiglas top. Mice were on a caloric restricted diet beginning 2-3 days prior to testing and maintained at 80-85% of starting body weight throughout the testing period. On the first day, mice were placed into one of the arms of the maze that was baited with mouse chow and Honey Nut Cheerios™. The central exit of the arm was blocked and the animal was left for 30 minutes prior to return to its home cage. Subsequently, testing was conducted over the next five days with two trials in the morning and two trials in the afternoon. The order of the arm separations – two different separations (see below), two trials per separation per day – was randomized across the days of the procedure. On the first two days of testing, mice were permitted to self-correct. Mice received one trial (each trial consisting of a sample phase + choice phase) and were returned to their home cages, and all other mice were tested before the second round of testing began.

In the delay non-match to place paradigm, mice were presented with a choice between a familiar and a novel arm where the correct choice was the novel arm. In the sample phase, only the start and sample arms were accessible to the mouse and the sample arm was baited. Mice were removed from the maze and placed in a holding cage if they had either spent 60s in the sample arm or had left the sample arm. During the choice phase, a new arm was open and baited with food while the start and sample arms were open but not baited. The correct choice arm varied in distance from the sample arm by either 2 (separation 2 or Sep 2) or 4 arms (separation 4 or Sep 4). Mice that entered the choice (food-baited) arm were scored as correct, while those who entered into the

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sample arm or returned to the start arm after exiting were scored as incorrect. Failure to make a selection in 3 minutes was also scored as incorrect, although this happened rarely.

The radial arm maze was rotated between sample and choice presentations such that the exact spatial location within the room for the start and sample arms were held constant during the trials but the arms themselves changed to reduce the possibility that odor cues were being utilized. The rotation took approximately 15 seconds.

4.2.10 Statistical Analysis

Statistical analyses were performed using GraphPad Prism 5 (San Diego, CA, USA). Measurements for model characterization were obtained from at least two different breeding rounds; for maternal measures, the reported n corresponds to the number of dams, while for offspring measures, the reported n represents the number of litters. Food and water consumption, maternal weights, litter size, pup weights, pup retrieval time, time on nest, delay fear conditioning, and locomotor activity were analyzed using a Student's two-tailed *t*-test. BECs were calculated using a linear regression and Pearson's correlation analysis was used to determine if the correlation was significant. Trace fear conditioning data were analyzed using a two-way ANOVA followed by Student's t-test with Bonferroni correction. Radial arm maze data were analyzed using two-way ANOVA and post hoc Student's t-tests. For the delay fear conditioning task, two animals from a litter were averaged and used as a single data point. For the trace fear conditioning task, two animals were used from a litter, one trained and one untrained. For the radial arm maze task, two animals from each litter were trained in the task and their data were averaged together. The number of correct

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arm entries for each trial per day across three days were determined and expressed as a percentage, and the number of correct arm choices for each day was analyzed for Sep 2 and Sep 4. In all cases, data are presented as mean ± SEM.

4.3 Results

4.3.1 Ethanol consumption and BEC levels

Ethanol consumption was monitored before and during mating, as well as throughout gestation, from three different breeding rounds (**Fig. 1**). Ethanol intake reached moderate – to – high levels before mating (8.36 ± 0.47 g ethanol/kg body weight/day), and continued throughout the mating period (7.47 ± 0.43 g ethanol/kg body weight/day).

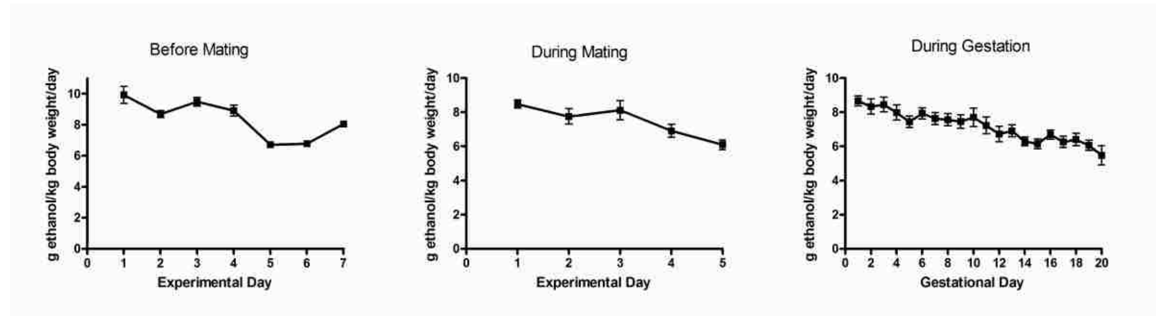

Figure 4.1. Average ethanol consumption

Average ethanol consumption (g ethanol/kg body weight/day) for C57Bl/6J mice before mating (A; n=23), during mating (B; n=21), and during gestation (C; n=30).

During gestation, ethanol consumption remained relatively constant with an average consumption of 7.17 ± 0.17 g ethanol/kg body weight/day. BECs of pregnant dams were measured during the last week of pregnancy, both two hours and four hours after ethanol access was initiated. After two hours drinking, BECs were 68.5 ± 9.2 mg/dL, and after four hours drinking, BECs reached 88.3 ± 11.5 mg/dL. Consumption of ethanol over

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both a 2-hr period and a 4-hr period significantly predicted BEC levels (2 hr period: Pearson's r = 0.57, p<0.05, 4 hr period: Pearson's r = 0.48, p<0.05; **Fig. 2A and 2B**).

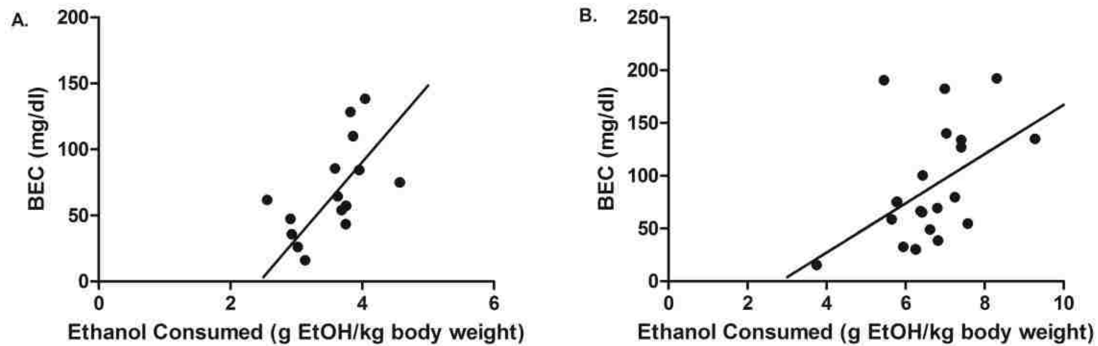

Figure 4.2. Correlation of blood alcohol concentrations (BECs) to the amount of ethanol consumed by female mice

(A) BEC levels significantly correlate with the amount of ethanol consumed at 2 hrs (n=15; r = 0.57, p<0.05). (B) BEC levels significantly correlate with the amount of ethanol consumed at 4 hrs (n=22; r = 0.48, p<0.05). BEC levels were determined from blood taken in a separate cohort of female mice after 2 or 4 hrs of drinking, using an enzymatic method described in the Materials and Methods.

4.3.2 Food and water consumption

We measured intake of standard chow and water both during the week of introduction of the solutions and during the last week of pregnancy for both the ethanol and control groups in three separate rounds of breeding (**Fig. 3**). Water consumption before mating did not differ significantly between the saccharin group (3.28 ± 0.22 mL/day) or the ethanol group (3.10 ± 0.11 mL/day) (t[17] = 0.74, n.s.) (**Fig. 3A**). Similarly, there was not a significant difference in water consumption between the saccharin group (3.81 ± 0.11 mL/day) or the ethanol group (4.16 ± 0.23 mL/day) in the last week of pregnancy (t[14] = 1.47, n.s.) (**Fig. 3A**). Before mating, there was no significant difference in food intake between the saccharin group (3.32 ± 0.2 g/day) and the ethanol group (3.70 ± 0.12 g/day) (t[18] = 1.66, n.s.) (**Fig. 3B**). Food consumption in

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the last week of pregnancy also did not significantly differ between the saccharin group (3.99 ± 0.07 g/day) and ethanol group (3.89 ± 0.16 g/day) (t[12] = 0.58, n.s.) (**Fig. 3B**).

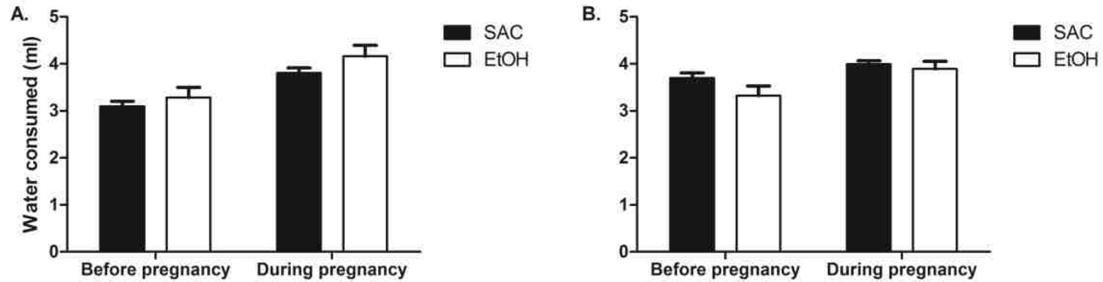

Figure 4.3. Food and water consumption by saccharin (SAC) control group and ethanol (EtOH) drinking group before and during pregnancy

(A) Amount of water consumed (mL) per day (20-hr access) before pregnancy (n=10) and during the last week of pregnancy (n=7). (B) Amount of food consumed (g) per day before pregnancy (n=9) and during last week of pregnancy (n=9 for SAC, n=7 for EtOH). No significant difference was found between SAC and EtOH groups at any time point for either food and water consumption.

The average caloric intake during the last week of pregnancy did not differ significantly between the saccharin group and the ethanol group (13.98 ± 0.25 kcal/day and 15.21 ± 0.53 kcal/day, respectively; t[12] = 2.10, n.s.), and the calorie equivalent of ethanol consumed was about 10% of the daily diet intake. The lack of a difference in food and caloric intake corresponded with no significant difference between the two groups for the change of maternal weight that occurred during pregnancy (**Table 1**).

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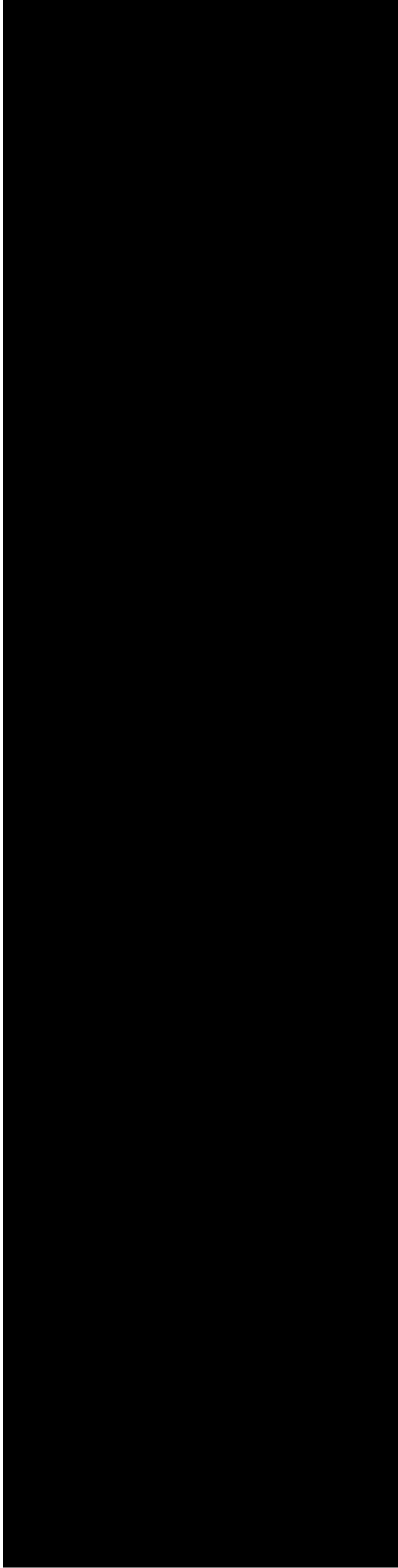

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4.3.3 Offspring weight and litter size

Litter size was determined for four separate breeding rounds at PD 7. The average litter size for the prenatal saccharin and prenatal ethanol groups were not significantly different ($t[62]=1.473$, n.s.; **Table 1**). Offspring weight was also determined at PD 7, 14, and at weaning (approximately PD 23) (**Table 1**). At PD 7 and 14 these were not significantly different between groups (PD 7: $t[11]=0.4517$, n.s.; PD14: $t[11]=0.3054$, n.s.) A similar result was obtained at weaning ($t[47]=1.641$, n.s.; **Table 1**).

4.3.4 Maternal care

Although pup weights and early postnatal growth are good indicators of maternal care, we also directly assessed maternal care. There was no significant difference in the amount of time taken for pup retrieval between saccharin-drinking and ethanol-drinking mothers ($t[19]=0.6804$, n.s.; **Table 1**), nor was there a difference in the amount of time mothers spent on the nest ($t[12]=0.07633$, n.s.).

4.3.5 Locomotor activity

Since locomotor activity could influence the level of activity of the mice, and therefore performance in the learning tasks we used, we determined the basal level of locomotor activity in PAE and saccharin control (SAC) control mice. In either a novel environment (day 1) or a familiar environment (day 2), PAE mice did not display differences in locomotor activity (measured as counts of beam interruptions) compared to SAC offspring (Day 1 – SAC 651.4 ± 63.02, PAE 768.7 ± 28.77; $t[10]=1.480$, n.s.; Day 2 – SAC 492.1 ± 53.27, PAE 514.6 ± 64.83; $t[10]=0.2681$, n.s.; **Fig. 4**).

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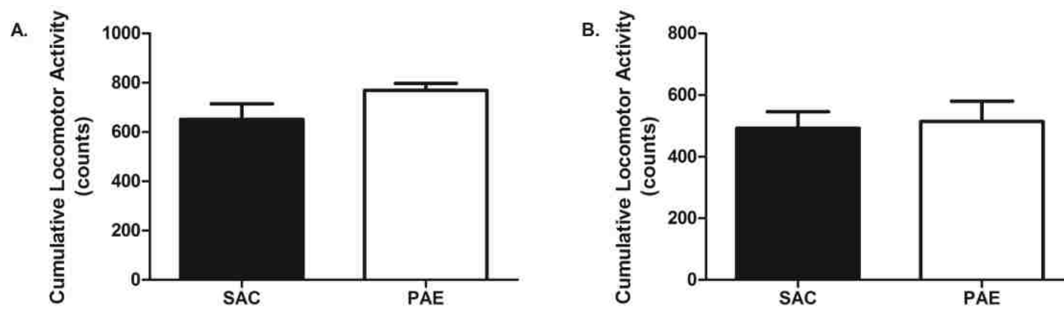

Figure 4.4. Effect of prenatal ethanol exposure on locomotor activity

Locomotor activity, measured as counts (beam interruptions), over two 15-min periods approximately 24 hrs apart, was determined in saccharin control (SAC) and prenatal alcohol exposure (PAE) mice, as described in Materials and Methods. (A) Locomotor activity for SAC (n=7) and PAE (n=5) mice on day one. (B) Locomotor activity for SAC (n=6) and PAE (n=6) mice on day two. No significant difference was found between SAC and PAE mice at either time point.

4.3.6 Delay fear conditioning

PAE models employing continuous access to ethanol throughout gestation have shown that the offspring display deficits in hippocampal-dependent tasks (Reyes et al., 1989; Savage et al., 2002; Allan et al., 2003). To determine if the offspring of our limited access model displayed similar behavioral deficits, we analyzed delay fear conditioned learning and memory in offspring from both groups. PAE and SAC control offspring were reared to adulthood and trained using a delay conditioning paradigm. Twenty-four hours later, mice were assessed for their conditioned responses to the training context by measuring freezing behavior following reintroduction into the training context (**Fig. 5**). PAE mice froze significantly less than did SAC controls (SAC 47.12 ± 4.89%, PAE 31.50 ± 4.94%; $t[17]=2.243$, $p<0.05$) when reintroduced into the context.

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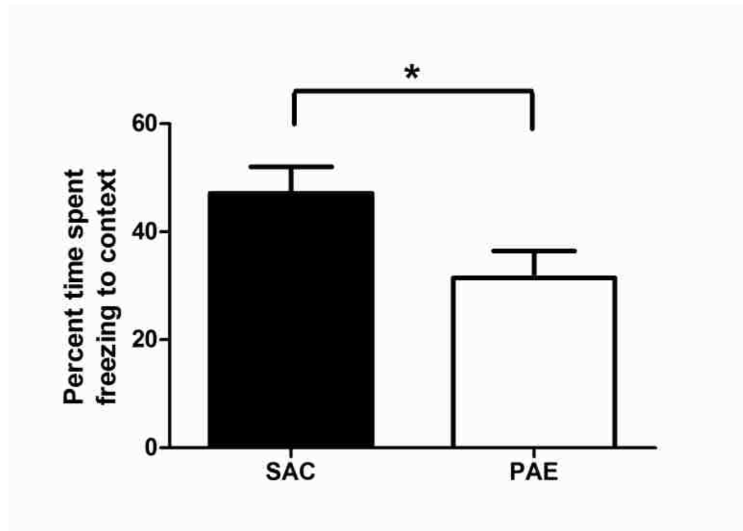

Figure 4.5. Effect of prenatal ethanol exposure on contextual fear learning and memory

Adult male saccharin control (SAC) and prenatal alcohol exposure (PAE) mice were trained in a delay fear conditioning paradigm as described in the Methods and Materials section. Approximately 24 hours later, the amount of time spent freezing in a 2 minute period after re-exposure to the training context was measured and expressed as a percentage (n=10 for SAC, n=9 for PAE;). Unpaired Student's *t*-test found a significant difference in percent freezing to the context between SAC and PAE offspring ($t[17]=2.243$, $p<0.05$).

