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Ethanol exposure impairs glutamatergic synaptic transmission and plasticity in the ca1 hippocampal region during the third trimester-equivalent of human pregnancy : implications for fetal alcohol spectrum disorder

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**ETHANOL EXPOSURE IMPAIRS GLUTAMATERGIC SYNAPTIC
TRANSMISSION AND PLASTICITY IN THE CA1 HIPPOCAMPAL REGION
DURING THE THIRD TRIMESTER-EQUIVALENT OF HUMAN PREGNANCY:
IMPLICATIONS FOR FETAL ALCOHOL SPECTRUM DISORDER**

By

Michael P. Puglia

B.S. Biochemistry, University of Nevada, 2004

DISSERTATION

Submitted in partial fulfillment of the requirements for the degree of

**Doctor of Philosophy
Biomedical Sciences**

The University of New Mexico
Albuquerque, New Mexico

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ABSTRACT OF DISSERTATION

Fetal alcohol spectrum disorder results from developmental exposure to ethanol. Although many organ systems are targeted by this teratogen, the central nervous system is exquisitely sensitive. Children with this disease often present with behavioral disorders as well as impairments in learning and memory. Ethanol exposure affects developmental processes throughout pregnancy; however, the third trimester-equivalent has demonstrated heightened sensitivity. Although the mechanism of action(s) of ethanol remains unknown, studies suggest that impairments in glutamatergic signaling and synaptic plasticity during the third trimester-equivalent period of development lead to alterations in synaptic formation and refinement. However, little is known about the developmental effects of ethanol in the CA1 hippocampal region, a brain region often studied in the context of learning and memory.

Studies presented in this dissertation address the acute and chronic effects of ethanol during the third trimester-equivalent period. First, characterization of the acute effects of ethanol demonstrated postsynaptic inhibition of both NMDA receptor (NMDAR) and AMPA receptor (AMPA) mediated synaptic responses, as well as the inhibition of long-term potentiation (LTP) induction. Then, an *in vivo* repeated ethanol exposure paradigm was used to mimic maternal drinking during this period. In contrast to the effects of acute ethanol exposure, repeated exposure did not affect AMPAR- or NMDAR-mediated synaptic strength or glutamate release; however, it impaired LTP expression and/or maintenance. Lastly, repeated *in vivo* third trimester-equivalent ethanol exposure did not affect expression of Ca²⁺-permeable AMPARs, or induce tolerance to the acute inhibitory effects of ethanol.

Studies in this dissertation add to a growing body of evidence indicating that significant differences exist between the effects of ethanol on the developing *versus* the mature brain. Furthermore, studies presented here support the notion that ethanol-mediated impairments in synaptic plasticity mechanisms during the third trimester-equivalent developmental period could lead to inappropriate wiring of neuronal circuitry, and set the stage for the long term deficits observed in children with fetal alcohol spectrum disorder.

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1. Introduction

Overview of Fetal Alcohol Syndrome and Fetal Alcohol Spectrum Disorders

Maternal ethanol (EtOH) use during pregnancy can lead to a broad range of clinical findings in children ranging from central nervous system impairments, such as learning and memory deficits, to full-blown fetal alcohol syndrome (FAS). The clinical hallmarks required for a diagnosis of FAS include characteristic facial dysmorphism, growth retardation and impaired central nervous system function (Sokol et al., 2003). Clinical cases that do not meet the diagnostic criteria for FAS are described under the umbrella term, fetal alcohol spectrum disorder (FASD) (Sokol et al., 2003; Jones and Smith, 1973). The prevalence of children with FASD in the U.S. was recently estimated to be 2-5%, whereas the prevalence of children with FAS is estimated to be 0.2-0.7% (Sokol et al., 2003; May et al., 2009).

In the general population, the majority of women stop drinking when they become pregnant (Muhuri and Gfroerer, 2009). However, a recent study by the National Survey on Drug Use and Health (2002-2006) found that 10.5% of women report EtOH use during pregnancy, and 3.2% engage in binge drinking (Muhuri and Gfroerer, 2009). Although the percentage of women reporting drinking decreases as pregnancy progresses, a significant portion continue to do so through the third-trimester; during this trimester, 6.9% report consuming EtOH, and 1% engage in binge drinking (Muhuri and Gfroerer, 2009).

Although a substantial percentage of women drink during pregnancy, not all have children afflicted by FAS or FASD (Maier and West, 2001). Factors that influence the consequences of maternal EtOH consumption and predict severity are: maternal drinking patterns (frequency and amount), metabolism, genetic susceptibility, timing of consumption during pregnancy, and increased vulnerability of some brain regions during certain stages of development (Maier and West, 2001; Livy et al., 2003a). In addition, EtOH use during pregnancy is often associated with other factors (i.e. poverty, poor nutrition/prenatal care, other drug use, etc) that enhance the neuroteratogenic effects of EtOH (Goodlett et al., 2005).

The clinical deficits associated with FAS and FASD are likely mediated by multiple mechanisms that depend on the timing of exposure. For example, the impairments in craniofacial features of children with FAS (i.e. short palpebral fissures, flat/indistinct philtrum, thin upper lip) have been ascribed to impaired cellular migration, including that of neural crest cells, during the first trimester period when organogenesis occurs (Goodlett and Horn, 2001). Exposure during the second trimester has also been associated with abnormalities in neuronal migration as well as cell survival, leading to changes in brain region volume (Goodlett et al., 2005). Exposure during the third-trimester has been linked to impaired neuronal survival/apoptosis, synaptic formation and synaptic refinement; these impairments are thought to be, in part, responsible for long-term deficits in learning, memory and other cognitive processes (Berman and Hannigan, 2000; Medina and Krahe, 2008; Olney, 2004). One brain region

known for its role in certain types of learning and memory is the hippocampus, which has been shown to be sensitive to the deleterious consequences of developmental EtOH exposure. The rest of this document will focus on the developmental effects of EtOH in the context of impaired hippocampal function.

Learning and memory alterations in children afflicted by FASD and in animal models of this disorder

Behavioral and cognitive impairments are a hallmark of FAS and FASD. Many brain regions are required for higher cognitive function, such as learning and memory, and have been shown to be targets of the teratogenic effects of EtOH. Several studies have assessed learning and memory in children with prenatal EtOH exposure, and found deficits thought to be attributable, in part, to impaired hippocampal function.

Hamilton and colleagues (2003) tested 8 adolescent males (9.5-16.5 y/o) diagnosed with FAS and 8 matched-controls in a virtual Morris water maze task, which is thought to probe hippocampal function. Children with FAS performed significantly worse in navigating to the hidden platform in the virtual maze; however, there was no difference in navigating to a cued platform, suggesting that deficits were attributable to the hippocampus, and not other brain regions controlling motor performance or motivation during the task (Hamilton et al., 2003). In a study by Uecker and Nadel (1998), 30 children, 15 with FAS and 15 matched controls (11 male, 4 female; average age 10 ± 2.3 y/o) were assessed for performance on object recognition/location tasks. Briefly, subjects were given cards of a dog, a kitten playing the guitar, a plant being watered, etc, which were

arranged on a large book. They were asked to remember one of the the cards and to locate it in the book. Both control and children with FAS could remember the cards; however, children with FAS demonstrated significant impairments in location recall (immediate and after 24 hrs) suggestive of hippocampal damage (Uecker and Nadel, 1998). Additional studies are summarized in Table 1.1.

Table 1.1. Summary of learning and memory deficits observed in children with prenatal EtOH exposure

Author (year)	Age (years)	Number of prenatally exposed subjects	Main finding
Mattson et al. 1998	5-16	25	Global-local visuospatial abnormalities
Steinhausen et al. 1982	3-15.5	49	Deficits in spatial relationships
Olson et al. 1998	14-16	226	Deficits in visuospatial skills
Uecker and Nadel 1996	10 ± 2.3	15	Spatial deficits and delayed recall
Uecker and Nadel 1998	10 ± 2.3	15	Deficits in spatial recall
Hamilton et al. 2003	9.5-16.5	8	Deficits in virtual water maze

Animal models have been developed to further our understanding of the pathophysiology of FASD. However, several challenges exist when attempting to model this human disorder. Maternal EtOH consumption varies in terms of timing, dose, duration, and amount; therefore, a single exposure paradigm cannot accurately model human maternal EtOH consumption patterns. Therefore, investigators have used several exposure paradigms to mimic this disease. In spite of this, the overwhelming consensus is that animal models of FAS and FASD accurately recapitulate deficits in higher cognitive function (Berman and Hannigan, 2000). These include learning and memory deficits attributed to impaired hippocampal function.

Savage and colleagues (2002) found that prenatal EtOH exposure (30-80 mg/dL) in rats (which models the human first and second trimester) led to impaired learning of a new platform location in the Morris water maze at postnatal days (P) 180-220 (Savage et al., 2002). Studies in the radial arm maze demonstrated impaired spatial learning in P60 rats gestationally exposed to EtOH (blood alcohol level = 170 mg/dL on gestational day 18) (Reyes et al., 1989). In addition, administration of 6g/Kg of EtOH on P6 (third trimester-equivalent period) resulted in impaired performance in a serial spatial discrimination reversal learning task in a t-maze at P40-42 (Thomas et al., 2002). Studies with animal models of the effect of developmental EtOH exposure on learning and memory are summarized in Table 1.2. Collectively, these studies indicate that developmental EtOH exposure leads to persistent impairments in hippocampal function, leading to alterations in learning and memory.

Table 1.2. Effects of prenatal or neonatal EtOH exposure on tests of learning and memory

Author (year)	Trimester-equivalent exposure period	Animal model	Type of behavioral test	Effect
Lochry and Riley (1980)	1st, 2nd	Rat	T-maze	impaired spatial acquisition
Zimmerberg et al. (1991)	1st, 2nd	Rat	T-maze	impaired reference memory
Blanchard et al. (1987)	1st, 2nd	Rat	Morris water maze	impaired spatial acquisition
Goodlett et al. (1997)	3rd	Rat	Morris water maze	impaired spatial acquisition
Savage et al. (2002)	1st, 2nd	Rat	Morris water maze	impaired "one trial" learning
Reyes et al. (1989)	1st, 2nd	Rat	Radial arm maze	impaired spatial memory
Thomas et al. (2002)	3rd	Rat	T-maze	impaired reversal learning

Many mechanisms of action of EtOH on the developing brain have been proposed and some of these may apply to the hippocampus. These include disrupted cellular energetics, cell migration, altered cellular acquisition and

timing, cell-cell interactions, cellular damage/apoptosis, and alterations in neuronal signaling (Goodlett et al., 2005; Goodlett and Horn, 2001). Although several mechanisms have been proposed, those that mediate persistent impairments in learning and memory are not fully understood. It is clear that EtOH affects multiple cellular processes during different stages of hippocampal development and several of these processes will be discussed in the next section.

Hippocampal development: effects of EtOH

In rats, hippocampal development begins early during the second trimester-equivalent and continues through the third trimester-equivalent (Kostovic et al., 1989; Andersen et al., 2007). As previously alluded to, rodent gestation mimics the first two human trimesters, whereas the first 10-12 rodent postnatal days mimic the gestational equivalent to the human third-trimester. Fig 1.1 provides an overview of critical neurodevelopmental events in humans and rats. Figure 1.2 shows brain growth as a function of gestational age in multiple mammalian species, emphasizing the period termed the “brain growth spurt”, which has shown heightened sensitivity to teratogens, especially EtOH (Livy et al., 2003a; Berman and Hannigan, 2000; Thomas et al., 2002; Galindo and Valenzuela, 2006; Krahl et al., 1999; Thomas et al., 2008; Olney et al., 2001; O’Leary-Moore et al., 2006; Hunt et al., 2009; Tsuji et al., 2008). The effects of EtOH on the different phases of hippocampal development will be discussed in more detail below.

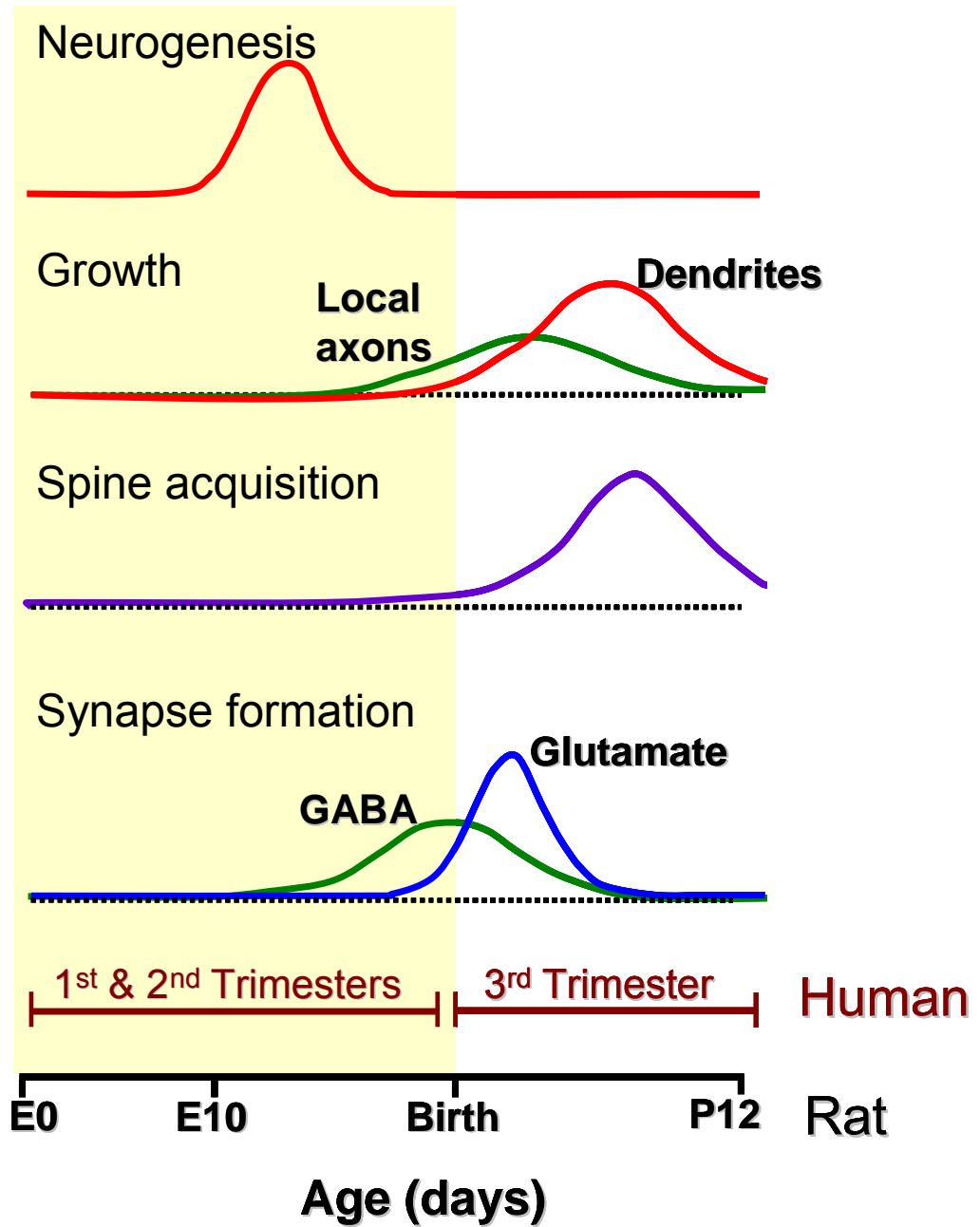


Figure 1.1. Time course of rat and human development showing significant neurodevelopmental events. Adapted from Ben-Ari (2002).

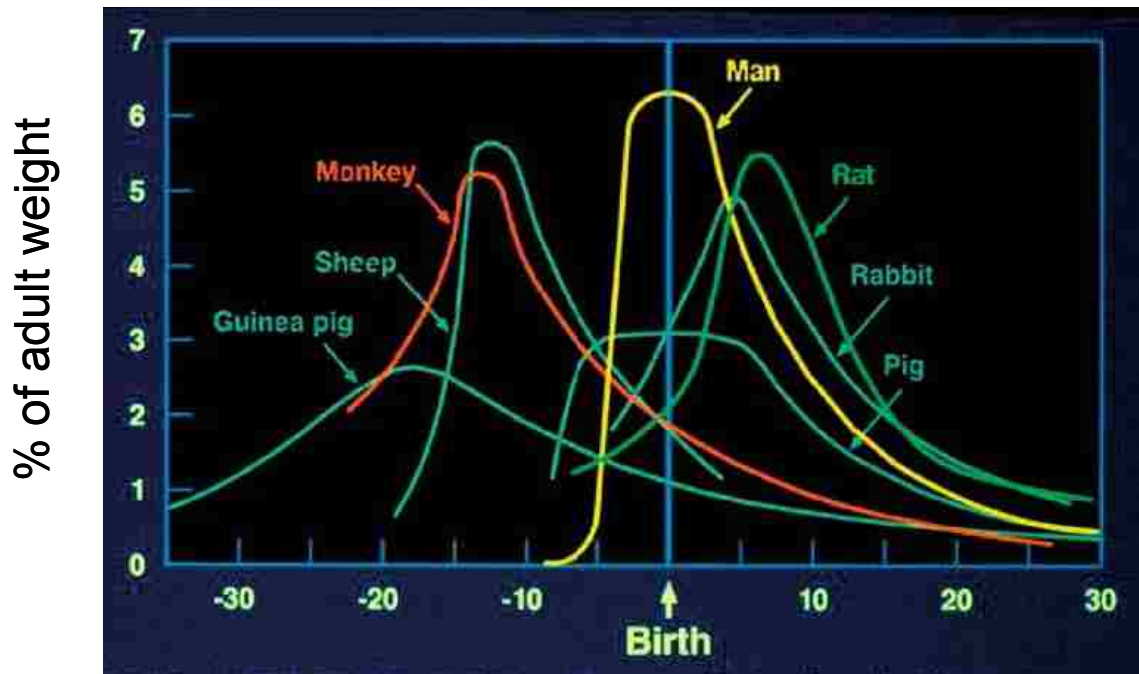


Figure 1.2. Brain growth spurt in several mammalian species showing the increase brain weight as a function of age. Units for x-axis vary by species, and are days for rat and guinea pig, 2 days for rabbit, 4 days for monkey, 5 days for sheep, weeks for pig, and months for humans. Adapted from Cudd, T. (2005).

Neurogenesis and neuronal migration

Stem cells from the ventricular germinative layer located between the ventricular wall and the unformed CA1 region migrate along two radial glial bundles (supragranular bundle and fimbrial bundle) to eventually lead to the formation of the hippocampus proper (Nakahira and Yuasa, 2005; Barry et al., 2008). In rats, stem cells differentiate into pyramidal cells at embryonic day(s) (E) 16-21, with peak generation occurring around E17 for CA3 neurons, and E18-19 for CA1 neurons (Andersen et al., 2007). Although CA3 neurons are generated earlier than CA1 pyramidal neurons— because of the longer distance they must travel to their final location relative to CA1 pyramidal neurons—the

CA3 region develops distinct layers after the CA1 region (Andersen et al., 2007). Early pyramidal cell layers contain 6-10 neuronal rows, which gradually decrease to 2-3 rows as the animal matures (Andersen et al., 2007). In the human, distinct layers of the hippocampal formation have been observed around the 16th week of embryonic development (Kostovic et al., 1989). In contrast to the generation of pyramidal cells, approximately 85% of the dentate granule cells are generated after birth in rats (Andersen et al., 2007). These neurons originate from a germinative layer of stem cells in the hilus (Andersen et al., 2007). The period of neurogenesis in this region is considerably longer, continuing into adulthood (Andersen et al., 2007).

GABAergic interneuron generation in the strata oriens and radiatum, molecular layer of the hilus, and lacunosum-moleculare have been observed approximately 1 day before the principle cells that will eventually form the CA1-3, and dentate gyrus (Andersen et al., 2007). It has been suggested that these early-generated interneurons guide future circuit formation, as will be discussed in more detail below (Andersen et al., 2007).

To the best of my knowledge, studies that directly address the effects of EtOH exposure on hippocampal cell migration are lacking; however, deficits have been shown in the cortex and cerebellum with both *in vitro* and *in vivo* EtOH exposure paradigms (Kumada et al., 2006; Miller, 1993). Furthermore, studies have shown long-lasting decreased neuronal number in the CA1 and dentate gyrus as a result of prenatal EtOH exposure in rats, suggesting impaired neurogenesis (Miller, 1995). Postnatal EtOH exposure did not affect neuronal

number, unless EtOH concentrations exceeded 300 mg/dL (Miller, 1995). Interestingly, postnatal EtOH exposure (~220 mg/dL) led to increased cell numbers in the dentate gyrus, suggesting possible region, dose, and timing

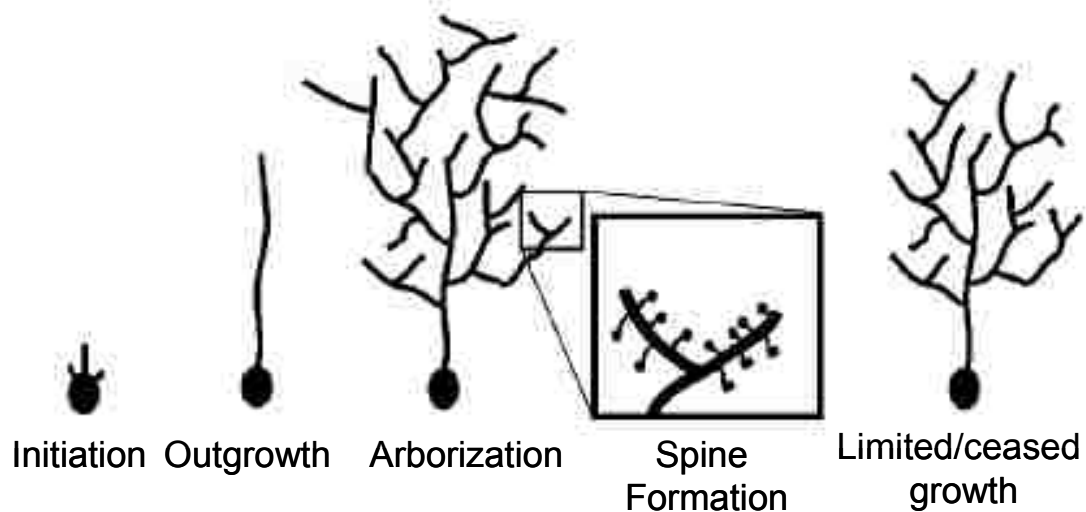


Figure 1.3. Milestones in dendritic development. Adapted from Scott and Luo (2001).

specific effects of developmental EtOH exposure (Miller, 1995).

Neuronal polarization; formation of axons and dendrites

Once neurons reach their destination, a process of polarization occurs where immature processes begin to form, eventually giving rise to axons and dendrites (Dotti et al., 1988). Dendritic development occurs in a series of regulated steps as shown in Fig 1.3. First, immature neurons develop polarity from which immature dendritic outgrowth occurs. Next, dendrites extend (often cue directed) and branch at specified intervals. This is followed by the generation of dendritic spines where the majority of excitatory synapses will be

established. Lastly, these dendrites reach their mature shape, and are characterized by defined borders and little further growth (Scott and Luo, 2001; Yoshimura et al., 2006).

Studies have shown that dendritic outgrowth is affected by developmental EtOH exposure. In the CA1 hippocampal region, a prenatal through lactation EtOH exposure paradigm (5.5g/Kg/day) resulted in blunted basilar dendrites in 14 day old mice (Davies and Smith, 1981). In addition, Ferrer and colleagues (1988) showed a reduction in dendritic spines in CA1-3 pyramidal cells in P15 offspring gestationally exposed to a 35% liquid EtOH diet; however, this effect was not present in P90 animals, suggesting possible compensatory actions (Ferrer et al., 1988). In a case report of a 4 month-old child born to an alcohol-dependent mother, there were reduced dendritic spines in cortical layer 5 pyramidal cells (Ferrer and Galofre, 1987). These data indicate that EtOH affects dendritic development, leading to improper formation of excitatory synaptic connections at dendritic spines (Haas et al., 2006; Cline and Haas, 2008; Fiala et al., 1998; Zhou et al., 2004; McKinney, 2005).

Axonal and dendritic development begins with immature neuronal projections called lamellipodia (Stage 1; Fig. 1.4). These projections then further develop into several protrusions called neurites, which will eventually give rise to an axon and several dendrites (Stage 2). However, at this point it is not determined which neurite will become the axon. As development progresses, the axon develops and its extension is guided by the growth cone (Stage 3). Further maturation of the remaining neurites occurs, and these will eventually become

the dendrites (Stage 4). This is then followed by formation of synaptic connections and incorporation into the neuronal network (Stage 5) (Dotti et al., 1988; Scott and Luo, 2001; Yoshimura et al., 2006; Miller, 2006).

Although not much is known about the effects of developmental EtOH exposure on hippocampal axonal projections, the consensus from studies to-date is that EtOH induces hypertrophy of these processes (Miller, 2006). Mossy fiber-to-CA3 pyramidal neuron synapses were found to be disorganized and have abnormal positioning in adult rats prenatally exposed to EtOH (West et al., 1981). In addition, prenatal EtOH exposure was shown to increase neuronal projections from the somatosensory cortex to the spinal cord in 3-4 month-old rats (Miller, 2006; Miller, 1987). However, children prenatally exposed to EtOH often exhibit decreased area in the corpus callosum, which contains a large density of cerebral cortical axons that cross into the contralateral hemisphere, suggesting that EtOH can preferentially affect this axonal population (Sowell et al., 2001).

Synaptogenesis

Cue directed signals (such as, NETRIN-1 and Sema3C) guide the axonal growth cone to the site of a potential synaptic connection (Miller, 2006; Sanes and Lichtman, 2001; Munno and Syed, 2003; Steup et al., 2000). In preparation for this partnership, partially differentiated pre- and postsynaptic components can be distinguished (Munno and Syed, 2003; Ahmari et al., 2000; Nimchinsky et al., 2002). Immature presynaptic “packets” contain a heterogeneous mixture of synaptic machinery molecules, including VAMP (synaptobrevin), Ca²⁺ channels, endoplasmic reticulum, and vesicular proteins (Ahmari et al., 2000).

Postsynaptic precursors have been shown to contain functional neurotransmitter receptors and cell-cell interaction molecules (Munno and Syed, 2003). These rudimentary synaptic components then make physical contact, and factors that help synaptic stabilization (such as neurotransmitters and agrin) are released from the presynaptic terminal (Munno and Syed, 2003). In addition to chemical signaling, cell adhesion, and cell-cell interacting molecules (i.e. Ephrin, N-cadherins, neuexins/neuroligins) act to further stabilize synaptic connections (Miller, 2006; Munno and Syed, 2003), in part, by facilitating the recruitment of scaffolding proteins such as PSD-95 or Sap-102, which recruit signaling molecules and glutamate receptors such as NMDA and/or AMPA receptors, as discussed in more detail below (Miller, 2006; Munno and Syed, 2003; Elias et al., 2008). Although synapse formation has been shown to occur in the absence of transmitter release, this process is required for synapse stabilization and maintenance (Verhage et al., 2000).

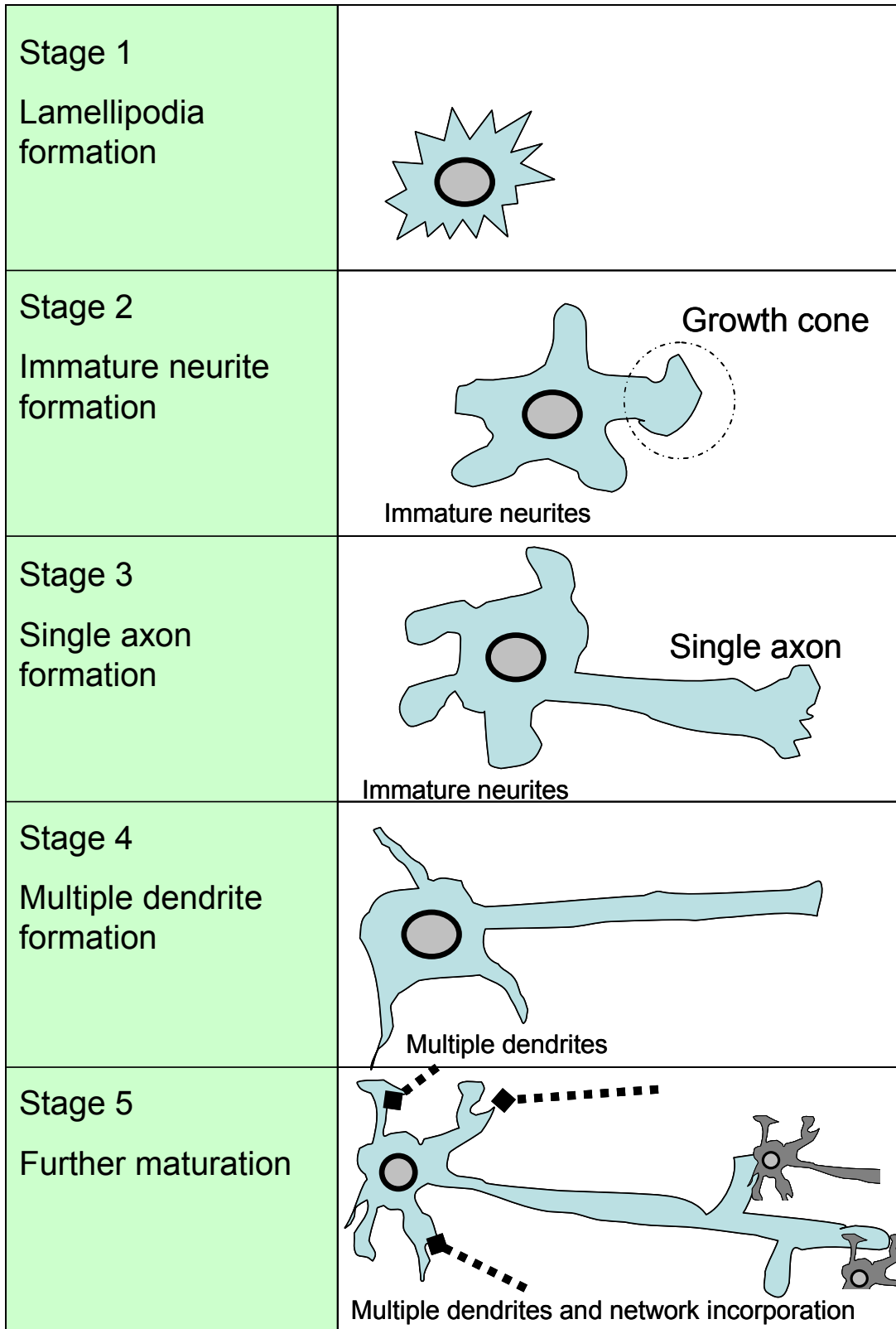


Figure 1.4. Stages of development neuronal polarization and development. From Yoshimura (2006).

Synaptic stabilization and refinement

Early glutamatergic synaptic transmission

Nascent glutamatergic synaptic connections are classically thought of those containing NMDARs and lacking AMPA receptors (AMPA). These glutamatergic synapses are considered silent because they are inactive at rest due to Mg^{2+} block of NMDARs that cannot be relieved as a consequence of the absence of AMPARs. Silent synapses have been identified throughout the developing brain, they and decrease with maturation (reviewed in Kerchner and Nicoll, 2008). In the neonatal rat hippocampal CA1, region almost all glutamatergic synapses are silent at birth (P0); over the course of the first two to three postnatal weeks, these synapses gradually acquire AMPARs (Durand et al., 1996). Studies have suggested that NMDARs play an active role in the maintenance of these silent synapses, by preventing functional AMPARs at the synapse (Hall et al., 2007; Adesnik et al., 2008). One proposed mechanism by which this occurs is the negative regulation of transmembrane AMPAR regulatory protein (TARP) expression (Hall et al., 2007). In addition, NMDARs have been shown to negatively regulate AMPAR transcription and translation, and also to increase AMPAR degradation (reviewed in Hall and Ghosh, 2008). Synapse silence is considered a mechanism that preserves the synapse in a naïve state until correlated activity selectively turns it on, as discussed in more detail below.