4.3.7 Trace fear conditioning

Although PAE-associated deficits in delay fear conditioning have been previously reported in both rats (Weeber et al., 2001) and in mice (Allan et al., 2003), we were unaware of any reports examining the effects of PAE on trace fear conditioning, a hippocampal-dependent task that is temporally dependent (McEchron et al., 1998), in a murine model. Thus, we determined if our model produced a deficit in trace fear conditioning. Twenty-four hours following conditioning, mice were assessed for their responses to the CS by measuring freezing behavior (**Fig. 6**) during the 10 sec tone and the subsequent 30 sec, corresponding to the trace interval in the training procedure. In addition, a group of untrained mice were assessed for their response to the tone by measuring freezing behavior during the same interval. Two-way ANOVA indicated a

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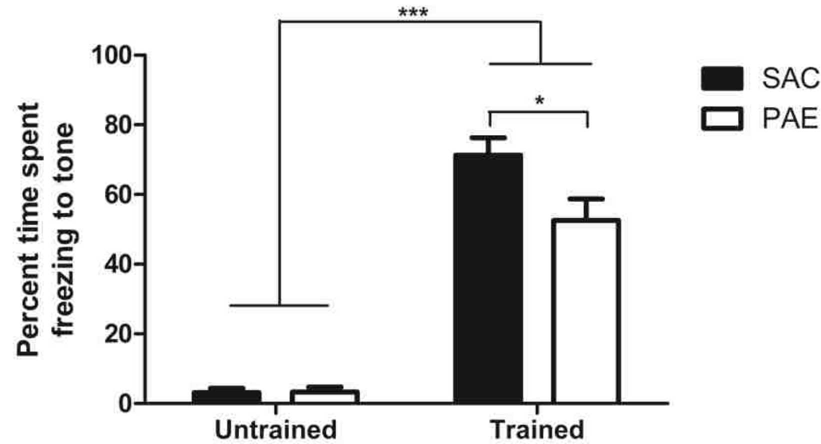

Figure 4.6. Effect of prenatal ethanol exposure paradigm on trace fear learning and memory

Adult male saccharin control (SAC) and prenatal alcohol exposure (PAE) mice were trained in a trace fear conditioning paradigm as described in the Methods and Materials. Approximately 24 hours. later, mice were placed in a novel context and the amount of time spent freezing during the 40 seconds after initiation of a 10 second tone, corresponding to the tone and trace periods in the training procedure, was measured and expressed as a percentage (mean ± SEM, n=7 for SAC, n=8 for PAE). A second (untrained) set of animals was exposed to all of the same conditions as the trained animals except for delivery of the unconditioned stimulus. Approximately 24 hours later, their freezing during the 40 seconds after initiation of the 10 second tone was recorded, similar to the trained animals. Two-way ANOVA indicated a significant main effect of training ($F[1,25]=186.3$, $p<0.001$) and prenatal exposure ($F[1,25]=4.670$, $p<0.05$), as well as an interaction of these factors ($F[1,25]=4.878$, $p<0.05$). Post hoc Student's t-test with Bonferroni correction revealed no significant difference between the untrained SAC and untrained PAE groups but a significant difference between the SAC and PAE animals that had undergone trace conditioning: $t[13]=3.141$, $p<0.01$.

significant main effect of training ($F[1,25]=186.3$, $p<0.001$) and prenatal exposure ($F[1,25]=4.670$, $p<0.05$), as well as an interaction of these factors ($F[1,25]=4.878$, $p<0.05$). Post hoc analysis using a Student's t-test with Bonferroni correction revealed a significant deficit in freezing in the trace-conditioned PAE animals compared to control animals ($t[13]=3.141$, $p<0.01$), but no difference between PAE and SAC offspring in the untrained group.

4.3.8 Radial Arm Maze

Since previous studies have demonstrated deficits in the dentate gyrus in PAE offspring (Sutherland et al., 1997; Samudio-Ruiz et al., 2009; Varaschin et al., 2010), we

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assessed dentate gyrus-dependent behavior in our PAE model. A recent study developed a delayed non-matching to place RAM task that is dentate gyrus-dependent (Clelland et al., 2009). Mice were tested for their ability to distinguish between two spatially separated locations, one of which was recently visited (the sample arm) and a second (the choice arm) which was previously unavailable, in an eight-way radial arm maze. Two-way ANOVA indicated a significant interaction of prenatal treatment and arm separation ($F[1,20]=5.924$, $p<0.05$). Post-hoc Student's *t*-test indicated that PAE offspring made significantly fewer correct arm entries in the dentate gyrus-dependent form of the task, Sep 2 (SAC 59.00 ± 3.18%, PAE 48.67 ± 2.86%; $t[10]=2.415$, $p<0.05$; **Fig. 7**), but no difference in performance (correct arm entries) for Sep 4 (SAC 48.83 ± 3.34%, PAE 54.33 ± 3.58%; $t[10]=1.123$, n.s.; **Fig. 7**), which does not require a functional dentate gyrus (Clelland et al., 2009).

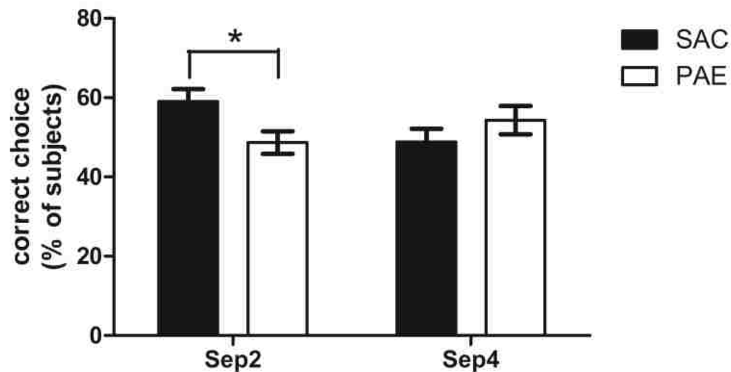

Figure 4.7. Effect of prenatal ethanol exposure paradigm on a delayed non-matching to place radial arm maze (RAM) task

Adult male saccharin control (SAC) and prenatal alcohol exposure (PAE) mice were trained in a RAM paradigm as described in the Methods and Materials section. The number of correct arm entries for each trial per day across three days were determined and expressed as a percentage (n=6). Two-way ANOVA indicated a significant interaction between prenatal treatment and separation ($F[1,20]=5.924$, $p<0.05$), and a

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post-hoc Student's *t*-test indicated a significant difference in correct arm entries for Sep 2 ($t[10]=2.415$, $p<0.05$), but not for Sep 4, between SAC and PAE animals.

4.4 Discussion

Prenatal exposure to alcohol is estimated to affect up to 5% of children born in the United States (May et al., 2009). Animal models are needed to help characterize the effects of prenatal ethanol exposure. We utilized a chronic, PAE paradigm, in which dams had access to a saccharin-sweetened 10% ethanol solution for four hours a day prior to and throughout the gestational period, to characterize the effects of PAE on hippocampal-dependent learning and memory. The model produced consistent drinking levels, averaging 7.17 ± 0.17 g ethanol/kg body weight/day, with BECs reaching 68.54 ± 9.24 mg/dL after 2 hrs of drinking and 88.32 ± 11.55 mg/dL after 4 hrs. The model allowed us to reach relevant BECs, with no apparent sign of malnutrition. We found no significant difference in either food or water consumption between dams offered saccharin-sweetened ethanol and dams offered saccharin-sweetened water. In addition, there was no significant difference in the overall weight gain of dams or in litter size, indicating that malnutrition is not a factor in our model. The limited access model yielded less variability in daily drinking than we previously observed (Caldwell et al., 2008) using a continuous access model: C57BL/6J dams given 22-hours / day access consumed 9.55 ± 2.57 g ethanol / kg body weight / day. Although the ethanol intake of the dams was significantly correlated with the BECs reached after 2 hours and 4 hours of drinking, inspection of the data shows that there is sufficient variability to necessitate measurements of BECs as the model is employed in the future.

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A confound in some PAE paradigms is that continued drinking during the postnatal period, or withdrawal symptoms during this period, may interfere with maternal care, leading to alterations in the offspring not directly due to PAE (Liu et al., 2000). However, we found no difference in pup weight (PD 7, PD 14, or at weaning), in pup retrieval times, or in time spent on the nest, indicating that tapering the nursing mothers off of the ethanol solution had minimal impact on maternal care.

A recent paper (Boehm et al., 2008) developed a similar model in C57Bl/6J mice, offering a 20% unsweetened ethanol solution for two hours a day, three hours into the dark cycle during pregnancy. Although there are some similarities, our model has key differences. We chose a 10% solution, rather than the 20% solution used by Boehm et al. (2008), in order to a) achieve moderate ethanol BECs in the dams and b) avoid malnutrition that may accompany higher doses of ethanol. By reducing the ethanol concentration to 10%, we found lower BECs than Boehm and colleagues (88.32 ± 11.55 mg/dL after 4 hrs drinking vs. approximately 130-170 mg/dL after 2 hrs drinking). In addition, malnutrition may be a confound in the Boehm et al. (2008) paper, since the model yielded a decrease in pup weight at PD 21, which we did not find at a similar time point (PD 23). Although other studies have shown decreases in pup weight, the lack of an impact on pup weight in our model may be due both to similar caloric intake between our control dams and our ethanol dams and to gradually reducing ethanol consumption after birth in the dams.

Boehm et al (2008) did not examine the effect of PAE on hippocampal-dependent learning and memory. The hippocampus is especially vulnerable to the effects of PAE, and previous studies have shown that prenatal ethanol-exposed offspring exhibit deficits

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in spatial tasks, similar to those seen in animals with hippocampal lesions (Berman and Hannigan, 2000). In our model, we observed behavioral deficits in PAE animals in a contextual delay fear conditioning task, which is hippocampal-dependent (McEchron et al., 1998; Quinn et al., 2002; Weitemier and Ryabinin, 2004). Prenatal ethanol-exposed offspring exhibited a decrease in the time spent freezing during the test period, when placed back into the training context, indicating a failure to learn the association between the context and the US shock. An important consideration in using an aversive conditioning paradigm to assess learning and memory deficits in PAE animals is the influence of pain perception on fear-conditioned learning, as studies have shown that animals prenatally exposed to alcohol exhibit hyperalgesia and increased analgesic response to morphine (Markel et al., 1986; Nelson et al., 1986). Young and Fanselow (1992) have described a negative-feedback model involving opioid-dependent analgesia that mediates, in part, the relationships between the perception of the intensity of a nociceptive US and the formation of the US-CS association. This mechanism is observed under conditions of low-to-moderate US intensity and becomes most apparent following more than one training session. We believe that the contribution of the feedback mechanism to conditioning in our animals would be minimal under the conditions that we used (moderate-to-high shock intensity and a single training session). In contrast to the study of Young and Fanselow (1992), Lehner et al. (2010) have shown that reactivity to the US foot shock is not correlated with conditioned responding (freezing) in the training context. Thus, our finding of reduced freezing upon return to the training context is likely to be due to learning and/or memory deficits in PAE offspring, rather than altered pain perception or analgesic responses. The identification of a learning / memory deficit

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in adult offspring demonstrates that our model produces learning and memory deficits that persist into adulthood, similar to FASD.

In addition to deficits in delay fear conditioning, studies in rats have shown a deficit in trace-conditioned learning and memory in animals exposed to alcohol prenatally (Wagner and Hunt, 2006; Hunt et al., 2009). In trace conditioning, an interval of time between the CS and the US creates a temporally non-contiguous relationship between the two stimuli. Learning the relationship between the CS and the US requires the hippocampus (McEchron et al., 1998; Quinn et al., 2002; Weitemier and Ryabinin, 2003, 2004). We found a deficit in freezing to the CS tone in prenatally-exposed mice as compared to saccharin controls, which appeared to be entirely dependent on learning the association between the CS and the US, since freezing to the CS tone in untrained animals was not different between the two groups. This novel result demonstrates that the PAE mouse model exhibits a deficit in learning and memory in the hippocampal-dependent trace conditioning task.

Recently, a study showed that irradiation to the dentate gyrus, which ablated neurogenesis, impaired performance in the Separation 2, but not Separation 4, version of the RAM, thus implicating the dentate gyrus in the former task (Clelland et al., 2009). Although several studies have examined the effects of PAE on performance in other versions of the RAM task (Reyes et al., 1989; Stone et al., 1996; Sluyter et al., 2005), to our knowledge, no one has demonstrated a behavioral deficit in PAE offspring directly attributed to the dentate gyrus; we therefore decided to examine the effects of PAE on performance in this modified RAM. We found that PAE offspring were selectively impaired in the Separation 2 version of the RAM, where the sample and choice arms are

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separated by 90°, with no apparent deficits in the Separation 4 task, where the sample and choice arm are 180° apart and an intact dentate gyrus function is not required. These results indicate that dentate gyrus function is impaired in PAE offspring in this model. It is believed that the dentate gyrus is important in spatial learning, especially more difficult pattern separations (Acsady and Kali, 2007; Hsu, 2007; Kesner, 2007; Leutgeb et al., 2007), and is also of interest because it is one of the two major sites of neurogenesis in the adult brain (Parent, 2007; Deng et al., 2010a). Studies examining the effects of PAE on the dentate gyrus have shown deficits in long-term potentiation (LTP), an electrophysiological model of the synaptic changes that underlie learning and memory, in prenatally-exposed rodents (Sutherland et al., 1997; Christie et al., 2005; Varaschin et al., 2010), impaired survival of newly generated dentate granule neurons in response to an enriched environment in adult mice (Choi et al., 2005), and decreased NMDA receptor-dependent activation of mitogen-activated protein kinase/extracellular signal regulated kinase 1/2 (ERK1/2) in the adult mouse dentate gyrus (Samudio-Ruiz et al., 2009). ERK activation has been shown to be necessary in learning tasks (Blum et al., 1999; Selcher et al., 1999), and alterations in adult neurogenesis in the dentate gyrus have been shown to affect performance in pattern separation tasks (Clelland et al., 2009; Sahay et al., 2011). Our findings provide a connection between dentate gyrus-specific biochemical and electrophysiological findings (Sutherland et al., 1997; Samudio-Ruiz et al., 2009; Varaschin et al., 2010) and dentate gyrus-specific behavioral deficits in PAE.

Although the paradigm that we detail appears to be a useful tool to study the effects of PAE, there is an important caveat that needs to be acknowledged. Studies have shown that the timing during gestation of PAE, as well as the chronicity of exposure,

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influences the severity the damage that is produced (Goodlett and Johnson, 1997; Sood et al., 2001; Mooney and Miller, 2009). Because the gestational period in mice is the equivalent of the first and second trimesters in humans, our model, by primarily targeting these two trimesters combined with reduced exposure during the initial half of the third trimester equivalent postnatally through breast milk, does not fully mimic PAE occurring throughout pregnancy in humans However, a recent study indicated that, from a cohort of about 95,000 women, most reported drinking in the first and second trimesters, with the levels of drinking, and particularly binge drinking (defined as more than 5 drinks in one sitting), decreasing greatly by the third trimester (Muhuri and Gfroerer, 2009). Therefore, the paradigm that we employed does model the most common pattern of PAE occurring in human populations.

In this study, we have described and characterized a mouse model of PAE. Dams exhibited moderate ethanol consumption during a 4hr. ethanol exposure period, achieving pharmacologically relevant BECs. This pattern of consumption produced learning deficits associated with hippocampal disruption in the offspring without any apparent influences from other confounds, such as malnutrition or alterations in maternal care. This model will facilitate the study of PAE, particularly in examining the preclinical efficacy of potential treatments that could ameliorate the long-lasting alterations in hippocampal function that are associated with FASD.

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5. Moderate Prenatal Alcohol Exposure Reduces Plasticity and Alters NMDA Receptor Subunit Composition in the Dentate Gyrus

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Abstract

Although it is well documented that heavy alcohol consumption during pregnancy impairs brain development, it is still controversial whether moderate alcohol consumption produces significant effects. Using a limited access, voluntary drinking paradigm, our laboratory recently demonstrated that moderate prenatal alcohol exposure (MPAE) results in long-lasting deficits in dentate gyrus-dependent learning and memory in adult offspring. Here, we identified a novel mechanism that may underlie this effect of MPAE. We found that MPAE mice exhibit deficits in GluN2B-containing *N*-methyl-D-aspartate receptor (NMDAR)-dependent long-term potentiation (LTP) in the dentate gyrus, with an associated reduced contribution of GluN2B-containing receptors to NMDAR-dependent field recordings. Using semi-quantitative immunoblotting techniques to characterize the levels and subcellular distribution of NMDAR subunits in the dentate gyrus, we found that the levels of C2´-containing GluN1 and GluN3A were increased in the synaptic membrane, while levels of GluN2B were decreased, in MPAE mice. These data suggest that MPAE results in profound alterations in the subunit composition of synaptic NMDARs, leading to impaired NMDAR-dependent LTP in the dentate gyrus.

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5.1 Introduction

The range of physical, cognitive, behavioral, and social dysfunctions that are associated with prenatal alcohol exposure (PAE) are collectively termed fetal alcohol spectrum disorders (FASDs), which have an estimated prevalence as high as 5% (Streissguth and O'Malley, 2000; May et al., 2009). The prevalence of FASDs is high, as many women and health care professionals erroneously believe that it is safe to drink in moderation during pregnancy (Valenzuela et al., 2012). However, a large body of experimental evidence suggests that the developing brain can be significantly altered by moderate PAE (MPAE) (Valenzuela et al., 2012). Notably, studies suggest that MPAE significantly impairs hippocampal-dependent learning and memory (Savage et al., 2002; Brady et al., 2011). Currently, there are no effective treatments for MPAE-induced learning and memory deficits; this is a consequence of our limited understanding of the mechanisms responsible for these effects of MPAE. The dentate gyrus (DG), the primary site of efferent inputs into the hippocampus, has been shown to be an important target of MPAE (Valenzuela et al., 2012). We recently showed that MPAE produces deficits in a DG-dependent delayed non-match to place task in adult offspring (Brady et al., 2011). We hypothesized that this could be a consequence of alterations in synaptic plasticity in the DG secondary to impairments in the function of *N*-methyl-D-aspartate receptors (NMDARs), which play a central role in DG synaptic plasticity and have been shown to be affected by MPAE (Costa et al., 2000; Malenka and Bear, 2004). Results of slice electrophysiological and biochemical techniques support this hypothesis, indicating that MPAE significantly impairs DG LTP, an effect that could be caused by changes in the subunit composition of synaptic NMDARs.

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5.2 Materials and Methods

5.2.1 Prenatal alcohol exposure paradigm

All of the procedures used in the current studies were approved by the University of New Mexico Health Sciences Center Institutional Animal Care and Use Committee.

C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) were maintained on a reverse 12-hour dark/12-hour light schedule (lights on at 20:00 hours) in group-housed cages. The MPAE paradigm has been previously described (Brady et al., 2011). All measurements were conducted on adult (2-5 months of age) offspring. For the MPAE paradigm, prior to and throughout gestation, dams voluntarily consumed either 0.066% (w/v) saccharin (SAC; Sigma-Aldrich, St. Louis, MO) or a 10% (w/v) ethanol (EtOH; DLI, King of Prussia, PA) solution sweetened with 0.066% (w/v) SAC for four hours (from 10:00 to 14:00 hours).

5.2.2 Electrophysiological Recordings

SAC and MPAE offspring were anesthetized with 250mg ketamine/kg body weight and coronal brain slices (400 μm) were prepared, as previously described (Samudio-Ruiz et al., 2009) Slices were allowed to recover in artificial cerebrospinal fluid (ACSF), containing (in mM) 119 NaCl, 4 KCl, 1.5 $MgSO_4$, 2.5 $CaCl_2$, 26.2 $NaHCO_3$, 1 NaH_2PO_4, and 11 glucose, equilibrated with 95% O_2/5% CO_2 at 28-30°C for at least 90 min. When indicated, ACSF containing low magnesium (0.1 mM $MgSO_4$) was used. Extracellular recordings from the medial perforant path (MPP) of the DG were made in slices maintained at 28-30°C perfused at 3.6 mL/min using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA). MPP recordings were confirmed by the

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observation of paired pulse depression in ionotropic receptor-dependent fEPSP recordings. Stimulation intensity was set to elicit 40% of the maximal response based on input/output curves. Stimulation duration was 75 μs and stimuli were delivered at 0.033 Hz, except when indicated. All recordings were done in the presence of 50 μM picrotoxin (Tocris, Ellisville, MO).

For NMDAR-mediated fEPSPs, recordings were done in the presence of low Mg^{2+}, 10 μM 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX) (Tocris), and 10 μM glycine (Bio-Rad, Hercules, CA). To determine GluN2B contribution to NMDAR-mediated fEPSPs, a 10 min baseline of NMDAR-mediated fEPSPs was recorded, and 10 μM ifenprodil (Tocris) was applied for 10 min and washed out. For LTP, recordings were performed as described previously (Malleret *et al*., 2010). Briefly, after a 10 min stable baseline was recorded in normal ACSF, four trains of theta burst stimulation (TBS; nine bursts of four pulses at 100 Hz, 200 ms interburst interval, 5 min intertrain interval) was delivered to the MPP. Recordings following TBS were made for 45 minutes. The fEPSP slope was measured as the response between the time points at which the response amplitude was 20 and 50% of the maximal amplitude for each fEPSP. In SAC animals, TBS stimulation was also recorded in in the presence of NMDAR antagonists [10μM MK-801 and 25μM DL-2-amino-5-phosphonovaleric acid (AP-5); Tocris] or 10μM ifenprodil.

5.2.3 Synaptic and extrasynaptic fraction preparation and immunoblotting of NMDA receptor subunits

Mice were anesthetized with ketamine (250 mg/kg body weight). The brain was rapidly removed and the DG was quickly microdissected and sonicated in

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homogenization buffer (Samudio-Ruiz et al., 2010) prior to storage at -80°C. Subcellular fractionation was performed as described by Goebel-Goody and colleagues (2009) with minor modifications as summarized in **Fig 2A**.

Protein concentrations of the TxP and TxS fractions were determined using the Bio-Rad (Hercules, CA) *DC* Protein Assay Kit with bovine albumin serum as a standard. Approximately 80% of the total homogenate protein was accounted for in the various subcellular fractions (data not shown).

Immunoblotting was performed as described in Samudio-Ruiz et al. (2010) using one of the following antibodies: anti-NR2A rabbit polyclonal antibody (1:1000 synaptic, 1:500 extrasynaptic, Phosphosolutions, Aurora, CO), anti-NR2B rabbit polyclonal antibody (1:1000 synaptic, 1:250 extrasynaptic, Phosphosolutions), anti-GluN1 C1, C2, or C2´ rabbit polyclonal antibody (1:500, a generous gift from Dr. Michael Browning), anti-NR3A rabbit polyclonal antibody (1:500, Santa Cruz Biotechnology Inc., Santa Cruz, CA), anti-PSD-95 rabbit polyclonal antibody (1:1000, Cell Signaling, Danvers, MA), anti-β-actin rabbit polyclonal antibody (1:2000, Cell Signaling) or anti-$GABA_AR$ α4 rabbit polyclonal antibody (1:500, Santa Cruz Biotechnology, Inc.). For each sample, the anti-NMDAR subunit immunoreactivity was normalized to either anti-PSD-95 or anti-β-actin immunoreactivity. Optical density values were obtained using Image J (NIH).

5.2.4 Sample collection and data analysis

For electrophysiological recordings, male offspring were used, with only one animal used from a litter to eliminate litter effects. For subcellular fractionation and

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subsequent immunoblotting experiments, tissue from two males and two females from each litter were pooled, with the reported number of determination (n) designating one litter; no significant gender differences were noted.

Electrophysiological data were acquired and analyzed with pClamp9/10 (Molecular Devices) and either GraphPad 4.0/5.0 (GraphPad software, San Diego, CA) or SPSS (SPSS Inc., Chicago, IL). For the ifenprodil block experiment, the area under the curve was calculated for baseline and ifenprodil recordings, and the percentage change was calculated for each recording. Electrophysiology data were analyzed by unpaired *t*-test (ifenprodil block experiments,), two-way ANOVA (input/output (I/O) curves), or repeated measures two-way ANOVA (LTP experiments). To compare immunoreactivities across multiple gels, each gel contained both SAC and MPAE samples, and optical densities of all bands were normalized to the average optical density of the SAC samples on that gel (presented in figures). Immunoblotting data were analyzed using a one sample t-test by comparing the data for the MPAE samples to a SAC population mean value of 1. Data are presented as mean ± SEM.

5.3 Results

We employed a limited access paradigm that we have shown to yield moderate alcohol intake in dams (Brady et al., 2011). In the present study, animals were derived from litters born to dams having an average consumption of 6.89 ± 1.27 g EtOH/kg body weight/day (n=18); blood ethanol concentrations measured in dams whose litters were not used for experiments were 90.5 ±11.61 mg/dL (n=11). In agreement with our previous study, we found no significant differences between SAC and MPAE maternal

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weight gain (maternal weight gain: SAC 9.4 ± 0.6 g, MPAE 10.0 ± 0.6 g, n.s.) or litter size: (SAC 7.1 ± 0.3 pups, MPAE 6.7 ± 0.3 pups, n.s).

Initial characterization of synaptic transmission in SAC and MPAE animals found no significant difference between SAC and MPAE input-output (I/O) curves for either ionotropic glutamatergic receptor-mediated fEPSPs (Two-way ANOVA, effect of treatment $F[1, 60] = 0.0993$, n.s., effect of input $F[7, 60] = 16.53$, $p<0.0001$, interaction $F[7, 60] = 0.3175$, n.s.) or NMDAR-mediated fEPSPs (Two-way ANOVA, effect of treatment $F[1, 72] = 2.248$, n.s., effect of input $F[8, 72] = 16.19$, $p<0.0001$, interaction $F[8, 72] = 0.2677$, n.s.), nor was a difference detected in ionotropic glutamatergic receptor-mediated fEPSP paired-pulse ratio between SAC and MPAE animals (SAC 0.95 ± 0.03, MPAE 0.90 ± 0.05, n.s; **Figure A10, Appendix A**.).

We examined whether MPAE altered NMDAR-dependent LTP in the DG. TBS elicited LTP in slices from the SAC group (**Fig 1A**). We confirmed that this LTP was NMDAR-dependent by blocking it with 10μM MK-801 and 25μM AP-5 (repeated measures two-way ANOVA: effect of time $F[36,360] = 2.489$, $p=0.0001$; effect of treatment $F[1,10] = 6.010$, $p<0.05$; interaction $F[36, 360] = 3.174$, $p=0.0001$; **Fig 1B** vs. **Fig 1A**). We also found that ifenprodil, a GluN2B subunit-specific antagonist, impaired LTP induction (repeated measures two-way ANOVA effect of time $F[36, 360] = 2.876$, $p=0.001$, effect of treatment $F[1,10] = 5.780$, $p<0.05$, interaction $F[36, 360] = 2.987$, $p=0.0001$), indicating that the TBS-elicited LTP was GluN2B subunit-dependent (**Fig 1C** vs. **Fig 1A**). Finally, we examined LTP in SAC and MPAE animals; repeated measures ANOVA found a significant effect of time ($F[36, 432] = 4.655$, $p=0.0001$), treatment

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(F[1, 12] = 5.365, p<0.05), and an interaction (F[36, 432] = 2.754, p=0.0001; **Fig 1D** vs. **Fig 1A**), thus suggesting that MPAE animals have GluN2B NMDAR-dependent LTP deficits.

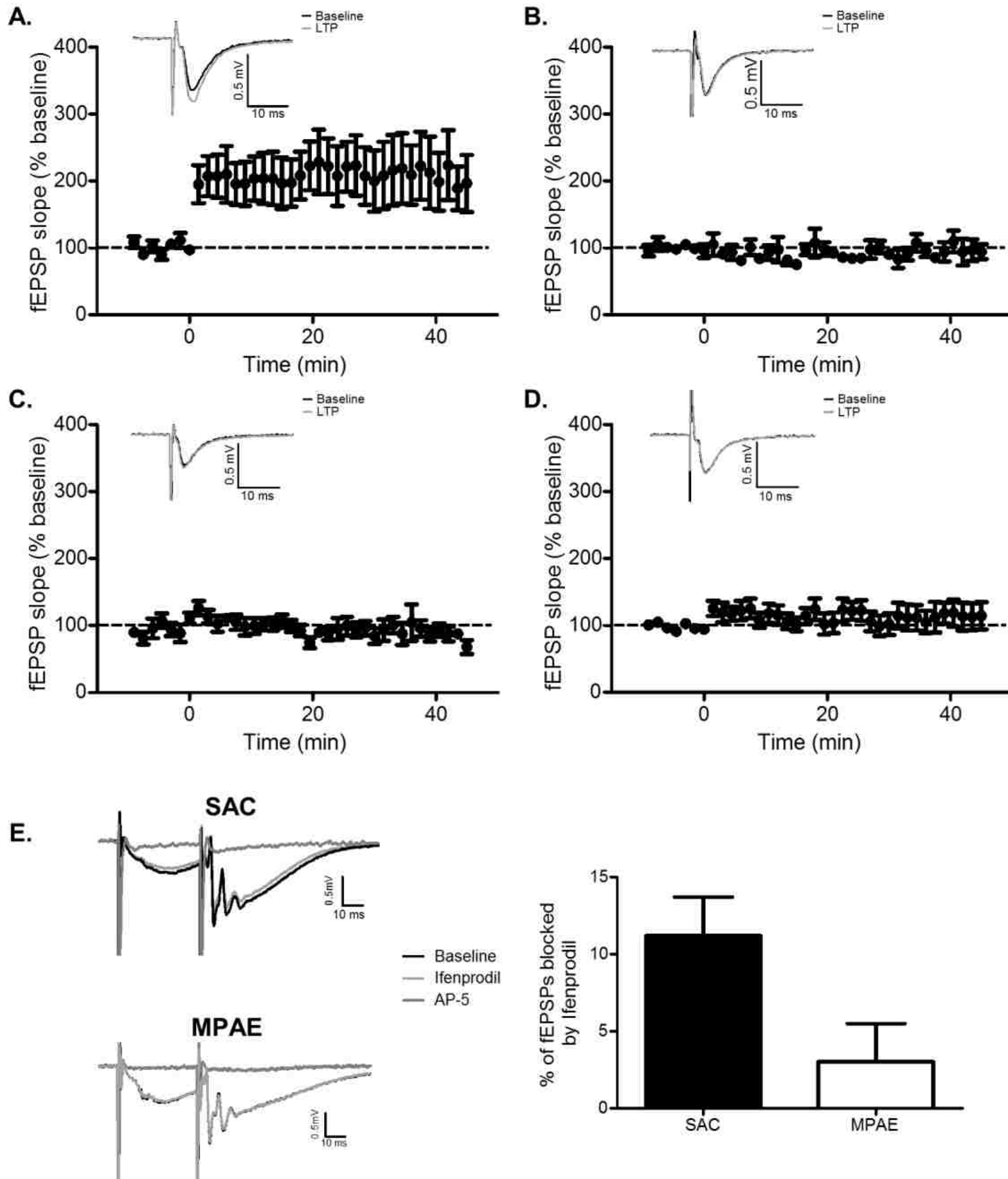

Figure 5.1Moderate prenatal alcohol exposure (MPAE) impairs *N*-methyl-D-aspartate receptor (NMDAR)-dependent long term potentiation (LTP) and reduces GluN2B-containing NMDAR contribution to fEPSPs in the dentate gyrus.

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A-C. LTP is elicited in the dentate gyrus in saccharin (SAC) mice (**A.**), and can be blocked using 10μM MK-801 and 25μM AP-5 (**B.**) and the GluN2B-specific antagonist ifenprodil (**C.**) **D.** LTP is impaired in MPAE mice. **E.** In the presence of ifenprodil, the change in area under the curve measured from NMDAR-dependent fEPSPs was significantly decreased in MPAE mice compared to SAC (n=7, *p<0.05)

Having observed a deficit in GluN2B-dependent LTP, we assessed the contribution of GluN2B-containing NMDARs to basal synaptic transmission in SAC and MPAE mice by measuring NMDAR-mediated fEPSPs in the absence and presence of ifenprodil. fEPSPs were blocked by 10 μM AP-5, confirming that they were NMDAR-dependent (**Fig 1E**). The amount of the first fEPSP blocked by ifenprodil (determined by measuring area under the curve) was significantly decreased in MPAE compared to SAC animals (SAC 11.19 ±2.51%, MPAE 3.01 ±2.47%, $t[12] = 2.326$, $p<0.05$; **Fig 1E**). No change in paired pulse ratio, either between SAC and MPAE or before and after ifenprodil, was observed (SAC without ifenprodil 2.54 ± 0.36, SAC with ifenprodil 2.62 ± 0.28, MPAE without ifenprodil 2.34 ± 0.21, MPAE with ifenprodil 2.53 ± 0.30).

We next determined whether the observed deficit in NMDAR-dependent synaptic plasticity was associated with alterations in NMDAR levels and/or synaptic localization in the DG. Toward this goal, subcellular fractions enriched in synaptic and extrasynaptic protein markers were prepared from control DG tissue. Postsynaptic density-protein 95 (PSD-95) was used as the synaptic fraction marker and the $GABA_AR$ α4 subunit was used as the extrasynaptic fraction marker (Chandra et al., 2006). As shown in **Fig 2B** and **2C**, PSD-95 was found only in the synaptic fraction and $GABA_AR$ α4 was enriched in the extrasynaptic fraction, indicating appropriate subcellular fractionation. Further evaluation found that PSD-95 and $GABA_AR$ α4 were found to be distributed similarly in

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both the SAC and MPAE tissue (**Fig 2D** and **2E**), demonstrating that MPAE does not lead to altered subcellular fractionation of these marker proteins.

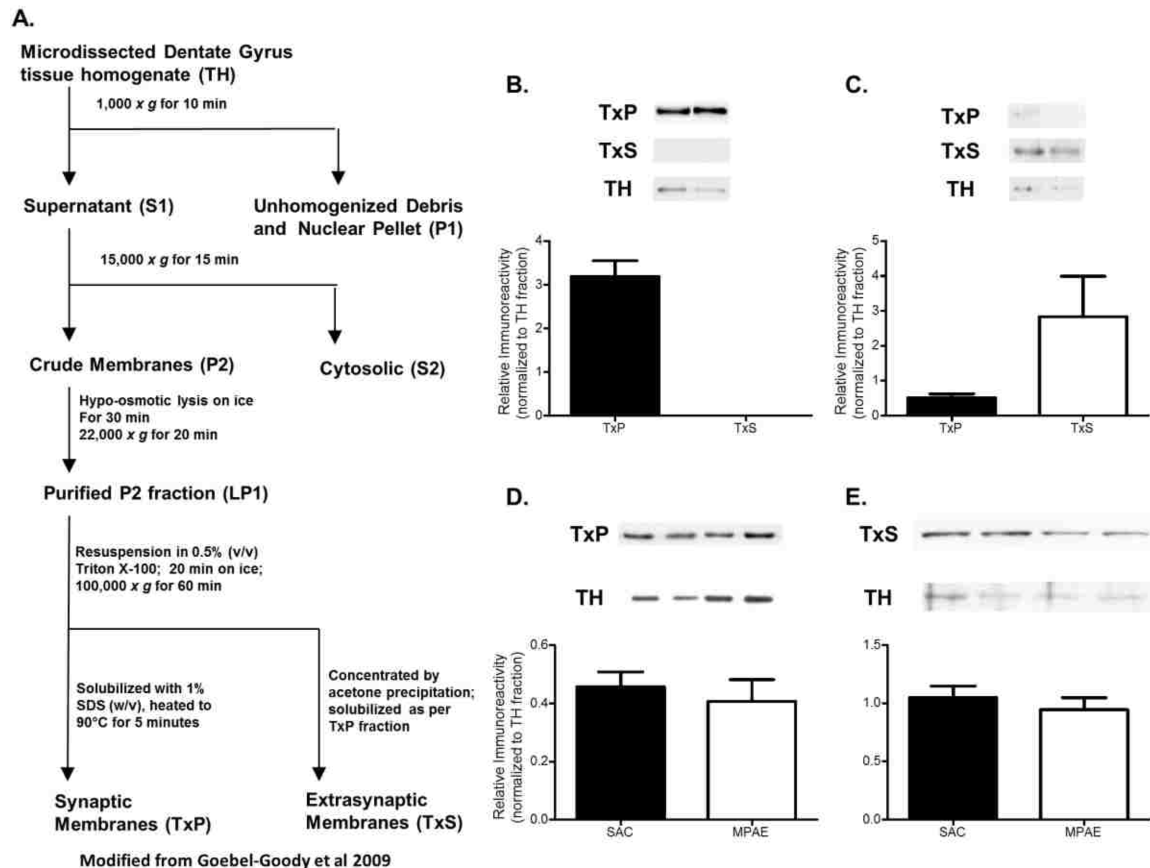

Figure 5.2. Method and characterization of subcellular fractionation technique.

A. Method used for obtaining synaptic and extrasynaptic fractions. **B-E.** Characterization of synaptic and extrasynaptic fractions in control (SAC) and moderate prenatal alcohol exposure (MPAE) tissue. **B** – In control tissue, PSD-95 is enriched in the synaptic (TxP) fraction, present about 3-fold higher than in extrasynaptic (TxS) fraction (n=4). **C** – In control tissue, $GABA_AR$ $\alpha4$ subunit is enriched in the TxS fraction, present about 2-fold higher than in the TxP fraction (n=4). **D** and **E** – In saccharin (SAC) vs. MPAE tissue, the level of PSD-95 in the TxP fraction (**D**) and $GABA_AR$ $\alpha4$ in the TxS fraction (**E**), normalized to the amount present in total homogenate, is not different, indicating no alteration in fractionation.

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The NMDAR is a tetramer composed of two obligatory GluN1 subunits and two modulatory GluN2 or GluN3 subunits (Prybylowski and Wenthold, 2004). Eight splice variants of the GluN1 subunit exist, four of which arise from alternate splice sites in the C-terminus (GluN1-1: C1 and C2 cassettes, GluN1-2: C2 cassette, GluN1-3: C1 and C2´ cassettes, GluN1-4: C2´ cassette); these C-terminal variants influence NMDAR trafficking (Horak and Wenthold, 2009). GluN2 subunits consist of four subtypes (A-D), which determine receptor kinetics and coupling to downstream signaling systems, while GluN3 subunits consist of two subtypes (A and B), which can convert the NMDAR into an excitatory glycine receptor (Smothers and Woodward, 2009). NMDAR GluN1, GluN2A, GluN2B, and GluN3A subunit protein levels in the TxP synaptic fraction and the TxS extrasynaptic fraction from DG tissue were determined by semi-quantitative immunoblotting. In initial studies, we found a significant increase in synaptic GluN1 subunit levels in MPAE DG (MPAE 1.87 ± 0.19, one sample t-test vs. 1 $t[4]=4.65$, $p<0.05$), leading us to examine the expression of the different GluN1 C-terminal splice variants by determining the levels of the C-terminal cassettes. We found an increase in C2´-containing GluN1 subunits (MPAE 1.48 ± 0.10; one sample t-test vs. 1 $t[8] = 4.698$, $p<0.01$), with no change in C1- or C2-containing GluN1 subunits (n=9, n.s., **Fig 3A**). When we assessed the levels of the GluN2 subunits, we found that GluN2B subunit levels were decreased (MPAE 0.65 ± 0.06; one sample t-test vs. 1 $t[6]=5.418$, $p<0.01$; **Fig 3C**) and GluN2A subunit levels were unchanged (n=6 SAC, 7 MPAE, ns; **Fig 3B**) in MPAE relative to SAC. Given that there was an increase in C2´ -containing GluN1 subunit expression with no associated increase in either GluN2A or GluN2B subunits, we examined GluN3A subunit levels in the synaptic fraction. In MPAE animals, GluN3A

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levels were found to be increased compared to SAC (MPAE 1.47 ± 0.18; one sample t-test vs. 1 t[7]=2.5843, p<0.05; **Fig 3C**). We found no significant differences in the levels of GluN1, GluN2A, or GluN2B in the extrasynaptic fraction in MPAE animals compared to SAC (**Fig A11, Appendix A**). Attempts to measure the expression of GluN3A expression in the extrasynaptic fraction were unsuccessful, presumably due to the very low, or absence of, expression of this subunit in this compartment.