Controversy has arisen as to the correct “location” of silent synapses during development. Evidence for postsynaptic silent synapses comes from electrophysiological recordings in the CA1 region of the hippocampus in neonatal

rats. Before a high-frequency induction paradigm, the only observable currents were NMDAR-mediated; after the induction paradigm, both NMDAR- and AMPAR-mediated currents could be observed (Durand et al., 1996). Immunogold labeling studies of glutamatergic synapses also showed a developmental increase in AMPARs with little change in NMDARs (Petrálie et al., 1999). In addition, laser-induced glutamate uncaging experiments—performed in the presence of the Na⁺ channel inhibitor tetrodotoxin to block action potential-dependent transmitter release—showed that glutamatergic synapses in layers 2/3 of the rats cortex had only NMDAR responses and these acquired AMPARs as development progressed (Busetto et al., 2008).

Evidence from cultured neuron and slice electrophysiology experiments suggests that presynaptic mechanisms can also explain the silent synapse phenomenon. Using immunohistochemical and electrophysiological methods, studies with hippocampal neurons in culture demonstrated that AMPARs and NMDARs were present in newly formed synapses (Cottrell et al., 2000; Friedman et al., 2000). In addition, spontaneous excitatory postsynaptic currents (sEPSCs) mediated by both NMDARs and AMPARs were detected in hippocampal slices from neonatal rats (Groc et al., 2002). Moreover, studies have shown that glutamate is released into immature synapses at a low level that is sufficient to activate only the high-affinity NMDARs but not AMPARs, resulting in silent synapses from the functional stand point (Choi et al., 2000). Silent synapses demonstrated AMPAR-mediated responses when methods to either increase the probability of glutamate release or glutamate actions at the AMPAR (i.e. paired-

pulse stimulation, increased temperature, or cyclothiazide application to inhibit desensitization) were used, suggesting that silent synapses express both AMPARs and NMDARs (Gasparini et al., 2000).

In addition to the above-described pre- and postsynaptic mechanisms of synapse silence, another mechanism of synapse inactivation involves the activity-dependent removal of AMPARs that exist in a “labile” state. Studies supporting this in neonatal animals showed that test frequencies typically used to induce evoked responses (0.05-1 Hz) caused a rapid decrease in the magnitude of AMPAR-mediated responses, thus suggesting that synapses are born with both NMDARs and AMPARs and that the AMPARs exist in a labile state that can be controlled in an activity-dependent manner (Xiao et al., 2004). Interestingly, when relatively prolonged interruption of these test-pulses (~40 min) occur, AMPAR-mediated field responses return to initial levels (Abrahamsson et al., 2007).

Only one study has addressed the effects of EtOH on silent synapses in the developing brain. Recordings from P3-4 rat pups exposed prenatally to EtOH (dam blood ethanol levels 0.08 g/dL) demonstrated decreased silent synapses in the CA1 hippocampal region (Mameli and Valenzuela. Unpublished observations). Although the mechanism remains unexplored, acute EtOH application was shown to strengthen AMPAR transmission in a pregnenolone sulfate (PREGS)-like neurosteroid manner in the CA1 hippocampal region of P3-4 rat pups (Mameli and Valenzuela, 2006). In addition, the levels of PREGS have been shown to be elevated by the same prenatal EtOH exposure paradigm

used for the silent synapses studies in brains of rat pups (up to P5) (Caldeira et al., 2004). These results suggest that EtOH exposure during the third trimester-equivalent period can prematurely stabilize synaptic connections, potentially limiting network connectivity (Voigt et al., 2005).

Besides studies of Mameli and Valenzuela, relatively few studies have addressed the acute effects of EtOH on NMDAR and AMPAR function in the hippocampal region during the third trimester-equivalent period. In mature animals, it is well documented that EtOH generally leads to inhibition of NMDARs, whereas AMPARs are relatively insensitive. Table 1.4 summarizes studies of EtOH on NMDAR- and AMPAR-mediated responses in the hippocampus. Studies from our laboratory have indicated that the effects of EtOH on glutamatergic function in the hippocampus are developmentally regulated, thus suggesting a critical period of vulnerability to EtOH-mediated insults (Mameli et al., 2005; Valenzuela et al., 2007).

Table 1.3. Summary of the effects of EtOH on AMPAR- and NMDAR-mediated responses in the mature and developing hippocampus

Receptor	Effect	EtOH Conc. (mM)	Brain region	Rodent Age	Author (year)
AMPA	↔	50	CA1	Adult	Lovinger et al. 1990
AMPA	↓	100	CA1	Adult	Lovinger et al. 1990
AMPA	↔	22	CA1	~P30 (130-160g)	Randall et al. 1995
AMPA	↔	80	CA1	P21-40	Carta et al. 2003
AMPA	↔	25-75	CA1	P12-20	Hendricson et al 2003
AMPA	↔	50	CA3	P21-26	Mameli 2005
AMPA	↓	50	CA3	P3-10	Mameli 2005
NMDA	↓	25-100	CA1	Adult	Lovinger et al. 1990
NMDA	↓	100	CA1	P4-7	Gordey et al. 2001
NMDA	↓	75	CA3	P5	Mameli et al. 2005
NMDA	↔	50	CA3	P5	Mameli et al. 2005
NMDA	↓	100	CA1	P20-25	Swartzwelder et al. 1995
NMDA	↓	80-100	CA1	P21-26	Suvarna et al 2005; Yaka 2003

Contributions of early network activity for synapse stabilization

Studies suggest that plasticity driven by correlated activity of developing neuronal networks is an important endogenous mechanism leading to synapse stabilization. In the hippocampus of rats and non-human primates, neurotransmitter-mediated synaptic signaling has been shown to begin with GABAergic transmission, followed by the acquisition of glutamatergic signaling (Ben-Ari, 2002; Khazipov et al., 2001; Tyzio et al., 1999). Contrary to the adult animal, GABA_A receptor-mediated transmission is depolarizing during the 3rd trimester-equivalent period of development, where immature neurons have high [Cl]_i (Ben-Ari, 2002). This developmental effect of GABAergic transmission can reduce the voltage-dependent Mg²⁺ block of NMDA receptors (NMDARs), thus facilitating increases of postsynaptic Ca²⁺ (Leinekugel et al., 1997). This depolarizing effect of GABA, however, is self-limiting preventing over excitation via increasing conductance across the plasma membrane and shunting of excitatory current (Ben-Ari et al., 2007). In addition, GABA_A receptor-dependent membrane potential depolarization can also lead to inactivation of Na⁺ channels resulting in reduced action potential generation (Zhang and Jackson, 1995). This depolarizing action of GABA_A receptors—along with the excitatory effects of glutamatergic transmission mediated by AMPARs and/or NMDARs—has been shown to drive large network oscillations termed giant depolarizing potentials (GDPs). GDPs have been found in many developing brain regions, including the hippocampus and cortex (Ben-Ari et al., 2007; Ben-Ari, 2001). GDPs are relatively slow recurrent waves of activity lasting several hundred milliseconds,

which generate large Ca^{2+} transients. The GDPs are thought to guide the development of synaptic connections and network formation, as suggested by a recent study. Kasyanov and colleagues (2004) found in brain slices from P1-P6 rat pups, that if a presynaptic stimulation of mossy fiber-CA3 synaptic connections was paired with a GDP, the result was “unsilencing” of synapses with low probabilities of transmitter release and long-lasting potentiation and strengthening of these connections. Furthermore, this potentiation was dependent on a postsynaptic rise in intracellular Ca^{2+} (Kasyanov et al., 2004). Thus, these results suggest that GDP-driven neuronal depolarizations facilitate, in part, Hebbian mechanisms of increased synaptic efficacy and network formation (Kasyanov et al., 2004).

Galindo and colleagues examined the effect of EtOH on GDPs and found that acute application at concentrations as low as 10 mM (legal intoxication limit ~17.4 mM) reversibly increases the frequency of these events in the CA3 hippocampal region in brain slices from P3-7 rat pups (Galindo et al., 2005). This effect was due to a positive feedback loop of interneuron-pyramidal cell firing driven by EtOH-mediated potentiation of GABAergic transmission via an increase in GABA release (Galindo et al., 2005). The increased GDP frequency by EtOH also led to increased intracellular Ca^{2+} levels and Ca^{2+} transients, suggesting that EtOH could affect Ca^{2+} -dependent processes that are essential for synapse stabilization, such as those discussed above (Galindo et al., 2005).

Synaptic plasticity mechanisms stabilizing synaptic connections

In addition to network-driven activity to stabilize and “unsilence” synaptic connections, there is a consensus that strongly correlated-activity also strengthens and stabilizes immature synaptic connections during the third-trimester equivalent period and throughout life. Experimentally, high-frequency stimulation paradigms have been shown to produce this effect. Classically, an increase in synaptic strength of AMPAR-mediated responses after such an induction paradigm is referred to as long-term potentiation (LTP), which has been shown, using NMDAR antagonists and/or intracellular Ca^{2+} chelation, to be dependent on NMDAR-mediated Ca^{2+} entry (Durand et al., 1996; Abrahamsson et al., 2007; Liao and Malinow, 1996; Puglia and Valenzuela, In press). This raises an interesting question: if synapses are indeed truly postsynaptically silent, how then is the voltage-dependent Mg^{2+} block of NMDARs alleviated in developing postsynaptic neurons? As previously mentioned, depolarizing GABAergic transmission and GDP's could be a potential mechanism (Leinekugel et al., 1997; Kasyanov et al., 2004). Another possible solution is the expression of NMDARs that have reduced sensitivity to Mg^{2+} blockade, such as those containing the NR2D subunit (Arvanian et al., 2004; Okabe et al., 1998). Additionally, the membrane potential at synapses may be more depolarized with respect to the soma, leading to removal of the Mg^{2+} block (Williams and Mitchell, 2008). In the hippocampus, Ca^{2+} -permeable AMPARs (i.e. GluR2-lacking) expression have also been shown to be developmentally regulated, with increased levels during the first 2 postnatal weeks (Zhu et al., 2000). This subset

of AMPARs has been shown to be inserted into the immature synapse in response to spontaneous activity and these AMPARs then get replaced in an activity-independent manner by Ca^{2+} -impermeable AMPARs (GluR2-containing), likely preserving synaptic strength (Zhu et al., 2000). Similar increases in synaptic Ca^{2+} -permeable AMPAR expression and replacement by Ca^{2+} -impermeable AMPARs have been observed in studies of LTP in more mature animals (Plant et al., 2006) but see (Adesnik and Nicoll, 2007). Ca^{2+} influx through this subset of AMPARs may provide additional signaling, facilitating the transition to the mature synaptic phenotype (Molnar et al., 2002; Pickard et al., 2000).

To the best of my knowledge, there is only one study on the effects of EtOH on synaptic plasticity mechanisms during the third-trimester equivalent period. Acute EtOH application was shown to strengthen AMPAR-mediated synaptic responses (Mameli and Valenzuela, 2006). This was done using a minimal stimulation paradigm that does not evoke a post-synaptic response (or very few responses); however, when acute EtOH (15 – 75 mM) was applied, there was an increase in AMPAR-mediated responses and a decrease in the failure rate of responses in the CA1 hippocampal region (Mameli and Valenzuela, 2006). This effect was developmentally regulated, and was not observed after P5 (Mameli and Valenzuela, 2006).

Summary

The third trimester-equivalent is a period of intense synaptic formation and refinement that has shown acute sensitivity to EtOH. Animal models of EtOH exposure during this period demonstrate lasting impairments in behavioral tests of learning and memory, which probe hippocampal function (Thomas et al., 2002; Thomas et al., 2004; Goodlett and Johnson, 1997). Several studies have suggested that plasticity mechanisms guide synaptic formation and refinement during this critical period of development (Medina and Krahe, 2008; Cline and Haas, 2008; Constantine-Paton and Cline, 1998; Garaschuk et al., 2000; Voronin and Cherubini, 2004; Nosyreva and Huber, 2005; Yasuda et al., 2008; Yasuda et al., 2003); however, the effects of EtOH on synaptic plasticity in the hippocampus during the third trimester-equivalent period are poorly understood.

Fundamental to synaptic plasticity is the modulation of ionotropic glutamate receptor function. Studies of EtOH's actions on glutamatergic transmission during the third trimester-equivalent period have mainly focused on the CA3 region. During the early portions of the third trimester-equivalent, EtOH was shown to increase the frequency of GDPs in the CA3 hippocampal region and this effect was driven by an enhancement in spontaneous GABA release at interneuron-to-CA3 pyramidal neuron synapses (Galindo et al., 2005). In addition, both during the early and late portions of the third trimester-equivalent period, EtOH has been shown to inhibit glutamate release as well as AMPAR-mediated transmission in the CA3 hippocampal region (Mameli et al., 2005). However, the effects of EtOH on glutamatergic synaptic transmission and plasticity in the CA1 region have only been assessed in the study of (Mameli and

Valenzuela, 2006), which only examined short-term modulation of AMPAR-mediated transmission under conditions of minimal stimulation during the early portion of the third trimester-equivalent. The studies described in this dissertation represent a more thorough characterization of the short- and long-term effects of EtOH on AMPAR and/or NMDAR-mediated synaptic transmission and plasticity in the CA1 region.

2. Goals of this study

1. Does acute EtOH exposure inhibit glutamatergic transmission and plasticity in the CA1 hippocampal region during the late portion of the third trimester-equivalent?

Previous studies have shown that acute EtOH application inhibits glutamate release as well as AMPAR-mediated (but not NMDAR-mediated) transmission in the CA3 hippocampal region during the late portion (i.e. P7-9) of the third trimester-equivalent period (Mameli et al., 2005). However, NMDAR- and AMPAR-mediated responses in pyramidal cells in layers 2/3 of the cortex were insensitive to acute EtOH application in brain slices from P7-9 rat pups, indicating region specific differences of EtOH sensitivity (Sanderson et al., 2009). Therefore, we examined the effect of acute EtOH exposure on NMDAR- and AMPAR-mediated responses and glutamate release in the CA1 hippocampal region. Since synaptic plasticity mechanisms are critical for synapse maturation, we further investigated the acute effect of EtOH exposure on LTP in this hippocampal region. These studies systematically explored the effect of EtOH on glutamatergic signaling and plasticity in the CA1 hippocampal region and provided baseline data necessary required for a better understanding of the chronic EtOH exposure studies described below.

2. Does chronic EtOH exposure impair glutamatergic transmission and plasticity in the CA1 hippocampal region during the late portion of the third trimester-equivalent?

Although fundamental, studies of acute EtOH application do not represent the gradual rise and fall of blood EtOH concentrations that occur with maternal consumption. To more closely mimic repeated *in vivo* EtOH exposure during the third trimester-equivalent period, we used a vapor inhalation paradigm (discussed in the next section). Results from goal 1 above showed that acute EtOH exposure inhibited both AMPAR- and NMDAR-mediated responses. Thus, we hypothesized that repeated *in vivo* third trimester-equivalent EtOH exposure would lead to a compensatory increase in NMDAR and AMPAR function that would result in increased synaptic strength and enhanced LTP during the withdrawal period. These EtOH-induced effects could result in abnormal synapse maturation and/or increased neuronal death in the CA1 region, leading to long-lasting cognitive deficits.

3. Does chronic EtOH exposure affect glutamatergic transmission in the CA1 hippocampal region during the early portion of the third trimester-equivalent?

Acute EtOH exposure has been shown to strengthen AMPAR-mediated responses in the CA1 hippocampal region during the early third trimester-equivalent period, as well as to potentiate network activity in the CA3 region, which provides excitatory input to the CA1 region (Mameli and Valenzuela, 2006; Galindo et al., 2005). During this early developmental period, studies have shown that increases in neuronal activity in hippocampal slice cultures induce synaptic insertion of Ca²⁺-permeable AMPARs into CA1 pyramidal neurons. These receptors are then gradually replaced in an activity independent manner by Ca²⁺-impermeable AMPARs (Zhu et al., 2000). Thus, during the early third

trimester, when synapses are beginning to form, we tested the hypothesis that repeated *in vivo* exposure to EtOH strengthens AMPAR-mediated neurotransmission in the CA1 region via an increase in synaptic expression of Ca²⁺-permeable AMPARs. Studies presented here address AMPAR-mediated synaptic strength, functional expression of Ca²⁺-permeable AMPARs, and acute EtOH effects of AMPAR-mediated responses during the early portion of the third trimester-equivalent. Strengthening of AMPAR-mediated transmission during this period could lead to premature stabilization of synapses and abnormal development of hippocampal circuitry.

3. Overview of approach

The aims of this study address the effects of EtOH during the human third trimester-equivalent period. In rats, this period occurs during the first 10-12 postnatal days of life, and studies presented here focus on this period. The rat model was chosen for several reasons. The majority of studies characterizing developmental hippocampal physiology and the effects of EtOH have largely been carried out in the rat model. In addition, rat metabolism of EtOH more closely resembles that of the human, as opposed to the mouse model where EtOH levels rapidly rise and fall (Livy et al., 2003b). The ease of housing and relative cost, compared to other appropriate models such as the guinea pig, pig, and non-human primate were additional factors for the choice of this model system.

Electrophysiological studies were carried out using the acute brain slice preparation. This procedure of preparing brain slices allows experimentation on otherwise difficult or inaccessible brain regions, including the hippocampus. Although this technique preserves some of the circuitry, inevitably some is lost due to the slicing procedure, and this will be discussed in the limitations section.

In a subset of studies, acute EtOH was bath applied to brain slices. Although the rapid rise of EtOH concentrations (over minutes) does not accurately mimic *in vivo* EtOH pharmacokinetics, this mode of application is a useful tool in determining the basic physiology of EtOH-mediated effects at the

synaptic and cellular levels. In addition, these studies provide data required for the interpretation of chronic *in vivo* EtOH exposure studies.

Various methods exist to model *in vivo* EtOH exposure during the third trimester-equivalent in rats. Several examples are the “pup in the cup” model where surgical implantation of an intragastric tube delivers an EtOH-containing liquid diet, intragastric gavage, and direct injection (Berman and Hannigan, 2000; Ikonomidou et al., 2000). The model we used was an EtOH vapor-inhalation paradigm. Advantages of this are that the dam and pups do not need to be separated, minimizing neonate and maternal stress. In addition, the gradual rise and fall of EtOH levels occurs over hours, mimicking EtOH pharmacokinetics during human consumption (Galindo and Valenzuela, 2006). Limitations exist with all exposure paradigms and those pertinent to studies presented here will be further addressed in the limitations section.

In all of my studies, the range of EtOH concentrations was 20-80 mM. As a reference, the standard legal intoxication limit is 17.4 mM (0.08 g/dL). Studies presented here represent a broad range of maternal consumption levels. If we infer from the studies of maternal EtOH consumption that approximately 1% of women report binge drinking during the third trimester, and this is defined as 5 or more drinks at the same time or over a couple of hours, then approximate maternal blood levels would be estimated to be greater than 33 mM (0.15 g/dL). Although some studies presented here use elevated EtOH concentrations, it is reasonable to estimate that drinking can produce these levels, particularly in alcoholic women. Case reports have shown maternal blood EtOH concentrations

of 57 - 65 mM (0.26 – 0.3 g/dL) at the time of birth, and 67 mM (0.31 g/dL) in the newborn (Fischer et al., 2003; Silva et al., 1987). Thus, studies here are modeling a range of EtOH concentrations achieved by some pregnant women during the third trimester.

4. Ethanol acutely inhibits ionotropic glutamate receptor-mediated responses and long-term potentiation in the developing CA1 hippocampus

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Abstract

Background- Developmental ethanol (EtOH) exposure damages the hippocampus, causing long-lasting alterations in learning and memory. Alterations in glutamatergic synaptic transmission and plasticity may play a role in the mechanism of action of EtOH. This signaling is fundamental for synaptogenesis, which occurs during the third-trimester of human pregnancy (first 12 days of life in rats).

Methods- Acute coronal brain slices were prepared from 7-9 day-old rats. Extracellular and patch-clamp electrophysiological recording techniques were used to characterize the acute effects of EtOH on α -amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptor (AMPA)- and N-methyl-D-aspartate receptor (NMDAR)-mediated responses and long-term potentiation (LTP) in the CA1 hippocampal region.

Results- EtOH (40 and 80 mM) inhibited AMPAR- and NMDAR-mediated field excitatory postsynaptic potentials (fEPSPs). EtOH (80 mM) also reduced AMPAR-mediated fEPSPs in presence of an inhibitor of Ca^{2+} permeable AMPARs. The effect of 80 mM EtOH on NMDAR-mediated fEPSPs was significantly greater in presence of Mg^{2+} . EtOH (80 mM) neither affected the paired-pulse ratio of AMPAR-mediated fEPSPs nor the presynaptic volley. The paired-pulse ratio of AMPAR-mediated excitatory postsynaptic currents was not affected either, and the amplitude of these currents was inhibited to a lesser

extent than that of fEPSPs. EtOH (80 mM) inhibited LTP of AMPAR-mediated fEPSPs.

Conclusions- Acute EtOH exposure during the third-trimester equivalent of human pregnancy inhibits hippocampal glutamatergic transmission and LTP induction, which could alter synapse refinement and ultimately contribute to the pathophysiology of fetal alcohol spectrum disorder.

Keywords: Ethanol, development, plasticity, glutamate, synaptic.

Introduction

Ethanol (EtOH) exposure during development can produce severe and long-lasting deficits in many organs, including the brain (Sokol et al., 2003; Warren and Foudin, 2001). Among the consequences of EtOH-induced brain damage are behavioral disorders, mental retardation, and learning and memory deficits (Warren and Foudin, 2001). Electrophysiological, structural, and behavioral studies performed with human and animal subjects suggest that learning and memory deficits are, in part, a consequence of damage to the hippocampal formation (Berman and Hannigan, 2000; Costa et al., 2000b). Spatial memory tests have demonstrated abnormalities in hippocampal function in children with fetal alcohol syndrome and in animal models of this condition (Hamilton et al., 2003; Johnson and Goodlett, 2002). However, the mechanisms by which EtOH exerts its deleterious effects on the hippocampal formation are not well understood.

Studies from several laboratories suggest that the mechanism of action of EtOH on hippocampal development involves alterations in glutamatergic synaptic transmission (Reviewed in Berman and Hannigan, 2000; Costa et al., 2000b). One study suggested that inhibition of NMDA receptors (NMDARs) induced by acute EtOH administration during the human third trimester-equivalent period of development (i.e., neonatal period in the rat) triggers widespread apoptotic neurodegeneration in many brain regions, including the CA1 hippocampal region (Ikonomidou et al., 2000). However, whether EtOH inhibits NMDAR function in

CA1 neurons during this developmental period has not been tested. Studies from our laboratory indicate that NMDARs in CA3 pyramidal neurons in acute brain slices from neonatal rats are relatively insensitive to EtOH; in contrast, AMPA receptor (AMPA) function and glutamate release were unexpectedly found to be particularly sensitive to EtOH in these neurons (Mameli et al., 2005). In light of these findings with CA3 pyramidal neurons, we hypothesized that acute EtOH exposure inhibits AMPAR function and glutamate release in CA1 pyramidal neurons, without affecting NMDARs.

Since glutamatergic neurotransmission is essential for certain forms of synaptic plasticity, a corollary of this hypothesis is that EtOH exposure should also affect plasticity in developing hippocampal neurons, where this process is thought to play a central role in synaptogenesis (Constantine-Paton and Cline, 1998). Many developing hippocampal Schaffer collateral-CA1 synapses are silent at resting membrane potentials; i.e., they exhibit currents mediated by NMDARs but not AMPARs (Kerchner and Nicoll, 2008; but see Groc et al., 2002). It has been suggested that expression of AMPARs is limited in these immature structures (Fiala et al., 1998; Matsuzaki et al., 2001). Recent evidence indicates that basal activation of NMDARs inhibits AMPAR-mediated responses during the synaptic maturation period (Adesnik et al., 2008; Hall et al., 2007). When NMDARs are activated by strong synchronous activity, they stimulate AMPAR recruitment to the postsynaptic density, contributing to synapse formation (Constantine-Paton and Cline, 1998; Leinekugel, 2003; Citri and Malenka, 2008; Durand et al., 1996; Maletic-Savatic et al., 1999; but see Zito et

al., 2009). Excessive spontaneous excitatory activity in cultured cortical neurons was shown to cause premature synaptic stabilization via AMPAR insertion into silent synapses and it was postulated that this could limit the range of connections in the neuronal network (Voigt et al., 2005). Chronic NMDAR blockade during early development in hippocampal organotypic slice cultures demonstrated a decreased threshold for LTP induction and a shift in baseline synaptic efficacy (Savic et al., 2003). Collectively, these studies suggest that if EtOH produced alterations in activity-dependent synaptic plasticity mechanisms, these could have a negative impact on the maturation of developing neuronal networks.

In this study, we used acute brain slices from postnatal day (P) 7-9 rats and electrophysiological techniques to characterize the acute effects of EtOH on AMPAR- and NMDAR-mediated responses in the CA1 region. We then investigated whether EtOH had any presynaptic effects by measuring the paired-pulse ratios of AMPAR-mediated responses and the presynaptic volley. Lastly, we tested the effects of acute EtOH on LTP induced via high-frequency tetanic stimulation.

Experimental Procedures

Tissue preparation and solutions

EtOH (99.8%) molecular biology grade from Sigma (cat #E7023) was used. Unless indicated, all other chemicals were from Sigma (St. Louis, MO) or Tocris Cookson (Bristol, UK). Timed-pregnant Sprague-Dawley rats were provided by Harlan (Indianapolis, IN). Both male and female rat pups (postnatal day (P) 7-9) were anesthetized with 250 mg/kg ketamine and coronal brain slices (300 μ m) were prepared using a vibratome, as previously described (Mameli et al., 2005). After a recovery period of 45 min at 35-36 °C, slices were stored in artificial cerebrospinal fluid (ACSF) for 1-8 hr at room temperature. ACSF contained the following (in mM): 126 NaCl, 2 KCl, 1.25 NaH₂PO₄, 1 MgSO₄, 26 NaHCO₃, 2 CaCl₂, 10 glucose, and 0.01 gabazine (also known as SR-95531) equilibrated with 95% O₂/5% CO₂. When indicated, the ACSF contained no MgSO₄ and 3 mM CaCl₂ (or 0.5 mM MgSO₄ and 2.5 mM CaCl₂), 50 μ M DL-2-amino-5-phosphonovaleric acid (AP5), 10 μ M 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX), 100 μ M 1-naphthylacetyl spermine trihydrochloride (NASPM), 50 μ M GYKI-53655, 0.5 μ M tetrodotoxin (TTX; Calbiochem, La Jolla, CA) and/or EtOH (20, 40, or 80 mM). Unless indicated, EtOH was bath-applied for 10 min. Animal procedures were approved by the Institutional Animal Care and Use Committee of the University of New Mexico Health Sciences Center and conformed to National Institutes of Health guidelines.

Electrophysiological recordings

Recordings using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA) were performed in the CA1 *stratum radiatum* at 32 °C perfused at 2 ml/min. Recording micropipette glass electrodes had resistances of 2-5 M Ω and were filled with ACSF. AMPAR and NMDAR-mediated fEPSPs were evoked using a concentric bipolar electrode (inner pole 25 μ m; outer pole 125 μ m; Frederick Haer Company, Bowdoinham, ME) placed in the vicinity of the Schaffer collateral fibers, approximately 200 μ m from the recording electrode. Input-output curves were measured at the start of all recordings, and the stimulation intensity was set to produce 40-50% of maximal responses for subsequent experiments. Stimulus duration was 75 μ s and stimuli were delivered at 0.033 Hz. For paired-pulse studies, the interpulse interval was 50 ms. LTP was induced via tetanic stimulation (100 Hz train for 1 s; this train was repeated a total of 3 times at 5 s intervals).

Whole-cell patch-clamp recordings were performed as previously described (Mameli et al. 2005). Recording micropipette glass electrodes had resistances of 4-7 M Ω , and were filled with an internal solution containing (in mM): 95 K-gluconate, 40 KCl, 10 HEPES, 2 MgCl₂, 3 Na₂ATP, 0.4 NaGTP, and 5 QX-314. The pH was 7.2 and the osmolarity was 270 mOsm. Access resistances were between 20-30 M Ω , and recordings were discarded if the access resistance changed more than 20%. The membrane potential was clamped at -65 mV (the liquid junction potential was 11.3; therefore, the corrected voltage was -76.3 mV). For evoked excitatory postsynaptic current

recordings (eEPSCs), stimulus duration, intensity, and frequency were the same as for fEPSP recordings. For perforated-patch recordings, we used the same internal solution (without QX-314) containing amphotericin-B (5-10 $\mu\text{g/ml}$). A stock solution of amphotericin-B (1 mg/mL in dimethylsulfoxide) was prepared every day, sonicated for 10-20 min and maintained under constant vortexing. Amphotericin-B from this stock solution was freshly added to aliquots of internal solution every 1-2 hours. The tips of the glass electrodes were pre-filled with amphotericin-B-free ACSF, and then filled with the amphotericin-B-containing internal solution. A 1-10 G Ω seal was obtained, then the voltage was clamped at -65 mV (the liquid junction potential corrected voltage was -76.3 mV), and the access resistance was monitored for perforation. When access resistances were between 60-80 M Ω , recordings were started and these were discarded if there were sudden changes in access resistance, indicating conversion to the whole-cell configuration. The ACSF contained 10 μM gabazine and 50 μM AP5 for whole-cell and perforated-patch recordings.

Data Analysis

Data were acquired and analyzed with pClamp 9 (Molecular Devices) and GraphPad Prizm 4.0 (San Diego, CA). The short time interval between the presynaptic volley and fEPSP prevented accurate measurement of the slope in some experiments. Therefore, fEPSP amplitude and area were measured. Recordings with greater inter-electrode separation distance ($\sim 400 \mu\text{m}$) were attempted to eliminate volley contamination of the fEPSP; however, the

amplitude of the fEPSP was substantially reduced, making it difficult to assess the inhibitory effect of EtOH. For each experiment, baseline and washout were defined as the average of 10-20 fEPSPs recorded immediately before EtOH application and the last 5-10 min of washout, respectively. For experimental conditions, 10 fEPSPs immediately preceding the start of the washout or further experimental procedures were averaged for analysis. For LTP studies, the percent change from baseline was measured after ≥ 30 min from induction and was the average of 20 fEPSPs. Except for the plasticity studies, the effect of acute EtOH exposure was quantified with respect to the average of baseline and washout responses. The paired-pulse ratio was calculated as the ratio of AMPAR-mediated fEPSP2/fEPSP1 (or eEPSC2/eEPSC1). Statistical analyses were performed by unpaired *t*-test, one sample *t*-test vs. a theoretical mean of zero or 100%, or one-way ANOVA followed by Bonferroni post hoc test; a $p \leq 0.05$ was considered to be statistically significant. Data are presented as mean \pm SEM.

Results

Characterization of input-output relationships for AMPAR- and NMDAR-mediated fEPSPs

To initially characterize AMPAR- and NMDAR-mediated fEPSPs, input-output curves were measured. AMPAR-mediated fEPSPs were recorded in the presence of gabazine (10 μ M; GABA_A receptor blocker) and AP5 (50 μ M; NMDAR blocker) with increasing stimulus intensities (Fig. 1A). NMDAR-mediated fEPSPs were recorded in the presence of gabazine (10 μ M), NBQX (10 μ M; AMPA/kainate receptor blocker) and in Mg²⁺-free ACSF (Fig. 1B). The average stimulus intensities that elicited a 50% of maximal stimulation response were 0.67 ± 0.08 mA and 0.41 ± 0.05 mA for AMPAR- and NMDAR-mediated fEPSPs, respectively (Figs. 1C-D). GYKI-53655 (50 μ M) blocked AMPAR fEPSPs leaving only the presynaptic volley spike (n=4; 1E), confirming that these were mediated by AMPARs. The non-NMDA antagonist, NBQX (10 μ M) produced a similar inhibitory effect (n=6; data not shown). NMDAR-mediated fEPSP responses were inhibited by 50 μ M AP5 (n=8; 1F).

Acute exposure to EtOH inhibited AMPAR- and NMDAR-mediated fEPSPs

To address the effects of acute bath application of EtOH on ionotropic glutamatergic signaling, we first characterized effects on pharmacologically isolated AMPAR-mediated responses. EtOH (80 mM) reversibly inhibited AMPAR-mediated fEPSPs (Fig. 2A). On average, inhibition was $15.88 \pm 1.04\%$ (n= 6; p<0.01 by one-sample *t*-test vs. zero; Fig 2B and 5A). To determine if

Mg²⁺ concentration affects EtOH mediated inhibition, AMPAR-mediated fEPSPs were recorded in the absence of Mg²⁺. EtOH (80 mM) reduced AMPAR-mediated fEPSP amplitude by 9.76 ± 3.60% (n= 6; p<0.05 by one-sample *t*-test vs. zero; Fig. 5A), and this was not different from the inhibition observed in the presence of Mg²⁺ (not significant (N.S.) by one-way ANOVA followed by Bonferroni post hoc test). We further tested the effects of EtOH (80 mM) in adult rats (P 40) and found that it did not significantly affect AMPAR-mediated fEPSP amplitude or area (n=5; N.S. by one-sample *t*-test vs. zero; p<0.01 by Bonferroni post hoc test vs P7-9; Fig. 5A and Table 1).

To determine if Ca²⁺ influx through Ca²⁺-permeable AMPARs alters EtOH sensitivity, we blocked these receptors with the synthetic polyamine NASPM (100 μM) and tested the effects of EtOH on the remaining responses (i.e. mediated by Ca²⁺ impermeable AMPARs). NASPM inhibited the fEPSP amplitude by 18.46 ± 2.85% (n=11; p<0.01 by one-sample *t*-test vs. zero; Fig. 3). In presence of NASPM, EtOH rapidly and reversibly inhibited fEPSP amplitude by 19.32 ± 1.66% (n=11; p<0.01 by one-sample *t*-test vs. zero; Fig. 3 and 5A).

NMDAR-mediated fEPSPs were recorded in Mg²⁺-free ACSF, as described above. Acute EtOH (80 mM) application reversibly inhibited NMDAR-mediated fEPSPs (Fig. 4A). The percent inhibition from the average of baseline and washout was 16.81 ± 2.91% (n=9; p<0.01 by one sample *t*-test vs. zero; Fig. 4C and 5B). Because Mg²⁺ has been shown to modulate NMDAR sensitivity to EtOH, we measured its effect on NMDAR-mediated fEPSPs in ACSF containing 0.5 mM Mg²⁺ and 2.5 mM Ca²⁺ (Fig. 4B) Under these conditions, we found that

80 mM EtOH inhibited the NMDAR-mediated fEPSPs by $29.18 \pm 3.16\%$ ($n=7$; $p<0.01$ by one-sample t -test vs. zero; Fig. 4D and 5B). This effect was significantly larger when compared to NMDAR-mediated fEPSPs recorded in Mg^{2+} -free ACSF ($p<0.05$ by one-way ANOVA followed by Bonferroni post hoc test; Fig. 5B).

Fig. 5 summarizes these effects of 80 mM EtOH on fEPSP amplitude, and also shows the effects of lower EtOH concentrations. Small but significant effects on AMPAR- and NMDAR-mediated fEPSP amplitudes were detected with 40 mM EtOH; however, no significant effects were observed with 20 mM EtOH. Dose dependent effects of EtOH revealed significant differences between 80 mM vs. 20 mM and 40 mM EtOH for both AMPAR- and NMDAR-mediated fEPSPs ($p<0.05$; by one-way ANOVA followed by Bonferroni post hoc test).

Table 1 summarizes the effects of EtOH on field, whole-cell and perforated patch fEPSP or eEPSC recordings analyzed for event area. In virtually all cases, these results were similar to those obtained with amplitude analysis, with the exception that NMDAR-mediated fEPSP area was significantly inhibited by 20 mM EtOH ($n=4$; $p<0.05$ by one-sample t -test vs. zero) but not by 40 mM EtOH exposure ($n=5$; N.S. by one-sample t -test vs. zero; Table 1) in ACSF lacking Mg^{2+} .

Acute EtOH does not affect glutamate release or the presynaptic volley

To assess the effects of EtOH on glutamate release, paired-pulse plasticity of AMPAR-mediated fEPSPs was assessed (Fig. 6). Recordings were

performed in the presence of gabazine (10 μ M) and AP5 (50 μ M). The percent change in the paired-pulse ratio of control recordings ($-2.9 \pm 11.6\%$; relative to the average of the baseline and washout periods in the same slice) was not significantly different from the percent change in the paired-pulse ratio ($4.33 \pm 1.71\%$) recordings obtained in presence of 80 mM EtOH ($n=7-10$; N.S. by unpaired *t*-test). Paired-pulse ratios of AMPAR-mediated eEPSCs were also recorded under whole-cell patch-clamp and perforated-patch conditions. EtOH (80 mM) did not significantly affect the paired-pulse ratios of AMPAR-mediated eEPSCs (whole cell = $0.1 \pm 5.2\%$ data not shown; perforated-patch = $-3.28 \pm 2.3\%$; Fig 6E; N.S. by one sample *t*-test v. zero; $n=4$ for each configuration).

In the whole-cell configuration, 80 mM EtOH did not significantly inhibit eEPSC1 amplitude ($-11.97 \pm 7.90\%$ from the average of baseline and washout by one sample *t*-test v. zero; $n=4$, data not shown). In the perforated-patch configuration, 80 mM EtOH had a small but significant inhibitory effect on eEPSC1 amplitude ($-4.84 \pm 1.3\%$ from the average of baseline and washout by one sample *t*-test v. zero; $n=4$, Fig. 6D-E) and area (Table 1). The percent inhibition of eEPSC1 amplitude (but not area) was significantly smaller than that observed in the AMPAR-mediated fEPSP recordings ($p<0.01$ by unpaired *t*-test). In perforated-patch experiments, EtOH did not significantly affect the holding current ($4.96 \pm 6.45\%$ of control), membrane time constant ($13.2 \pm 8.57\%$ of control) and membrane resistance ($4.9 \pm 2.95\%$ of control) from the average of baseline and washout ($n=4$; N.S. by one sample *t*-test v. zero; membrane time constant and membrane resistance were measured from responses to a 10 mV

hyperpolarizing step of 10 ms duration). Similar results were found for the holding current (6.7 ± 8.9 % of control), membrane time constant (13.57 ± 6.69 % of control) and membrane resistance (4.38 ± 2.36 % of control) in whole-cell recordings ($n=4$; N.S. by one sample t -test v. zero).

To assess the effect of EtOH on presynaptic excitability, measurements of the presynaptic volley were performed (Fig. 7). Recordings were performed in the presence of gabazine (10 μ M), NBQX (10 μ M), and AP5 (50 μ M). EtOH (80 mM) did not significantly affect the presynaptic volley (21.9 ± 10.10 %; $n=7$; N.S. by one-sample t -test vs. zero). As expected, the presynaptic volley was blocked by the Na⁺ channel antagonist, TTX (0.5 μ M). After TTX application, a residual stimulus artifact could be observed, which was subtracted to obtain the isolated presynaptic volley response.

EtOH acutely inhibited LTP induction

To determine the effect of acute EtOH exposure on the induction of LTP, a high-frequency stimulation protocol was used. Experiments were performed in the presence of gabazine (10 μ M) in standard ACSF. LTP induction was blocked by AP5 (50 μ M) plus MK-801 (50 μ M), demonstrating that it was NMDAR-dependent (10.2 ± 14.92 % change in fEPSP amplitude; $n=9$; N.S. by one-sample t -test vs. zero; data not shown). In the absence of EtOH, high-frequency stimulation increased the amplitude of the AMPAR-mediated fEPSP by 25.3 ± 7.33 % from baseline ($n=6$; $p<0.05$; by one-sample t -test vs. zero; Fig. 8A). For EtOH studies, the LTP induction protocol was delivered in the middle of a 10 min

EtOH application. EtOH 40 mM (Fig. 8B) did not significantly inhibit the induction of LTP; the fEPSP amplitude increase in the presence of EtOH (40 mM) was $22.5 \pm 4.18\%$ ($p < 0.01$; $n = 7$ by one-sample *t*-test vs. zero). The LTP induction protocol was repeated in the same slice in the absence of EtOH, and it induced an increase of $29.7 \pm 8.11\%$ from baseline, which was not significantly different than the increase in the presence of 40 mM EtOH (N.S. by paired *t*-test).

For 80 mM EtOH (Fig. 8C), the percent change in AMPAR-mediated fEPSP amplitude was $6.0 \pm 5.39\%$ ($n = 8$; N.S. by one-sample *t*-test vs. zero); the LTP induction protocol was then repeated in the same slice in the absence of EtOH, resulting in a $24.8 \pm 8.43\%$ increase in the amplitude of the fEPSP from baseline ($p < 0.05$ by one-sample *t*-test vs. zero).

We also measured the effect of longer exposure to 80 mM EtOH on LTP. A stable baseline of AMPAR-mediated fEPSPs was recorded and EtOH was bath-applied (Fig. 8D), causing a $14.01 \pm 3.94\%$ reduction (at $t = 13-15$ min) in the fEPSP amplitude ($n = 6$; $p < 0.05$ by one sample *t*-test from baseline). This effect was followed by gradual run-down of the responses, which was observed independently of whether or not high-frequency stimulation was delivered (Fig. 8D). High-frequency stimulation failed to potentiate the fEPSP under these conditions (Fig. 8D).

Table 1 summarizes the effects of EtOH on LTP of fEPSP area. In all cases, the effects of EtOH were similar to its effects on fEPSP amplitude.

Discussion

To the best of our knowledge, this is the first characterization of the acute effects of EtOH on glutamatergic transmission and plasticity in the rat CA1 hippocampal region during the third trimester-equivalent period of development. Two effects of EtOH were demonstrated: 1) inhibition of both AMPAR- and NMDAR-mediated responses and 2) inhibition of NMDAR-dependent LTP. These findings add to growing evidence indicating that alterations in excitatory amino acid-mediated neurotransmission play a role in the pathophysiology of fetal alcohol spectrum disorder (Berman and Hannigan, 2000; Costa et al., 2000b; Kimura et al., 2000; Olney, 2004; Thomas and Riley, 1998; Valenzuela et al., 2007).

Acute EtOH exposure inhibits AMPAR-mediated fEPSPs

Acute inhibitory effects of EtOH have been observed on AMPAR-mediated responses evoked by exogenous agonist application in developing and mature neurons in different brain regions (Carta, et al, 2003; Costa, et al, 2000; Hsiao, and Frye, 2003; Lovinger, et al, 1989; Lu, and Yeh, 1999; Martin, et al, 1995; Moykkynen, et al, 2003; Nie, et al, 1994; Valenzuela, et al, 1998). EtOH has also been shown to acutely inhibit the function of non-NMDA glutamate receptors in central amygdala synapses from adult rats (Zhu et al., 2007; Roberto et al., 2004). In contrast to these studies, AMPAR function has been shown to be relatively insensitive to acute EtOH exposure in CA3 pyramidal and dentate granule neurons from juvenile or adult rats (Weiner et al., 1999; Ariwodola et al.,

2003). Similar findings have been obtained in the CA1 hippocampal subfield, where Lovinger et al. (1990) were the first to study the effect of EtOH on synaptic AMPAR function using the acute slice preparation; these investigators reported that 50 mM EtOH exposure did not significantly affect non-NMDAR-mediated fEPSPs and that 100 mM EtOH reduced the amplitude of these events by only ~10%. Similarly, Randall et al. (1995) found that non-NMDAR-mediated synaptic responses were not affected by 22 mM EtOH at Schaffer collateral-to-CA1 synapses from 130-160 g rats, and Carta et al. (2003) demonstrated that 80 mM EtOH did not inhibit AMPAR-mediated EPSCs in CA1 pyramidal neurons from P21-40 rats. Durand et al. (1981) also demonstrated that EtOH (100 mM) had little effect on the amplitude of fEPSPs that were, in part, mediated by AMPARs in the CA1 hippocampal region of adult rats. A 30 min application of 100 mM EtOH inhibited non-NMDAR-mediated fEPSPs by ~20% in the CA1 hippocampus of C56Bl/6 mice (Gordey et al., 2001). In addition, EtOH (25-75 mM) did not affect the amplitude of AMPAR-mediated miniature EPSCs in CA1 pyramidal neurons of the rat hippocampus from P12-20 rats (Hendricson et al., 2003).

In contrast to the findings of the studies described above, we found that acute EtOH exposure inhibits non-NMDAR-mediated fEPSPs in the CA1 region of neonatal (but not adult) rats, where EtOH had a small but significant effect at a concentration as low as 40 mM. Under our recording conditions, non-NMDAR-mediated fEPSPs were abolished by the selective AMPAR antagonist, GYKI-53655, indicating that kainate receptors do not contribute to the generation of these events. It should be noted, however, that the effects of EtOH on AMPAR

function were either undetectable or less robust under somatic voltage-clamp conditions. In the whole-cell configuration, 80 mM EtOH had no significant effect on eEPSC amplitude, and, in the perforated-patch configuration, it had a smaller action than in dendritic field recordings. These findings suggest that: 1) intracellular signal transduction pathways that are disrupted during whole-cell recording are required for EtOH's action, 2) EtOH may preferentially act on dendritic AMPARs that are not sampled under somatic patch-clamp recordings (Williams, and Mitchell, 2008), and/or 3) EtOH may affect non-AMPAR-mediated components of the fEPSP—for instance, those mediated by leak channels or capacitive currents. This possibility is unlikely given that EtOH did not affect the holding current, membrane time constant, or membrane resistance.

Previous studies have suggested that AMPAR subunit composition and postsynaptic proteins involved in AMPAR function are developmentally regulated in the hippocampus (CA1), which may explain the increased EtOH sensitivity of AMPARs at P7-9 (Elias et al., 2008; Zhu et al., 2000; Fukaya et al., 2006; Rouach et al., 2005; Morimoto-Tomita et al., 2009; Sager et al., 2009). In addition, other factors, such as neurosteroids, play a role in the effects of EtOH in developing CA1 pyramidal neurons. Studies from our laboratory previously showed that EtOH strengthens AMPAR-mediated synaptic transmission in CA1 pyramidal neurons from P3-4 (but not P6-10 rats) under conditions of minimal stimulation; this effect was mediated by an endogenous pregnenolone sulfate-like neurosteroid that acts at the presynaptic level (Mameli et al., 2005; Mameli and Valenzuela, 2006; reviewed in Valenzuela et al., 2007). Thus, EtOH can

either potentiate or inhibit CA1 AMPAR-mediated synaptic transmission during the early or late portions of the third trimester-equivalent period of rat development. We previously detected relatively potent inhibitory effects of EtOH on AMPAR-mediated synaptic transmission in CA3 pyramidal neurons of the developing hippocampus (Mameli et al., 2005), suggesting that immature CA1 and CA3 AMPARs may share some of the properties that confer EtOH sensitivity and this should be explored in the future. However, synaptic AMPARs are not sensitive to EtOH in all developing brain regions and under all experimental conditions, as shown by the voltage-clamp studies presented here and our recently reported results with neocortical layer II and III pyramidal neurons from P7-9 rats (Sanderson et al., 2009).

Ca²⁺ influx through AMPARs is not required for modulation by EtOH

A previous study with recombinant AMPARs expressed in *Xenopus* oocytes found that EtOH inhibited Ca²⁺-permeable (i.e. containing GluR1 + GluR3 subunits) and Ca²⁺-impermeable (containing GluR2 + GluR3 subunits) receptors to a similar extent. However, for GluR1 + GluR3 receptors, greater EtOH inhibition was observed when the only permeant ion was Ca²⁺ (Dildy-Mayfield and Harris, 1995). These findings suggest that increased intracellular Ca²⁺ levels modulate AMPAR sensitivity to EtOH. Consequently, we tested the effect of EtOH on synaptic AMPARs in the presence of an antagonist of Ca²⁺-permeable AMPARs. The functional expression ratio of Ca²⁺-permeable to -impermeable AMPARs was approximately 1/5, consistent with a previous report

(Pickard et al., 2000). In the presence of NASPM, an inhibitor of Ca^{2+} -permeable AMPARs, EtOH reduced the fEPSP amplitude to a similar extent to controls. These results suggest that EtOH does not preferentially inhibit Ca^{2+} -permeable AMPARs in developing CA1 hippocampal synapses, and Ca^{2+} influx through Ca^{2+} -permeable AMPARs does not affect the sensitivity to EtOH. However, we were unable to directly test the effects of EtOH on Ca^{2+} -permeable AMPARs due to the lack of availability of pharmacological agents to isolate these receptors. Studies using molecular biological approaches and/or genetically modified mice could be used to further address this issue.

Acute EtOH exposure inhibits NMDAR-mediated fEPSPs

We found that acute EtOH inhibits NMDAR-mediated fEPSPs in the CA1 region of neonatal rats; small but significant effects were detected at EtOH concentrations of 40 mM (~5%) and 80 mM (~15%). Our findings are in general agreement with those of Gordey et al. (2001) who showed that 100 mM EtOH inhibits NMDAR-mediated fEPSPs by ~20% in the CA1 region of slices from P4-7 C56Bl/6 mice. Other slice electrophysiological studies performed in our laboratory have investigated the modulation of NMDARs by EtOH in pyramidal neurons from another hippocampal subfield; i.e., the CA3 region. In CA3 pyramidal neurons from P5 rats, currents evoked by exogenous application of NMDA were significantly inhibited (~10%) by 75 mM EtOH, but were unaffected by lower EtOH concentrations (Mameli et al., 2005). In slices from older rats (P9-10), 50 mM EtOH significantly inhibited (~8%) exogenous NMDA-evoked

currents in these neurons (Mameli et al., 2005). Although these results are in general agreement with the findings of the present study, it must be kept in mind that currents evoked by exogenous NMDA are likely mediated by synaptic and extrasynaptic NMDARs. We examined the effect of EtOH on NMDAR currents triggered by synaptic glutamate release in CA3 pyramidal neurons and found that these are inhibited (up to 40%) by 50 mM EtOH in slices from P3-10 rats; however, this effect is likely a consequence of an EtOH-induced decrease of glutamate release as discussed in more detail below (Mameli et al., 2005). In the present study, we did not detect an effect of EtOH on glutamate release in the CA1 region, but did find an effect on NMDAR-mediated fEPSPs. These findings indicate that the mechanism by which EtOH modulates NMDARs in the neonatal hippocampus is regionally specific; i.e., it predominantly inhibits NMDAR-mediated responses in the CA1 and CA3 region via postsynaptic and presynaptic mechanisms, respectively.

The magnitude of inhibition of synaptic NMDAR-mediated responses in neonatal CA1 pyramidal neurons reported here is slightly lower than that observed in some studies with acute slices from juvenile or adult animals. For instance, Lovinger et al. (1990) demonstrated that 25-100 mM EtOH inhibits NMDAR-mediated fEPSPs by ~20-50% in slices from adult rats. Swartzwelder et al. (1995) reported that CA1 NMDAR-mediated fEPSPs were inhibited by ~30% and 40% in P20-25 rat slices acutely exposed to 30 and 100 mM EtOH, respectively. Similarly, 80-100 mM EtOH was shown to inhibit CA1 NMDAR-mediated fEPSPs by ~50% in slices from P21-26 C57BL/6J mice (Suvarna et al.,

2005; Yaka et al., 2003) and by ~20-30% in slices from adult $+/-fyn^Z$ mice (Miyakawa et al., 1997). The relative insensitivity to EtOH of NMDARs in neonatal CA1 pyramidal neurons could be attributed to expression of NR2D-containing NMDARs, which have been shown to be less sensitive to EtOH inhibition (Chu et al., 1995). However, NMDARs containing the NR2D subunit may not be present in the CA1 hippocampus from rats older than P6 (Mameli et al., 2005). Therefore, differences in the subunit composition, phosphorylation state or association with other proteins between NMDARs in neurons from neonatal vs. more mature animals could be responsible for age-dependent differences in EtOH sensitivity in the CA1 region (Elias et al., 2008; Yasuda et al., 2003). Another reason could be related to Mg^{2+} concentration in the ACSF. Morrisett et al. (1991) reported that EtOH inhibits NMDAR-mediated fEPSPs in slices from adult rats with an EC_{50} of ~50 mM in the presence of 1 mM Mg^{2+} and an EC_{50} of 100 mM in its absence. Jin et al. (2008) also observed enhanced inhibition of recombinant NMDARs in the presence of Mg^{2+} . In agreement with these findings, we found that 80 mM EtOH had a significantly greater inhibitory effect on NMDAR-mediated fEPSPs when recorded in the presence of 0.5 mM Mg^{2+} than under 0 mM Mg^{2+} conditions.

Lack of Presynaptic Effects of EtOH

We show here that EtOH acutely reduces the amplitude of both AMPAR- and NMDAR-mediated fEPSPs in neonatal CA1 pyramidal neurons. However, it did not affect the paired-pulse ratio of AMPAR-mediated fEPSPs or eEPSCs

under whole-cell patch-clamp and perforated-patch clamp conditions. Since alterations in paired-pulse plasticity are an indicator of changes in transmitter release (Zucker and Regehr, 2002), these findings suggest that EtOH does not affect glutamate release at Schaffer collateral-to-CA1 pyramidal neuron synapses from P7-9 rats. As mentioned above, EtOH induced a neurosteroid-dependent increase in glutamate release at these synapses in slices from younger rats (P3-4) (Mameli et al., 2005; Mameli and Valenzuela, 2006; Reviewed in Valenzuela et al., 2007). Therefore, acute EtOH exposure exerts developmentally-regulated actions on glutamate release in this hippocampal region. A similar observation was made in the CA3 hippocampal region where EtOH acutely inhibits glutamate release in slices from rats younger than P10; this effect is mediated by inhibition of presynaptic N-type Ca^{2+} channels (Mameli et al., 2005). Previous studies have shown that EtOH affects glutamate release in several brain regions, including the CA1 hippocampal region from more mature rats (reviewed in Siggins et al., 2005). In cultured hippocampal CA1 neurons, EtOH decreased mEPSC frequency without affecting amplitude, suggesting that it inhibits glutamate release; this effect was shown to be mediated by endocannabinoid release triggered by an elevation in postsynaptic Ca^{2+} levels (Basavarajappa et al., 2008). EtOH inhibited KCl-induced vesicular FM1-43 destaining in the CA1 *stratum radiatum* of P21-28 rats, and this effect was occluded by antagonists of N-type and P/Q-type Ca^{2+} channels; EtOH also reduced frequency of AMPAR-mediated mEPSCs in the presence, but not in the absence of KCl (Maldve et al., 2004). Moreover, EtOH decreased both the

frequency of asynchronous NMDAR-mediated mEPSCs and the paired-pulse ratio of NMDAR-mediated EPSCs in CA1 pyramidal neurons (Hendricson et al., 2004). Future experiments should determine whether the lack of a presynaptic effect of EtOH in the neonatal CA1 region is, in part, a consequence of a unique pattern of voltage-gated Ca^{2+} channel expression in glutamatergic axonal terminals.

Acute EtOH Exposure Inhibits NMDAR-dependent LTP

We found that the induction of LTP was suppressed by application of 80 mM EtOH. This was not observed at a concentration of 40 mM. These findings are in agreement with the literature and indicate that exposure to relatively high EtOH concentrations is required to inhibit LTP in the CA1 hippocampal region (Izumi et al., 2007; Schummers et al., 1997; Sinclair and Lo, 1986; Sugiura et al., 1995; Tokuda et al., 2007; Zhang and Morrisett, 1993; Zhang et al., 2005). One paper showed that LTP was virtually abolished by 60 mM EtOH in slices from P15-25 rats, but not P70-100 rats (Swartzwelder et al., 1995). However, other studies have reported more potent effects of EtOH on LTP in the rat CA1 hippocampal region than those reported here. This could be a consequence of differences in experimental conditions (i.e., slice preparation protocol, recording temperature, LTP induction paradigm, and age of rats) (Randall et al., 1995; Blitzler et al., 1990; Pyapali et al., 1999; Fujii et al., 2008). For instance, in some but not all cases, LTP was induced by theta burst stimulation, and in other studies there were variances in the number of pulses in the LTP induction train

(Pyapali et al., 1999; Fujii et al., 2008). Future studies should address the effect of EtOH on neonatal LTP evoked by different patterns of stimulation.

The mechanism by which EtOH blocks LTP in the developing CA1 hippocampus may involve inhibition of NMDARs (Blitzer et al., 1990) because the dose-dependency of EtOH's effect on NMDAR-mediated fEPSPs matches that of its effect on LTP. Since our studies were performed in presence of gabazine, we did not investigate the contribution of an EtOH-induced increase in GABA_A receptor-mediated transmission. However, future studies should examine a potential participation of GABA_A receptors in the mechanism of action of EtOH. Studies suggest that EtOH could indirectly inhibit NMDAR-mediated responses and LTP by increasing GABAergic transmission (Schummers et al., 1997; Schummers and Browning, 2001). GABAergic neurosteroids were recently shown to mediate the effects of EtOH on LTP (Izumi et al., 2007). During the third-trimester equivalent, GABA_A receptors will likely have a dual excitatory and inhibitory action and may actually facilitate LTP induction under some conditions (Caillard et al., 1999). In addition, the inhibitory actions of EtOH on LTP were occluded by an angiotensin 1 receptor blocker, and this receptor may also be involved in the mechanism by which EtOH affects LTP during the neonatal period of rat development (Wayner et al., 1993). Inhibition of AMPARs could also contribute to LTP blockade because these receptors are needed for removal of Mg²⁺ block from NMDARs (reviewed in Kerchner and Nicoll, 2008). LTP during this neonatal period is not dependent on CaMKII as in more mature animals, but requires protein kinase A (Yasuda et al., 2003). EtOH may also interfere with

these pathways uniquely involved in developmental LTP (Newton and Messing, 2006).

A limitation of our study is that the EtOH concentration increased in the recording chamber over a short period of time (2-3 min). Human consumption of alcoholic beverages does not cause such a sudden increase in brain EtOH levels, even during binge drinking. Tokuda et al., (2007) showed that a gradual increase in EtOH concentration induces the emergence of an NMDAR- and Ca^{2+} channel-independent form of synaptic plasticity that depends on Ca^{2+} release from internal stores. Future studies should examine if EtOH produces a similar effect in developing neurons. Moreover, to further assess the effects of gradual increases in EtOH concentrations, the effects of EtOH exposure on developmental LTP *in vivo* should also be explored in the future.

Implications of Findings

Plasticity of glutamatergic transmission may contribute to the maturation of developing neuronal networks, for example, by stabilizing newly formed synapses (Constantine-Paton and Cline, 1998; Leinekugel, 2003; Zhu et al., 2000; Molnar et al., 2002). Alterations in these processes likely contribute to the long-lasting effects of EtOH on the CA1 hippocampus of rodents exposed to EtOH during the third trimester-equivalent of human pregnancy. For instance, abnormal circuit formation secondary to LTP blockade in the CA1 region could explain the deficits in input/output curves detected in this hippocampal subfield in P45-60 rats that were exposed to EtOH vapor at P4-6 (Bellinger et al., 1999).

EtOH inhibition of NMDAR-mediated responses could trigger apoptosis in the hippocampal CA1 region (Ikonomidou et al., 2000), although this mechanism may not apply to other regions such as layer II/III of neocortex or CA3 pyramidal neurons (Mameli et al., 2005; Sanderson et al., 2009). Alternatively, EtOH-induced alterations in glutamatergic transmission could contribute to the neurogenesis or neuronal proliferation deficits that may be responsible for decreased pyramidal neuronal numbers in the CA1 hippocampal region of rats exposed to EtOH during the neonatal period (Livy et al., 2003; Miller, 1995; Martel et al., 2009). In addition, EtOH-induced NMDAR inhibition during the neonatal period could contribute to the compensatory upregulation of these receptors that has been detected during EtOH withdrawal (Thomas et al., 2004). It can be concluded that therapeutic interventions that prevent the EtOH-induced inhibition of glutamatergic transmission and plasticity in the CA1 region during the third trimester of pregnancy could mitigate the learning and memory alterations associated with fetal alcohol spectrum disorder.

Figure Legends

Figure 1. AMPAR- and NMDAR-mediated fEPSP input-output curves in the CA1 hippocampus. Sample traces of (A) AMPAR- and (B) NMDAR-mediated fEPSPs evoked at increasing stimulus intensities via stimulation of the Schaffer collateral fibers. Events were recorded in the *stratum radiatum* in the presence of gabazine (10 μ M) and AP5 (50 μ M) for AMPARs, and in the presence of gabazine (10 μ M), NBQX (10 μ M), and absence of Mg^{2+} for NMDARs. Pooled data for (C) AMPAR- and (D) NMDAR-mediated fEPSP input-output curves. (E) AMPAR- and (F) NMDAR-mediated fEPSPs were blocked with GYKI-53655 (50 μ M) and AP5 (50 μ M), respectively.

Figure 2. EtOH (80 mM) inhibits AMPAR-mediated fEPSPs. (A) Sample traces of inhibitory effects of EtOH on AMPAR-mediated fEPSP amplitudes recorded in the presence of gabazine (10 μ M) and AP5 (50 μ M). (B) Time course of the effect of EtOH.

Figure 3. EtOH sensitivity of AMPAR-mediated fEPSPs is not affected by inhibition of Ca^{2+} permeable AMPARs. (A) Sample traces demonstrating the effects of NASPM (100 μ M) on AMPAR-mediated fEPSPs (10 μ M gabazine and 50 μ M AP5). Also shown is the EtOH (80 mM)-induced reversibly inhibition of fEPSPs in the presence of NASPM. (B) Time course of the effect of NASPM and EtOH.

Figure 4. EtOH inhibits NMDAR-mediated fEPSPs. Sample traces of inhibitory effects of 80 mM EtOH on the amplitude of NMDAR-mediated fEPSPs recorded in the presence of gabazine (10 μ M), NBQX (10 μ M), in Mg²⁺-free ACSF (A) and 0.5 mM Mg²⁺ ACSF (B). Time courses of the effects of EtOH in Mg²⁺-free ACSF (C) and 0.5 mM Mg²⁺ ACSF (D).

Figure 5. EtOH dose dependently inhibits AMPAR- and NMDAR-mediated fEPSPs. (A) The effects of 20, 40, and 80 mM EtOH are shown as percent inhibition from the average of baseline and washout. AMPAR-mediated fEPSPs were recorded in the presence of gabazine (10 μ M) and AP5 (50 μ M) in standard ACSF. In addition, AMPAR-mediated fEPSPs were recorded in Mg²⁺-free ACSF. Also illustrated is the effect of EtOH on AMPAR-mediated fEPSPs in presence of NASPM, as well as the effect of EtOH on AMPAR-mediated fEPSPs in slices from adult rats. (B) Effect of EtOH on NMDAR-mediated fEPSPs recorded in Mg²⁺-free ACSF, and NMDAR fEPSPs recorded in 0.5 mM Mg²⁺ ACSF. *p<0.05, and **p<0.01 by one-sample t-test vs. zero. See text for ANOVA results.