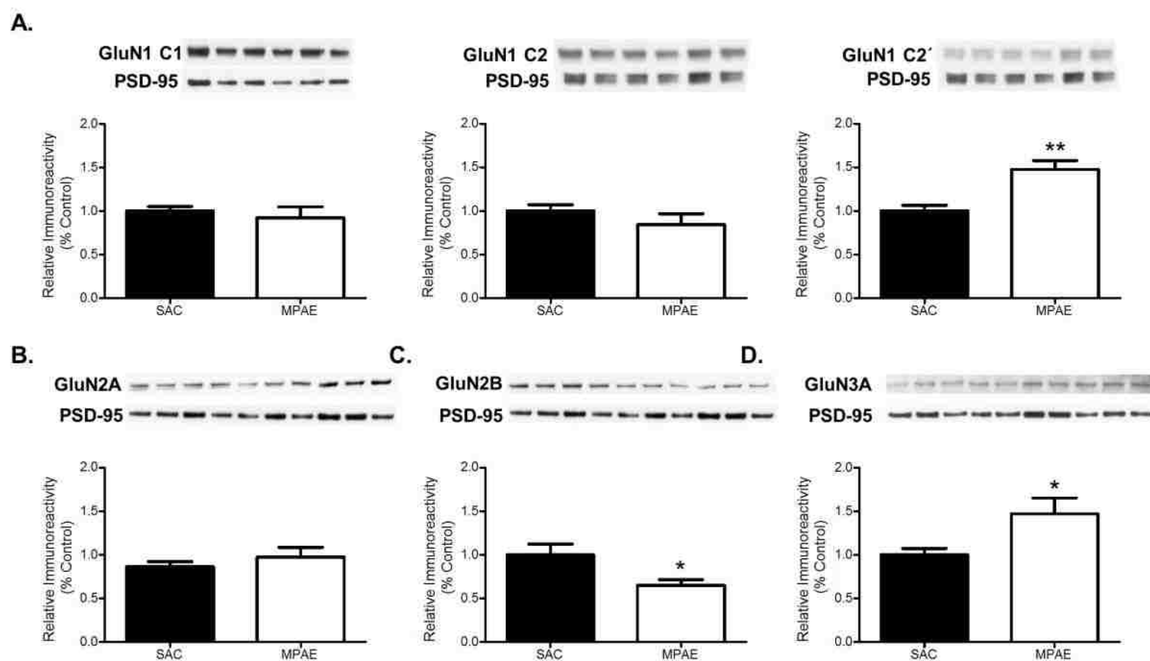

Figure 5.3. Moderate prenatal alcohol exposure (MPAE) leads to alterations of *N*-methyl-D-aspartate receptor (NMDAR) subunit levels in the synaptic fraction of the dentate gyrus.

A. C2´-containing GluN1 subunit levels are significantly increased in MPAE mice compared to saccharin (SAC; n=9, **p<0.01), with no changes in C1- or C2-containing GluN1 subunits. **B.** GluN2A subunit levels are unchanged in MPAE mice compared to SAC. **C.** GluN2B subunit levels are significantly decreased in MPAE mice compared to SAC (n=7, *p<0.05). **D.** GluN3A subunit levels are significantly increased in MPAE mice compared to SAC (n=7, *p<0.05).

5.4 Discussion

We have shown that MPAE produces neurophysiological and neurochemical alterations in the adult mouse DG. While MPAE did not affect basal transmission, it was

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associated with impaired NMDAR-dependent LTP and decreased contribution of GluN2B-containing NMDARs to fEPSPs. To our knowledge, although other studies have examined the effect of MPAE on LTP in the DG (Sutherland et al., 1997; Varaschin et al., 2010), none determined if observed deficits were NMDAR-dependent. Using immunoblotting techniques, we found that GluN2B levels were decreased, while C2´-containing GluN1 and GluN3A levels were increased, in an enriched synaptic fraction.

The decrease in synaptic localization of GluN2B in MPAE animals, seen both in immunoblotting studies and in electrophysiological recordings, is likely to be a contributing factor to the LTP deficits, as previous research has implicated GluN2B as important for LTP expression in the DG (Vasuta et al., 2007). Further, emerging research has implicated downstream signaling from GluN2B as being important for LTP. While alterations to the GluN2B C-terminal tail disrupted LTP, conversions of the GluN2A C-terminal tail to the GluN2B C-terminal tail restored LTP (Barria and Malinow, 2005; Foster et al., 2010). In addition, disruption of GluN2B levels at the synapse, either through dissociation from PSD-95 (Gardoni et al., 2009) or through genetic manipulation (Brigman et al., 2010), impairs LTP. A recent study found that early in development, switching the GluN2B subunit with the GluN2A subunit to restore NMDAR-mediated currents did not rescue GluN2B loss of function, primarily due to loss of signaling downstream of GluN2B (Wang et al., 2011). A decrease in GluN2B receptor levels in MPAE animals may alter downstream signaling in these animals, leading to the observed LTP deficits. Our earlier study (Samudio-Ruiz et al. 2009), demonstrating reduced NMDAR-dependent activation of extracellular signal-regulated kinase 1/2 in the DG of MPAE mice, supports this possibility,

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Another mechanism by which the GluN2B deficit may affect LTP in MPAE animals is through its role in metaplasticity. Several papers have indicated that the ratio of GluN2A to GluN2B may play a role in determining the threshold for LTP or LTD induction, with an increase in the GluN2A/GluN2B ratio resulting in an increase in the threshold for LTP induction (Xu et al., 2009; Lee et al., 2010). *In vivo* studies examining the effects of MPAE on LTP in the DG have found that LTP is impaired in lower stimulus tetanization protocols, similar to our results, but is intact under high stimulus tetanization protocols (Varaschin et al., 2010). Further examination of the immunoblotting shows that the GluN2A/GluN2B ratio in the synaptic fraction is significantly increased in MPAE animals compared to SAC controls (**Fig A7, Appendix A**), which may shift the threshold for LTP in these animals and, thus, account for the observed electrophysiological deficit.

The increases in GluN1 and GluN3A subunits may also affect LTP expression in MPAE animals. The increase in synaptic C2´-containing GluN1 subunit levels, with no associated change in C1-containing subunits, suggests that the GluN1-4 splice variant, one of the two predominant splice variants in the hippocampus (Laurie and Seeburg, 1994), is increased in MPAE mouse DG. The GluN1-4 splice variant lacks the C1 cassette, which contains two endoplasmic reticulum retention signals that impede forward trafficking, and contains the C2´ cassette, which has an enhancing effect on forward trafficking (Horak and Wenthold, 2009). Thus, an increase in C2´-containing GluN1 subunits may account for the observed increase in synaptic, total GluN1subunit levels. GluN3A subunits have been shown to preferentially traffic to the surface with GluN1-4

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(Smothers and Woodward, 2009), potentially leading to the increase in synaptic GluN3A levels in these animals.

Diheterotetrameric GluN1/GluN3A NMDARs are excitatory glycine receptors, generally considered to be impermeable to Ca^{2+}. Alterations in the levels of GluN1/GluN3A-containing NMDARs may modify the Ca^{2+} load in a cell after glutamatergic stimulation, having effects on downstream signaling of NMDARs. However, although GluN1/GluN3A receptors have been found to traffic to the surface in transfected *Xenopus* oocytes, GluN3A requires the presence of either GluN3B or GluN2 to traffic to the surface in HEK293 cells (Low and Wee, 2010), indicating that GluN1/GluN3A diheterotetrameric receptors may not be present at the surface in mammalian cells. Unfortunately, we were unable to immunoblot for GluN3B using commercially available antibodies in our system, and could not determine whether GluN3B levels were increased. GluN3A incorporation into GluN1/GluN2-containing receptors would alter the function of the receptor by decreasing Ca^{2+} permeability and Mg^{2+} blockade (Perez-Otano et al., 2001). GluN3A levels are high soon after birth, and quickly decrease during the first 21 days, corresponding to the time of synaptic maturation (Perez-Otano et al., 2001). Although the role of GluN3A in synaptic maturation and plasticity has not been fully examined, overexpression of GluN3A in the CA1 of the hippocampus into adulthood has been reported to limit synapse potentiation (Roberts et al., 2009), which is of note given that animals prenatally exposed to ethanol have synaptic plasticity impairments.

Although other factors may be involved in the LTP deficits seen in adult MPAE animals, our study implicates MPAE alterations in NMDAR subunit composition at the

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synaptic membrane as a contributor to LTP deficits. Given the role that NMDARs play in LTP, it is likely that the GluN2B deficit and/or the C2′-containing GluN1 and GluN3A increases contribute to the LTP deficits seen in MPAE animals, and may be an avenue of exploration for therapeutic intervention.

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6. General Discussion

6.1 Summary of Findings

The research detailed in this dissertation was conducted in order to test the hypothesis that a limited access, PAE model produces dentate gyrus-specific learning deficits that are associated with NMDA receptor-dependent LTP deficits and NMDAR subunit composition alterations.. This hypothesis was based on literature indicating that the dentate gyrus is a target for the damaging effects of PAE, exhibiting LTP deficits *in vivo* and NMDA receptor-dependent ERK 1/2 activation impairment *in vitro* (Sutherland et al., 1997; Samudio-Ruiz et al., 2009; Titterness and Christie, 2010; Varaschin et al., 2010).

The first portion of the hypothesis states that a limited access PAE model produces dentate gyrus-specific learning deficits. Although neurochemical and neurophysiological alterations in the dentate gyrus had been observed in other PAE models, a dentate gyrus-dependent behavioral correlate had not been reported. In order to test this hypothesis, a limited access model of PAE was developed and characterized. The limited access model was chosen in order to more closely mimic human behavior (discussed further below) than the voluntary, two-bottle choice paradigm previously used in the lab. The studies detailed in Chapter 4 show that four hour access to a 10% (w/v) ethanol solution sweetened with 0.066% (w/v) saccharin yielded dams with a mean daily ethanol intake of 7.17 ± 0.17 g ethanol/kg body weight/day and an average BEC of 88.3 ± 11.5 mg/dL at the end of the four hour exposure. Compared to SAC, there were no differences in gross measures of offspring health (e.g., litter size) and no alterations in measures of maternal care (e.g., pup retrieval time), which can confound interpretation of

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results obtained in the offspring. Differences between SAC and PAE adult mice were found in contextual and trace fear conditioning, two hippocampal-dependent learning tasks. These behavioral deficits have been observed in other PAE models with similar BECs (Weeber et al., 2001; Allan et al., 2003; Hunt et al., 2009), and correlate with hippocampal-dependent deficits seen in human studies with moderate BECs (Willford et al., 2004). Additionally, in a dentate gyrus-dependent, delayed non-match to place (DNMP) radial arm maze (RAM) task (Clelland et al., 2009), performance impairment was observed in PAE offspring in the limited access model, implicating PAE as having a deleterious effect on the dentate gyrus.

The second portion of the hypothesis states that NMDAR-dependent LTP in the dentate gyrus is impaired in PAE mice compared to controls. Although other studies have shown that LTP is impaired in PAE animals, to my knowledge none have provided evidence that links this deficit to NMDAR functioning. Initially, synaptic transmission was examined in SAC and PAE animals using *in vitro* slice electrophysiology. Analyses of fEPSPs revealed that there were no alterations in glutamate ionotropic receptor-dependent input/output (I/O) curves, indicating no overall change in glutamate ionotropic receptor levels and/or function, nor was there any change in NMDAR-dependent I/O curves, indicating no overall change in NMDAR levels and/or function. LTP was then elicited using a sub-maximal theta-burst stimulation (TBS), which has been shown to produce moderate LTP in the dentate gyrus *in vitro* (Malleret et al., 2010). The use of sub-maximal LTP was chosen because previous *in vivo* studies had shown that moderate PAE leads to deficits in LTP elicited by sub-maximal, but not maximal, stimulation (Sutherland et al., 1997; Varaschin et al., 2010). Application of AP-5 and MK-801

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prevented LTP in control animals, confirming that LTP was NMDAR-dependent. In addition, ifenprodil blocked LTP induction, indicating that the GluN2B subunit was important for this LTP paradigm in the dentate gyrus. Importantly, LTP induction was impaired in PAE mice compared to SAC mice. Ifenprodil blocked a lower percentage of NMDAR-dependent fEPSPs in PAE animals compared to controls, indicating that fewer NMDARs present at the synapse contained the GluN2B subunit. This is consistent with the observed deficit in LTP which, as noted above, was shown to be GluN2B-dependent.

The third portion of the hypothesis states that PAE alters NMDA receptor subunit composition in the dentate gyrus. To examine this question, NMDA receptor subunit levels were measured in subcellular fractions prepared from microdissected dentate gyrus tissue from control and PAE animals. A previous paper showed that synaptic and extrasynaptic fractions can be isolated from a synaptosomal membrane preparation based on solubility in a nonionic detergent, such as Triton X-100 (Goebel-Goody et al., 2009). As shown in chapter 5, I was able to confirm this separation by immunoblotting each fraction for a protein marker characteristic of that fraction: PSD-95 for the synaptic fraction, $GABA_A$ receptor α4 subunit for the extrasynaptic fraction. Further, I demonstrated that PAE does not alter the distribution of these markers after subcellular fractionation. Although no differences in NMDAR subunit levels in the extrasynaptic fraction were found in the present studies, it was determined that, in the synaptic fraction, there was an increase in the levels of the C2´-containing GluN1 and the GluN3A subunits, with a decrease in GluN2B subunit levels.

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6.2 Interpretation and Significance of Results

6.2.1 Significance of PAE model

As mentioned in the Introduction, the development of an animal model for PAE must try to mimic human behavior as much as possible without introducing confounds that may interfere with the results and analyses of the results. Early PAE studies focused on fetal alcohol syndrome (FAS), a medical diagnosis in which there is a confirmation of maternal alcohol consumption and the child exhibits: 1) evidence of a characteristic pattern of minor facial anomalies, 2) evidence of prenatal and/or postnatal growth retardation, and 3) evidence of deficient brain growth (May et al., 2009). Although FAS is the most severe result of PAE, it may not be the most common. Children who have less severe manifestations of FASD are often less likely to be diagnosed and are rarely presented to those capable of making such diagnoses (May et al., 2009). As a consequence, these less severe cases are often estimated, based on reports of maternal drinking. A recent report of pregnant women showed that use of any alcohol during pregnancy is about 12% (Floyd et al., 2009), with the prevalence of FASD estimated as high as 5% of school children (May et al., 2009). Another problem encountered in human studies examining the effects of moderate ethanol exposure is that it is difficult to correlate the levels of ethanol intake with behavioral effects. Some studies have suggested that low amounts of maternal ethanol intake result in no discernible deficits in offspring (O'Leary et al., 2010), while maternal ethanol intake as low as 1 drink per week has been implicated in adverse child outcomes (Sood et al., 2001).

One of the goals of the present studies was to develop a PAE paradigm that modeled low-to-moderate ethanol intake, resulting in moderate BECs, and yielded

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offspring exhibiting hippocampal-dependent learning deficits, as seen in human offspring exposed to moderate levels of ethanol *in utero* (Willford et al., 2004; Mattson et al., 2011). One of the initial considerations was to determine the most appropriate timing and route of ethanol administration. Some studies have linked first trimester exposure to ethanol in humans with learning and memory deficits (Richardson et al., 2002b; Willford et al., 2004), indicating that this may be a time to target intervention for treating PAE. Interestingly, at this time period, the hippocampus, including the dentate gyrus, has not begun formation, indicating that dysregulation of these regions originates before migration of granule cells into this region. As mentioned before, the gestational period of the rodent, in our case the mouse, is the equivalent of the human first and second trimesters, with the early postnatal period of the mouse being the third trimester equivalent (Gil-Mohapel et al., 2010). Thus, while human maternal ethanol consumption may continue throughout the entire gestational period, this is difficult to model in rodents, because of this fundamental difference in the rodent third trimester equivalent compared to the third trimester in humans- i.e., in rodents it occurs postpartum, whereas in humans it occurs *in utero*. Consequently, it was decided to limit ethanol exposure to gestational stages and model first and second trimester exposure in humans. This decision is supported by a recent study showing that, of a cohort of about 95,000 women, most reported drinking in the first and second trimesters, with the levels of drinking decreasing greatly by the third trimester (Muhuri and Gfroerer, 2009)

Another factor to be considered was the mode of administration in the model. Previous models used various methods of administration, including liquid diet, intragastric intubation or gavage, and vapor inhalation. However, each model has its own

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pitfalls (reviewed in Gil-Mohapel et al., 2010). Although liquid diets are effective for self-administration, mimicking the normal route of administration in humans, there may be large variations in consumption between mice, they may produce alterations in maternal behaviors, and they may elicit stress responses which may confound results. Gavage, while ensuring similar levels of consumption, elicits high BECs and significant stress responses that question its utility for studying moderate exposures, which can be achieved with less stressful paradigms. Although vapor inhalation may not elicit as great of a stress response as gavage, it does not mimic the oral route of administration used by humans. A recent model, previously used in our lab, used a voluntary, two-bottle choice paradigm where the dams were offered both water and either a 10% (w/v) ethanol solution (5% w/v in later studies) sweetened with 0.066% (w/v) saccharin or the 0.066% (w/v) saccharin solution alone (Allan et al., 2003). Although this was an improvement over previous models, my studies sought to update this paradigm to better model human behavior. First, the solutions were always available in the previously used model, thus allowing the dam to drink at any time during the day. Although this may give consistent BECs throughout the day, it may not mimic human behavior, where pregnant mothers consuming low-to-moderate levels of ethanol do so in a shorter time period: a recent study found that 38.8% of women who were pregnant in the last year reported consuming fewer than 4 drinks in one sitting (Caetano et al., 2006). It was decided, instead, to offer the ethanol solution [10% (w/v)] or saccharin control over a four hour time period during the dark cycle. Indeed, the previous model found that most of the ethanol consumption occurred during the dark period (Allan et al., 2003). By switching to a limited access paradigm, similar BECs [78 mg/dL with 5% (w/v) ethanol (Caldwell et al., 2008) vs. 88

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mg/dL in the limited access model] were achieved, while more appropriately modeling human drinking behavior. In addition, there was greater variability in drinking among dams in the previous model compared to the limited access model [9.55 ±2.57 g EtOH/kg body weight/day (Caldwell et al., 2008) vs. 7.17 ± 0.17 g EtOH/kg body weight/day], which should allow for less variability in offspring measures.

Similar to hippocampal-dependent learning and memory deficits that have been shown to be produced by other models of PAE, the offspring of mothers who had limited access to alcohol prior to and throughout gestation exhibited deficits in hippocampal-dependent contextual and trace fear conditioning. This correlates well with the hippocampal-dependent spatial learning deficits that have been recorded in children that were exposed to alcohol *in utero* (discussed above), demonstrating the face validity of the model. One of the more interesting aspects of the model, however, is the demonstration of PAE deficits in the DNMP RAM task, which has been shown to be dentate gyrus-dependent (Clelland et al., 2009). Although this was not entirely unsurprising, based on other neurochemical and electrophysiological data implicating this region in PAE effects, to my knowledge there is no evidence in the literature indicating dentate gyrus-specific effects of PAE in humans. Although imaging data has shown volume reductions in the hippocampal formation (Chen et al., 2003), and behavioral studies have shown hippocampal-dependent deficits, none of these studies has attempted to isolate the dentate gyrus as a precise target of PAE in humans. The role of pattern separation as a dentate gyrus-dependent task was based primarily on computer models and animal studies, (Treves et al., 2008), limiting studies on the effects of PAE on pattern separation in humans. However, a recent study set out to examine whether a pattern separation task

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was correlated with activity of the dentate gyrus/CA3 pathway using fMRI (Bakker et al., 2008). The researchers found a strong bias toward pattern separation was observed in the dentate gyrus/CA3, lending support to the dentate gyrus' role in pattern separation in humans. In addition, humans with mild cognitive impairment and who scored poorly on pattern separation tasks exhibited altered BOLD fMRI signals in the dentate gyrus/CA3 region during those tasks (Yassa et al., 2010). Based on the findings in the mouse FASD model and these human studies, I predict that children with FASD may exhibit deficits in pattern separation. The deficit in pattern separation seen in the mouse PAE model may also be the behavioral correlate of the LTP and ERK1/2 deficits seen in the dentate gyrus in other PAE models (Sutherland et al., 1997; Samudio-Ruiz et al., 2009; Varaschin et al., 2010), further strengthening the need to examine this region of the brain.

6.2.2 Significance of the NMDA receptor subunit alterations and LTP deficit

The alterations in synaptic membrane NMDAR subunit composition combined with the associated LTP deficits may help to explain the learning and memory deficits seen in the PAE mouse model. The NMDAR-dependent I/O curves were unchanged in PAE mice compared to SAC controls, indicating no gross alterations in NMDAR number and/or function. However, there were alterations in NMDAR subunit expression in immunoblotting experiments, specifically increases in C2´-containing GluN1 subunits and GluN3A subunits, with a corresponding decrease in GluN2B subunit levels. NMDAR-dependent fEPSPs also exhibited a decreased contribution of GluN2B subunits in PAE mice. Together, these alterations in subunit expression, with no change in NMDAR-dependent I/O curves, suggest a shift in subunit composition, rather than a shift

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in overall NMDAR levels. It is this shift in subunit composition that may be responsible for the LTP deficits seen in PAE animals (summarized in Figure 6A).

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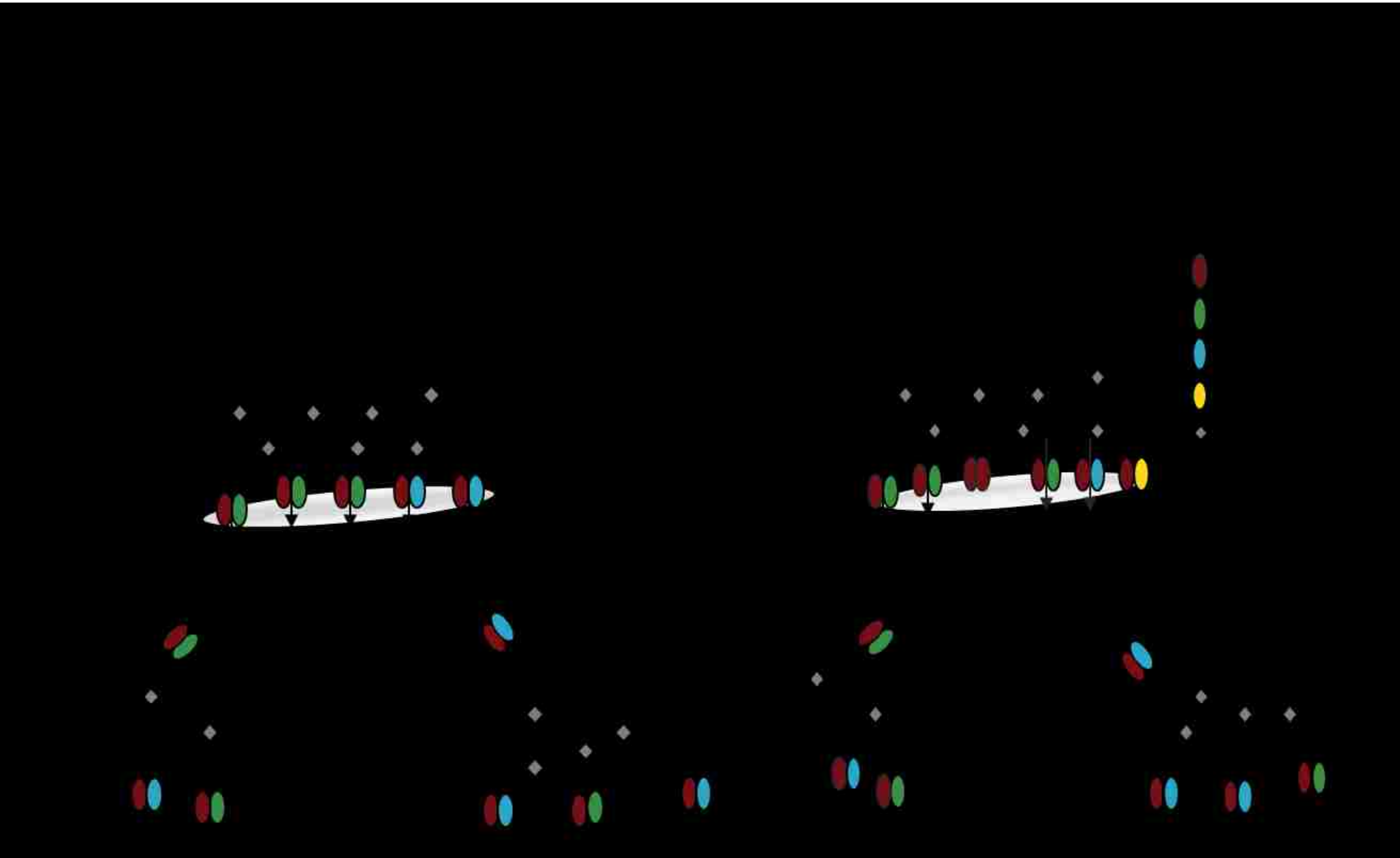

Figure 6.1. Model of PAE effects on NMDAR subunit composition and LTP in the dentate gyrus

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6.2.2.1 Changes seen in the GluN1 splice variants and GluN3A subunit levels in the synaptic membrane

Although the increases seen in the levels of C2´-containing GluN1 subunits, as well as the increase in GluN3A subunits, were surprising, the current literature supports a potential role in producing LTP deficits in PAE mice. Studies have shown that GluN3A surface expression is increased with expression of C2´-containing GluN1 subunits (Smothers and Woodward, 2009), and so it is likely that these two subunits are complexed together at the synaptic membrane in the dentate gyrus, although their precise location (i.e., inner, middle or outer molecular layer or in the hilus), or whether they form diheterotetramers or triheterotetramers with GluN2 subunits, cannot be determined from these studies. Predicting the consequence of increased surface expression of GluN3A-containing NMDARS is somewhat limited, since the exact role of these receptors is not entirely clear. Studies in heterologous systems have shown that diheterotetramers (consisting of GluN1 and GluN3A) are excitatory glycine receptors, exhibiting low Mg^{2+} sensitivity and little Ca^{2+} permeability (Low and Wee, 2010), which would indicate that their presence may alter the signaling profile. A shift from GluN2- to GluN3-containing NMDARs, therefore, would lead to a decreased Ca^{2+} load, but because these receptors remain permeable to Na^{+} and K^{+}, they contribute to the excitability of the cell. The low Mg^{2+} block would also allow these receptors to activate upon release of glycine from the presynaptic terminal without prior membrane depolarization. Consequently, these receptors would no longer act as a coincidence detector, as other non-GluN3-containing NMDARs would, but may contribute to overall depolarization of the membrane, similar to AMPARs, although perhaps not a significant amount, depending on the level of

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receptor increase compared to AMPAR levels. However, there are several reasons why these subunit increases may not lead to alterations in NMDAR fEPSPs in PAE animals. First, the measured K_d of rodent GluN3A NMDARs for glycine is 40nM, and therefore GluN3A is likely saturated by glycine *in vivo* (Yao and Mayer, 2006), which may mean it keeps the cell in a more depolarized state rather than responding to synaptic transmission (Henson et al., 2010) Release of further glycine may then bind to the GluN1 subunit, which has been shown to increase desensitization and thus have an antagonistic effect on the receptor (Pachernegg et al., 2012). In addition, and perhaps more importantly, although GluN1/GluN3A diheterotetrameric currents have been measured in *Xenopus* oocytes, there is controversy as to whether they exist endogenously. Studies have shown that GluN3A-dependent currents do not occur in HEK293 cell unless also co-transfected with GluN3B or GluN2 subunits, (Chatterton et al., 2002; Smothers and Woodward, 2009), and studies examining excitatory glycine receptors endogenously have thus far failed to detect currents (Tong et al., 2008), indicating that mammalian cells may not express functional GluN1/GluN3A heterodimers. However, a recent study, using electrophysiological recordings and Ca^{2+} imaging, detected the presence of GluN1/GluN3 receptors in mature optic nerve myelin (Pina-Crespo et al., 2010). This may indicate that the increase in GluN1 and GluN3A results in GluN1/GluN3A receptors, possibly occurring with a concomitant increase in GluN3B, which couldn't be measured in the present studies using the commercially available antibodies, or that GluN3A is incorporating itself into GluN1/GluN2 complexes.

The presence of GluN3A in GluN1/GluN2/GluN3A triheterotetramers imparts different functional and pharmacological characteristics in these receptors compared to

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GluN1/GluN2-containing receptors (reviewed in Henson et al., 2010). Single channel recordings in heterologous systems co-expressing GluN1, GluN2A, and GluN3A show two distinct populations of NMDA-evoked currents– the typical GluN1/GluN2A currents, characterized by a large conductance, and currents that have much smaller conductances, likely due to the inclusion of GluN3A subunit. In GluN3A knockout mice, early postnatal cortical neurons exhibit enhanced NMDA responses and increased dendritic spine (Das et al., 1998), indicating that the presence of GluN3A produces a dominant negative effect on NMDARs. Another study has shown that overexpression of GluN3A in the forebrain of juvenile mice, which would produce higher expression of GluN3A later in development than normal, produces functional GluN1/GluN2/GluN3 receptors at the synaptic membrane, as well as decreased spine densities, fewer synapses, and impaired LTP (Roberts et al., 2009). Although spine densities and synapse number were not measured in the present studies, an LTP deficit was found in PAE mice, perhaps related to the increase in GluN3A. If PAE induces higher expression of GluN3A later in life, and leads to incorporation of GluN3A into NMDARs , it may be altering synapse development, and thus playing a role in LTP and learning and memory deficits.

If the GluN3A subunit is incorporated into GluN1/GluN2 NMDA receptors, it may be taking the place of the GluN2B subunits, which would explain why GluN2B subunit levels are decreased without a corresponding decrease in GluN1 subunit levels or increase in GluN2A levels. However, if this is the case, then the observed increase in GluN1 subunit levels would be unaccounted for. Studies have shown that in heterologous systems, GluN1-2, GluN1-3, and GluN1-4 splice variants are able to traffic to the surface without the presence of GluN2 or GluN3 subunits (Okabe et al., 1999;

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Horak and Wenthold, 2009), indicating that it is possible that the increased expression of C2´-containing GluN1 subunits is due to non-complexed GluN1-4 subunits. Whether these subunits would exist solo or as homodimers, or whether this is actually possible in an endogenous system, is unknown.

6.2.2.2 Significance of the decreases in GluN2B subunit levels and its possible effect on LTP in PAE animals

In contrast to the paucity of information about the GluN3A subunit, the role of, and potential effect of decreased levels of, synaptic GluN2B subunits is much more established in the literature. In Chapter 2 it was noted that GluN2B antagonists, as well as genetic deletion of the GluN2B receptor, block LTP induction in various regions of the brain (Barria and Malinow, 2005; Zhao et al., 2005; Brigman et al., 2010). Studies employing receptor mutants have shown that the C-terminal tail of the GluN2B subunit is critical for its role in LTP (Kohr et al., 2003; Gardoni et al., 2009; Foster et al., 2010), particularly its interaction with CaMKII (Barria and Malinow, 2005; Zhou et al., 2007). Therefore, it stands to reason that reductions in GluN2B levels in the PAE mouse dentate gyrus, measured both in immunoblotting and electrophysiological studies, are a strong candidate for the mechanism underlying the LTP deficits that were recorded in these animals.

Another interesting possibility is that the GluN2B deficit exerts an effect on metaplasticity, rather than LTP itself. Metaplasticity has been defined as "the plasticity of plasticity", or the ability of neural circuits to alter the expression of LTP and long-term depression (LTD) after a subsequent bout of activity (Abraham, 2008). Metaplasticity could result in a sliding threshold for LTP or LTD induction, depending on previous

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activity at that synapse. LTP inhibition, a form of metaplasticity, occurs when a stimulus frequency that is known to induce LTP no longer does, probably due to untimely NMDAR activation (Zorumski and Izumi, 2012). Low concentrations of NMDA prior to LTP induction, as well as weak tetanic stimulation, have been shown to inhibit LTP, with no overall change in basal synaptic transmission (reviewed in Zorumski and Izumi, 2012) This inhibition has been shown to be dependent on NMDARs, adenosine A2 receptors, p38 MAPKs, and the protein phosphatases 1A, 2A, and calcineurin (Abraham, 2008).

Although the precise role of the NMDARs in metaplasticity is unknown, many studies have implicated the GluN2A/GluN2B ratio as important for bidirectional synaptic plasticity, or the threshold at which LTP or LTD is induced, possibly due to the differences in open probabilities and decay times between GluN2A and GluN2B receptors, leading to different Ca^{2+} profiles (Lau and Zukin, 2007). The GluN2A/GluN2B ratio can be altered in a number of ways following neuronal activity (reviewed in Yashiro and Philpot, 2008). Early in development, there is an increase in GluN2A transcription, possibly due to NMDAR/PKA/CREB pathway activation, while older cortical cultures exhibit an increase in GluN2B protein after AP5 blockade. In addition, as discussed in Chapter 2, synaptic activity may lead to GluN2A accumulation in the synapse, and may alter the phosphorylation levels of the subunits, leading to either increased retention in the synaptic membrane or increased endocytosis, depending on the site of subunit phosphorylation. These activity-dependent changes may alter the ratio of GluN2A/GluN2B, thus changing the synaptic threshold for LTP or LTD.

Whole cell recordings from CA1 pyramidal cells found that priming stimuli that produce one-fifth maximal fEPSPs were able to shift the threshold for LTP/LTD (Xu et

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al., 2009). After determining a stimulation protocol, termed the threshold protocol, that did not produce either LTP or LTD, the investigators found that priming stimuli of low frequencies (1 and 5 Hz for 600 pulses) triggered LTP at the threshold protocol, while priming stimuli of high frequencies (50 and 100 Hz) led to LTD at the threshold protocol, confirming that previous stimulation at a synapse can alter the subsequent synaptic plasticity. These priming stimuli altered the GluN2A/GluN2B ratio, with low frequency priming decreasing the GluN2A/GluN2B ratio and high frequency priming increasing the GluN2A/GluN2B ratio, indicating that a low GluN2A/GluN2B ratio lowers the threshold for LTP, while a high GluN2A/GluN2B ratio lowers the threshold for LTD. Xu and colleagues (2009) also found that low dose ifenprodil block, which increases the GluN2A/GluN2B ratio, elicits LTD, rather than LTP, at the threshold protocol. Another study also found that prolonged suppression of transmitter release, which in effect would create a "silent" postsynaptic synapse, resulted in increased GluN2B-containing NMDA receptors (and thereby a decreased GluN2A/GluN2B ratio) on the dendritic spine, as well as a lowered threshold for potentiation of the synapse (Lee et al., 2010).

PAE animals exhibited a decreased level of GluN2B subunits in the synaptic fraction, and further analysis determined that the GluN2A/GluN2B ratio is increased in these animals (Figure A7 in Appendix A; $t[12]=2.196$, $p<0.05$; $n=7$). This indicates that, in PAE animals, a stronger stimulus may be required in order to produce LTP, since the increased GluN2A/GluN2B ratio would slide the threshold towards LTD rather than LTP. This would also indicate that NMDAR-dependent LTD may be easier to elicit in PAE animals. This is supported by the fact that in dentate gyrus recordings *in vivo*, Sutherland and colleagues (1997) found LTP deficits in PAE animals at submaximal, but not at

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maximal, LTP stimuli. Neither the Sutherland et al. (1997) nor the studies detailed in this dissertation examined the ability to induce LTD in PAE animals. In the dentate gyrus, homosynaptic LTD has been shown to be independent of the NMDAR; however, heterosynaptic LTD is NMDAR-dependent (Poschel and Stanton, 2007). Heterosynaptic LTD occurs when LTP that is induced in one perforant pathway, such as the medial perforant pathway, results in LTD in the other pathways (in this example, the lateral perforant pathway). This interplay of LTP and LTD in these pathways may help the dentate gyrus to encode pattern separation information: when one pathway is activated, the resulting inhibitory effect on the other pathway may prevent the activity in this second pathway from eliciting synaptic strengthening. Based on the results of this dissertation, I would predict that heterosynaptic LTD may be elicited at a lower threshold in PAE mice. If GluN2B levels in the dentate gyrus are altering the LTP and LTD thresholds, this may have profound effects on the ability of the dentate gyrus to encode information for pattern separation in the proper way.

The NMDAR also has a role in regulating adult neurogenesis, an important function of the dentate gyrus (reviewed in Parent, 2007; von Bohlen und Halbach, 2011). Dentate gyrus neural stem cells reside in the subgranular zone (SGZ), on the border of the granule cell layer and the hilus. There are three classes of neurogenic cells: Type 1, Type 2, and Type 3. Type 1 cells exist as radial glial-like astrocytes, and express glial fibrillary acidic protein (GFAP) and nestin. These cells give rise to fast proliferating intermediate precursors called transient amplifying precursor cells (TAPs). TAPS exist as two types, Type 2a and 2b. Type 2 cells express the stem cell marker Sox2, and may or may not express doublecortin (DCX; type 2a and 2b, respectively). These cells

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transition into Type 3 cells, which at first are able to slowly proliferate, but soon differentiate into postmitotic immature neurons and then slowly migrate into the granule cell layer. Within this time period, usually about a week after birth, these cells are tonically activated by ambient GABA, which results in neuronal depolarization, rather than hyperpolarization (Deng et al., 2010b). During the second week, they begin to extend processes, with dendrites extending into the molecular layer and axons extending via the mossy fiber pathway to the CA3 region. In the third week, they begin to integrate into the existing neural circuitry, and respond to glutamatergic inputs. However, they are still quite different than mature neurons– they exhibit high membrane resistance and high resting potentials, which may contribute to increased excitability. Adult-born granule cells, at 4-6 weeks of age, exhibit a lower threshold for LTP than do older granule cells (Wang et al., 2000).