Figure 6. Paired-pulse ratios of AMPAR-mediated fEPSPs and eEPSCs were not affected by acute EtOH (80 mM) exposure. Recordings were performed in the presence of gabazine (10 μ M) and AP5 (50 μ M). The interpulse interval was 50 ms. (A) Sample fEPSP traces from control (i.e. no EtOH) and (B) EtOH groups. (C) Time course of the fEPSP paired-pulse ratio for these groups. (D) Sample traces of perforated-patch eEPSC paired-pulse recordings. (E) Summary

of the EtOH-induced percent change of eEPSC1 amplitude and paired-pulse ratio. * $p < 0.01$ by one sample t -test vs. 100%.

Figure 7. Acute EtOH (80 mM) application did not affect the presynaptic volley amplitude. Recordings were performed in the presence of gabazine (10 μ M), NBQX (10 μ M), and AP5 (50 μ M). (A) Sample traces and time course (B) are shown. Presynaptic volleys were blocked by TTX (0.5 μ M). The residual signal corresponds to a portion of the stimulus artifact.

Figure 8. Acute EtOH (80 mM, but not 40 mM) inhibited the induction of LTP in the CA1 hippocampus of P7-9 animals. (A) LTP induction in control slices. The LTP induction protocol (represented by the dashed lines) consisted of 3 one second 100 Hz trains (trains were separated by 5s). Recordings were obtained in the presence of gabazine (10 μ M). Effect of 40 mM (B) and 80 mM (C) EtOH on LTP. The first LTP induction protocol was delivered in the presence of EtOH, and repeated in its absence. (D) Effects of continuous 80 mM EtOH exposure on LTP induction; also shown are control recordings obtained from slices in which LTP was not induced. Note different time scales in x-axis (A-D).

Figure 1

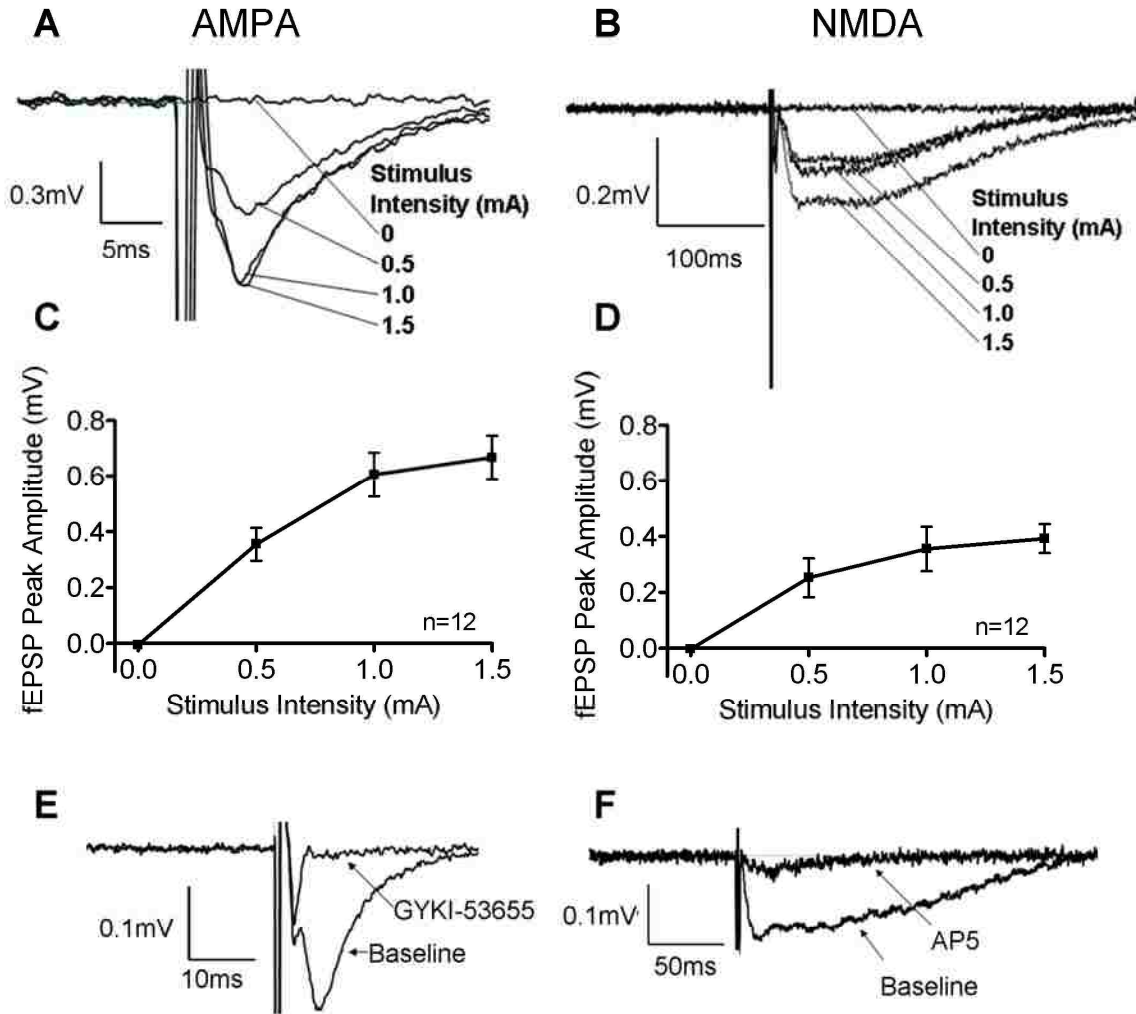


Figure 2

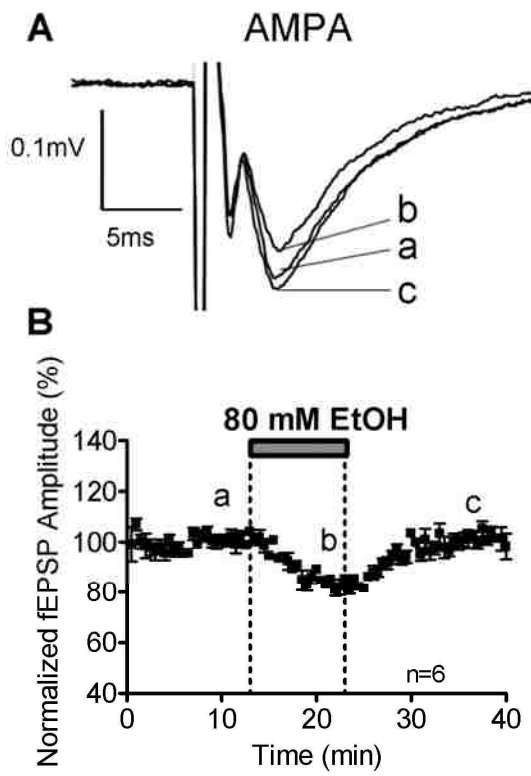


Figure 3

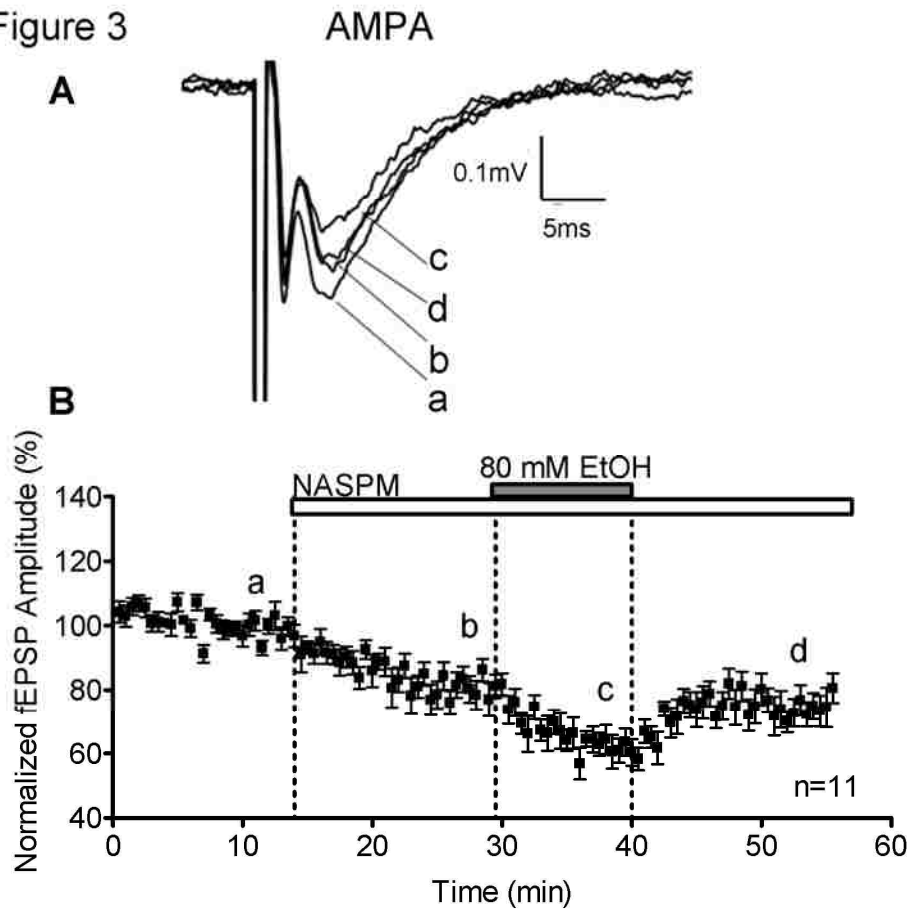


Figure 4

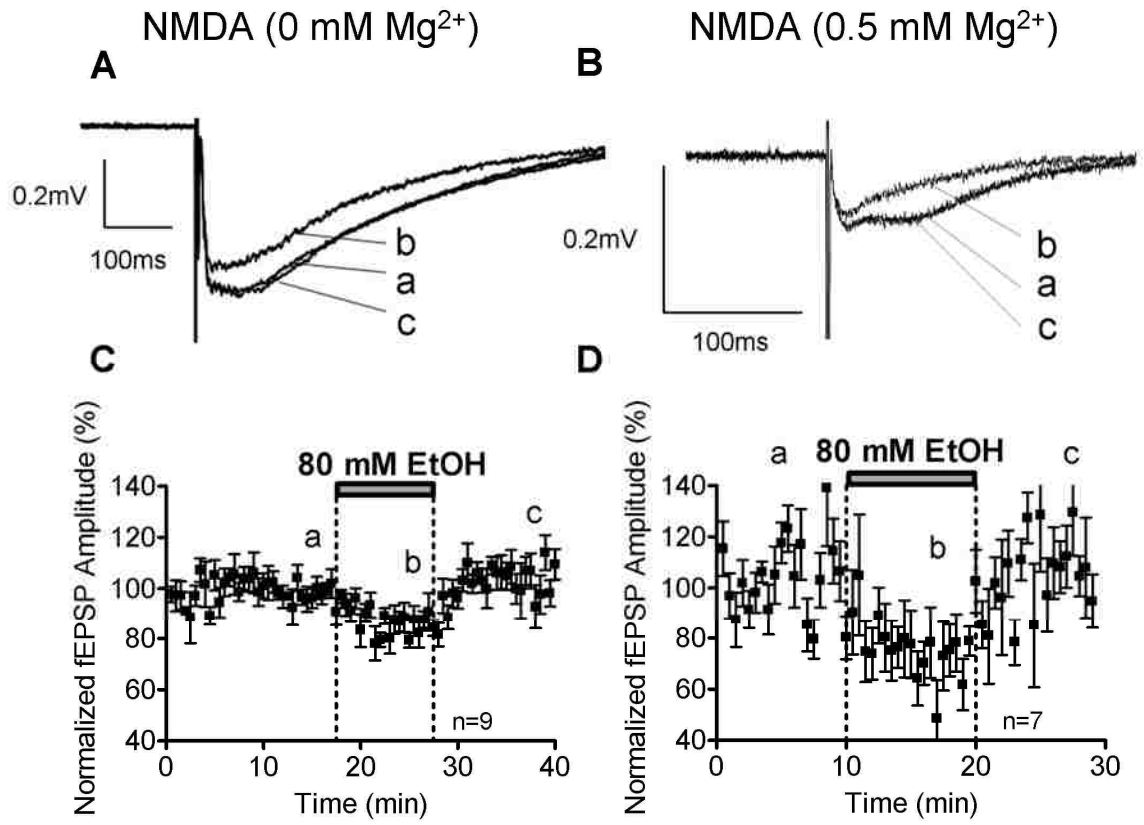


Figure 5

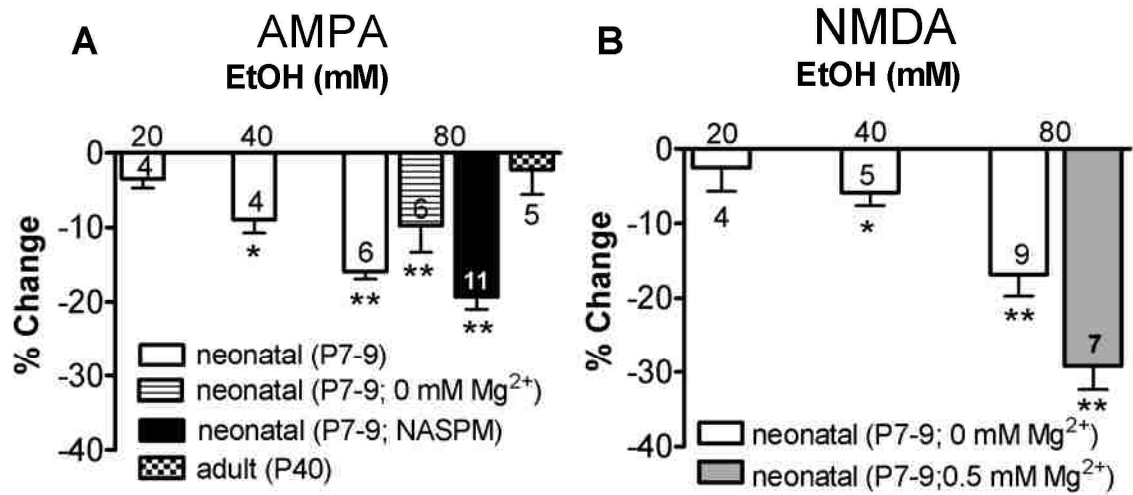


Figure 6

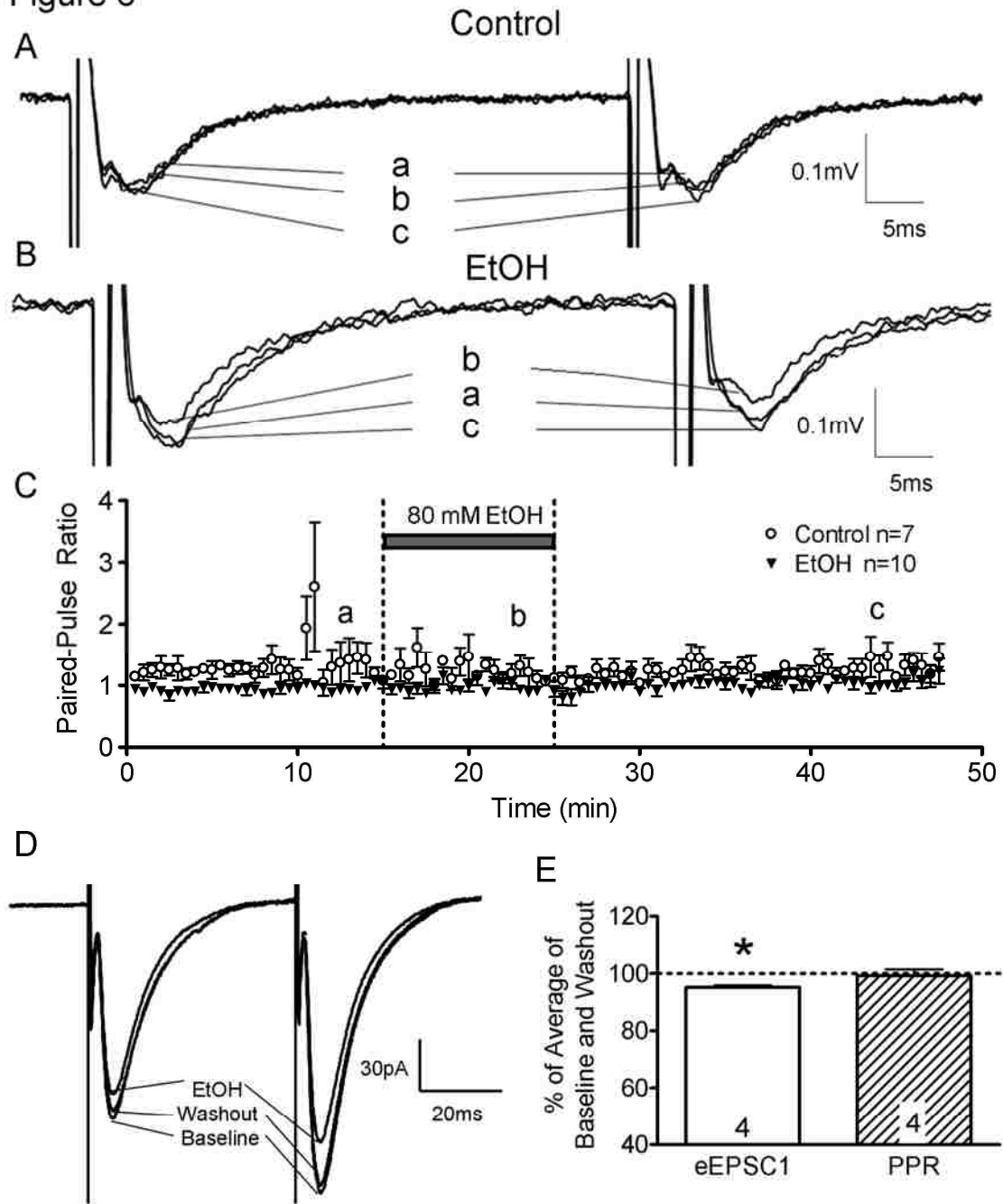


Figure 7

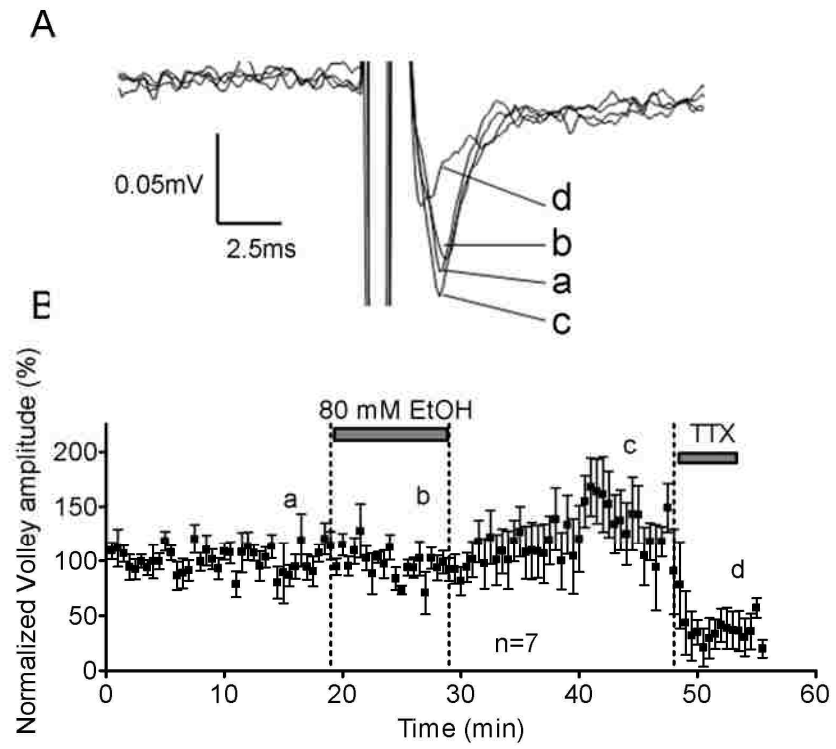


Figure 8

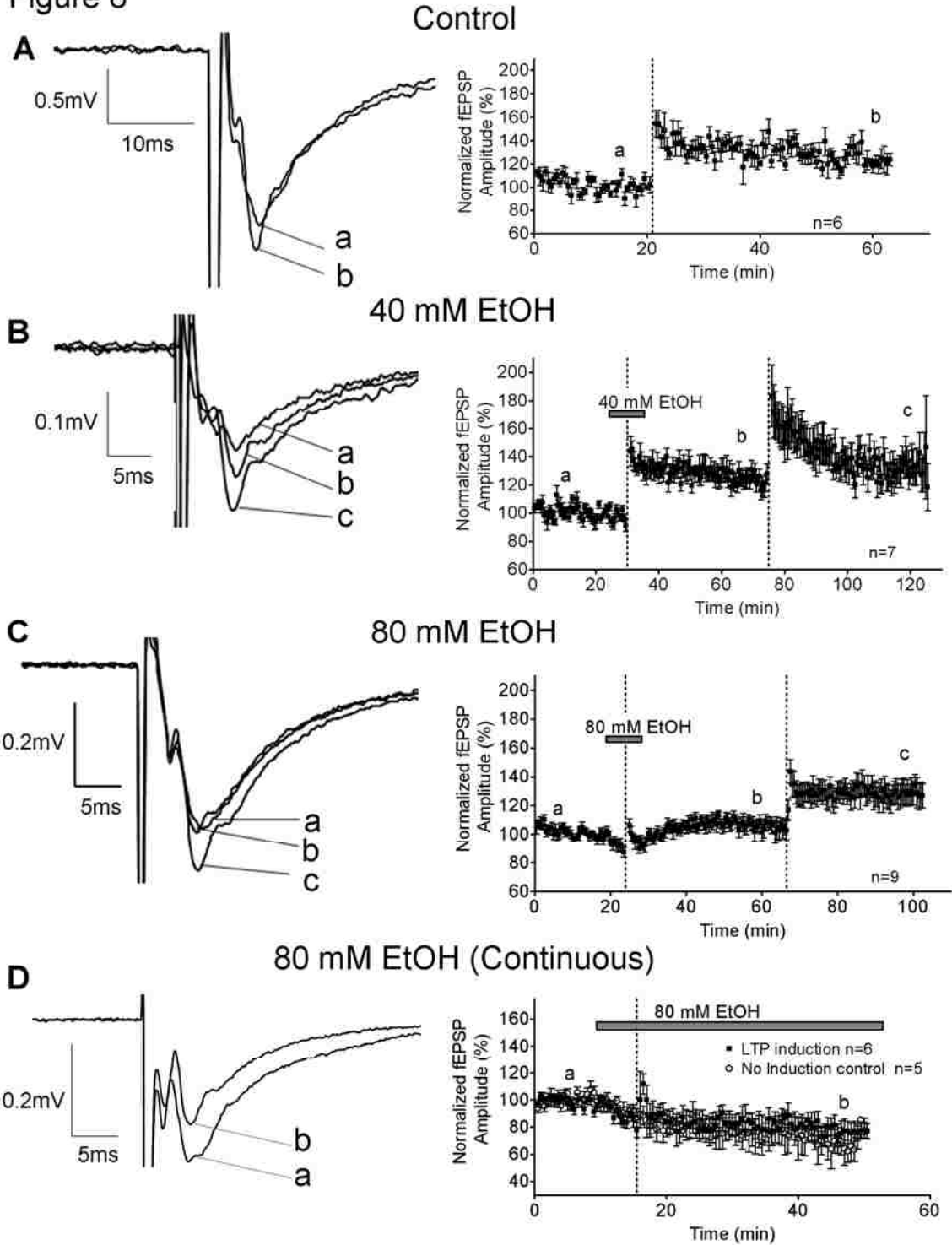


Table 1-Summary of Effects of EtOH on Synaptic Event Area

Experiment	Event	% change	SEM	One sample t-test vs. zero
AMPA				
20 mM EtOH	fEPSP	-3.77	1.70	N.S.
40 mM EtOH	fEPSP	-8.61	0.83	p<0.01
80 mM EtOH	fEPSP	-16.10	1.30	p<0.01
80 mM EtOH (0 Mg ²⁺)	fEPSP	-15.37	4.12	p<0.05
80 mM EtOH (NASPM)	fEPSP	-21.38	1.57	p<0.01
80 mM EtOH (P40 Animal) ^a	fEPSP	-3.17	3.22	N.S.
80 mM EtOH Perforated-Patch	eEPSC	-9.59	1.20	p<0.01
80 mM EtOH Whole-cell	eEPSC	-3.97	9.72	N.S.
AMPA (Pulse 2 / Pulse 1)				
80 mM Control	fEPSP	-2.89	3.18	N.S.
80 mM EtOH ^b	fEPSP	-5.63	2.39	p<0.05
80 mM EtOH (Perforated-Patch)	eEPSC	-3.28	2.30	N.S.
80 mM EtOH (Whole-Cell)	eEPSC	0.10	5.20	N.S.
NMDA				
20 mM EtOH (0 Mg ²⁺)	fEPSP	-6.15	0.72	p<0.01
40 mM EtOH (0 Mg ²⁺)	fEPSP	-23.26	10.80	N.S.
80 mM EtOH (0 Mg ²⁺)	fEPSP	-21.55	2.34	p<0.01
80 mM EtOH (0.5 Mg ²⁺) ^c	fEPSP	-34.40	2.49	p<0.01
LTP				
Control	fEPSP	13.20	4.51	p<0.01
40 mM EtOH ^b	fEPSP	9.30	2.49	p<0.01
40 mM EtOH (washout) ^d	fEPSP	13.50	5.39	p<0.05
80 mM EtOH	fEPSP	2.40	3.28	N.S.
80 mM EtOH (washout) ^e	fEPSP	18.50	6.00	p<0.05

^aAll Other recordings were performed in slices from P7-9 rat pups

^bNot significantly different from control (by unpaired t-test)

^cSignificantly different from 80 mM EtOH 0 Mg²⁺ (by unpaired t-test)

^dNot significantly different than LTP in the presence of 40 mM EtOH (by paired t-test)

^eSignificantly different than LTP in the presence of 80 mM EtOH (by paired t-test)

5. Repeated third trimester-equivalent ethanol exposure inhibits long-term potentiation in the hippocampal CA1 region of neonatal rats

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Keywords: Fetal alcohol syndrome, development, plasticity, glutamate, synaptic.

Abbreviations: α -amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA), N-methyl-D-aspartate (NMDA), Ethanol (EtOH), Long term potentiation (LTP), Postnatal day (P), Artificial cerebrospinal fluid (ACSF), field excitatory postsynaptic potentials (fEPSPs), fetal alcohol syndrome (FAS), fetal alcohol spectrum disorder (FASD), Serum ethanol concentrations (SECs), 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX), DL-2-amino-5-phosphonovaleric acid (AP5), number of determinations (n), protein kinase A (PKA)

(Ready to be submitted. Neurotoxicology and Teratology)

Abstract

Ethanol (EtOH) exposure during development damages the hippocampus, causing long-lasting learning and memory deficits. Synaptic plasticity mechanisms (e.g. long term potentiation—LTP), contribute to synapse formation and refinement during development. We recently showed that acute EtOH exposure inhibits glutamatergic synaptic transmission and N-methyl-D-aspartate (NMDA) receptor-dependent LTP in the CA1 hippocampal region of postnatal day (P) 7-9 rats. Inhibition of NMDA receptors by EtOH during development has been postulated to induce compensatory upregulation of these receptors during EtOH withdrawal periods, leading to excitotoxicity and hippocampal damage. We therefore hypothesized that LTP should be enhanced with repeated *in vivo* EtOH exposure, due to increased NMDA receptor function during periods of early EtOH withdrawal. To test this hypothesis, rat pups were exposed to EtOH vapor (2 or 4.5 g/dL in air; serum EtOH concentrations = 22-32 or 70-86 mM) from P2-9 (4 hrs/day). Brain slices were prepared immediately after the end of the 4 hr exposure on P7-9 and extracellular electrophysiological recordings were performed 1-7 hrs later under EtOH-free conditions to model early withdrawal. LTP was not different than group-matched controls in the 22-32 mM group; however, it was abolished in the 70-86 mM group. Neither α -amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA)/NMDA receptor function nor glutamate release was affected in the 70-86 mM EtOH exposure group. These data suggest that repeated *in vivo* exposure to elevated doses of EtOH during the third trimester-equivalent period impairs synaptic plasticity, which may alter

maturation of hippocampal circuits and ultimately contribute to the long-lasting cognitive deficits associated with fetal alcohol spectrum disorder.

Introduction

Ethanol (EtOH) exposure during fetal development can result in a spectrum of clinical findings (termed fetal alcohol spectrum disorder—FASD), ranging from relatively mild central nervous system impairments, such as learning and memory deficits, to the most severe manifestation of the disorder—fetal alcohol syndrome (FAS). The clinical hallmarks of FAS include characteristic facial dysmorphism, growth retardation, and impaired central nervous system function. EtOH has been shown to affect many brain regions, and one that has been shown to be particularly sensitive is the hippocampus (Berman and Hannigan, 2000). Children and animals developmentally exposed to EtOH exhibit deficits in learning and memory that are, in part, a consequence of impaired hippocampal function (Hamilton et al., 2003; Johnson and Goodlett, 2002).

Although many mechanisms for the neuroteratogenic actions of EtOH have been put forth, one that has received significant research efforts is impaired glutamatergic signaling and synaptic plasticity (Berman and Hannigan, 2000; Medina and Krahe, 2008; Medina et al., 2003; Costa et al., 2000b; Swartzwelder et al., 1995a). During the third trimester-equivalent period (approximately postnatal day (P) 0-10 in rats), intense glutamatergic synapse formation and refinement occur throughout the developing brain, including the hippocampal region, and these processes are thought to be mediated by activity-dependent mechanisms (Durand et al., 1996; Zhu et al., 2000; Constantine-Paton and Cline, 1998; Yasuda et al., 2003; Leinekugel, 2003). The majority of studies addressing

the effects of developmental EtOH exposure on glutamatergic signaling and learning and memory have focused on characterizing long-lasting changes in more mature animals. However, relatively few studies have studied the effects of EtOH during the third trimester-equivalent period.

We have previously shown that acute EtOH exposure inhibits ionotropic glutamate receptor-mediated responses and long term potentiation (LTP) induction in the CA1 hippocampal region of P7-9 rat pups (Puglia and Valenzuela, In press). We hypothesized that repeated EtOH exposure would lead to a compensatory increase in NMDA receptor (NMDAR) and AMPA receptor (AMPA) function that would result in increased NMDAR synaptic strength during the withdrawal period and, as a consequence, enhanced LTP. This hypothesis is supported by studies in which, 6 g/Kg of EtOH was administered on P6 in a binge-like fashion, followed 21 hrs later by MK-801 (an NMDAR antagonist) or saline (Thomas et al., 2002). The inhibition of NMDARs by MK-801 protected against EtOH-mediated deficits in a spatial discrimination reversal learning task, suggesting that NMDAR-mediated upregulation during periods of EtOH withdrawal contributes to the long-term deficits observed in FAS and FASD (Thomas et al., 2002).

An EtOH vapor exposure paradigm was used to model human third trimester EtOH consumption, where rat dams and pups were repeatedly exposed to EtOH vapor from P2-9. Acute brain slices were prepared from P7-9 rat pups and slice electrophysiological techniques were used to investigate LTP and ionotropic glutamate receptor-mediated synaptic transmission.