Several studies have implicated a role of adult neurogenesis in learning and memory. Enriched environment or exercise, both of which have been shown to increase adult neurogenesis, enhance performance on certain hippocampal-dependent tasks (Bruel-Jungerman et al., 2005; van Praag et al., 2005), whereas ablation and/or depletion of adult-born neurons disrupts certain forms on learning (Shors et al., 2001; Shors et al., 2002), suggesting a connection between neurogenesis in the dentate gyrus and hippocampal-dependent learning/memory. Given the hypothesized role of the dentate gyrus in pattern separation, there is a particular interest in the role of neurogenesis in pattern separation. As described in Chapter 4, (Clelland et al., 2009) reported that ablation of neurogenesis by irradiation of the dentate gyrus impaired radial maze performance of mice trained to differentiate between an initial sample arm and a

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subsequent test arm that were closely separated but had no effect on performance when the mice were trained in a version of the task in which the arms were further apart in space. The same study also assessed performance in a touch screen spatial discrimination task, which required the mice to identify the correct location of an illuminated box, when provided with two choices, with the spatial location of the incorrect location varying in distance from the correct location. Similar to the DNMP task, mice with neurogenesis ablation were impaired in the tasks when the incorrect location was close to the correct location. Sahay and colleagues (2011) have shown that irradiation of the dentate gyrus, with consequent reduction in neurogenesis, impaired context discrimination: while control mice were able to discriminate between a context paired with an aversive stimulus (a footshock) and a similar context that was not paired with the aversive stimulus, as evidenced by reduced freezing (fear responding) in the second context, mice in whom the dentate gyrus had been irradiated displayed similar levels of freezing in the two contexts. However, by genetically increasing the level of neurogenesis, animals were better able to distinguish these two contexts. These studies implicate a role for neurogenesis in learning, specifically in pattern separation tasks that may be dependent on the dentate gyrus.

The NMDAR has been implicated in neurogenesis, as well as the incorporation of new neurons into an existing network, although its downstream effects may depend on the timing of activation. Some studies have found that NMDAR antagonism increases precursor cell proliferation (Cameron et al., 1995; Hu et al., 2008; Petrus et al., 2009). The mechanism by which this occurs, however, is unknown, since several studies have found that NMDAR subunits are not present on young granule cells or in precursor cells

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until differentiation occurs (Nacher and McEwen, 2006; Muth-Kohne et al., 2010), although there is a possibility that they are expressed on the radial glial-like precursors (Nacher et al., 2007). A possible explanation is that the NMDAR is acting via indirect means, such as controlling GABA release from interneurons that may act on the excitatory GABA receptors present on these precursor cells.

Approximately two weeks following integration into the circuitry of the dentate gyrus, immature granule cells begin to receive glutamatergic inputs and have a lower threshold to elicit LTP. NMDARs are expressed by this point, and studies suggest that NMDAR activation is important for regulating circuit integration. Ifenprodil inhibits LTP in immature granule cells, indicating that LTP is GluN2B-dependent (Snyder et al., 2001b), and inhibition of NMDARs prevented LTP-dependent progenitor cell proliferation (Chun et al., 2006) and precursor differentiation (Babu et al., 2009). These studies indicate that the NMDAR is important for functional roles in immature granule cells, particularly in a positive feedback role after LTP induction. Similarly, NMDAR antagonist infusion into the lateral ventricles of rats during the training period of a delayed match to place (DMP) Morris water maze task resulted in learning impairments, associated with decreased cell survival, cell proliferation, and dendritic arborization of immature neurons compared to controls, indicating that the NMDAR is important for learning-induced changes in neurogenesis (Tronel et al., 2010). The inconsistencies with previous studies, which found that NMDARs played an inhibitory role in cell proliferation, may be the activation signal of the NMDAR; those studies that found an inhibitory role of the NMDAR simply blocked the receptor, with no other activation of

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the circuitry, whereas the latter studies were examining the role of the NMDAR after LTP and/or learning.

PAE has been shown to affect neurogenesis in the adult dentate gyrus in animal models, although the results of the studies have not been consistent, possibly due to the use of differing paradigms of alcohol exposure. Choi and colleagues (2005), using a moderate voluntary PAE model, found no change in the number of proliferating progenitors, but did find an impaired response to an enriched environment compared to controls, whereas other investigators (Redila et al., 2006; Boehme et al., 2011), who have employed a liquid diet paradigm, have reported that PAE was associated with decreased proliferation and neurogenesis. Klintsova and colleagues (2007) demonstrated that postnatal exposure to alcohol on PD 4-9 resulted in a reduced survival rate for new neurons in the rat dentate gyrus. In regards to this dissertation, alterations in NMDAR subunit composition at the synaptic membrane may help to account for these changes. A decrease in GluN2B-containing NMDARs, leading to decreased LTP in the dentate gyrus, may impair activity-dependent increases in neurogenesis and cell proliferation, similar to effects seen in several studies (Snyder et al., 2001b; Chun et al., 2006; Babu et al., 2009; Tronel et al., 2010), and lead to the impaired response in enriched environment reported by Choi and colleagues (2005). However, this would not account for the other studies that saw basal decreases in proliferation and neurogenesis. Overall decreases in NMDAR levels would presumably lead to increases in proliferation, since previous studies have shown NMDAR antagonism under basal conditions also lead to increases. However, PAE has also been shown to affect the GABAergic system, and since these cells are primarily GABA responsive, this may account for these changes.

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A final consideration is whether alterations in the NMDAR subunits would lead to alterations in circuit incorporation, and thus contribute to LTP and learning deficits. If GluN2B subunit levels are decreased in a population of cells, including newborn neurons that are incorporating into the circuitry, this decrease (possibly combined with an increase in GluN3A) may increase the threshold for LTP, causing these cells to be pruned because they never get activated and incorporated into the existing circuitry. If this were the case, this would not only account for the observed decrease in LTP, but also for the impairment in pattern separation, since these newborn cells would no longer be able incorporate and provide new cells for encoding. However, whether the decrease in GluN2B (and increase in GluN3A) exists in the entire dentate gyrus, or whether it exists in a subset of cells, is unknown from these studies. It is unlikely that it would be limited to newborn neurons, since they only account for approximately 6% of granule cells in the dentate gyrus (Parent, 2007), but alterations to that 6% may have a profound impact on dentate gyrus function.

6.3 Future Directions

Analyses of the studies in this dissertation indicate that several avenues of research deserve further investigation, some of which have been discussed already. One drawback of the current studies is that the association between NMDAR subunit alterations, LTP deficits, and dentate gyrus-dependent learning deficits are corroborative, rather than definitive. Further investigation is needed to determine whether the LTP deficits are directly due to the observed NMDAR subunits changes, and are responsible for the learning deficits. There are several ways that this could be examined. First, restoration of NMDAR subunits to normal levels in PAE animals would determine

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whether these changes are responsible for the LTP deficits. Short interfering RNA (siRNA) directed against C2´-containing GluN1 and/or GluN3A could be stereotaxically injected into the dentate gyrus of PAE mice to decrease these levels. Electrophysiology could then determine if LTP is recovered. It would be necessary to perform preliminary studies to determine exactly how much siRNA would need to be injected to restore normal levels. Restoration of GluN2B levels in the dentate gyrus at the synapse would also be important to determine the contribution of these reductions to LTP deficits in PAE mice. Transgenic mice containing a Cre-Lox GluN2B receptor could restore GluN2B receptor levels in PAE mice. This method, however, would have several pitfalls. First, since the current studies were performed in young adult animals, the increased expression of GluN2B would also need to occur at this point. The levels would also need to be expressed at specific levels, and therefore the promoter for the transgene would need to be considered carefully. In addition, an increase in GluN2B transcription may not necessarily result in increased expression at the synaptic membrane, since there are multiple levels of regulation for surface expression, including both at translational and trafficking levels. Another mechanism to increase GluN2B level expression would be to stereotaxically inject a vector, such as adeno-associated virus (AAV) or lentivirus, to overexpress GluN2B levels. This method would also encounter difficulties similar to transgenic mice, but the method would not be as complex. Restoration of GluN1, GluN2B, and GluN3A subunit levels in PAE to SAC levels would help confirm that the alterations in NMDAR subunit levels at the synapse are, at least partly, responsible for the LTP deficits in PAE mice. It is important to note, however, that altering levels of these subunits at the time points studied in this dissertation (2-5 months) would not adjust

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for any alterations that the GluN2B deficits may cause at earlier time points in development, such as synaptic maturation and circuit integration, which may also contribute to later deficits.

Another mechanism that may underlie changes in receptor subunit levels at the synaptic membrane would be alterations in the phosphorylation states of the different subunits. As discussed in Chapter 2, there are a number of serine/threonine and tyrosine sites on the C-terminals of GluN1, 2A, and 2B that are responsible for regulating synaptic targeting and endocytosis. Previous analysis of tyrosine phosphorylation levels in synaptic and extrasynaptic fractions found that phosphorylation of Tyr1472 on GluN2B was associated with synaptic membrane localization, while Tyr1336 phosphorylation on GluN2B was associated with extrasynaptic membrane localization (Goebel-Goody et al., 2009). Analysis of these phosphorylation sites would help determine if alterations in phosphorylation are consistent with the observed decrease in GluN2B in the synaptic membrane, with an expectation that Tyr1472 phosphorylation would be decreased in PAE animals. Tyr1472 has been shown to be phosphorylated by Src family kinases (SFKs); thus analysis of SFK levels, both total and activated, would be useful. Reductions in Tyr1472 phosphorylation could also (or alternatively) be due to an increase in phosphatase activity. Although the phosphatase that would be responsible for this is currently unknown, the striatal-enriched protein tyrosine phosphatase (STEP) is a strong candidate (Kurup et al., 2010). There are also several other pathways that may account for a decrease in Tyr1472. Inhibition of Cdk5, a proline-directed serine/threonine kinase, leads to increased Src association with PSD-95 and increased Tyr1472 phosphorylation, indicating a role of Ckd5 in negative regulation of GluN2B phosphorylation (Zhang et

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al., 2008). In addition, mice deficient in protein tyrosine phosphatase alpha (PTPα) show decreases in SFK activity, as well as decreased autophosphorylation of proline-rich tyrosine kinase 2 (Pyk2), which is responsible for phosporylating and activating SFKs (Le et al., 2006). PAE alterations in any of these pathways could result in decreases in GluN2B Tyr1472 phosphorylation, thereby decreasing levels in the synaptic membrane. If Tyr1472 phosphorylation levels were decreased in PAE animals, an analysis of protein levels and activity levels of these kinases and phosphatases in SAC vs. PAE animals may indicate the cause of the decrease. In addition, rescue of these pathways using pharmacological methods *in vitro* may also show an increase in both Tyr1472 and GluN2B levels in PAE animals: for example, pharmacological inhibition of Ckd5 in PAE mice may lead to increased Tyr1472 phosphorylation and increased GluN2B expression in the synaptic membrane in these mice, and may lead to recovery of LTP deficits.

Another avenue to be researched further is the association of subunits at the surface. Although the current studies suggest that there is an increase in C2´-containing GluN1/GluN3A receptors, with a decrease in GluN2B-containing receptors, the exact stoichiometry of subunits in the receptors are unknown. There are several ways to examine this question. First, co-immunoprecipitation could determine if GluN3A was present with C2´-containing GluN1 alone or in combination with GluN1/GluN2A. Also, to determine whether the loss of GluN2B is from triheterotetrameric or diheterotetrameric receptors, a series of serial co-immunoprecipitations could be performed, as described by Al-Hallaq and colleagues (Al-Hallaq et al., 2007). For this method, an initial immunoprecipitation would be performed; for example, GluN2A would be immunoprecipitated (IP1). The unbound fraction would then be subjected to another

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immunoprecipitation by anti-GluN2A (IP2) antibody. These two immunoprecipitations have been shown to remove the majority of GluN2A from the sample (Al-Hallaq et al., 2007). The unbound fraction from this step would be subjected to yet another immunoprecipitation (IP3), but this immunoprecipitation would be for another protein, for example GluN2B. Anything associated with this last fraction would then likely be receptors that do not contain GluN2A, but would contain GluN2B. The initial two IPs (IP1 and IP2) would likely contain GluN1/GluN2A diheterotetrameric and GluN1/GluN2A/GluN2B triheterotetrameric NMDARs. Using this method, the levels of GluN2B-containing triheterotetrameric and diheterotetrameric NMDARs could be determined in both SAC and PAE tissue, and this may elucidate whether the decrease in GluN2B is specific to an NMDA receptor complex type or simply an overall decrease.

It is not known whether the effects of PAE on GluN2B levels, and the apparent increase in the GluN2A/GluN2B ratio, exert an effect on thresholds for LTP and LTD in these animals. It has been suggested that there is a limit to the extent that changes in the GluN2A/GluN2B ratio can exert an effect on subsequent plasticity (Xu et al., 2009), with any increase in the GluN2A/GluN2B ratio higher than a 77% increase showing no effect. Based on the immunoblotting data, we found about a 65% increase in the GluN2A/GluN2B ratio, which would be in the appropriate range to exert an effect. To determine if the ratio is playing a role in the LTP deficits, it would be possible to attempt a preferential blockade of GluN2A to lower the GluN2A/GluN2B ratio, and then test the effects on LTP. Although NVP-AAM077, when used at higher concentrations, has been shown to be non-specific for the GluN2A receptor (Berberich et al., 2005; Neyton and Paoletti, 2006), lower concentrations have been shown to elicit a partial block of GluN2A

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with no effect on GluN2B (Berberich et al., 2005; Xu et al., 2009). Using NVP to elicit a partial block of GluN2A would therefore decrease the GluN2A/GluN2B ratio, and consequently may lower the threshold for LTP in PAE animals. Whole cell recordings could be used to determine the appropriate GluN2A block to normalize the ratio, and field recordings could be used to examine LTP in treated and non-treated PAE animals.

As discussed earlier, the decrease in GluN2B subunits, as well as the increase in GluN3A subunits, specifically on newly born neurons may decrease the ability of these cells to incorporate into the existing circuitry, thereby altering the ability of the dentate gyrus to undergo LTP and/or learning. A new strain of mice described by Lagace and colleagues (2007) would allow for examination of newly born neurons and their incorporation into the circuitry in SAC and PAE mice. Briefly, Nestin-CreER^{T2} founder mice, containing a Cre recombinase linked to the estrogen receptor, were bred with R26R-YFP mice, which contain a STOP codon between two *loxP* sites next to YFP. Upon tamoxifen (TAM) administration, CreER^{T2}, which is under control of the Nestin promoter, can dimerize and enter the nucleus, where the Cre recombinase can interact with the *loxP* site to generate DNA recombination and remove the STOP codon, leading to expression of YFP. Since CreER^{T2} is under the control of the Nestin promoter, only cells that are actively expressing Nestin at the time of TAM injections (i.e., dividing cells) would undergo recombination and express YFP. YFP expression would continue throughout the life of the cell. Therefore, 4-6 weeks after TAM injections, immunohistochemistry (IHC) and electrophysiological recordings can assess cells expressing YFP, which can help to characterize these cells in SAC and PAE mice. Using IHC, the levels of GluN2B and GluN3A can be examined and co-localized with cells

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expressing YFP, giving an indication as to whether these cells also exhibit subunit alterations. Whole cell recordings, using YFP to locate newborn neurons can determine whether GluN2B currents are altered in the cells, and can also determine if these cells have altered thresholds for LTP.

6.4 Critique of the Current Studies

Although every study is planned carefully, retrospective analysis often shows either limitations to the study or alternative methods to approach the study. In the case of my dissertation studies, one of the biggest limitations is the use of an animal model, and trying to compare data across animal models. An animal model is very useful for studying molecular mechanisms related to a human condition or disorder, such as FASD; however, at this point, no animal model can fully encompass all aspects of FASD seen in humans. Although similarities occur, allowing for limited study, there are a number of areas of the human disorder difficult to mimic in an animal model. For example, gestational exposure in our mouse model only covers the first and second trimester equivalent of a human gestational period. As noted, it has been reported in a recent study that, from a cohort of about 95,000 women, most reported drinking in the first and second trimesters, with the levels of drinking decreasing greatly by the third trimester (Muhuri and Gfroerer, 2009), allowing our model to encompass a large percentage of PAE cases; however, there are still instances in which women drink into the third trimester, which could not be assessed without modifying the model to include a postnatal exposure paradigm (e.g., inhalation chamber exposure). In addition, typically women who drink during pregnancy also have other factors present that could influence outcomes, such as other substance abuse or stress (Rubio et al., 2008; Muhuri and Gfroerer, 2009).

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Although isolating the effect of prenatal ethanol exposure is important, these factors may also play a role in prenatal development, and may need to be considered and studied in a future model.

Another limitation of this study is the focus on young adult offspring. Many studies have demonstrated that PAE causes effects in humans into adolescence and young adulthood, providing the rationale for conducting our studies in mice that were the 2-5 month range. However, a drawback to this is that we don't know the developmental timeline for the changes seen. GluN2B levels decrease during development, as do GluN3A levels. It is possible that GluN2B levels are at normal expression throughout the beginning of development, and they decrease to below normal levels, or that they start out at lower levels, and undergo normal reduction. Similarly, during development, GluN3A could begin higher than normal, and reduce its subunit numbers by the same proportion, or it could begin at normal levels, and the mechanism for subunit level reduction is impaired, leading to later increased expression. The differences between these two scenarios could have a profound effect on development and regulation of the system. Further studies of subunit levels throughout the course of development would help to answer these questions.

The present studies were largely focused on PAE effects on the NMDAR, and did not take into account NMDAR effects on other receptors, or those receptors' effects on the NMDAR. As mentioned in Chapter 2, a number of receptors, including dopamine, histamine, opioid, and metabotropic glutamate receptors, are able to modulate NMDAR function. Alterations in these receptors by PAE could, therefore, have an influence on NMDARs, in addition to that done by PAE. For example, mGluR5 has been shown to

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activate PKC and lead to NMDAR phosphorylation, and may be physically connected via scaffolding proteins (Skeberdis et al., 2001; Heidinger et al., 2002). PAE has been shown to decrease mGluR5 levels in the dentate gyrus (Galindo et al., 2004), and preliminary studies in Appendix A.1 show that mGluR5 association with PSD-95 is significantly decreased in PAE offspring compared to controls in the whole hippocampus, which may alter it role in modulating NMDAR function. Further studies on this may not only help elucidate the full effect of PAE on the glutamatergic system, but may also provide another target for therapeutic intervention.

Another consideration in evaluating the current studies is the technique of *in vitro* hippocampal slice preparation. Although slice preparation can be very useful in isolating the hippocampus to perform electrophysiological studies, the process of preparing the slices may be quite damaging, which may influence the studies. Previous studies have shown that *in vitro* slice preparation results in very rapid spine retraction in dendrites, which then recover in an hour or so, typically during the slice recovery period (Kirov et al., 2004). The faithful recovery of the synaptic connections, however, is unknown. It is possible that alterations in spine recovery may give an inaccurate picture of LTP in hippocampal slices. When comparing SAC and PAE slice preparations, this may have two major implications that would influence the interpretation of results. First, both sets of slices undergo similar spine retraction and recovery; thus, if any alterations in synaptic connections do occur during recovery, they are similar between the two groups, and therefore LTP comparisons between the two groups are not compromised. However, it is possible the PAE may result in spine recovery different than that seen in SAC slices, and

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it is this that account for the difference between LTP, rather than PAE effects on LTP induction itself.

Lastly, our studies did not examine the effect of PAE on the hypothalamic-pituitary-adrenal (HPA) axis, which is a key component of the stress response system. The HPA axis regulates a number of endocrine changes, including cortisol (corticosterone in rodents; CORT) release, which has been implicated in learning and memory mechanisms in the hippocampus. Chronic CORT exposure has been shown to lead to GluN2B subunit reductions in rats (Gourley et al., 2009), inhibition of NMDAR currents (Liu et al., 2007), and reduced neurogenesis. Studies have shown that PAE causes HPA tone to be increased throughout life, and is associated with higher basal and post-stress cortisol in humans, as well as higher post-stress corticosterone levels in animal studies (Weinberg et al., 2008). This may indicate that PAE effects on CORT may have a direct and/or indirect impact on the effects of PAE on NMDARs, and that the effect of PAE on both factors may interact to induce the phenotype seen in FASD.

6.5 Summary

In summary, this dissertation was designed and carried out to uncover a potential mechanism that may explain dentate gyrus-dependent learning deficits seen in PAE animals. These studies initially focused on developing and characterizing a moderate, limited access PAE model, in an attempt to both better mimic human drinking patterns during pregnancy and to determine whether dentate gyrus-dependent deficits do exist, which would be consistent with the literature. As shown in Chapter 4, animals achieved moderate BECs with no alterations in maternal care, and their offspring displayed

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impairments in hippocampal-dependent learning tasks, similar to those seen in previous models. Prior literature has found electrophysiological and biochemical alterations in the dentate gyrus that would support deficits in dentate-gyrus dependent learning, and our model confirms that these deficits are present. Further studies set out to determine a cellular mechanism for the observed learning deficits in the dentate gyrus. Previous literature has implicated a role of the NMDAR in these alterations. Examination of NMDAR subunit levels in the synaptic and extrasynaptic membrane in the dentate gyrus found an increase in C2´-containing GluN1 subunits, an increase in GluN3A subunits, and a decrease in GluN2B subunits in the synaptic fraction, with no differences in the extrasynaptic fraction. The deficit in GluN2B subunits was confirmed by electrophysiological recording using pharmacological blockade, and NMDAR-dependent LTP was found to be impaired in the dentate gyrus of PAE animals, suggesting that the deficit in GluN2B may be responsible. These alterations may underlie the dentate gyrus-dependent learning deficits seen in our model. Further, these studies provide a basis for later studies that may help to delineate the role of PAE in alterations in the NMDAR and its subsequent effects on metaplasticity and neurogenesis.

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Appendix A: Supplemental Data

Figure A1:

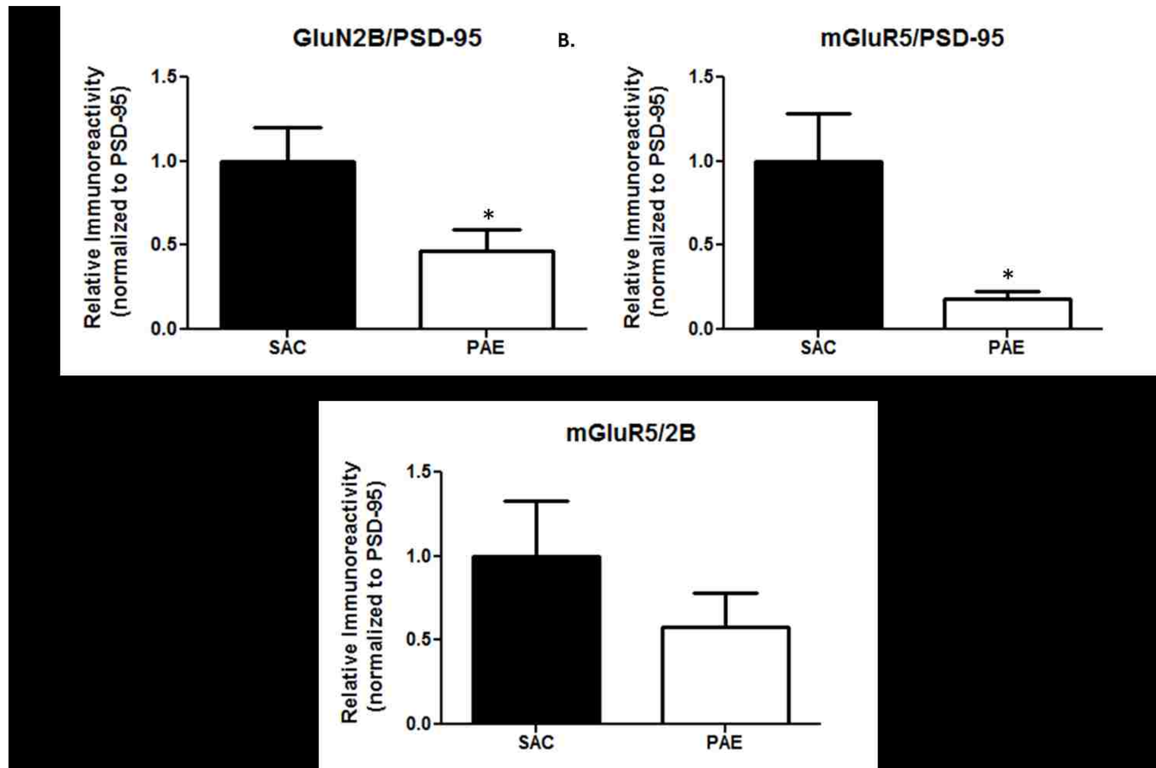

Figure A1. PAE alters the association of mGluR5 and GluN2B with PSD-95 in the hippocampus.

The hippocampus was dissected from SAC and PAE animals and homogenized. A P2 fraction was obtained as described in (Samudio-Ruiz et al., 2010), solubilized with 1% DOC, and incubated with Protein A Sepharose beads and antibodies directed against PSD-95. Immuno-complexes were processed as described by Samudio-Ruiz *et al.*(2010) and immunoblotting for levels of GluN2B, mGluR5, and PSD-95 was performed. A)PAE animals have decreased association of GluN2B with PSD-95 compared to controls, consistent with results seen in the lab's previous model. $t[13]=2.193$, $p<0.05$, n=8 SAC, 7 PAE. B)PAE animals have decreased association of mGluR5 with PSD-95 compared to controls. $t[13]=2.690$, $p<0.05$, n=8 SAC, 7 PAE. C)A comparison of the ratio of mGluR5/PSD-95 to the GluN2B/PSD-95 ratio shows that there is no significant difference in mGluR5/GluN2B ratio, indicating that although both exhibit decreased association with PSD-95, their relative levels coupled together via scaffolding are unaltered.

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Figure A2:

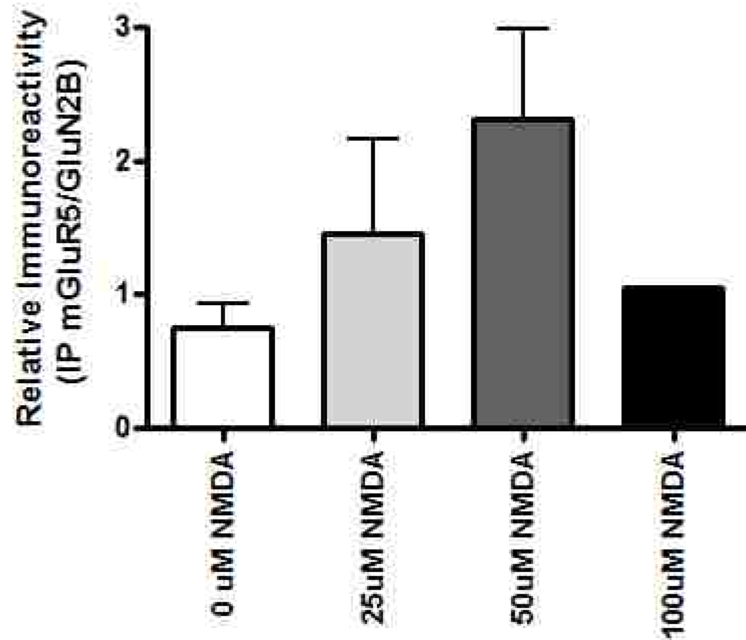

Figure A2. Dose response of mGluR5/GluN2B association as measured by co-immunoprecipitation following hippocampal slice activation with 0, 25, 50, and 100µM NMDA.

300µM slices were prepared as described in Chapter 5, and incubated with the appropriate NMDA dose for 3 min before the hippocampus was dissected out and homogenized. A P2 fraction was prepared, the membrane was solubilized in 1% deoxycholate (DOC), and the sample was then incubated with Protein G microbeads and antibodies directed against GluN2B were used to immunoprecipitate the protein. After eluting the protein from the beads, a Western blot was performed and the levels of mGluR5 and GluN2B were determined. n=2 for 0, 25, and 50 µM NMDA and n=1 for 100µM NMDA.

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Figure A3:

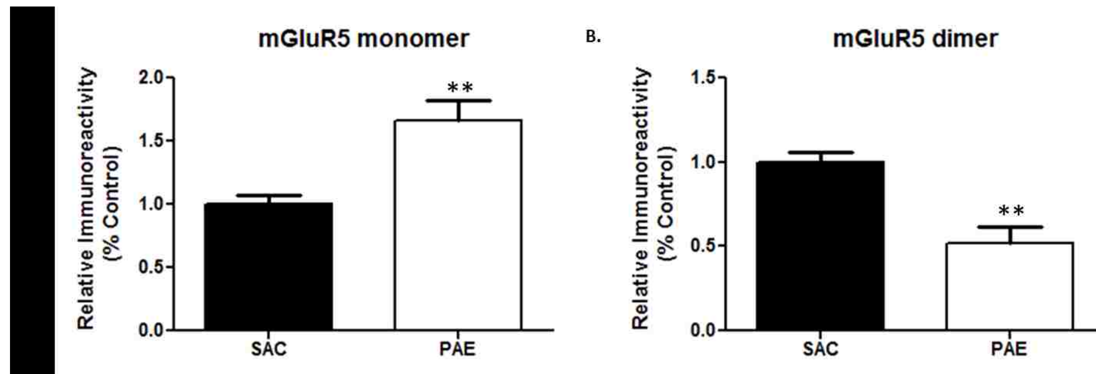

Figure A3. Semiquantitative immunoblotting analysis for mGluR5 levels in the dentate gyrus from SAC and PAE animals.

Microdissection and Western blot analysis was performed as described in Chapter 5. After immunoblotting, two distinct bands were observed, one at the predicted molecular weight (130 kD) and one at twice the predicted molecular weight (260 kD). Further research indicated that mGluR5 forms dimers that may not completely dissociate upon SDS solubilization. A) Analysis of monomer band of mGluR5. $t[10]=4.282$, **$p<0.01$ SAC n=7 PAE n=5. B) Analysis of dimer band of mGluR5. $t[6]=4.222$, **$p<0.01$ n=4

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Figure A4:

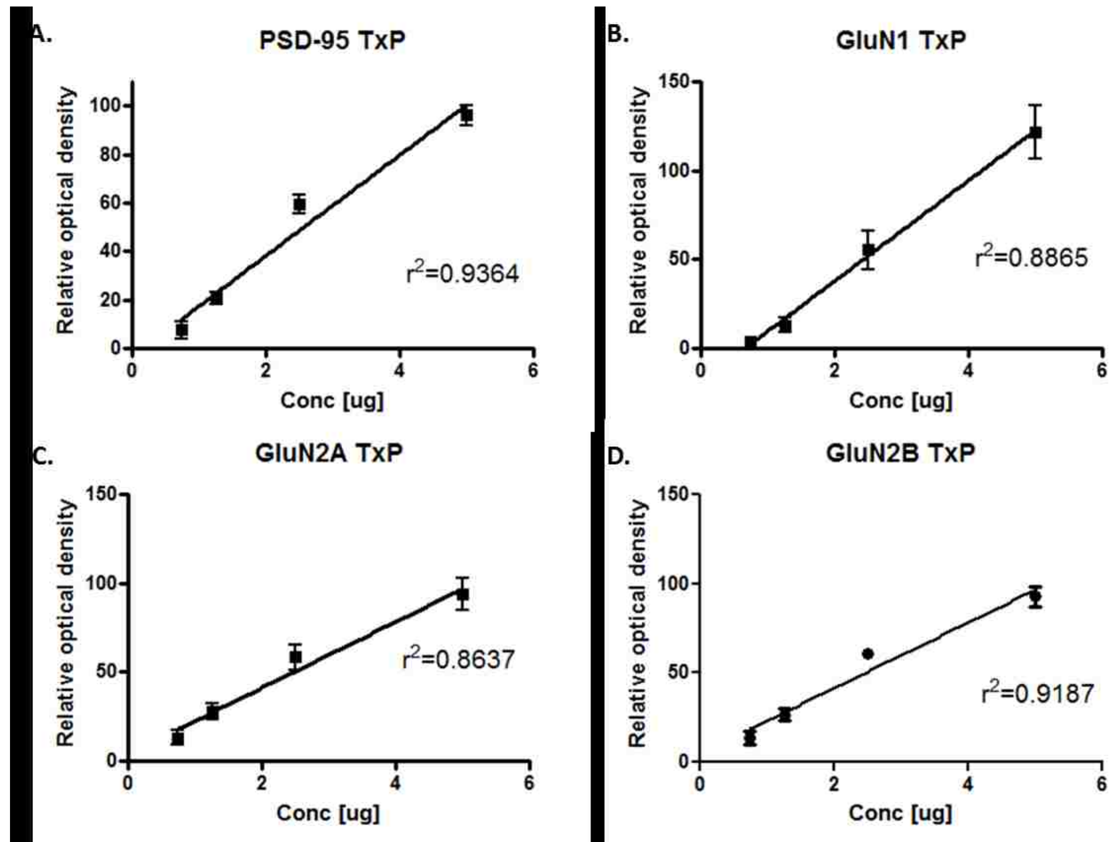

Figure A4. Linear range determination in TxP fractions for GluN1, GluN2A, GluN2B, and PSD-95.

Subcellular fractionation was performed on naïve tissue as described in Chapter 5, and the TxP fraction was loaded onto a gel in increasing protein concentrations and immunoblotted for the appropriate protein. The optimum total protein concenntration was determined to be 3µg for GluN1, 2A, 2B, and PSD-95 and was used for TxP immunoblotting experiments.

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Figure A5:

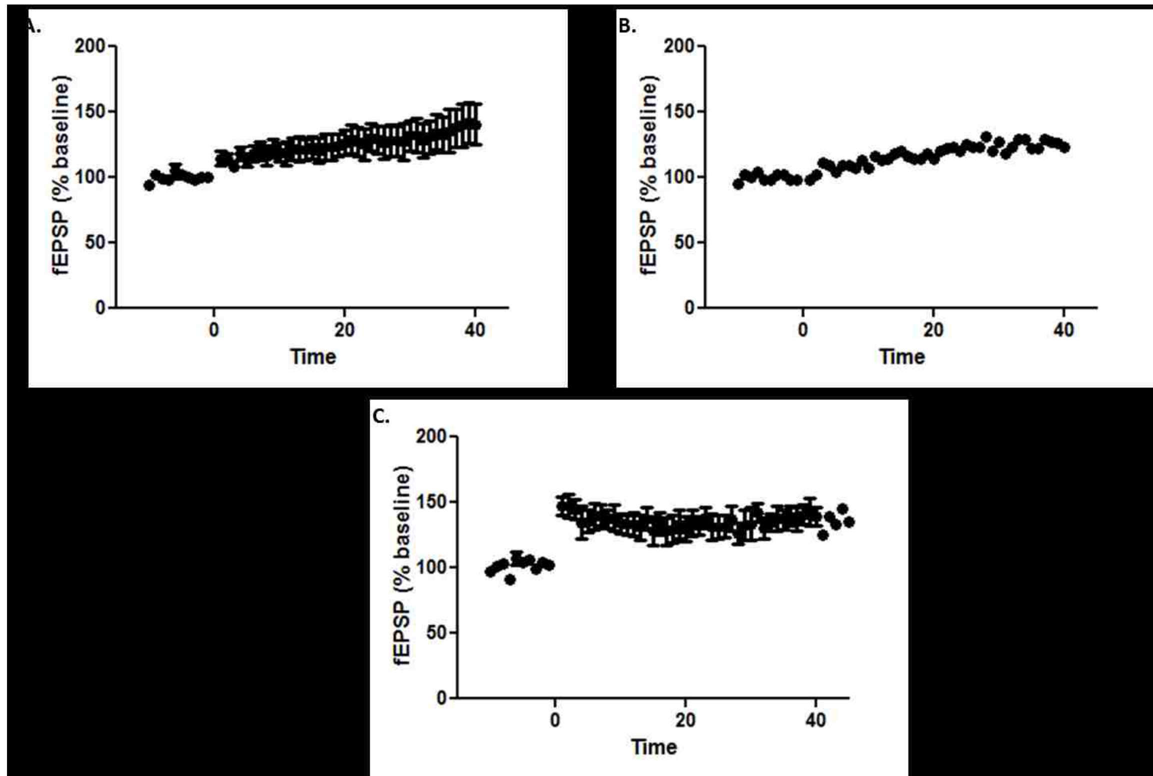

Figure A5. LTP protocols

A number of LTP protocols were tested in *in vitro* slices from naïve animals to determine the protocol which would produce the most consistent results in the medial perforant path-dentate gyrus pathway. A) LTP was elicited using 5 trains of 4 bursts of 4 pulses at 100Hz, with an interburst interval (IBI) of 200ms and an intertrain interval of 30s (n=5). B) 1 train of 100Hz for 1s (n=1). C)The LTP protocol chosen for use in the experiments in Chapter 5: 4 trains of 9 bursts of 4 pulses at 100Hz, IBI 200ms, ITI 5 min (n=4).

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Figure A6:

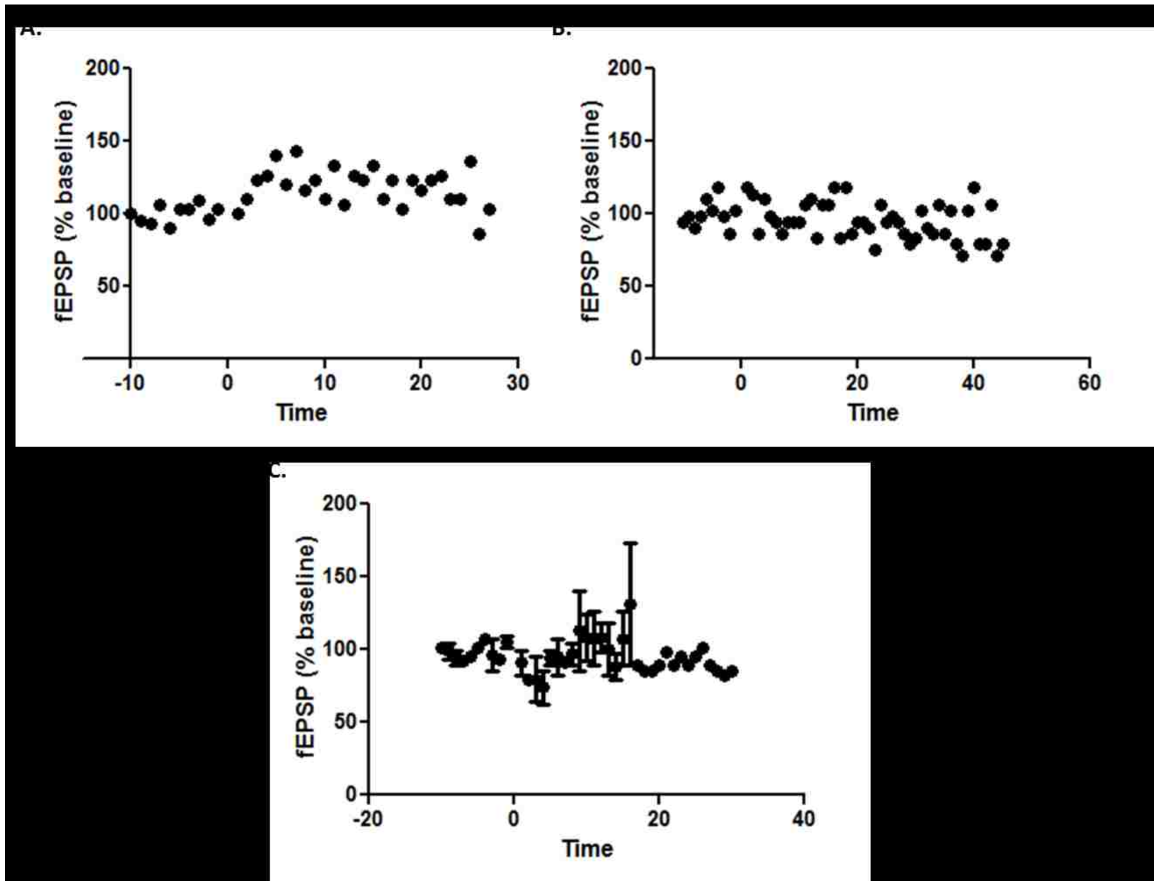

Figure A6. A number of LTD protocols were tested in *in vitro* slices from naïve animals in the MPP-DG pathway.