Experimental Procedures

EtOH vapor chamber exposure paradigm

Animal procedures were approved by the Institutional Animal Care and Use Committee of the University of New Mexico Health Sciences Center and conformed to National Institutes of Health guidelines. Timed-pregnant Sprague-Dawley rats (gestational days 9-17) were obtained from Harlan Laboratories Inc. (Indianapolis, IN). Neonatal rat pups and dams were exposed to EtOH vapor as previously described (Galindo and Valenzuela, 2006; Puglia and Valenzuela, In press). Briefly, litters were culled to 10 pups on P2 and dams and rat pups were exposed daily for 4 hrs per day until P9 (Fig. 1A). Exposures were started at 07:00 hrs (lights on at 06:00 hrs and lights off at 18:00 hrs). Ninety-five percent EtOH (Cat #801VWR Tarr, Phoenix, AZ) was vaporized with a heating flask that was regulated with a peristaltic pump. EtOH vapor mixed with air, or air alone, was delivered to the exposure chamber for the EtOH and control groups, respectively. Control and EtOH litters were paired based upon exposure round, where birthdays were 0-3 days apart, and further referred to as pair-matched. The only mortality in any of the 6 pair-matched groups was 3 pups in one litter from the 70-86 mM EtOH exposure group.

Tissue preparation and solutions

Unless otherwise indicated, chemicals were purchased from Sigma (St. Louis, MO) or Tocris Cookson (Ellisville, MO). Rat pups (male and female) were deeply anesthetized with 250 mg of ketamine / Kg of body weight and euthanized

by decapitation immediately after the 4 hr exposure to air or EtOH on P7-9. Serum was obtained by collecting trunk blood samples, allowing them to coagulate and centrifuging at 2.3 x g for 15 min at 4° C. Serum EtOH concentrations (SECs) were determined using an alcohol dehydrogenase-based assay as previously described (Puglia and Valenzuela, In press). Coronal brain slices (400 µm) were prepared using a vibratome, as previously described (Mameli et al., 2005). After a recovery period of 45 min at 35-36°C, slices were maintained for 1-7 hrs at room temperature. Artificial cerebrospinal fluid (ACSF) contained the following (in mM): 126 NaCl, 2 KCl, 1.25 NaH₂PO₄, 26 NaHCO₃, 1 MgSO₄, 2 CaCl₂, 10 glucose, and 0.01 gabazine (also known as SR-95531) equilibrated with 95%O₂/5%CO₂. When indicated, the ACSF also contained 50 µM DL-2-amino-5-phosphonovaleric acid (AP5), and/or 10 µM 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX) in 0 mM Mg²⁺ (3 mM Ca²⁺).

Electrophysiological recordings

Recordings were performed in the CA1 *stratum radiatum* with a perfusion rate of 2 ml/min at 32°C using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA). Recording micropipette glass electrodes had resistances of 3-5 MΩ and were filled with ACSF. A concentric bipolar electrode (inner pole 25 µm; outer pole 125 µm; Frederick Haer Company, Bowdoinham, ME) was placed in the vicinity of the Schaffer collateral fibers, approximately 200 µm from the recording electrode, to evoke fEPSPs. Input-output curves were measured at the

start of all recordings, and the stimulation intensity was set at 40-50% of that required to produce maximal responses for subsequent experiments. Stimulus duration was 75 μ s and stimuli were delivered at 0.033 Hz. LTP was induced via tetanic stimulation (100 Hz train for 1 s; this train was repeated a total of 3 times at 5 s intervals). For paired-pulse studies, the interpulse interval was 50 ms.

Data Analysis

Data were acquired and analyzed with the aid of pClamp 9 (Molecular Devices) and GraphPad Prism 4.0 Softwares (San Diego, CA). The short time interval between the presynaptic volley and the fEPSP prevented accurate measurement of the slope in most experiments; therefore, data were analyzed using the fEPSP amplitude. For each experiment, baseline was defined as the average of 20 fEPSPs immediately before drug application. For LTP quantification, the percent change from baseline was measured after ≥ 35 min from induction and was the average of 10 fEPSPs.

Statistical analyses of pooled data were performed using GraphPad Prism. A $p \leq 0.05$ was considered to be statistically significant. Shown data are presented as mean \pm SEM, and the number of determinations (n) represents the number of recordings, which were performed in slices from 6-10 pups from 2-3 different litters per treatment condition.

Results

To model EtOH exposure during the equivalent to the human third trimester of pregnancy, rat dams and pups were exposed to air (control) or EtOH via an inhalation paradigm (Fig 1A). Exposures occurred for 4 hrs/day and chamber EtOH vapor levels were 2 or 4.5 g/dL (Fig. 1B), which yielded SEC's of 27 ± 1.7 (21 - 32) and 74 ± 1.9 (70 - 86) mM, respectively (Fig. 1C). This exposure paradigm did not affect rat pup weight gain in the 21-32 mM group; however, in the 70-86 mM group, there was a trend towards reduced pup weights that reached significance at P8 (Fig. 1D).

We addressed the effects of repeated *in vivo* EtOH exposure on LTP in brain slices of P7-9 rat pups in the presence of gabazine (10 μ M; Figs. 2-3). In the 21-32 mM EtOH group, a high frequency LTP induction paradigm significantly increased AMPAR-mediated fEPSP amplitude to 120.4 ± 3.7 % of baseline (at $t = 50-55$ min; Figs. 2A-C). Group matched controls similarly showed an increase to 113.7 ± 5.7 % in AMPAR-mediated fEPSP amplitude with respect to baseline (at $t = 50-55$ min; Figs. 2A-C). The magnitude of LTP induction was not significantly different between 21-32 mM EtOH group and group-matched controls (Fig. 2C).

In rat pups where repeated *in vivo* EtOH exposure led to SECs of 70-86 mM, LTP was significantly impaired, with AMPAR-mediated fEPSP amplitude being 96.1 ± 1.8 % of baseline (at $t = 50-55$ min; Figs. 3A-C). Group-matched controls showed a significant increase to 116.0 ± 5.9 % in AMPAR-mediated fEPSP amplitude with respect to baseline (at $t = 50-55$ min; Figs. 3A-C). The

magnitude of LTP induction was significantly different between the 70-86 mM EtOH group and group-matched controls (Fig. 3C).

To investigate whether the deficits in LTP induction in the 70-86 mM EtOH group were a consequence of alterations in ionotropic glutamatergic signaling, NMDAR- and AMPAR-mediated input-output curves were recorded (Fig. 4). AMPAR-mediated input-output curves were recorded in the presence of gabazine (10 μ M) and AP5 (50 μ M; Fig. 4A). There were no significant differences in the input-output curves between the EtOH and group-matched controls (Fig. 4B). NMDAR-mediated input-output curves were recorded in the presence of gabazine (10 μ M), NBQX (10 μ M) and in the absence of Mg^{2+} (Fig. 4C). There were no significant differences in the input-output curves between the EtOH and group-matched controls (Fig. 4D).

Finally, we investigated whether the impaired LTP in the 70-86 mM EtOH group was a result of alterations in glutamate release by measuring paired-pulse plasticity of AMPAR-mediated fEPSPs (Valenzuela et al., 2007). The paired-pulse ratio is typically inversely related to the probability of glutamate release (Mameli et al., 2005). Paired-pulse ratios were not significantly different between the EtOH and group-matched controls (Fig 5).

Discussion

This study was undertaken to determine if *in vivo* EtOH exposure during the third trimester-equivalent period in rat pups alters glutamatergic signaling and plasticity. The main findings of this study are that exposure to high levels of EtOH inhibits LTP in the developing CA1 hippocampal region and that this is not a consequence of alterations in glutamatergic synaptic efficacy or glutamate release.

We chose an exposure paradigm that resembles repeated maternal EtOH consumption where blood EtOH concentrations gradually rise and fall (Galindo and Valenzuela, 2006). The EtOH vapor exposure paradigm is advantageous because it minimizes stress in dams and neonates by eliminating the need for maternal separation. EtOH exposure leading to SECs of 21-32 mM did not significantly affect pup weight gain over the course of postnatal days 2-9; however, exposure leading to SECs of 70-86 mM reduced weight gain over this period, indicating that this paradigm effectively models FAS, where growth retardation is a requisite for diagnosis. These results are consistent with previous vapor-inhalation paradigms that caused growth retardation following high EtOH exposure during the third trimester-equivalent period (Bellinger et al., 2002). In contrast, the lower exposure dose did not affect weight, possibly mimicking the less severe end of FASD.

Contrary to our hypothesis, LTP was impaired during the early withdrawal period following repeated EtOH exposure. The impaired LTP was observed only in the EtOH exposure group with SECs of 70-86 mM, suggesting that relatively

high doses of EtOH exposure are needed to abolish synaptic plasticity mechanisms during the third trimester-equivalent. These results are consistent with studies of acute EtOH application in brain slices from P7-9 rat pups, where 80 mM application (but not 20 or 40 mM application) inhibited the induction of LTP (Puglia and Valenzuela, In press). In addition, acute EtOH (60 mM) application inhibited CA1 hippocampal LTP in slices from P15-25 rats, but this effect was not observed in P70-100 rats (Swartzwelder et al., 1995a). Furthermore, LTP was not impaired in P40-80 guinea pigs gestationally exposed to EtOH during the third trimester-equivalent period (maternal blood EtOH concentration 245 mg/dL) (Byrnes et al., 2004). Collectively, these studies suggest that LTP is sensitive to acute EtOH exposure in preparations from young rats and that, in the case of neonatal rats, repeated EtOH exposure causes an LTP deficit that persists during withdrawal.

We found no difference in synaptic strength measured by AMPAR- and NMDAR-mediated input-output curves, suggesting that the proposed enhanced excitation occurring during the withdrawal period is not observed in this model of EtOH exposure. This further indicates that synaptic strength is preserved with our repeated EtOH exposure paradigm, possibly through homeostatic plasticity mechanisms (Turrigiano and Nelson, 2004; Hou et al., 2008; Carpenter-Hyland and Chandler, 2006; Swann, 2004). These homeostatic plasticity mechanisms do not appear to be engaged when EtOH is administered in a binge-like fashion to neonatal rats, where excitotoxicity mediated by NMDAR upregulation is the likely mechanism of action of EtOH (Thomas et al., 2002). Future studies should

examine whether exposure paradigms, which involve gradual administration of EtOH, result in persistent learning and memory alterations and if these can be ameliorated by NMDAR antagonists. Importantly, previous studies in P45-60 rats exposed to similar levels of EtOH (approximately 80 mM) during the third trimester-equivalent period (P4-9), exhibited a persistent decrease in fEPSP input-output curves in the CA1 hippocampal region of (Bellinger et al., 1999). Taken together with our results, these findings suggest that homeostatic plasticity mechanisms are only able to transiently maintain glutamatergic synaptic strength and that it eventually becomes impaired during the juvenile period of rat development.

The lack of an effect on glutamate release measured by paired-pulse ratios is consistent with our previously reported effects of acute EtOH in the CA1 hippocampal region in brain slices from P7-9 rat pups (Puglia and Valenzuela, In press). This study, along with the results presented here, suggests that acute or repeated EtOH exposure during the third trimester-equivalent period does not affect glutamate release in the CA1 hippocampal region. However, acute EtOH exposure has been shown to inhibit glutamate release in the CA3 hippocampal region of P3-10 rat pups (but not >P15) by means of inhibition of an N-type Ca^{2+} channel, indicating that there are region specific differences in the effects of EtOH on presynaptic function (Mameli et al., 2005).

We investigated the effects of EtOH on glutamatergic synaptic strength, as well as presynaptic glutamate release, and found that third trimester-equivalent EtOH exposure did not affect either of these parameters. How then might EtOH

be impairing LTP? The time-course of LTP showed that for the first 15-20 min after LTP induction, there was no difference between the EtOH (70-86 mM) and control group; however, after about 20 min, the fEPSPs in the EtOH group rapidly decayed to or below baseline levels, while those in the control group remained potentiated. This indicates that EtOH potentially impairs a process involved in LTP maintenance. A candidate factor that has been shown to be involved in LTP induction and maintenance during this developmental period is protein kinase A (PKA) (Yasuda et al., 2003). The dependence of LTP on PKA activity has been shown to be developmentally regulated, where inhibition of this signaling enzyme leads to impaired LTP in P7-9 rat pups, but not in more mature animals (Yasuda et al., 2003). The time course of LTP in the presence of a PKA inhibitor closely resembles the time course of LTP in the EtOH group (70-86) (Yasuda et al., 2003). Moreover, EtOH has been shown to affect PKA signaling, potentially implicating this molecule as a target of developmental EtOH exposure (Yao et al., 2002).

Future studies should address the mechanism by which this third-trimester EtOH exposure paradigm leads to impaired LTP in brain slices from P7-9 rat pups. We chose to focus on the third trimester-equivalent period because it is associated with intense synaptic formation and refinement; however, since women who consume EtOH are likely to do so throughout pregnancy, future studies should model EtOH exposure through all trimesters of pregnancy. In addition, studies presented here were performed in the presence of gabazine, which blocks GABA_A receptors. Previous studies in mature animals have shown

that effects of EtOH on GABAergic signaling can play a role in alterations in LTP (Schummers and Browning, 2001). Thus, future studies should address developmental effects of EtOH with GABAergic transmission intact.

In conclusion, the studies presented here suggest that a third trimester-equivalent EtOH exposure paradigm leads to impaired synaptic plasticity in the developing CA1 hippocampal region. In addition, these data indicate that not all third trimester-equivalent EtOH exposure paradigms result in enhanced NMDAR function during the withdrawal period. Synaptic plasticity mechanisms are thought to be fundamental for the generation of appropriate synaptic connections during development. Alterations in these processes present one mechanism that could underlie the long-term deficits associated with FASD.

Figure Legends

Figure 1. Third trimester-equivalent EtOH exposure paradigm. (A) Schematic representation of the exposure paradigm where rat pups and their respective dams were repeatedly exposed to EtOH vapor or air (control) for 4 hrs per day, starting on P2 and continuing to P9. Brain slices for electrophysiology studies were prepared from P7, P8 or P9 rat pups, immediately after the 4 hr exposure period. (B) EtOH concentrations in the exposure (vapor) chamber. Note that in most cases error bars are smaller than the symbol. (C) Serum EtOH concentrations measured from trunk blood samples collected immediately after euthanasia. The dashed line represents the legal intoxication limit (0.08 g/dL = 17.4 mM). (D) Average pup weight of control and EtOH vapor-exposed groups as a function of postnatal day. * $p < 0.05$, by Two-way ANOVA followed by Bonferroni post hoc test.

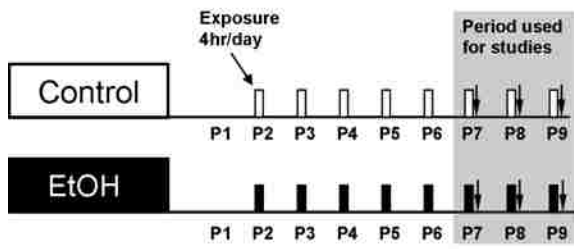
Figure 2. Repeated *in vivo* EtOH exposure does not inhibit LTP in rat pups with SEC's of 21-32 mM. (A) Sample traces of glutamatergic fEPSP-responses from brain slices of control and EtOH-exposed rat pups. Note that letters correspond to time points indicated by the lines in Panel B. Scale bars 0.2 mV/5 ms. (B) Time course of AMPAR-mediated fEPSPs before and after LTP induction in the EtOH and control groups. (C) Summary of AMPAR-mediated fEPSP amplitude after LTP induction ($t = 50-55$ min). * $p < 0.05$ from one-sample t-test from 100%. NS, not significant by unpaired t-test.

Figure 3. Repeated *in vivo* EtOH exposure inhibits LTP in rat pups with SEC's of 70-86 mM. (A) Sample traces of glutamatergic fEPSP-responses from brain slices of control and EtOH-exposed rat pups. Note that letters correspond to time points indicated by the lines in Panel B. Scale bars 0.2 mV/5 ms. (B) Time course of AMPAR-mediated fEPSPs before and after LTP induction in the EtOH and control groups. (C) Summary of AMPAR-mediated fEPSP amplitude after LTP induction ($t = 50-55$ min). * $p < 0.05$ from one-sample t-test from 100%. # $p < 0.05$ by unpaired t-test.

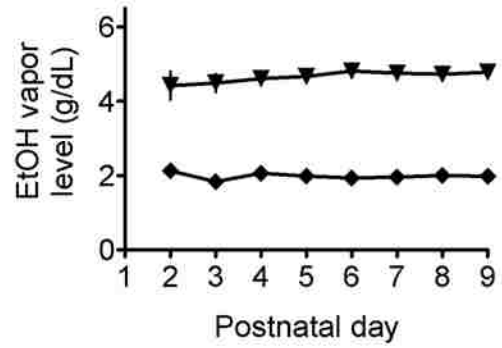
Figure 4. Repeated *in vivo* EtOH exposure does not alter AMPAR- and NMDAR- mediated input-output curves in rat pups with SEC's of 70-86 mM. Sample traces and input-output curves for AMPAR- (A, B; scale bar = 0.2 mV/5 ms) and NMDAR-mediated fEPSPs (C, D; scale bar = 0.5 mV/25 ms).

Figure 5. Repeated *in vivo* EtOH exposure does not affect AMPAR-mediated fEPSP paired-pulse ratios. Sample traces of control (A) and EtOH (B) groups. Scale bars 0.2 mV/10 ms (C) Scatter plot of paired-pulse ratios (AMPA-mediated fEPSP2/fEPSP1).

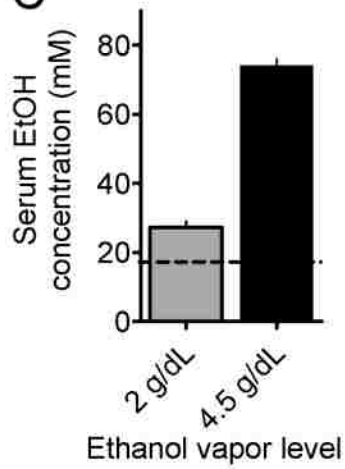
A Figure 1



B



C



D

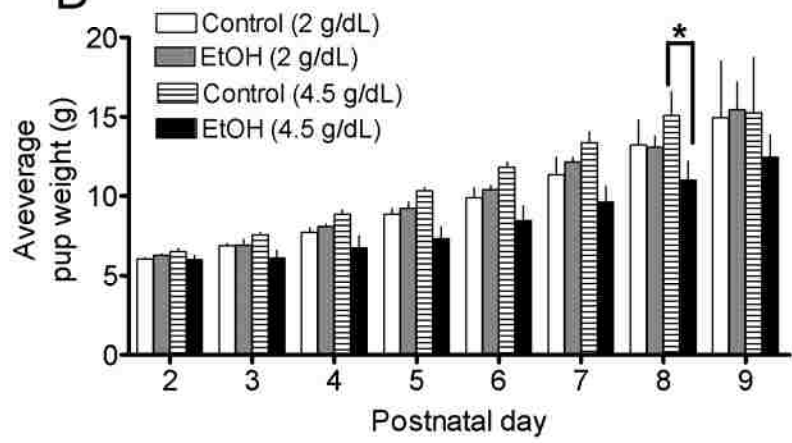


Figure 2

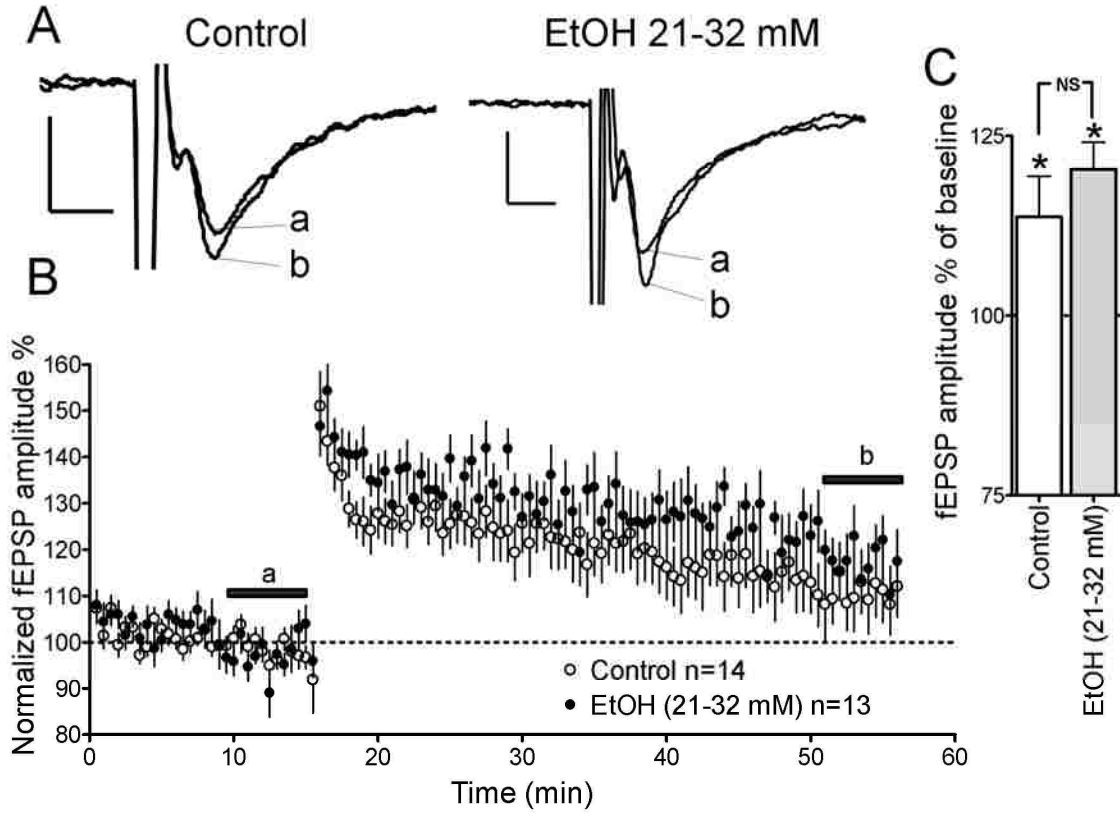


Figure 3

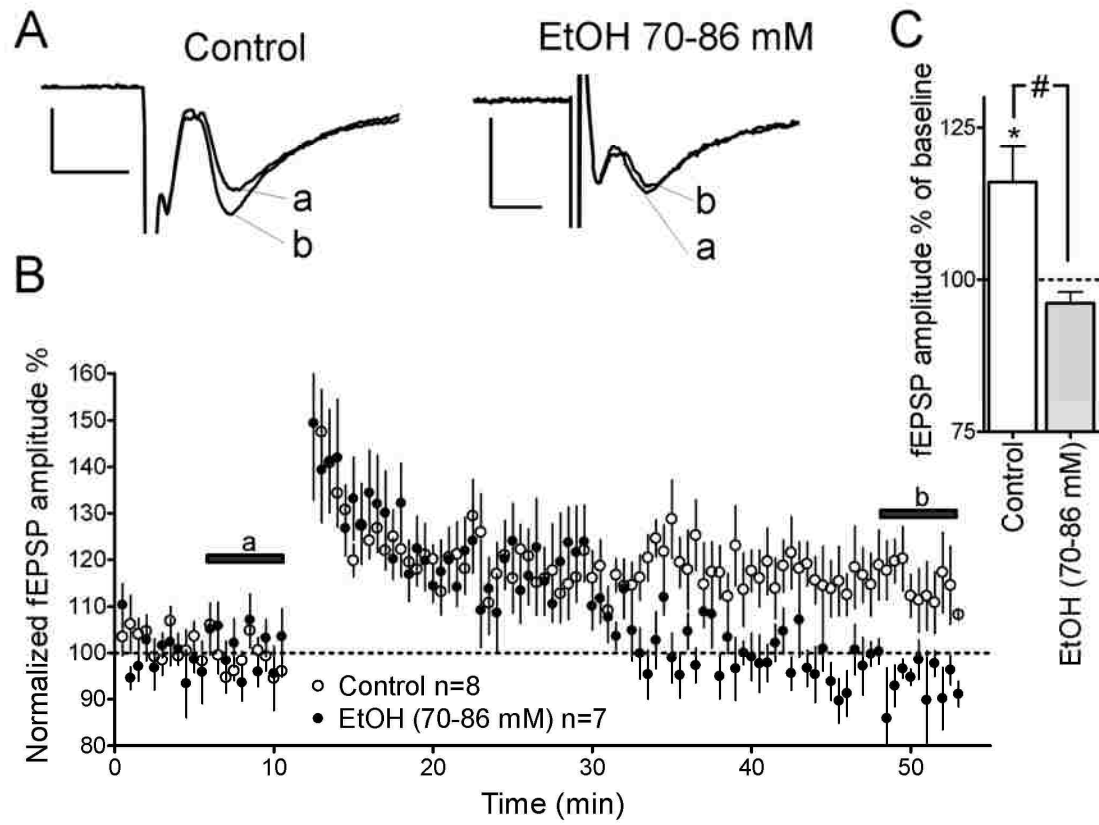


Figure 4

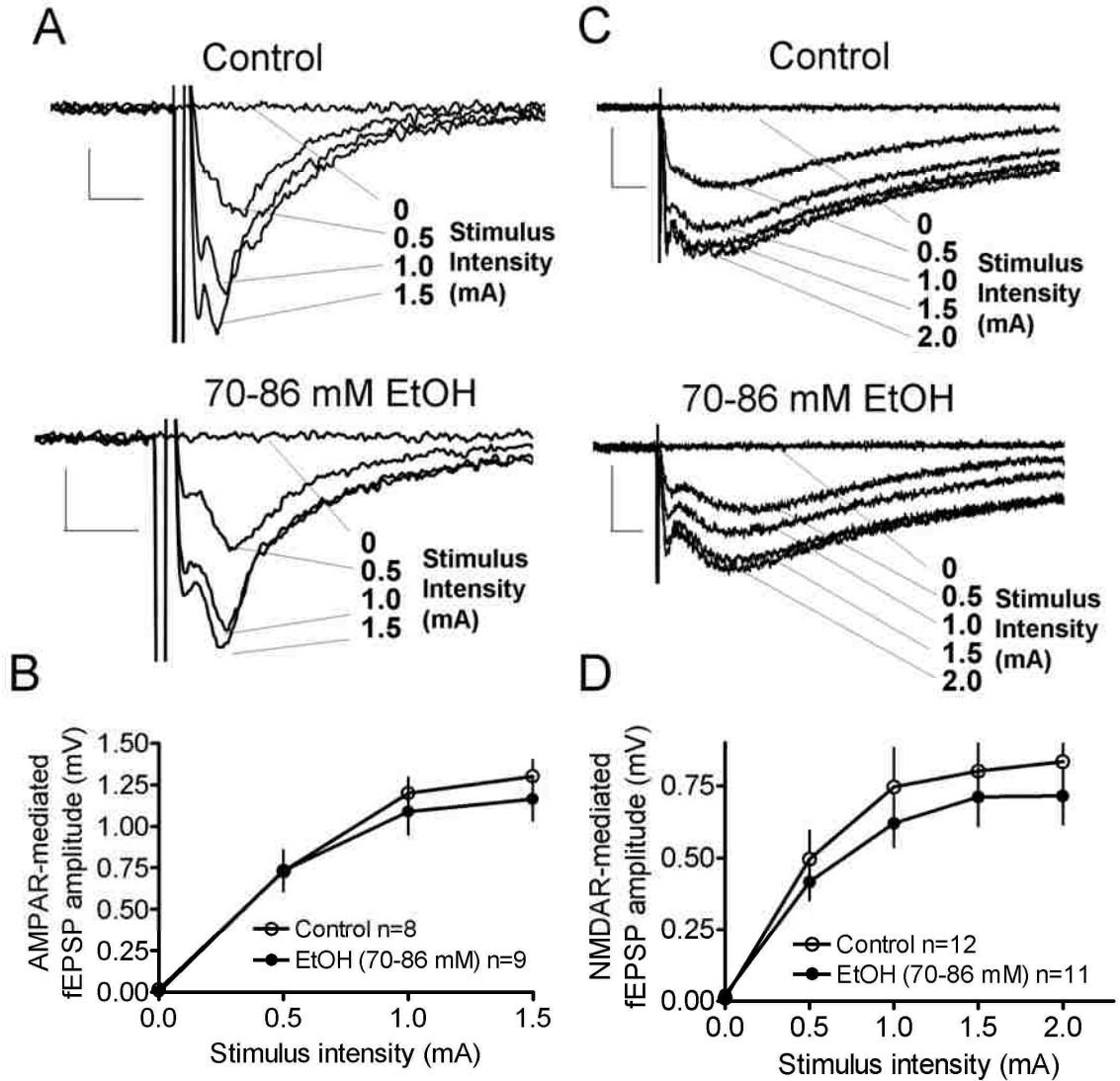
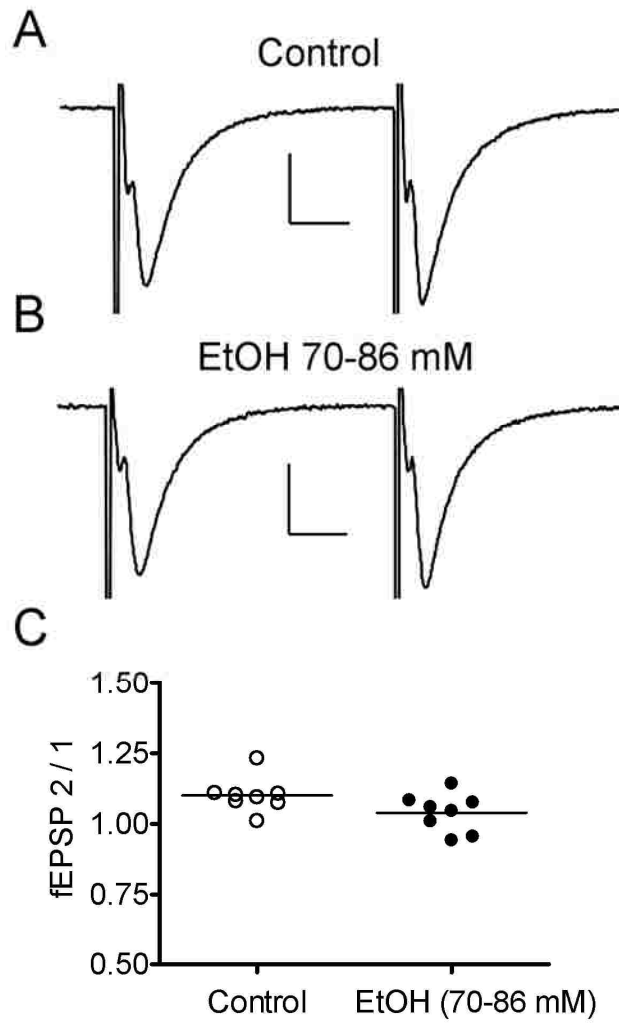


Figure 5



6. AMPA receptor-mediated synaptic transmission in the CA1 hippocampal region of neonatal rats: unexpected resistance to repeated ethanol exposure

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(Alcohol; in press)

Abstract

AMPA glutamatergic receptors (AMPA) mediate the majority of fast excitatory synaptic transmission in mature neurons. In contrast, a number of developing synapses do not express AMPARs; these are gradually acquired in an activity-driven manner during the first week of life in rats, which is equivalent to the third trimester of human pregnancy. Neuronal stimulation has been shown to drive high conductance Ca^{2+} -permeable AMPARs into the synapse, strengthening glutamatergic synaptic transmission. Alterations in this process could induce premature stabilization or inappropriate elimination of newly formed synapses and contribute to the hippocampal abnormalities associated with fetal alcohol spectrum disorder. Previous studies from our laboratory performed with hippocampal slices from neonatal rats showed that acute ethanol exposure exerts potent stimulant effects on CA1 and CA3 neuronal networks. However, the impact of these *in vitro* actions of acute ethanol exposure is unknown. Here, we tested the hypothesis that repeated *in vivo* exposure to ethanol strengthens AMPAR-mediated neurotransmission in the CA1 region via an increase in synaptic expression of Ca^{2+} -permeable AMPARs. We exposed rats to ethanol vapor (serum ethanol concentration ~40 mM) or air for 4 hr/day from postnatal day (P) 2 to 6. In brain slices prepared at P4-6, we found no significant effect of ethanol exposure on input-output curves for AMPAR-mediated field excitatory postsynaptic potentials (fEPSPs), the contribution of Ca^{2+} -permeable AMPARs to these fEPSPs, or the acute effect of ethanol on fEPSP amplitude. These results suggest that homeostatic plasticity mechanisms act to maintain glutamatergic

synaptic strength and ethanol sensitivity in response to repeated developmental ethanol exposure.

Introduction

Ethanol exposure during development can result in fetal alcohol spectrum disorder (FASD), which represents a broad range of clinical alterations (Green, 2007). These include learning and memory deficits that are, in part, a consequence of damage to the hippocampal formation (Berman and Hannigan, 2000). Approximately 10% of pregnant women consume ethanol and ~2% binge drink (Centers for Disease Control and Prevention (CDC), 2009). In addition, about half of pregnant women who consume ethanol continue to do so through the third trimester, a period of intense glutamatergic synaptic formation and refinement (Khazipov et al., 2001; U.S. Department of Health and Human Services, 1998). Ionotropic glutamatergic signaling is largely mediated by α -amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptors (AMPA) and N-methyl-D-aspartate receptors (NMDARs) (Dingledine et al., 1999). AMPARs mediate synaptic transmission at the resting membrane potential and contribute to removal of Mg^{2+} block from the NMDAR (Dingledine et al., 1999). The AMPAR is a tetrameric receptor composed of various combinations of subunits (GluR1-4) and the functional properties of the receptor are highly dependent on its subunit composition. Receptors containing GluR1, 3 and/or 4 subunits are Ca^{2+} -permeable and susceptible to polyamine blockade (Donevan and Rogawski, 1995). When AMPARs contain a GluR2 subunit, they become Ca^{2+} -impermeable

as a result of the presence of a positively-charged arginine in the trans-membrane 2 domain, which blocks Ca^{2+} entry and prevents blockade by polyamines (Bassani et al., 2009).

Increases in spontaneous neuronal activity in hippocampal slice cultures were shown to induce synaptic insertion of Ca^{2+} -permeable (GluR4-containing; higher conductance) AMPARs into CA1 pyramidal neurons, which are then gradually replaced in an activity independent manner by Ca^{2+} -impermeable (GluR2-containing; lower conductance) AMPARs (Zhu et al., 2000). Work from our laboratory indicates that during the neonatal period of rat development, which is equivalent to the third trimester of human pregnancy, ethanol paradoxically acts as a potent stimulant of neuronal activity both in the CA3 and CA1 hippocampal regions (reviewed in Valenzuela et al., 2008). Acute ethanol application in brain slices increased spontaneous network activity of CA3 hippocampal pyramidal neurons, which provide glutamatergic input to CA1 hippocampal pyramidal neurons (Galindo et al., 2005). Moreover, acute ethanol exposure of slices from postnatal day (P) 3-5 rats induced the production and/or release of a pregnenolone sulfate-like neurosteroid retrograde messenger that decreases failures of AMPAR-mediated excitatory postsynaptic currents (EPSCs) at Schaffer collateral-to-CA1 pyramidal neuron synapses under conditions of minimal stimulation (Mameli et al., 2005; Mameli and Valenzuela, 2006). Based on these results and those of Zhu et al. (2000), we hypothesized that repeated *in vivo* exposure to ethanol would strengthen AMPAR-mediated neurotransmission

in the CA1 region via an increase in synaptic expression of Ca²⁺-permeable AMPARs.

To test this hypothesis, we used field recording techniques in the CA1 hippocampal region in brain slices from animals developmentally exposed to ethanol vapor or air. We measured AMPAR-dependent field excitatory postsynaptic potential (fEPSP) input-output curves to assess changes in synaptic strength, and used a pharmacologic inhibitor of Ca²⁺-permeable AMPARs to assess changes in the functional expression of these receptors. We also re-examined the acute effects of ethanol on AMPAR-mediated synaptic responses and tested whether these acute effects were affected by repeated ethanol vapor exposure.

Experimental Procedures

Ethanol Vapor Chamber Exposure Paradigm

Animal procedures were approved by the Institutional Animal Care and Use Committee of the University of New Mexico Health Sciences Center and conformed to National Institutes of Health guidelines. Timed-pregnant Sprague-Dawley rats were obtained from Harlan (Indianapolis, IN). Neonatal rat pups and dams were exposed to ethanol vapor as previously described (Galindo and Valenzuela, 2006). Starting on P2, animals were transported to a room housing the ethanol vapor chamber apparatus and weighed prior to exposure. Air tight lids were then placed on the animals' home cages (La Jolla Alcohol Research Inc, La Jolla, CA) and were perfused with air (control group) or an air/ethanol vapor mixture (ethanol group). Ethanol was vaporized using a heating flask that receives a constant drip of 95% liquid ethanol (Tarr LLC, Phoenix, AZ) regulated with a peristaltic pump. Ethanol vapor (or air) was continuously removed by an exhaust hose connected to a vacuum line, maintaining constant ethanol vapor levels. Litters were culled to 10 pups on P2 and exposed daily for 4 hrs per day until P6 (Fig. 1A). Exposures were started at 07:00 hrs (lights on at 06:00 hrs and lights off at 18:00 hrs). The P2-6 developmental period was chosen because it encompasses the time frame where the acute stimulatory actions of ethanol were detected in the CA1 and CA3 hippocampal regions (Galindo et al., 2005; Mameli et al., 2005; Mameli and Valenzuela, 2006).

Tissue preparation and solutions

Unless indicated, chemicals were from Sigma (St. Louis, MO) or Tocris Cookson (Ellisville, MO). Both male and female rat pups were deeply anesthetized with 250 mg/kg of ketamine and euthanized by decapitation immediately after the 4 hr exposure on P4-6. Trunk blood samples were allowed to coagulate and centrifuged at 2.3 x g for 10 min to obtain serum. Serum ethanol concentrations were determined using an alcohol dehydrogenase-based assay involving the reduction of NAD⁺ (β -NAD, free acid, grade I, Roche, Indianapolis, IN). NADH absorbance was read at a wavelength of 340 nm. Coronal brain slices (400 μ m) were prepared using a vibratome, as previously described (Mameli et al., 2005). After a recovery period of 45 min at 35-36°C, slices were stored for 1-8 hr at room temperature. Artificial cerebrospinal fluid (ACSF) contained the following (in mM): 126 NaCl, 2 KCl, 1.25 NaH₂PO₄, 1 MgSO₄, 26 NaHCO₃, 2 CaCl₂, 10 glucose, and 0.01 gabazine (also known as SR-95531) equilibrated with 95%O₂/5%CO₂. When indicated, the ACSF also contained 100 μ M 1-naphthylacetyl spermine trihydrochloride (NASPM), 50 μ M D,L-2-amino-5-phosphonovaleric acid (AP5), 50 μ M GYKI-53655 and/or 10 μ M 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX).

Electrophysiological recordings

Recordings were performed in the CA1 *stratum radiatum* at 32°C with a perfusion rate of 2 ml/min using an Axopatch 200B amplifier (Molecular Devices, Sunnyvalley, CA). Recording micropipette glass electrodes had resistances of 3-5 M Ω and were filled with ACSF. AMPAR fEPSPs were evoked using a

concentric bipolar electrode (inner pole 25 μm ; outer pole 125 μm ; Frederick Haer Company, Bowdoinham, ME) placed in vicinity of the Schaffer collateral fibers. Input-output curves were measured at the start of all recordings, and, for subsequent experiments, the stimulation intensity was set at 40-50% of the intensity required to elicit maximal responses. Stimulus duration was 75 μs and stimuli were delivered at 0.033 Hz.

Data Analysis

Data were acquired and analyzed with pClamp 9 (Molecular Devices, Sunnyvale, CA) and GraphPad Prizm 4.0 (San Diego, CA). The short time interval between the presynaptic volley and fEPSP prevented accurate measurement of the slope in most experiments. Therefore, data were analyzed using the fEPSP amplitude. For each experiment, baseline was defined as the average of 20 fEPSPs immediately before drug application. For experimental conditions, 10 fEPSPs immediately preceding the start of the ethanol washout or the application of NBQX or GYKI-53655 were averaged for analysis. Statistical analyses of pooled data were performed by Students *t* test, one sample *t* test vs. a theoretical mean of zero or 100%, or repeated measure two-way ANOVA. A $p \leq 0.05$ was considered to be statistically significant. Data are presented as mean \pm SEM with the number of determinations (n) representing the number of recordings. Data shown in Figs. 2-3 were obtained in slices from 9-10 pups from 5 different litters per treatment condition. Data shown in Fig. 4 were obtained in slices from 2 pups from 2 different litters. Data shown in Fig. 5 were obtained in slices from 4 pups from 1 litter each for the control and ethanol groups.

Results

To model exposure to ethanol during the third trimester of human pregnancy, rats were exposed to ethanol vapor during the neonatal period of development (Fig 1A). Dams and rat pups were exposed to ethanol vapor daily for 4 hr per day with the average chamber ethanol concentration ranging from 1.51 to 1.65 g/dL (Fig. 1B). This exposure resulted in elevated ethanol concentrations in neonatal pups, and very low levels in dams (Fig. 1C). Daily litter average pup weights were not different between the control and ethanol groups (n=6 for control and n=6 for ethanol; not significant (N.S.) by repeated measures two-way ANOVA; Fig. 1D).

In brain slices from control and ethanol-exposed animals, we recorded AMPAR-mediated fEPSP input-output curves in the presence of gabazine (10 μ M) and AP5 (50 μ M) to measure changes in synaptic strength. There was no significant difference between the control and ethanol-exposed groups (N.S. by repeated measure two-way ANOVA; Fig. 2).

We then tested the effects of NASPM (a blocker of Ca^{2+} -permeable AMPARs) on AMPAR-mediated fEPSPs in slices from control and ethanol exposed animals. Figs 3A-B show that NASPM reduced amplitudes of AMPAR-mediated fEPSPs to a similar extent in the control and ethanol groups. As expected, the events were abolished by the AMPAR antagonist, GYKI-53655 (50 μ M). The residual potential recorded in the presence of GYKI-53655 (or NBQX; data not shown) corresponds to the presynaptic volley, which was not significantly different between the control (0.12 ± 0.02 mV; n=12) and ethanol

(0.12 ± 0.02 mV; $n=14$) groups (N.S. by unpaired t-test). The time course of the effect of NASPM is shown in Fig. 3C, where after a stable baseline was recorded in the presence of gabazine (10 μ M) and AP5 (50 μ M), NASPM (100 μ M) was applied for 15 min. In a subset of experiments, NASPM application was followed by application of GYKI-53655 or the non-NMDA antagonist, NBQX (10 μ M) ($n=12$ for control and $n=14$ for ethanol) confirming that the fEPSPs were AMPAR-mediated. The percent of functional Ca^{2+} -permeable AMPARs (i.e. those lacking GluR2) corresponds to the magnitude of inhibition of fEPSPs by NASPM, and was $21 \pm 1.9\%$ ($n=19$) for the control and $17.8 \pm 1.5\%$ for the ethanol ($n=21$) groups (N.S. by unpaired t-test). NASPM did not differentially inhibit the AMPAR-mediated fEPSPs at P4, P5 or P6 (i.e. 3, 4, or 5 ethanol exposures; N.S. by two-way ANOVA; Fig 3D). In addition, the effect of NASPM was not correlated with the serum ethanol concentration (by linear regression; $R^2=0.02$; slope not significantly different from zero; Fig. 3E).

Mameli et al. (2006) showed previously that acute exposure to ethanol, at concentrations as low as 15 mM, significantly decreased the failure rate of AMPAR-mediated EPSCs in CA1 pyramidal neurons from P3-4 rats under conditions of minimal stimulation; this effect was dependent on the release of a pregnenolone sulfate-like neurosteroid. Here, we re-examined the acute effect of ethanol on AMPAR-mediated synaptic transmission in the developing CA1 region using extracellular recording techniques. Ethanol was bath-applied after a stable baseline was obtained in the continued presence of gabazine (10 μ M); note that AP5 was omitted because the actions of the pregnenolone sulfate-like

neurosteroid are NMDAR dependent (Mameli and Valenzuela, 2006).

Unexpectedly, we found that ethanol (40 mM) induced a small but significant inhibition of the AMPAR-mediated fEPSP ($-7.53 \pm 1.49\%$ with respect to the average of baseline and washout; $p < 0.01$; by one sample t-test v. zero, $n=6$; Fig. 4A-B).

Finally, we investigated whether ethanol vapor exposure affected the acute sensitivity to ethanol of AMPAR-mediated fEPSPs. Since the acute effect of 40 mM ethanol was small, we tested the acute effect of a higher ethanol concentration (80 mM). In the presence of gabazine (10 μ M) and AP5 (50 μ M), ethanol (80 mM) was bath-applied for 10 minutes (Fig. 5A-B). Acute ethanol application significantly inhibited AMPAR-mediated fEPSPs by $13.07 \pm 1.35\%$ and $10.02 \pm 2.29\%$ (from the average of baseline and washout) in slices from the control and ethanol groups, respectively ($n=8$; $p < 0.01$ by one sample t-test from zero for both conditions). These values were not significantly different from each other (N.S. by unpaired t-test).

Discussion

A number of studies have demonstrated that developmental ethanol exposure produces persistent alterations in AMPAR function in CA1 hippocampal and medial septum/diagonal band neurons (Bellinger et al., 1999; Hsiao and Frye, 2003; Wijayawardhane et al., 2007). However, to the best of our knowledge, this is the first characterization of the effect of *in vivo* third trimester-equivalent ethanol exposure on AMPAR function in the CA1 hippocampal region of neonatal rats. We used a vapor chamber ethanol exposure paradigm that models repeated moderate-to-heavy maternal ethanol use during the third trimester of human pregnancy. An advantage of this exposure paradigm is that serum ethanol concentrations in the dams are low, resulting in undetectable alterations in maternal care (Galindo and Valenzuela, 2006). Averaged individual pup weight was not different between control and ethanol-exposed litters suggesting that this exposure paradigm models FASD more closely than fetal alcohol syndrome, which involves growth retardation. This exposure paradigm focuses on a critical period of brain development, termed the brain growth spurt, where most glutamatergic synapses are generated and refined. A limitation of our study is that it models a relatively short period of the third trimester-equivalent of human pregnancy. Maternal ethanol use typically spans all three trimesters and pregnant women rarely start drinking during the third trimester (Centers for Disease Control and Prevention (CDC), 2009). However, this study was undertaken to selectively assess the impact of repeated ethanol exposure during the third trimester-equivalent because of the critical importance of this

period for neuronal circuit maturation. Future studies should re-examine the impact of ethanol exposure during all three trimesters on glutamatergic synaptic transmission in the developing CA1 region.

Contrary to our hypothesis, the strength of AMPAR-mediated synaptic transmission did not differ between control and ethanol-exposed animals. This is surprising in light of the previous finding from our laboratory that acute exposure to 15-75 mM ethanol induced the production and/or release of a pregnenolone sulfate-like neurosteroid retrograde messenger that strengthened AMPAR-mediated synaptic transmission in the CA1 region of neonatal rats (Mameli and Valenzuela, 2006). However, when we re-examined this effect of ethanol using field recording techniques, we found that AMPAR-mediated fEPSPs were not potentiated, but rather inhibited, by acute exposure to ethanol. Inhibitory effects of ethanol on AMPARs have previously been observed in both the CA3 and CA1 hippocampal regions of neonatal rat pups (Mameli et al., 2005; Puglia and Valenzuela, Submitted). Therefore, it is possible that the acute effects of ethanol reported by Mameli and Valenzuela (2006) can only be observed under certain experimental conditions *in vitro*—i.e. under minimal stimulation in the whole-cell voltage-clamp configuration with K⁺ channel blockers present in the internal solution. These conditions could favor the detection of the neurosteroid-dependent stimulatory actions of ethanol because of two reasons. First, excitatory postsynaptic currents evoked under conditions of minimal stimulation are likely mediated by AMPARs located in the proximal dendrites and/or soma of CA1 pyramidal neurons and these receptors could be affected by

ethanol differently than the more distal receptors sampled in the fEPSP recordings. Second, K⁺ channel inhibition could facilitate ethanol-induced dendritic release of the pregnenolone sulfate-like neurosteroid. It is also possible that the stimulatory effects of ethanol on CA3 network activity that were previously reported by Galindo et al. (2005) are also dependent on the experimental conditions. However, this possibility is unlikely given that these effects were observed with whole-cell electrophysiological recordings in single neurons, and also with Ca²⁺ imaging techniques in ensembles of CA3 neurons.

An alternative explanation for the lack of an effect of neonatal ethanol vapor exposure on AMPAR-mediated transmission in the CA1 region is that ethanol stimulates CA1 and CA3 neuronal activity *in vivo*, but neurons adapt to these effects; i.e., homeostatic plasticity mechanisms may act to restore synaptic strength (Carpenter-Hyland and Chandler, 2006). Our finding that the acute effect of ethanol on AMPAR-mediated fEPSPs is not affected by vapor chamber ethanol exposure suggests that these homeostatic mechanisms do not involve the development of AMPAR tolerance to ethanol. This conclusion is also supported by the finding of Galindo et al. (2006) that CA3 pyramidal neurons did not develop tolerance to the excitatory actions of ethanol after repeated ethanol vapor exposure. Moreover, lack of development of AMPAR tolerance to the acute effects of ethanol was also observed in a study with medial septum/diagonal band neurons from P15-25 and P32-35 rats that were exposed to ethanol on P4-9 (blood ethanol level = 352 ± 9 mg/dL) (Hsiao and Frye, 2003).

It was also unexpected that third trimester-equivalent ethanol exposure did not alter the functional expression of Ca²⁺-impermeable AMPARs. In hippocampal slice cultures from neonatal rats, it has been shown that increased spontaneous excitatory activity induces synaptic trafficking of Ca²⁺-permeable, GluR4-containing AMPARs (Zhu et al., 2000). Consistent with this, we predicted that our ethanol exposure paradigm would repeatedly excite CA1 and CA3 neuronal networks, thus resulting in increased expression of Ca²⁺-permeable AMPARs at CA1 pyramidal neuron synapses. As discussed above, repeated ethanol exposure could initiate homeostatic plasticity mechanisms that would prevent changes in synaptic expression of Ca²⁺-permeable AMPAR (Lissin et al., 1998; Watt et al., 2000). Alternatively, it is possible that the paradigm of slice preparation immediately after ethanol exposure led to GluR4 insertion and subsequent exchange of this subunit with the Ca²⁺-impermeable GluR2 subunit. Although the activity-independent exchange of GluR4 with GluR2 has indeed been documented, this possibility is unlikely because this is an activity-independent slow process that can take up to 30 hrs and we measured expression of Ca²⁺-permeable AMPARs less than 8 hrs after ethanol exposure (Zhu et al., 2000). Moreover, if this exchange process had taken place, we should have observed an increase in AMPAR fEPSP input-output curves mediated by increased synaptic expression of GluR2-containing receptors.

In conclusion, our findings show that the *in vitro* acute stimulatory actions of ethanol on developing CA1 pyramidal neurons occur under some, but not all, experimental conditions. If these actions of ethanol take place *in vivo*, the long-

lasting impact on AMPAR-mediated synaptic transmission in the CA1 region remains unknown. Moreover, AMPAR-mediated responses can be inhibited by acute ethanol exposure in this region and this effect is not affected by long-term ethanol vapor exposure. This finding confirms that AMPAR-mediated responses are important targets of the developmental actions of ethanol and that these responses are not subject to the acquisition of ethanol tolerance. Future studies should examine whether ethanol alters the normal functioning of other neurotransmitter receptors or intracellular signaling pathways during the third trimester of pregnancy, as these could have deleterious consequences on the maturation of hippocampal neuronal networks and play a role in the learning and memory alterations that characterize FASD.

Figure Legends

Figure 1. Third trimester-equivalent ethanol exposure paradigm. (A) Schematic representation of the exposure paradigm where rat pups and their respective dams were repeatedly exposed to ethanol vapor for 4 hr per day. Exposure(s) started at P2 and continued to P6. Brain slices for electrophysiology studies were prepared from P4, P5 or P6 rats, immediately after the 4 hr exposure period. (B) Ethanol concentrations in the exposure (vapor) chamber. (C) Serum ethanol concentrations measured from trunk blood samples collected immediately after euthanasia. Line represents the legal intoxication limit (17.4 mM). (D) Average pup weight of control and ethanol vapor-exposed pups as a function of postnatal day.

Figure 2. Third trimester-equivalent ethanol exposure does not alter synaptic efficacy in the CA1 hippocampus of P4-6 rat pups. (A) Representative traces of AMPAR fEPSPs recorded at increasing stimulus intensities. (B) Pooled data of control and ethanol exposed animals.

Figure 3. Third trimester-equivalent ethanol exposure does not alter functional expression of Ca²⁺-permeable AMPARs. Representative traces of fEPSP recordings from the control (A) and ethanol (B) groups obtained in the presence of gabazine (10 μM) and AP5 (50 μM), followed by NASPM (100 μM) and then by GYKI-53655 (50 μM). (C) Time course of the effect of NASPM application followed by GYKI-53655 (or NBQX 10 μM) application. Letters above the time

course correspond to letters in sample traces (a, b and c). (D) NASPM-mediated inhibition of fEPSP amplitude as a function of postnatal day. (E) NASPM-mediated inhibition of fEPSP amplitude as a function of the serum ethanol concentration.

Figure 4. Acute Ethanol (40 mM) inhibited fEPSP responses in slices from naïve P4 rats. (A) Sample traces from fEPSP recordings and (B) time course.

Recordings were performed in the presence of gabazine (10 μ M) and fEPSPs were inhibited by NBQX (10 μ M). Letters above the time course correspond to letters in sample traces (a, b, c, and d).

Figure 5. Third trimester-equivalent ethanol vapor exposure does not induce tolerance to the acute inhibitory effects of ethanol on the amplitude of AMPAR-mediated fEPSPs. Ethanol (80 mM) was bath-applied to brain slices from P4-6 rat pups. (A) Sample traces from fEPSP recordings and (B) time course.

Recordings were performed in the presence of gabazine (10 μ M) and AP5 (50 μ M). Letters above the time course correspond to letters in sample traces (a, b, and c).

Figure 1

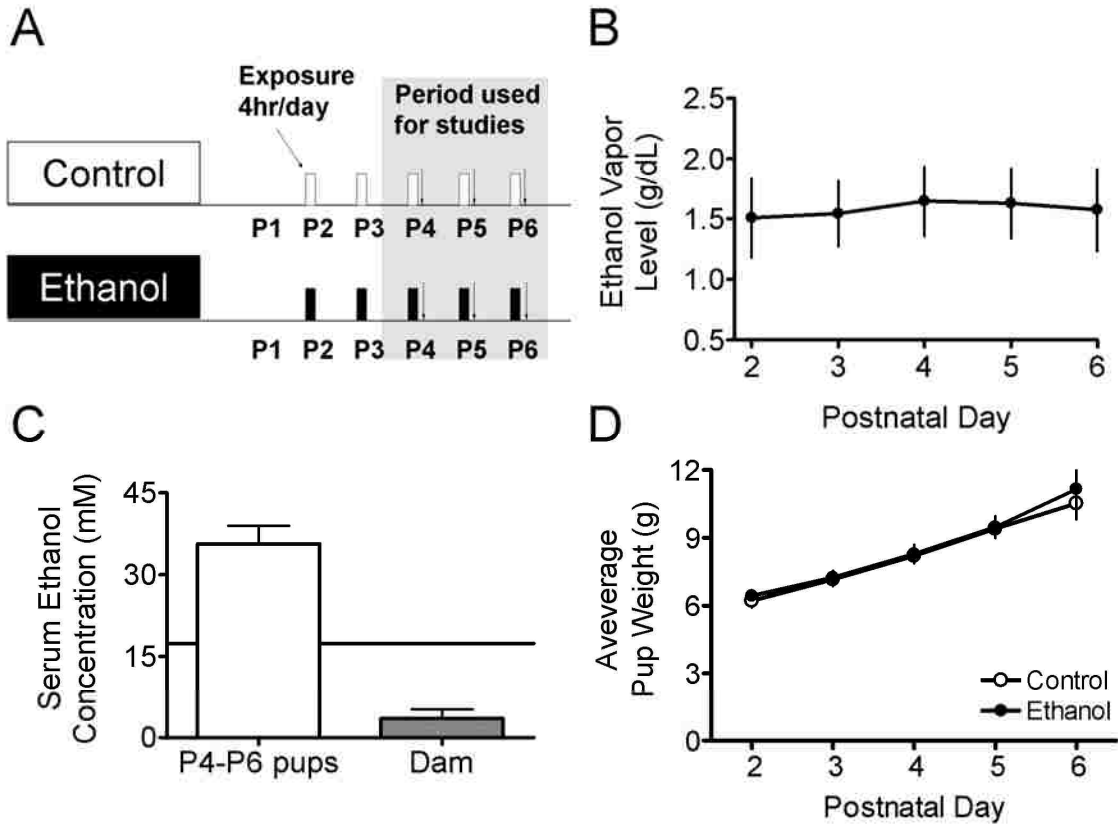


Figure 2

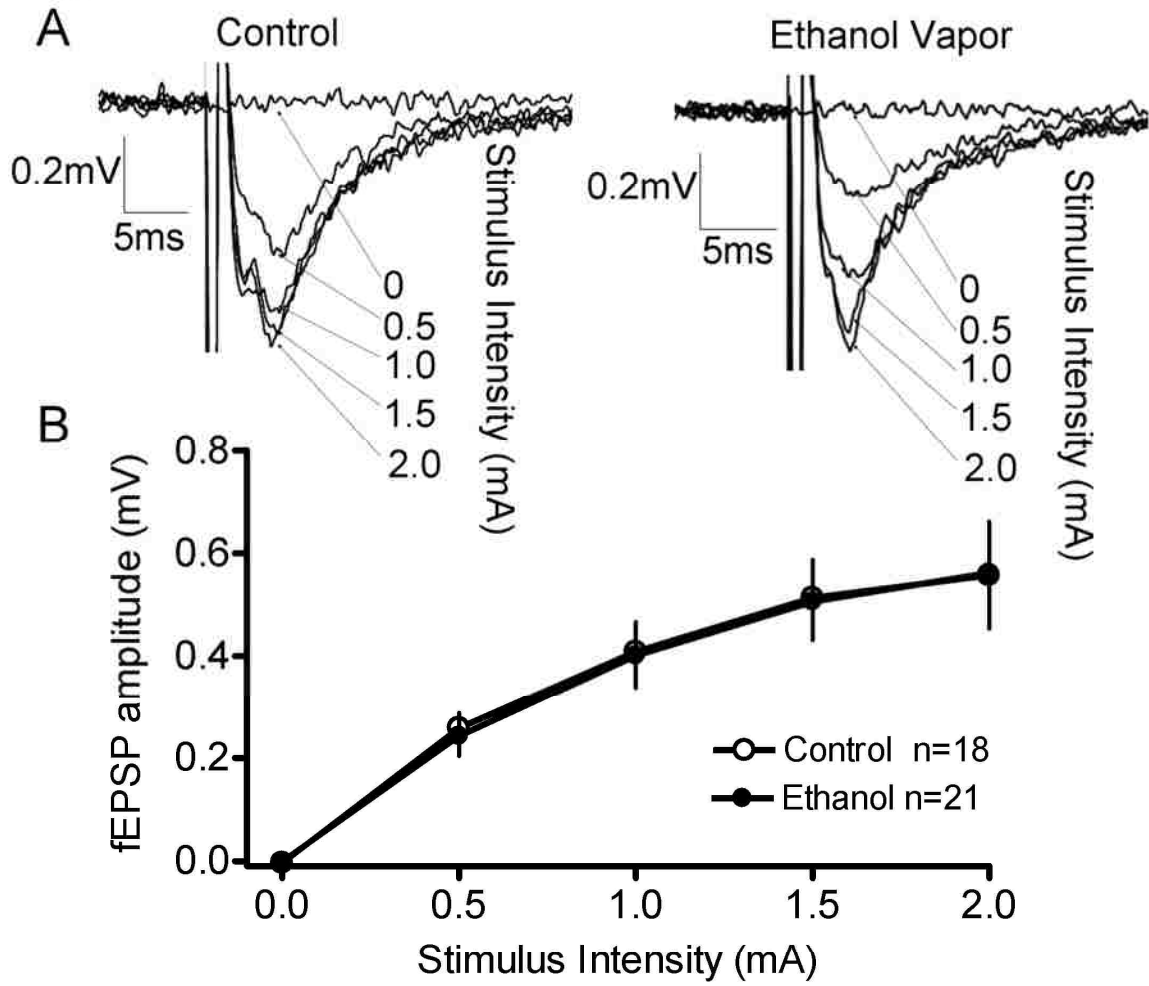


Figure 3

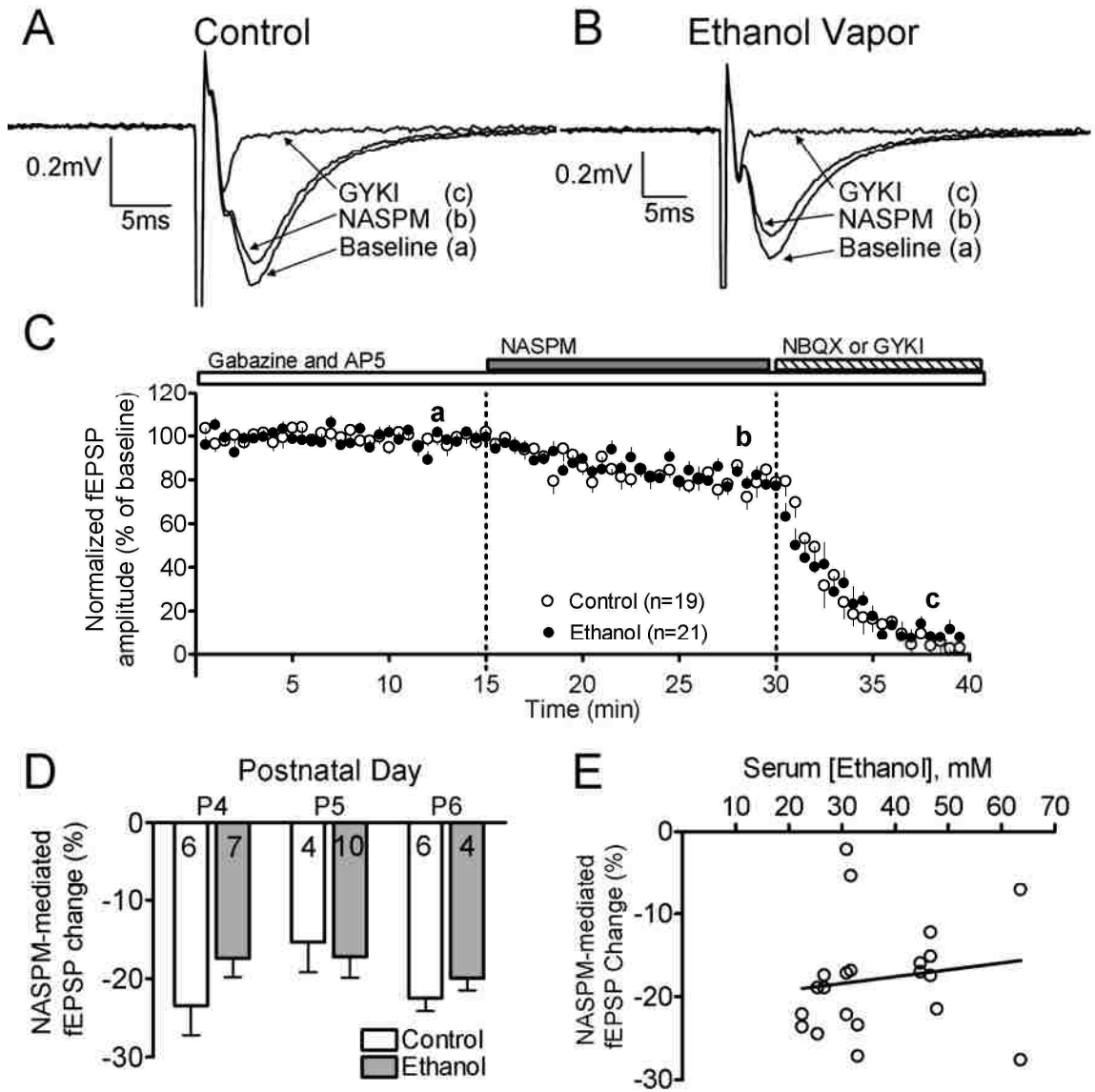
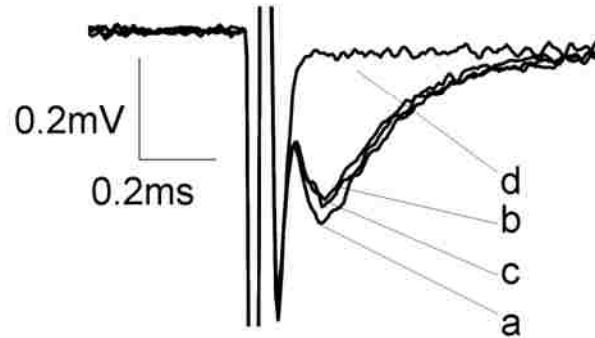