A) Stimulation protocol of 5Hz for 120s (n=1). B) Stimulation protocol of 2Hz for 600s (n=1). C. Stimulation protocol of 1 Hz for 900s (n=2)**.** Further research found that homosynaptic LTD was not NMDAR-dependent (see Chapter 6), so these studies were not pursued.

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Figure A7

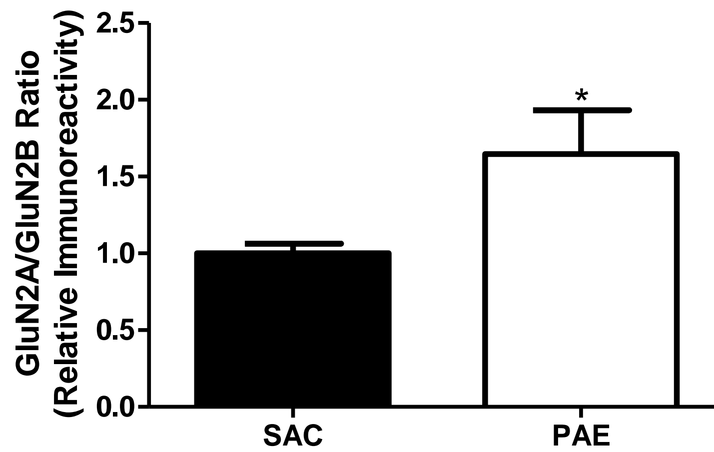

Figure A7. The ratio of GluN2A/GluN2B in the synaptic membrane in PAE animals in increased.

The levels of GluN2A and GluN2B were determined in the synaptic membrane using immunoblotting, and were normalized to PSD-95 levels, as described in Chapter 5. The ratio of GluN2A to GluN2B was then determining, normalized to control levels. $t[12] = 2.196$,* $p<0.05$, $n=7$

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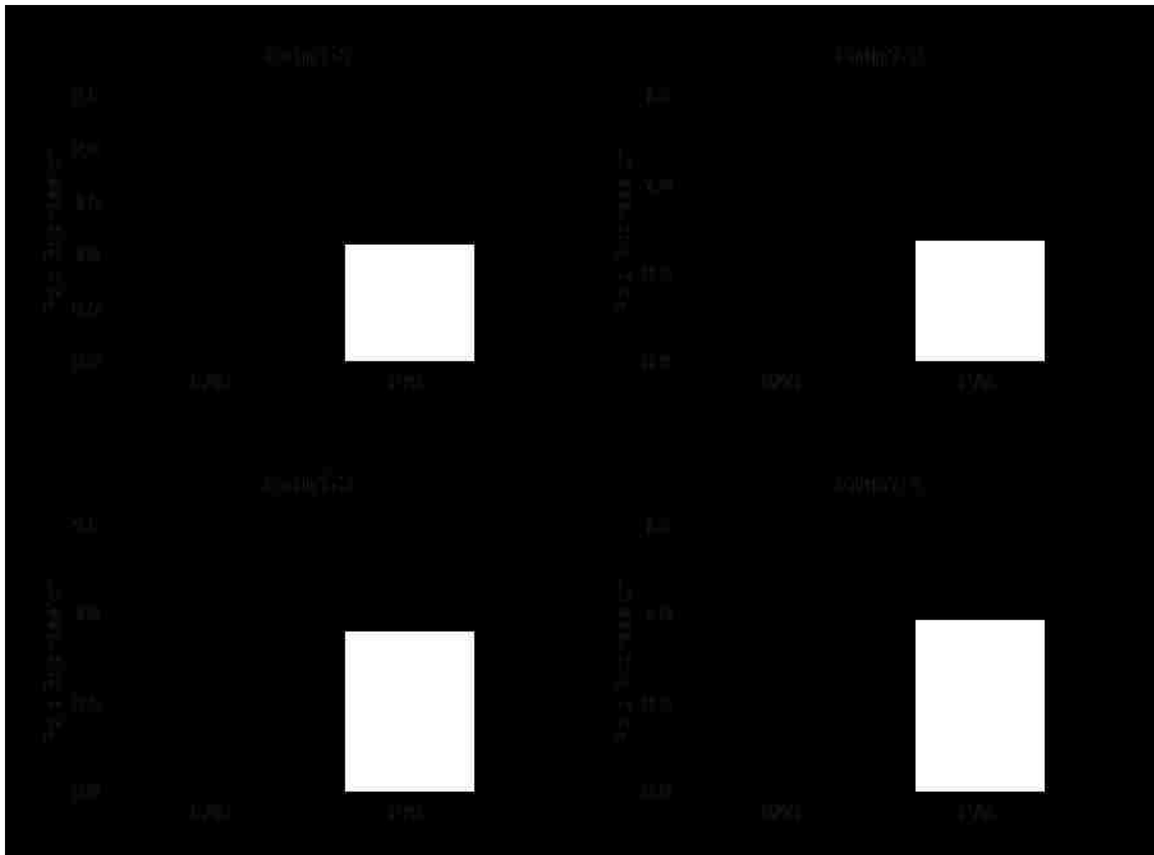
Figure A8

Table A8.1 – Primers used for RT-PCR study

Target	Forward Primer	Reverse Primer
Grin1-1	GCCCGACCCTAAAAAGAAAG	TGCTCGTGTCTTTGGAGGAC
Grin1-2	ACGTGTGGAGGAAGAACAGC	CTCTCCCTATGACGGGAACA
Grin1-3	CCCGACCCTAAAAAGAAAGC	TGGTACTGCGTGTCTTTGGA
Grin1-4	AGATCGCCTACAAGCGACAC	TGGTACTGCTGCAGGTTCTTC
Grin2A	ACGTGACAGAACGCGAACTT	ATCTCCAAACACCAAGCCAT
Grin2B	GCCATGAACGAGACTGACCC	CCATTATCATAGATGAGCCC
Grin3A	TCAGAATCCCCTGCACCTAC	GACTCGAGGTGGAACTTGGA
HPRT1	GCCGAGGATTTGGAAAAAGT	ATCCAGCAGGTCAGCAAAGAA
Cyclophilin A	TGCTGGACCAAACACAAACG	GCCTTCTTTCACCTTCCCAAA

A.



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B.

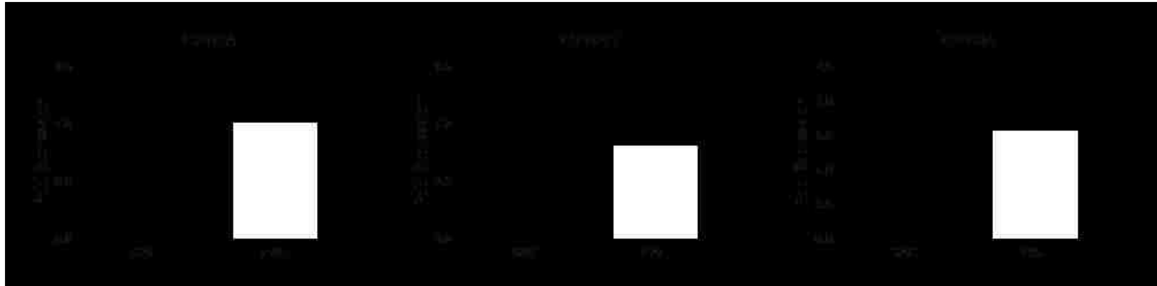

Figure A8. PAE does not alter NMDAR subunit mRNA levels in the dentate gyrus of the hippocampus.

The dentate gyrus was microdissected from each animal [approximately 5-10µg of tissue, prepped with RNAlater (Life Technologies, Grand Island, NY) per manufacturer's instructions] and was transferred to a QIAshredder™ homogenizer (Qiagen, Valencia CA) for thorough lysis and used according to manufacturer's protocol. RNA was extracted using an RNeasy® Plus Mini Kit (Qiagen) exactly as described by manufacturer. The mRNA concentration was determined (OD 260 nm) using a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Isolated mRNA was stored at -80°C. Reverse transcription was performed using a QuantiTect Reverse Transcription Kit according to the manufacturer's protocol (Qiagen) and a PTC-200 Peltier Thermal Cycler. Primers for the different NMDAR subunit mRNAs (Table A8.1) were designed as follows: Grin1-1, Grin1-2, and Grin1-4 based on sequences from GenBank and Hollmann and colleagues (1993), Grin1-3 from Manta and colleagues (2011), and Grin2A, Grin2B, and Grin3A from GenBank. PCR products for each primer were confirmed both with DNA gel electrophoresis and DNA sequencing. Primer efficiencies were validated. RT-PCR was performed using a QuantiFast SYBR Green PCR Kit (Qiagen) on a 7300 Real-Time PCR System (Applied Biosystems). Proper controls, i.e. no template controls and reverse transcription controls, were used and a dissociation curve was evaluated. Results were assessed using the ΔΔCT method (n=7 for all mRNAs tested). A.) No significant difference was found between SAC and PAE mice for Grin1 splice variant mRNA levels. B.) No significant difference was found between SAC and PAE mice for Grin 2A, 2B, or 3A mRNA levels.

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Figure A9

Figure A9. NMDAR subunit levels are altered in a crude synaptosomal membrane fraction from the dentate gyrus of PAE animals.

The dentate gyrus from SAC and PAE mice was microdissected, and a P2 fraction was obtained as described in (Samudio-Ruiz et al., 2010). Western blot analysis was performed and analyzed as described in Chapter 5. A.) GluN1 subunit levels are increased in PAE animals compared to SAC (SAC 1.00, PAE 3.10 ± 0.78; $t[9] = 2.6982$, $p<0.05$). B.) GluN2A levels are increased in PAE animals compared to SAC (SAC 1.00, PAE 4.48 ± 0.59; $t[5] = 5.9053$, $p<0.01$). C.) GluN2B levels are not significantly different in PAE animals vs. SAC (n=7).

Discussion/Interpretation of results from Appendices 8 and 9:

The data from Chapter 5 indicate that there is an alteration of NMDAR subunit levels at the synaptic membrane. These alterations could arise for several reasons; alterations in subunit transcription, translation, and/or trafficking would all possibly lead to altered surface expression. Appendix 8 examined mRNA levels for the different NMDAR subunits, and no significant differences were found between SAC and PAE animals, indicating that the dysregulation does not occur at the transcriptional level. It should be noted, however, that these values had a large amount of variability associated with them, which may interfere with interpretation of the results. Examination of the P2 fraction shows an increase in GluN1 subunit levels and GluN2A subunit levels, with no change in GluN2B subunit levels. This information leads to several different conclusions. First, the increase in GluN1 levels seen at both the P2 fraction and the

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synaptic membrane fraction would indicate that the increased synaptic membrane levels in PAE animals may be due to overall increases in GluN1 expression, possibly though altered GluN1 translation. If the increase in GluN1 P2 subunit levels mimics the increase seen at the synaptic membrane and is GluN1-4 specific (C2´-containing GluN1), then the increase at the P2 fraction level may allow for increased trafficking to the surface, since this splice variant does not have the ERR signal found in the C1 cassette. In addition, if GluN3A subunit levels are increased at the P2 fraction (not determined in this study), this may also aid in forward trafficking of the GluN1 subunit. The GluN2A subunit increase in the P2 fraction, with no concurrent increase either mRNA levels or subunit levels in the synaptic or extrasynaptic membranes, is intriguing. This would imply that GluN2A translation is increased in the endoplasmic reticulum (which is also fractionated with the P2 fraction), but is not forward trafficked to the synaptic membrane. The likelihood of this is unknown. GluN2A forward trafficking is activity dependent (Barria and Malinow, 2002; Storey et al., 2011), so it is possible that while GluN2A translation is increased in PAE animals, they do not reach the surface due to overall decreased synaptic activity in PAE animals, possibly due to the observed decreased GluN2B synaptic levels. The required activation for GluN2A incorporation is unknown; most studies examine activity-dependent incorporation by blocking all NMDAR activity using AP5. The decrease in GluN2B synaptic levels, with no associated decrease in mRNA or P2 levels, would indicate that the decrease is due to altered trafficking of the GluN2B subunit. GluN2B synaptic incorporation is constitutive, indicating that alterations to the constitutive exocytic pathway may occur in PAE animals. Recently, an *N*-glcosylation site in the M3-M4 loop of the GluN2B N-terminus, which is not present in the GluN2A subunit, has

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been shown to be responsible for the constitutive trafficking of the GluN2B subunit (Storey et al., 2011). Alterations to glycosylation could alter forward trafficking of the GluN2B subunit. Further studies are needed to determine the level of dysregulation for the NMDAR subunits.

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Figure A10

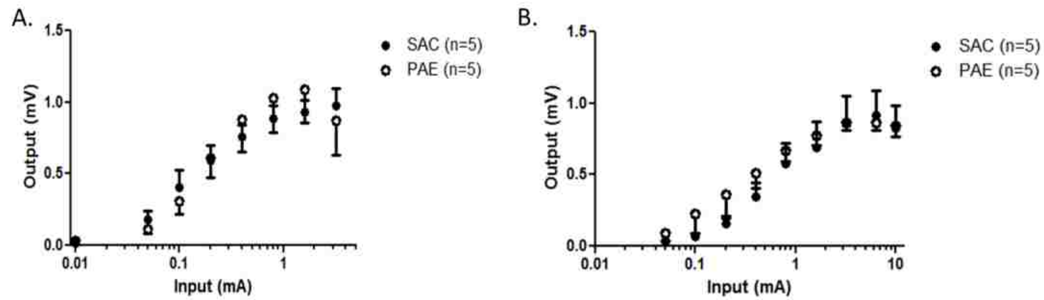

Figure A10. Basal synaptic transmission is unaltered in PAE mice.

A. Input/Output (I/O) curves for ionotropic receptors in SAC and PAE mice. Two-way ANOVA indicated no significant difference between I/O curves. **B.** NMDAR-mediated I/O curves in SAC and PAE mice. Two-way ANOVA indicated no significant difference.

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Figure A11.

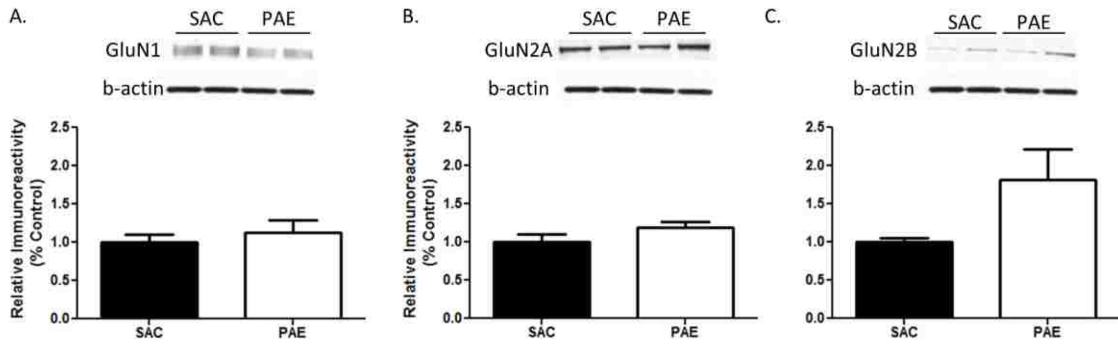

Figure A11. Prenatal alcohol exposure does not significantly alter NMDAR subunit levels in the extrasynaptic fraction.

A. GluN1 (n=10), **B.** GluN2A (n=7), and **C.** GluN2B (n=7) subunit levels are unaltered in PAE mice compared to SAC. No significant difference was found for any subunits.

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Abbreviations Used

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AP5	2-amino-5-phsophonopentanoate
ARND	alcohol-related neurodevelopmental disorder
AUC	area under the curve
BDNF	brain derived neurotrophic factor
BEC	blood ethanol concentration
CA	cornu ammonis
Ca^{2+}	calcium ion
CASK	calcium/calmodulin-dependent serine protein kinase
CaMKII	calcium/calmodulin activated kinase II
cAMP	cyclic adenosine monophosphate
Cd^{2+}	cadmium ion
CK2	casein kinase II
CREB	cAMP response element binding protein
CS	conditioned stimulus
D1	dopamine 1
DNMP	delayed non-match to place
DPC	deoxycholate
E	embryonic day
EDTA	ethylene diaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
EPSC	excitatory postsynaptic current
ERK 1/2	extracellular signal-regulated kinase 1/2
ER	endoplasmic reticulum
ERR	endoplasmic reticulum retention
EtOH	ethanol
FAS	fetal alcohol syndrome
FASD	fetal alcohol spectrum disorder
fEPSP	field excitatory postsynaptic potential
GABA	γ-aminobutyric acid

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GABAR	γ-aminobutyric acid receptor
GD	gestational day
HB	homogenization buffer
HEK293	human embryonic kidney cells 293
HFS	high frequency stimulation
IBI	interburst interval
I/O	input/output
ITI	intertrain interval
LPP	lateral perforant pathway
LTP	long term potentiation
LTD	long term depression
MEK	MAP kinase/ERK kinase
Mg^{2+}	magnesium ion
mGluR	metabotropic glutamate receptor
MOPP	molecular layer perforant path-associated cell
MPAE	moderate prenatal alcohol exposure
MPP	medial perforant pathway
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
nAChR	nicotinic acetylcholine receptor
NAD	nicotinamide adenine dinucleotide
NBNI	norbinaltorphimine
NMDA	*N*-methyl-D-aspartate
NMDAR	*N*-methyl-D-aspartate receptor
PAE	prenatal alcohol exposure
PD	postnatal day
PDZ	PSD-95/Dlg1/zo-1 protein
PKA	protein kinase A
PKC	protein kinase C
PKM	protein kinase M
PSD	postsynaptic density

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PTP	protein tyrosine phosphatase
RAM	radial arm maze
RT-PCR	real time polymerase chain reaction
SAC	saccharin
SAP	synapse associated protein
SFK	Src family kinase
SGZ	subgranular zone
SNARE	soluble NSF attachment protein receptor
STEP	striatal-enriched tyrosine phosphatase
TBS	theta burst stimulation
TGN	trans-Golgi network
TM	transmembrane
TxP	triton pellet fraction (synaptic fraction)
TxS	triton soluble fraction (extrasynaptic fraction)
US	unconditioned stimulus
VGCC	voltage gated calcium channel
Zn^{2+}	zinc ion

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