Figure 4

A



B

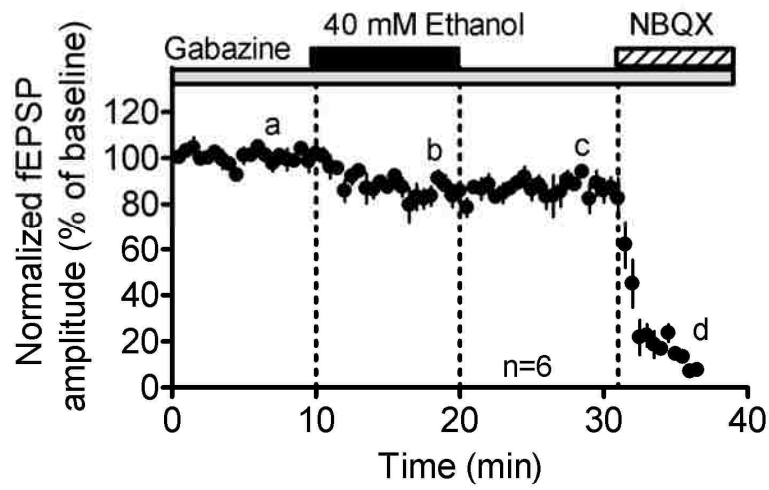
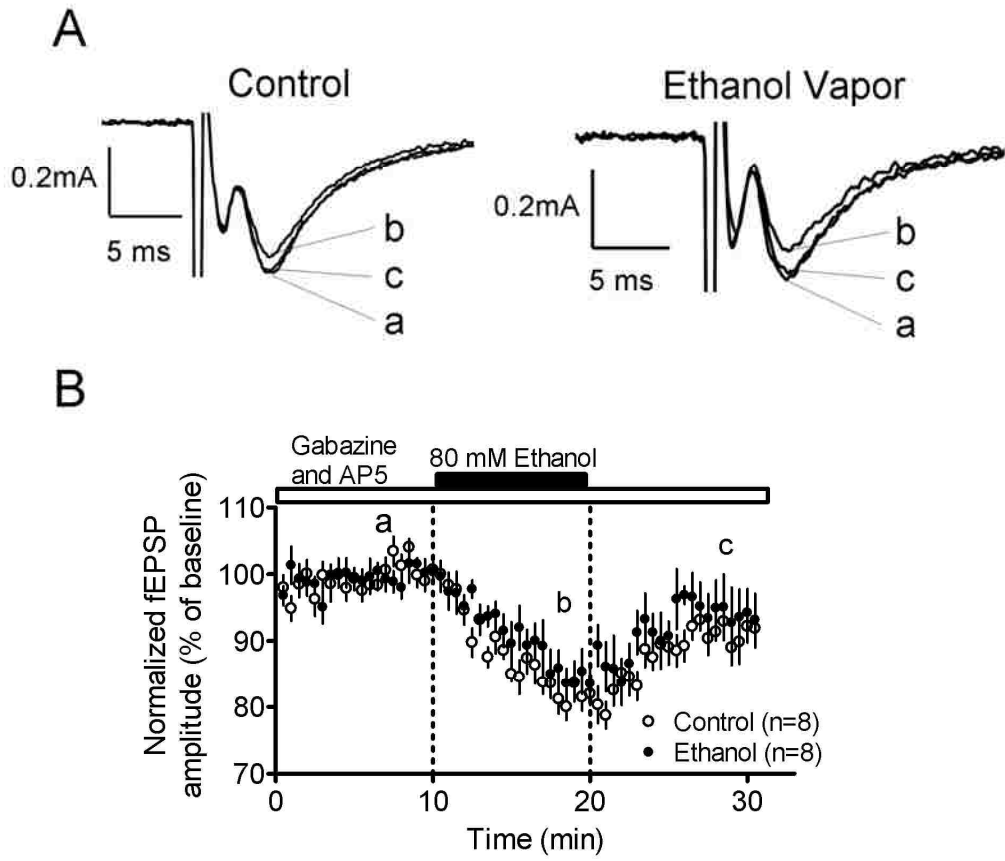


Figure 5



7. Conclusions

7.1 Summary of main findings

The main findings of this dissertation project can be summarized as follows. First, acute EtOH application reduced AMPAR- and NMDAR-mediated responses, as well as LTP induction, in the CA1 hippocampal region during the late portion of the third trimester-equivalent period (P7-9). In addition, acute EtOH application did not affect glutamate release. Second, at P7-9, LTP was impaired during early withdrawal following a repeated *in vivo* third trimester-equivalent EtOH exposure paradigm. Measures of NMDAR- and AMPAR-mediated synaptic strength were not different than controls, nor were measures of presynaptic glutamate release. Lastly, repeated *in vivo* third trimester-equivalent EtOH exposure did not affect AMPAR-mediated synaptic strength, expression of Ca²⁺-permeable AMPARs, or induce tolerance to the acute inhibitory effects of EtOH during the early portion of the third trimester-equivalent period (P4-6). Main findings from chapters 4-6 are summarized in table 7.1.

Table 7.1. Summary of the effects of EtOH on the neonatal CA1 hippocampal region.

	Acute	Chronic
AMPA responses	<ul style="list-style-type: none"> ↓ fEPSPs (P4-9; 40-80mM; Total=Ca^{2+}-impermeable) ↑ EPSCs (P3-4; >15mM; minimal stimulation; Mameji et al. (2006) ↓ EPSCs (P7-9; 80mM; perforated-patch) 	<ul style="list-style-type: none"> ↔ fEPSPs I/O curve (P4-9; 23-86 mM) ↔ Ca^{2+}-permeable AMPAR expression (P4-6; 23-63mM) ↔ Tolerance to acute EtOH (P4-6; 80mM)
NMDAR responses	<ul style="list-style-type: none"> ↓ fEPSPs (P7-9; 40-80mM; increased effect in presence of Mg^{2+}) 	<ul style="list-style-type: none"> ↔ fEPSPs I/O curve (P7-9; 70-86 mM)
Glutamate release	<ul style="list-style-type: none"> ↔ PPR AMPAR-fEPSP; (P7-9; 80mM) 	<ul style="list-style-type: none"> ↔ PPR AMPAR-fEPSP (P7-9; 70-86mM)
LTP (3x100Hz)	<ul style="list-style-type: none"> ↓ Induction (P7-9; 80mM) 	<ul style="list-style-type: none"> ↓ Expression/maintenance (P7-9; 70-86mM)

7.2 Overall conclusions

Glutamatergic synaptic transmission and plasticity are sensitive targets of EtOH during the third trimester-equivalent. Studies in this dissertation provide insight as to the developmentally-regulated and brain region specific effects of EtOH on glutamatergic signaling and plasticity during the third trimester-equivalent period. These data add to a growing body of evidence indicating that significant differences exist between the effects of EtOH on the developing versus the mature brain, confirming that conclusions from studies in mature animals may not be applicable to those during development.

One example is the developmental effects of EtOH on AMPAR-mediated transmission in the CA1 hippocampal region. Studies in chapter 4, as well as work from others, demonstrate that AMPAR-mediated transmission in this region is relatively insensitive to the actions of EtOH in mature animals (Randall et al., 1995; Lovinger et al., 1990; Carta et al., 2003). However, during the third trimester-equivalent period, we found AMPAR-mediated responses to be sensitive to the acute actions of EtOH. These results were consistent with previous studies from our lab showing acute inhibitory effects of EtOH in the CA3 region, but are in contrast to results obtained in cortical layer 2/3 neurons during this period (Mameli et al., 2005; Sanderson et al., 2009). Another example is the effects of EtOH on NMDAR-mediated transmission. Studies presented here found inhibition of NMDAR-mediated transmission during the third trimester-equivalent, consistent with what is found in the mature animal in the CA1 hippocampal region. However, during the third trimester-equivalent in the CA3

hippocampal region and cortical layers 2/3, NMDAR-mediated transmission was relatively insensitive to EtOH. These data suggest that the effects of EtOH are developmentally regulated in some but not all brain regions (Mameli et al., 2005; Sanderson et al., 2009). Additionally, the effects of EtOH on glutamate release during the third trimester-equivalent period also demonstrate brain region specific differences. Previous studies found inhibition in the CA3 region, but in the CA1 region there was no effect. Collectively, these studies highlight not only developmental differences in the sensitivity to the acute effects of EtOH, but also brain region specific differences.

Interestingly, the chronic effects of EtOH exposure are different from what occurs in more mature animals, in some, but not all regards. In mature animals, chronic EtOH exposure results in neuroadaptation of NMDAR-mediated responses (Thomas and Riley, 1998). Roberto and colleagues (2004) found that glutamate release, as well as NMDAR-mediated postsynaptic responses, were enhanced in the central amygdala during early EtOH withdrawal from EtOH vapor exposure (Roberto et al., 2004). Furthermore, sensitization to acute EtOH was observed in NMDAR-mediated responses (Roberto et al., 2004). Nelson and Gruol (2005) found increased NMDAR-mediated responses and expression in the CA1 hippocampal region after 7 days of EtOH withdrawal (Nelson et al., 2005). However, studies presented here demonstrate a lack of adaptation after repeated rounds of *in vivo* EtOH exposure for all parameters tested (glutamate release, NMDAR-, and AMPAR-mediated synaptic strength, and tolerance of AMPAR-

mediated responses to acute EtOH exposure), suggesting differences in response to repeated EtOH-mediated neuronal insults during development.

LTP expression and/or maintenance in the CA1 hippocampus was impaired with a third trimester-equivalent exposure paradigm. This was consistent with studies of Roberto and colleagues (2002) with mature animals chronically exposed to EtOH showing impaired LTP in the CA1 hippocampus (Roberto et al., 2002). However, Fujii and colleagues (2008) demonstrated a decreased threshold for LTP induction in the CA1 hippocampus of mature rats exposed chronically to EtOH and this appeared to be a result of decreased GABAergic tone and increased NMDAR-activity (Fujii et al., 2008). The authors address the discrepancy between their findings and those of Roberto and colleagues (2002) as attributable to differences in EtOH exposure paradigm and blood EtOH levels. In adults, impairments in glutamatergic signaling and synaptic plasticity by chronic and acute EtOH intoxication are likely to contribute to memory loss, anxiety, seizures, dependence, neuronal damage, and cognitive impairments (Saitz, 1998; Siggins et al., 2003; Vengeliene et al., 2008). However, EtOH exposure during the third trimester-equivalent period is likely to cause impaired synapse formation and refinement in the immature brain, and impaired network formation (among other processes) likely contributing to the spectrum of mental retardation, behavioral problems and cognitive impairments observed in children afflicted by FASD (Berman and Hannigan, 2000; Voigt et al., 2005; Bellinger et al., 1999).

7.3 Basic and Clinical implications

Deficits in learning and memory and behavior disorders have been observed in children with exposure levels of less than 2 standard drinks per day (Olson et al., 1997; Sood et al., 2001). Similarly, an animal study found that prenatal exposure to low concentrations of EtOH (blood alcohol levels near 0.03 g/dL) caused relatively subtle deficits in learning and memory that were detected in a modified version of the Morris water maze test (Savage et al., 2002). Studies proposed in this dissertation support the notion that alterations in glutamatergic signaling and plasticity could contribute to long-term learning and memory deficits in adult animals; however, why do we observe impairments in LTP during development only with elevated doses although cognitive impairment can be detected in animals exposed to lower doses? This may be related to the recording conditions used in our studies. For example, the paradigm that we used to induce LTP does not mimic patterns of neuronal activity that induce plasticity under physiological conditions, and it is possible that the latter is more sensitive to EtOH than experimentally-induced LTP. Alternatively, EtOH-induced behavioral deficits could be a consequence of alterations in multiple neurotransmitter systems across developing neuronal networks.

An elegant study of cultured hippocampal neurons demonstrated that when neurons were subjected to prolonged inhibition of AMPARs by NBQX—which possibly mimics what occurs with chronic *in vivo* EtOH exposure in the CA1 region—there was compensatory increases in postsynaptic AMPAR

expression (Thiagarajan et al., 2005). This raises the question of why we did not observe compensatory upregulation in AMPAR-mediated responses in our chronic EtOH exposure studies. An indication of what is potentially occurring comes from studies of developing hippocampal neurons in culture. When individual neurons were transfected with a K_{ir} potassium channel, which hyperpolarized the neuron, there was homeostatic regulation normalizing firing rates in neurons with existing synaptic connections. However, when transfection occurred in neurons before synapse formation, the result was an overall reduction in synaptic inputs (Burrone et al., 2002). Therefore, it is possible that homeostatic increases in AMPAR expression at the single synapse level occurred during our EtOH exposure paradigm but that the impact of this effect was offset by a reduction in synapse number, resulting in an undetectable change in synaptic strength. This is supported by the studies of Bellinger and colleagues (1999) as previously discussed, where a third-trimester EtOH exposure paradigm led to decreased synaptic efficacy in mature animals, which was interpreted to be a consequence of a decrease in synapse number (Bellinger et al., 1999).

Homeostatic plasticity mechanisms act to preserve synaptic strength, yet we did not observe NMDAR “upregulation” in response to EtOH-induced inhibition of these receptors, as proposed by Thomas and Colleagues (2002 and 2004). As discussed in Chapter 5, this could be due to differences in the EtOH exposure paradigm (binge vs. gradual EtOH administration). However, a question is how does NMDAR antagonism protect against learning and memory

deficits in mature animals that were exposed to EtOH in a binge-like fashion during the 3rd trimester-equivalent (Thomas et al., 2002; Thomas et al., 2004)? Clues to this may come from the negative regulatory effects of NMDARs on AMPAR transcription and translation, degradation, association with accessory proteins, and maintenance of silent synapses (Hall and Ghosh, 2008). The use of these NMDAR-inhibiting agents may allow synapses that were not stabilized due to EtOH-mediated impairments, a “second chance” to become stabilized via increases in AMPAR-mediated transmission and expression of accessory proteins involved in synaptic formation and maintenance, presenting a possible clinical therapeutic target in FASD (Hall et al., 2007; Adesnik et al., 2008).

Although many mechanisms have been proposed for EtOH-mediated impairments in CNS function, it is becoming clear that alterations in the normal assembly of neuronal circuitry plays an important role in the mechanism of action of EtOH. Studies presented here, as well as work from others, support the notion that impairments in glutamatergic signaling and synaptic plasticity during the third trimester-equivalent period are a target of EtOH, and in part, contribute to the long-term deficits observed in FASD.

FASD is often described as a preventable disease (Medina and Krahe, 2008; Ripabelli et al., 2006; Payne et al., 2005; Krulewitch, 2005), and indeed efforts to heighten awareness about the consequences of EtOH consumption during pregnancy have had some limited success (Abel, 1998). In spite of this, a substantial portion of women drink during pregnancy (Muhuri and Gfroerer, 2009). Therefore, therapeutic interventions for FASD need to target several

areas such as reducing/preventing maternal ethanol consumption, ameliorating the effect of EtOH during fetal development and treating the long-lasting consequences of EtOH exposure. Clinical therapies for children afflicted with FASD have shown some efficacy and these include behavioral, social, educational, and pharmacologic interventions (Reviewed in Peadon et al., 2009). New therapeutic interventions are being developed with the aid of animal models of FASD; for example, studies with rats have shown improvements in learning and memory and hippocampal dendritic spine density with environmental enrichment and voluntary exercise (Thomas et al., 2008; Hannigan et al., 1993; Berman et al., 1996). However, these interventions have yet to be translated into the clinic.

The data support that the long-term deficits induced by developmental EtOH exposure are founded in alterations in developmental processes such as glutamatergic signaling and network formation as addressed here, and “*post hoc*” treatments may have limited efficacy. Encouragingly, animal models of prenatal EtOH exposure have shown reversal of learning and memory deficits when the nootropic agent, aniracetam (positive allosteric modulator of AMPARs) was administered during early life after prenatal EtOH exposure (Vaglenova et al., 2007). Furthermore, another pharmacologic agent, vinpocetine (phosphodiesterase 1 inhibitor), was been shown to restore impairments in ocular dominance plasticity induced by third trimester-equivalent EtOH exposure (Medina et al., 2006). Clearly, future research is needed toward the development of effective therapeutic interventions against FASD.

Attempts to mitigate EtOH-mediated deficits during *in utero* development are potentially one of best avenues of treatment. Within this developmental window, the third trimester is believed to be the period of highest susceptibility to the teratogenic effects of EtOH (Livy et al., 2003a; Berman and Hannigan, 2000; Thomas et al., 2002; Galindo and Valenzuela, 2006; Krahl et al., 1999; Thomas et al., 2008; Olney et al., 2001; O'Leary-Moore et al., 2006; Hunt et al., 2009; Tsuji et al., 2008). Choline supplementation during prenatal and early postnatal development has been shown to be protective in a number of areas, including growth retardation as well as learning and memory impairments (Thomas et al., 2000; Thomas et al., 2009). In addition, studies have shown that NMDAR inhibition after third trimester-equivalent EtOH exposure protects against learning and memory deficits (Thomas et al., 2002). However, the use of pharmacotherapy against the effects of EtOH described here, such as, restoring synaptic plasticity with a positive modulator of AMPARs, presents inherent problems. Maturation of neuronal circuits occurs at different time points in the brain, and unless region specific targeting of the ameliorative agent can be achieved, benefit is questionable due to the likelihood that unwanted side effects may occur. Furthermore, compliance issues would likely be encountered with pregnant women who use/abuse EtOH. Nonetheless, understanding the basic pathophysiology of the developmental actions of EtOH remains paramount in designing effective therapies against FASD.

What are the first steps in dealing with maternal EtOH consumption?

When a pregnant woman seeks prenatal care, the first thing that needs to be

addressed is maternal substance abuse. Although it is advocated by the American College of Obstetricians and Gynecologists that all pregnant women are screened for this, approximately 35-55% of women in two studies reported that questions related to substance abuse were not asked during prenatal care visits (Hankin et al., 2000). Pregnancy presents a unique opportunity to provide interventions to alcoholic women due to increased maternal motivation to have a “healthy” baby (Hankin et al., 2000). Although FAS and FASD have been described as “preventable”, the underlying pathology is often EtOH-dependence and addiction, which are difficult to prevent and treat (Rayburn and Bogenschutz, 2004). In non-pregnant women, several approaches, including pharmacotherapy, have shown clinical efficacy in treating alcohol-dependence; however, the majority of these pharmacologic agents negatively affect the offspring, effectively making them unsafe for use while pregnant (Rayburn and Bogenschutz, 2004). Thus, in addition to counseling by the primary health care provider, alternative psychosocial interventions include Alcoholics Anonymous and family service agents (Lui et al., 2008). If women are unable to quit drinking, data presented here, support the notion that any attempt to reduce maternal EtOH consumption likely has beneficial effects with regard to the developing fetus.

7.4 Critique and future directions

Slice preparation and recording conditions

For the preparation of the brain slices used in the studies described here, animals were euthanized after anesthesia with ketamine (NMDAR-antagonist). In addition to its sedative/hypnotic effects, ketamine has also been shown to induce cell death in the hippocampal region, potentially affecting electrophysiological recordings and this must be kept in mind when interpreting our results (Hayashi et al., 2002; Young et al., 2005). The acute slice preparation has many advantages as discussed in Chapter 3; however, neuronal connections are inevitably severed, and synaptic inputs that can be studied are limited to those preserved in the brain slice. In addition, because the sections are relatively thin, there is an increased exposure of cells to the ACSF; therefore, diffusible neuromodulators that affect glutamatergic signaling and plasticity (e.g. BDNF, nitric oxide, endocannabinoids) could potentially be washed out.

The recordings presented here were performed under pharmacologically isolating conditions, which included inhibitors of GABAergic transmission. Studies in mature animals have shown that effects of EtOH on GABAergic signaling can play a role in alterations in LTP (Schummers and Browning, 2001). Thus, future studies should address developmental effects of EtOH with GABAergic transmission intact, as well as the roles of alterations in other neurotransmitter systems in the mechanism of action of EtOH. In addition, pharmacological agents were used to elucidate the effects of EtOH on Ca²⁺-impermeable AMPARs, and inferences were made as to the effects on Ca²⁺-permeable AMPARs by using the effect on the total population as a reference. Experiments using genetic manipulation techniques, such as point mutations in

the GluR2 subunit of AMPARs making all AMPARs Ca²⁺-permeable, would be required to more directly address the effects of EtOH on this subpopulation of receptors. Alternative approaches could include miRNA approaches aimed at knocking down the enzyme (adenosine deaminase) that causes mRNA editing of the GluR2 subunit, leading to the insertion of an arginine instead of a glutamine in the intracellular portion of the M2 domain.

The rapid rise and fall in EtOH concentrations that occurs with bath perfusion is not physiologic, and studies have shown differential effects of synaptic plasticity with gradual (over 75 min) increases in EtOH concentrations in the CA1 hippocampal region (Tokuda et al., 2007a). In addition to this, acute studies of EtOH actions, such as those described in this dissertation, do not assess the potential contributions of products of EtOH metabolism, such as acetaldehyde, which has been postulated to contribute to the deficits observed in FASD (Hard et al., 2001). We attempted to address this issue by using an *in vivo* exposure paradigm; however, this shortcoming must be kept in mind when interpreting data from our acute EtOH application studies.

Recording techniques

The majority of studies in this dissertation use the field recording technique. Advantages of this approach include that data are collected as an average of responses over a population of neurons. On the other hand, this very advantage is a limitation in regards to not being able to detect what is occurring at the level of the individual neuron. Furthermore, field recordings during the

early developmental period have inherent problematic issues. In the majority of recordings, the fiber volley overlaps with the field response, thus making the preferred method of quantification (e.g. initial fEPSP slope) difficult to implement. In addition, the fiber volley was significantly contaminated by the rising phase of the fEPSP, making it difficult to ascertain whether EtOH affected the presynaptic volley, leading to indirect inhibition of the fEPSP. This is addressed in detail in Chapter 8.6 and Appendix fig 8.6; briefly, exploration of this issue revealed that the presynaptic volley is not directly affected by EtOH and that the decrease in its amplitude is a consequence of EtOH-induced inhibition of the fEPSP.

Another uncertainty that must be kept in mind when interpreting our results is that measures of glutamate release were performed using paired-pulse plasticity that, although commonly used, do not directly measure glutamate release (Hanse and Gustafsson, 2001). Future studies using quantum dots or FM dyes should be employed to confirm that EtOH does not affect glutamate release at developing CA1 synapses.

In our studies, synaptic plasticity was induced using a high frequency stimulation paradigm (HFS; e.g. 100Hz). Although this HFS paradigm replicates *in vivo* changes at the level of glutamate receptors that occur with learning and memory in the hippocampus, LTP is not induced by this type of stimulation under physiological conditions in the intact developing brain (Whitlock et al., 2006). The physiological mechanism that is thought induce plasticity during development involves large GABAergic and glutamatergic driven bursting patterns thought to originate in the CA3 region and propagate to the CA1. These

bursting patterns provide the correlated network-activity and neuronal depolarization necessary to stabilize synaptic connections. (Leinekugel et al., 1997; Ben-Ari, 2001; Leinekugel, 2003). Elegant studies have shown that pairing afferent stimulation with this bursting activity leads to induction of LTP. It would be interesting to assess the effect of EtOH on plasticity induced by this type of stimulation in the developing hippocampus (Kasyanov et al., 2004; Mohajerani et al., 2007). In addition, exciting future experiments would be to further investigate the effects of developmental EtOH exposure on plasticity using *in vivo* recording techniques in neonatal animals; however, these studies must await the development of less invasive recording techniques that minimally disrupt neonatal behavior and maternal-pup interactions. Furthermore, experiments addressing changes at the individual synapse level using 2-photon imaging and laser-induced glutamate uncaging could be useful in establishing whether synaptic strength at individual synapses is increased.

In studies presented in this dissertation, it was concluded that the inhibitory effects of EtOH on LTP induction were likely postsynaptic due to the lack of presynaptic changes in glutamate release probability as discussed in chapters 4 and 6. This was based upon data from paired-pulse plasticity studies; however, it is also a possibility that the effects occur as a result of EtOH-mediated impairments in the LTP induction paradigm. Future studies should address the effects of EtOH on the LTP induction paradigm using whole-cell or perforated patch-clamp recording techniques and measure Ca^{2+} influx triggered by the induction paradigm in the presence and absence of EtOH. There is a

relationship between Ca^{2+} concentration and the change in synaptic strength, by which high synaptic concentrations lead to LTP, whereas low synaptic concentrations (as well as extrasynaptic) leads to LTD. Thus, measuring the effects of EtOH on this parameter would assess effects on the induction paradigm.

Additionally, the conclusion that repeated in vivo EtOH exposure does not affect synaptic strength was based on data from AMPAR- and NMDAR-mediated input-output curves. However, as described in chapters 4-6, our studies did not account for changes in the presynaptic volley and therefore should be addressed as synaptic excitability rather than synaptic strength. To address this, NMDAR-mediated fEPSPs do demonstrate a clear presynaptic volley and data were reanalyzed after normalization to this parameter (Chapter 8.8). There was no significant difference in NMDAR-mediated input-output curves between control and EtOH exposed animals suggesting that there is no change in synaptic strength, but uncertainty remains with regard to AMPAR-mediated responses. Future experiments as previously discussed above should be used to address this uncertainty.

Exposure paradigm

Although advantageous in many respects, the vapor chamber inhalation paradigm has limitations with regards to the route of EtOH administration. For instance, the role of the maternal-fetal interface in the mechanism of action of EtOH during the 3rd trimester-equivalent cannot be assessed with this method. Although we did not observe differences in growth progression, the long-term

effects of EtOH on lung development and gas exchange have not been studied. Additionally, this route of EtOH entry into the developing animal bypasses hepatic metabolism that occurs with other third trimester-equivalent models of EtOH exposure (“pup in the cup” and intragastric gavage); however, vapor chamber exposure more closely mimics the flow of blood from placenta→umbilical vein→ductus venosus→inferior vena cava→→brain in the developing human fetus. Thus, it is important to keep this point in mind when comparisons across studies are being made.

It is also important to note that the exposure paradigm modeled here is only one of many drinking patterns pregnant women engage in—others include low-dose consumption with meals and heavy binge drinking during weekends. In addition, our studies only addressed effects during the early withdrawal period. It is possible that adaptive changes are delayed in their expression, and future experiments should address the effects of prolonged withdrawal in these animals (Nelson et al., 2005). In addition, future studies should also investigate EtOH effects throughout pregnancy including the first and second trimester-equivalent periods. Furthermore, future studies modeling co-drug use such as nicotine, marijuana, cocaine, etc. may more accurately describe what occurs in the human population. During pregnancy, 16.8% of women report cigarette use, 2.8% report marijuana, and 0.3% report cocaine (including crack) use (Muhuri and Gfroerer, 2009). Another caveat associated with FASD is that environmentally-induced stress is often associated with developmental EtOH exposure, which likely

enhances the detrimental effects of EtOH on the developing fetus (Choi et al., 2008; Weinberg et al., 2008; Kinsella and Monk, 2009).

Behavioral relevance and long-lasting effects of this paradigm

Studies presented here only address synaptic function. An interesting future direction would be to replicate the learning and memory deficits observed by Thomas and colleagues (2002) using the vapor chamber exposure paradigm (Thomas et al., 2002). Furthermore, studies aimed at observing deficits with lower doses of EtOH using more rigorous behavioral testing methods—such as those developed in the Morris laboratory—coupled with more sensitive assays of neuronal function should be performed in the future (Bast et al., 2005).

8. Appendix

8.1. The effects of acute EtOH exposure on Long Term Depression (LTD) in the CA1 hippocampus of P7-9 rat pups

8.1.1. Purpose of the experiment

EtOH (80mM) was shown to affect the induction of LTP in the hippocampus in acute slices of P7-9 rat pups. These experiments examined whether EtOH also affected the induction of LTD.

8.1.2. Methods

Field EPSP slice recordings from the CA1 hippocampal region were performed as described in chapter 4. When indicated, the ACSF contained gabazine (10 μ M) AP5 (50 μ M), and/or LY 367385 (LY; 50 μ M; mGluR1 antagonist). LTD induction was performed by delivering a 5 Hz stimulus train for 3 min. Test pulses were delivered every 30 s. Stimulus duration was 75 μ s, and the paired-pulse interval was 50 ms. Data were analyzed using a one sample t-test v. 100%. Baseline corresponded to the 20 sweeps immediately preceding the application of EtOH, and the test condition corresponded to the last 20 sweeps of the recording, which was at least 35 min from the LTD induction protocol. The fEPSP peak amplitude was used for analysis. Data are presented \pm SEM.

8.1.3. Results

Figure 8.1.A shows the time course of the LTD induction paradigm, and 8.1.B shows the time course for the paired-pulse ratio. Fig 8.1.C. shows that, under control (gabazine) conditions, the LTD protocol significantly reduced fEPSP amplitude to $80.5 \pm 5.3\%$ of baseline ($n=7$; $p<0.05$ by one sample t-test v. 100%). In addition, there was a significant increase of 113.6 ± 2.3 ($p<0.01$; by one sample t-test v. 100%) in the paired-pulse ratio. We then tested if EtOH (80 mM) affected this LTD induction paradigm and glutamate release. When this LTD protocol was administered in the middle of the EtOH application (in the presence of gabazine), fEPSPs were reduced to 74.4 ± 3.2 of baseline ($n=7$; $p<0.01$ by one sample t-test v. 100%). There was a significant increase of 116.2 ± 4.5 ($p<0.05$; by one sample t-test v. 100%) in the paired-pulse ratio. LTD in the presence of EtOH was not significantly different from LTD in its absence; the increase in the paired-pulse ratios was also not significantly different (NS by unpaired t-test).

We then attempted to elucidate the mechanism for this LTD induction. We first attempted to see if it was NMDAR dependent. Using gabazine and AP5, we found that fEPSPs were significantly inhibited to $85.3 \pm 2.3\%$ of baseline ($n=6$; $p<0.01$ by one sample t-test v. 100%). However, there was no significant change in the paired-pulse ratio ($100.3 \pm 2.4\%$; NS by one sample t-test v. 100%). We then tested if this effect was mGluR1 dependent and found that an antagonist of this receptor did not affect LTD recorded in presence of AP5 and gabazine;

fEPSPs were significantly reduced to 85.0 ± 4.3 ($n=8$; $p<0.05$ by one sample t-test v. 100), and the paired-pulse ratio was not affected $103.4 \pm 3.0\%$ (NS by one sample t-test v. 100%).

8.1.4. Conclusions

Studies have shown that LTD induction mechanisms are developmentally regulated; in P8-15 rats, the mechanism by which LTD is induced is largely presynaptic (Nosyreva and Huber, 2005). EtOH (80 mM) did not affect the induction of LTD, and this was not entirely surprising in light of the fact that EtOH did not affect glutamate release in this brain region in previous studies in immature animals (see Chapter 4; Fig. 6). However, it was surprising that we observed a decrease in fEPSP responses (LTD) with no change in the paired-pulse ratio with AP5 and gabazine present. This is suggestive of both pre- and postsynaptic LTD induction mechanisms. The presynaptic component identified as a decrease in glutamate release by the paired-pulse ratios is potentially NMDAR- and/or GABA_AR-dependent, due to the lack of a change in the paired-pulse ratio when these receptors were blocked. The mechanism of the postsynaptic LTD component remains unidentified; we thought it was likely mGluR mediated, but LY failed to reduce LTD.

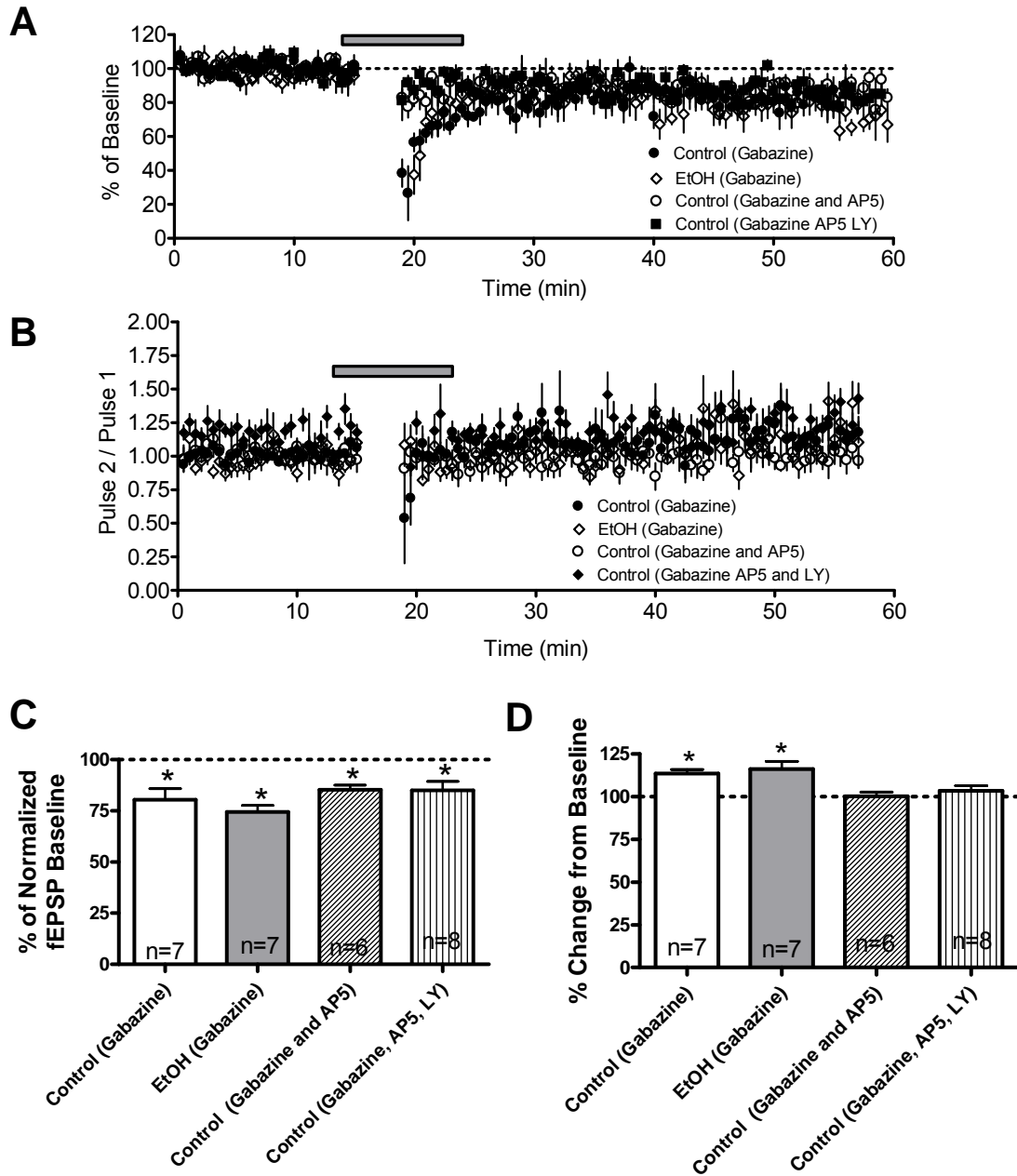


Figure 8.1.1. Effects of acute EtOH on LTD induction in the CA1 region of P7-9 rats. (A) Time course of fEPSP amplitude under identified conditions. LTD induction protocol was a 5Hz stimulus for 3 min, and is represented by the gap in data points. (B) Time course of the paired-pulse ratio of the LTD induction protocol. Bar indicates when 80 mM EtOH. (C) Bar graph illustrating the percent decrease in fEPSPs normalized to the baseline (D) Percent change in the paired pulse ratio from baseline. $*=p<0.05$.

8.2. In vitro and in vivo effects of EtOH on MeCP2 levels, phosphorylation, and BDNF mRNA.

8.2.1. Purpose of the experiment

Mutations in the gene that codes for the protein MeCP2 lead to Rett syndrome. Alterations in brain derived neurotrophic factor (BDNF) levels have been detected in animal models of FASD and Rett syndrome. The BDNF gene is regulated by multiple factors, including MeCP2, and has been shown to be induced by neuronal activity. One of the functions of MeCP2 is to repress transcription by binding to methylated DNA sites, which is attenuated by MeCP2 phosphorylation at specific sites. We have previously shown that EtOH potentiates neonatal CA3 hippocampal neuronal circuits resulting in increased Ca^{2+} transients (Galindo et al., 2005). Therefore, we hypothesized that increased EtOH-induced Ca^{2+} influx into the neonatal CA3 hippocampal region leads to activity-dependent phosphorylation of MeCP2, leading to an increase in BDNF mRNA levels.

8.2.2 Methods

Western Blotting

Brain slices (400 μ m) were prepared as previously described (Chapter 4). P4-6 brain slices were exposed to EtOH (50 mM; 30 min), Mg^{2+} -free ACSF, and Mg^{2+} -free ACSF + NMDA (10 μ M; 5 min exposure, followed by 25 min chase in Mg^{2+} -free ACSF) at 32°C. Then the CA3 hippocampal region was microdissected, and western blotting studies with CA3 homogenates were performed. Primary antibodies against total MeCP2 (1:1000; Upstate

Biotechnologies, Lake Placid, NY) and phospho-S421-MeCP2 (1:1000; generously provided by Michael Greenberg, Harvard University) were used. Samples (10 µg) were run on a precast 7.5% ready gel (Bio Rad, Hercules, CA). Data are presented as the ratio of phospho-S421-MeCP2 over total MeCP2. Western blot studies were normalized to the average intensity of 3 coomassie stained bands or actin.

Immunohistochemistry and Microscopy

Brain slices were also paraformaldehyde fixed (4% PFA; 24-48 hrs), sucrose protected (24 hrs), frozen in isopentane, and re-sectioned (12-14 µm thick) using a cryostat. A pap pen was used to draw a circle around the tissue, which was then permeabilized with 0.2% Triton-X-100 in PBS (~20 min) followed by blocking in 2% bovine serum albumin (BSA) in PBS (30+ min). Then, the primary antibody was applied in PBS w/ 2% BSA (Overnight at 4° C). Slides were then rinsed in PBS-T 5x10min, followed by incubation with the secondary antibody in 2% BSA at room temp for 2 hr. Slides were then rinsed in PBS-T 5x10 min, and counterstained with DAPI 1:500 (10-20 min). Slides were then rinsed again, and 1-2 drops of Vectashield (Burlingame, CA) were applied and cover-slipped. Microscopy images were taken with a Zeiss Meta 510m confocal microscope at 40x.

Vapor exposure

Vapor exposures were performed as previously described (chapter 5). Neonatal rats were placed in vapor chambers; EtOH or air was perfused for 4 hrs/day starting at P2 and ending at P8. Samples were taken at P2, P4, P6 and P8 (SECs= 40-80 mM).

RT-PCR

RT-PCR was used to measure BDNF mRNA levels in neonatal rats exposed to air or EtOH using a vapor chamber exposure paradigm. mRNA was isolated from the hippocampus using the Oligotex Direct mRNA Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol and stored at -80 °C prior to use. The mRNA concentration was measured (OD 260 nm) using a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). cDNA synthesis reactions were performed using 10 ng mRNA, and RT-PCR was performed as described (Caldwell et al., 2008). The relative quantification of the target gene was determined using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

8.2.3 Results

Figure 8.1 shows western blot data of p-MeCP2 and total MeCP2 levels in the CA3 hippocampus microdissected from brain slices exposed to ACSF (control), EtOH (50 mM; 30 min), Mg²⁺-free ACSF, and NMDA in Mg²⁺-free ACSF (10 μm; 5 min followed by 25 min chase). Although there were large increases in both p-MeCP2 levels (Fig. 8.2.1a) and total MeCP2 (Fig. 8.2.1b) levels these

were not significantly different from baseline. However, when analyzed as a ratio of p-MeCP2 to total MeCP2, EtOH caused a significant decrease in this parameter (n=4; p<0.05 by one sample t-test v. 100%). As expected, NMDA treatment led to a significant increase in the ratio (n=4; p<0.05 by one sample t-test v. 100%).

To address these results using a different method, we performed immunohistochemistry. Figure 8.2.2 shows sample 40x images from (control), EtOH, Mg²⁺-free ACSF, and NMDA in Mg²⁺-free ACSF conditions. No differences with respect to control in DAPI staining (pixel intensity) were observed for either the EtOH or NMDA conditions, indicating that cell counts were not affected by these treatments (Fig. 8.2.3A,C). When analyzed for changes in total MeCP2 levels, no significant effects of EtOH or NMDA were detected (Fig. 8.2.3B,D). Similar results were obtained when data were normalized to DAPI staining (Fig. 8.2.3E).

We then proceeded to study the effect of *in vivo* EtOH exposure on MeCP2 levels. Rat pups and dams were exposed to EtOH vapor reaching SEC's of 40-80mM starting on P2, and continuing through P8 (Fig. 8.2.4A-B). Pup weights were not significantly different by two-way ANOVA (n=3; Fig. 8.2.4C). As shown in Fig. 8.2.5A-B, *in vivo* EtOH exposure did not consistently change MeCP2 levels.

Samples were then processed for BDNF mRNA level analyses in the laboratory of Dr. Caldwell (Fig. 8.2.6A-B). Exon VI represents the coding sequence, and is representative of changes in all splice variants. There was a

significant decrease in the total (exon VI) on all postnatal days tested for the EtOH group compared to controls (n=3; $p<0.05$ by two-way ANOVA followed by Bonferroni's post hoc test). This significant difference on all postnatal days tested was also seen with exon IV, and from P4-8 with exon V.

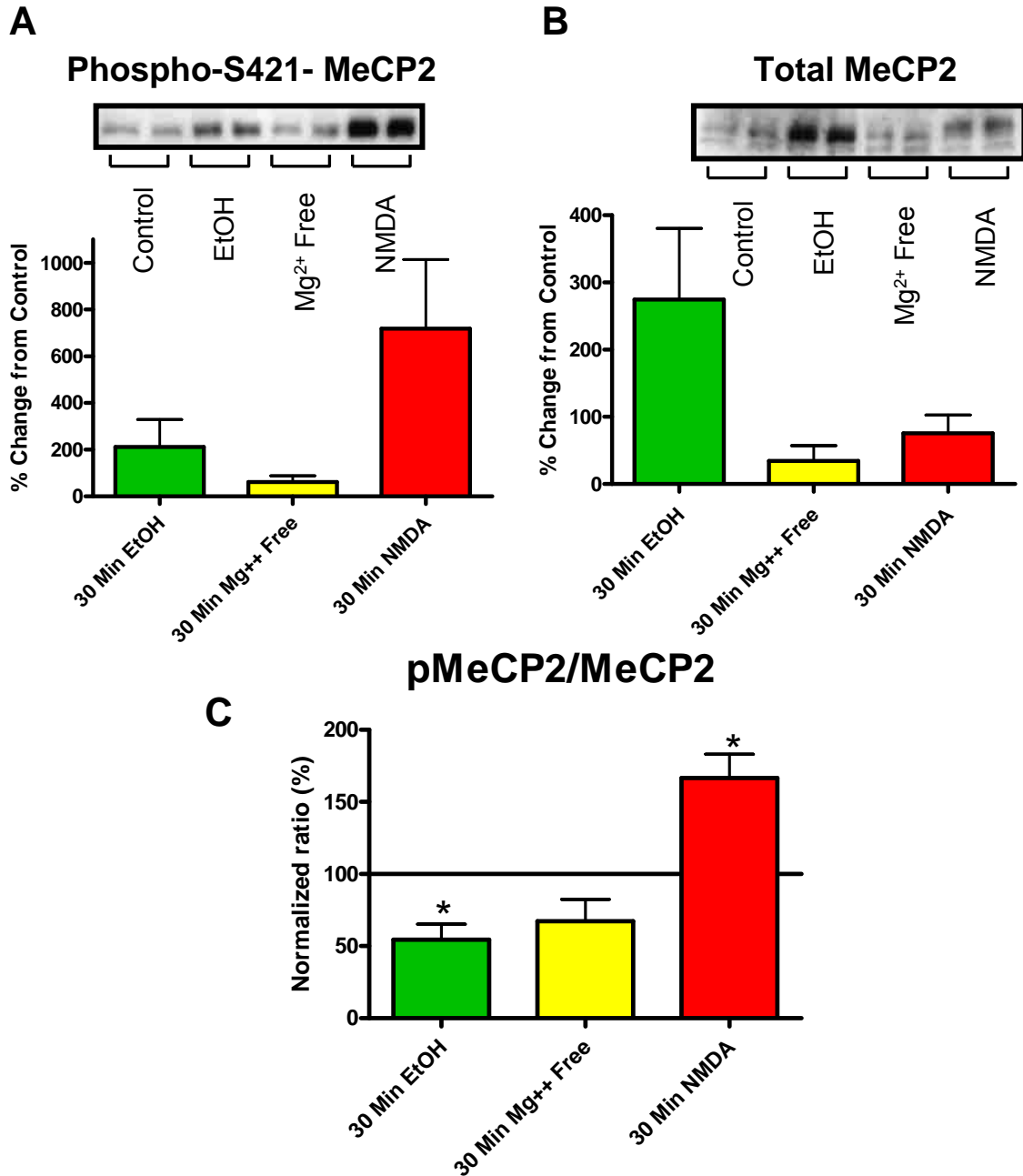


Figure 8.2.1. Western blot data of *in vitro* CA3 microdissected slice experiments. Effects of phospho-S421-MeCP2 (A) and total MeCP2 (B) levels expressed as a percent change from ACSF controls. Insets are representative western blots for each condition. (C) ratio of phospho-S421-MeCP2 to total MeCP2 levels normalized to ACSF only controls. $n = 4$, $*p < 0.05$ by one sample t-test from 0 or 100%.

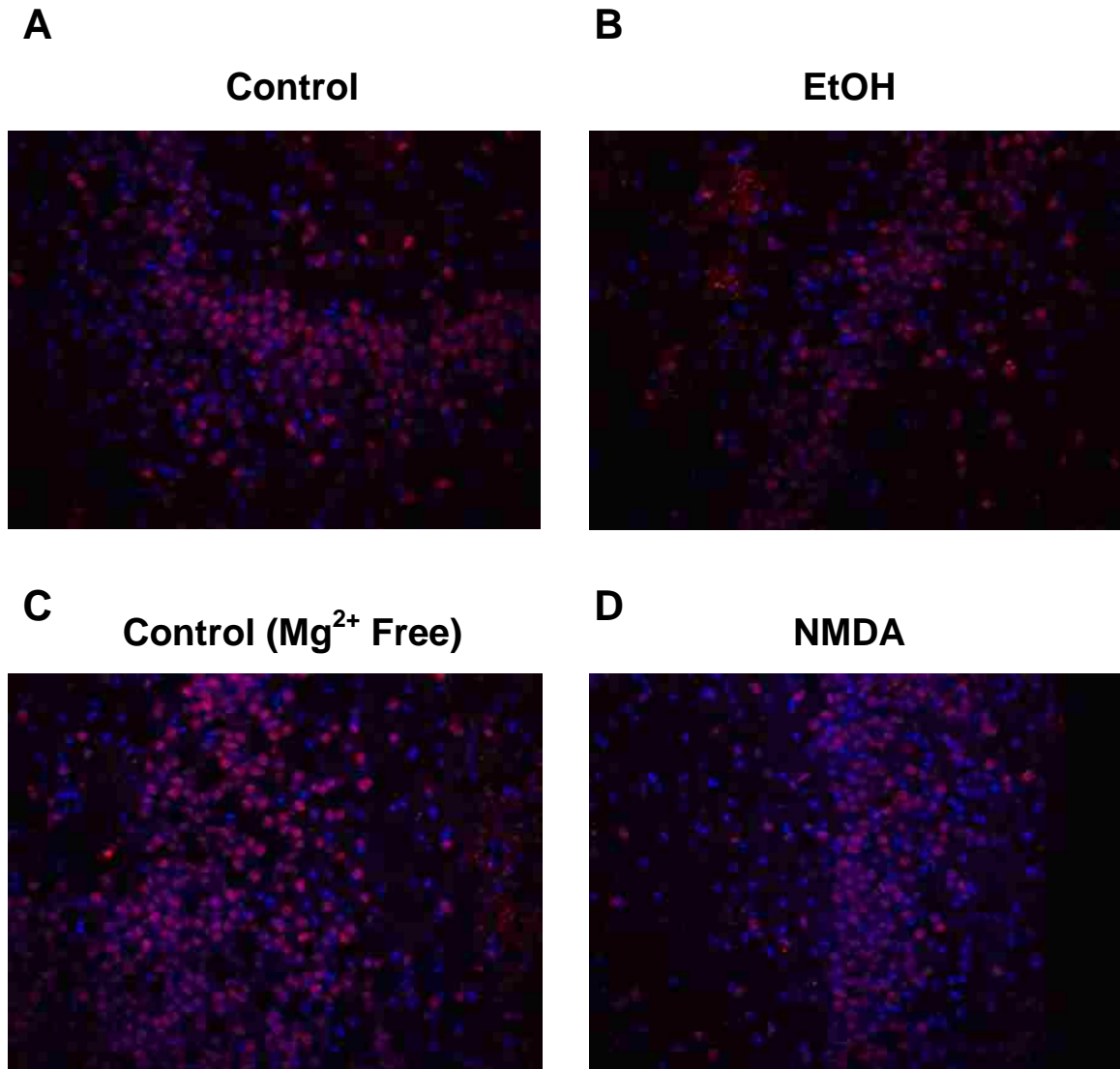


Figure 8.2.2. Immunohistochemical images of total MeCP2 levels from resected brain slices. (A) ACSF control, (B) EtOH exposed slices (50 mM) (C) control in Mg²⁺ free ACSF, and (D) NMDA (10 μM) in Mg²⁺ free ACSF. Red staining (Cy3) is 2^{ry} Antibody to total MeCP2, and blue DAPI nuclear DNA. Images taken at 40x.

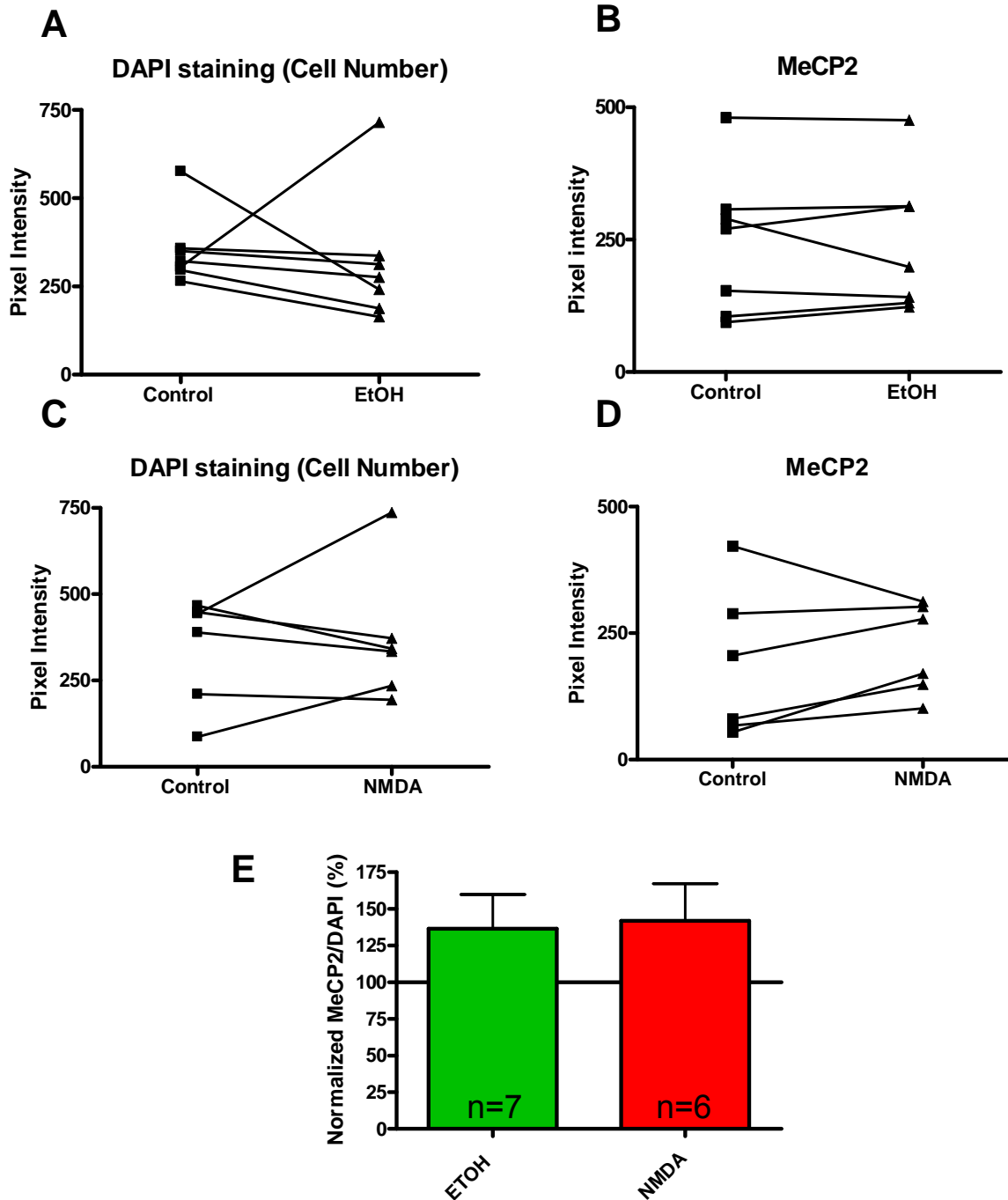
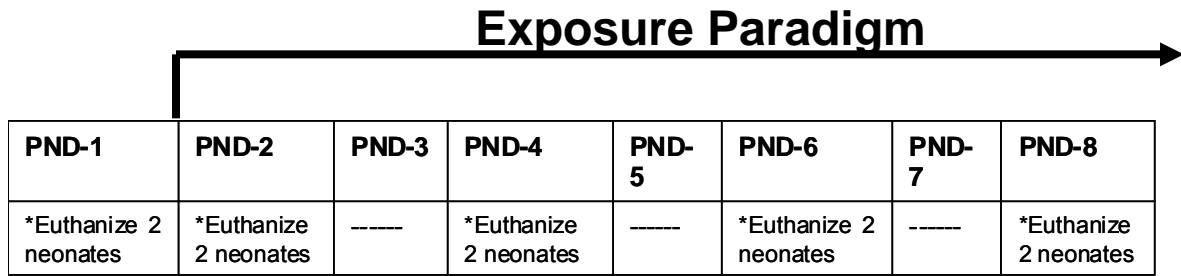
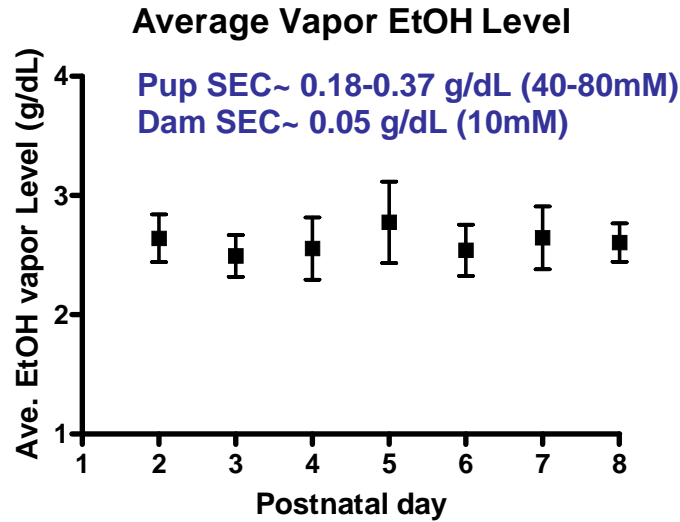


Figure 8.2.3. Total MeCP2 levels are not altered with EtOH or NMDA exposure in brain slices when analyzed by immunohistochemistry. Pixel intensity for DAPI stained slides for control and EtOH (50 mM) treated slices (A), and control and NMDA (10 μ M) treated slices (C). Pixel intensity of total MeCP2 levels in control and EtOH treated slices (B), and control and NMDA treated slices (D). Summary bar graph showing the total MeCP2 pixel intensity normalized to that of the DAPI staining (E). MeCP2 and DAPI staining were performed in the same slice for each data point.

A



B



C

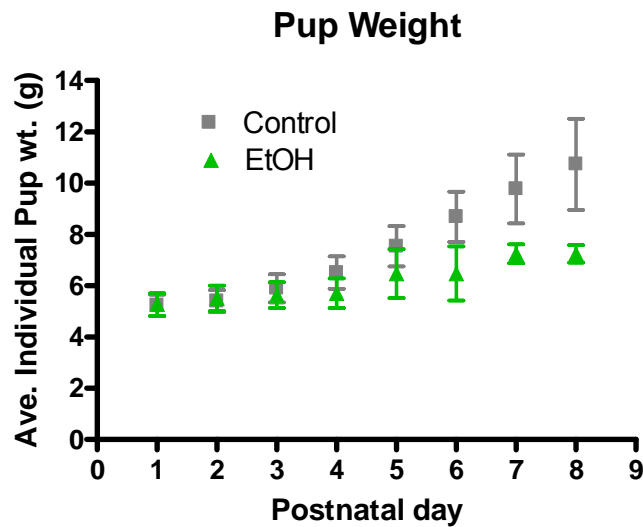


Figure 8.2.4. Vapor exposure paradigm. (A) Exposure paradigm where animals were exposed to vapor levels for 4hrs / day starting on P2 and continuing to P8. (B) EtOH vapor level measured in the chamber. This led to pup SECs of 40-80 mM, and dam SECs of 10 mM (n=2). (C) Pup weight measured on each postnatal day for control and EtOH exposed animals.

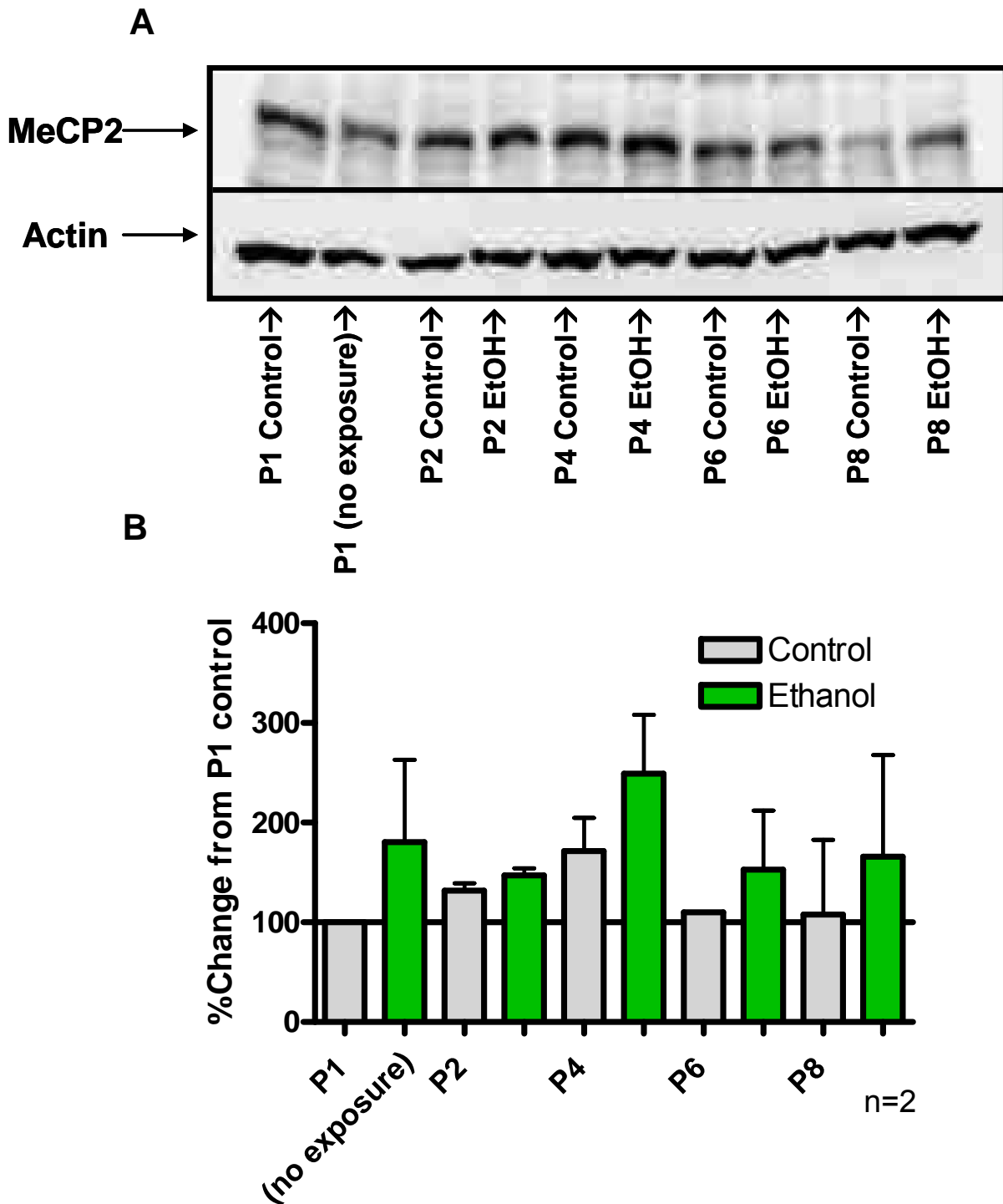


Figure 8.2.5. *In vivo* EtOH vapor exposure does not alter hippocampal total MeCP2 levels. (A) Representative western blot for total MeCP2 levels from whole hippocampus. (B) Percent change of total MeCP2 levels normalized to 100% of P1 controls. Data was normalized to Actin. N=2.

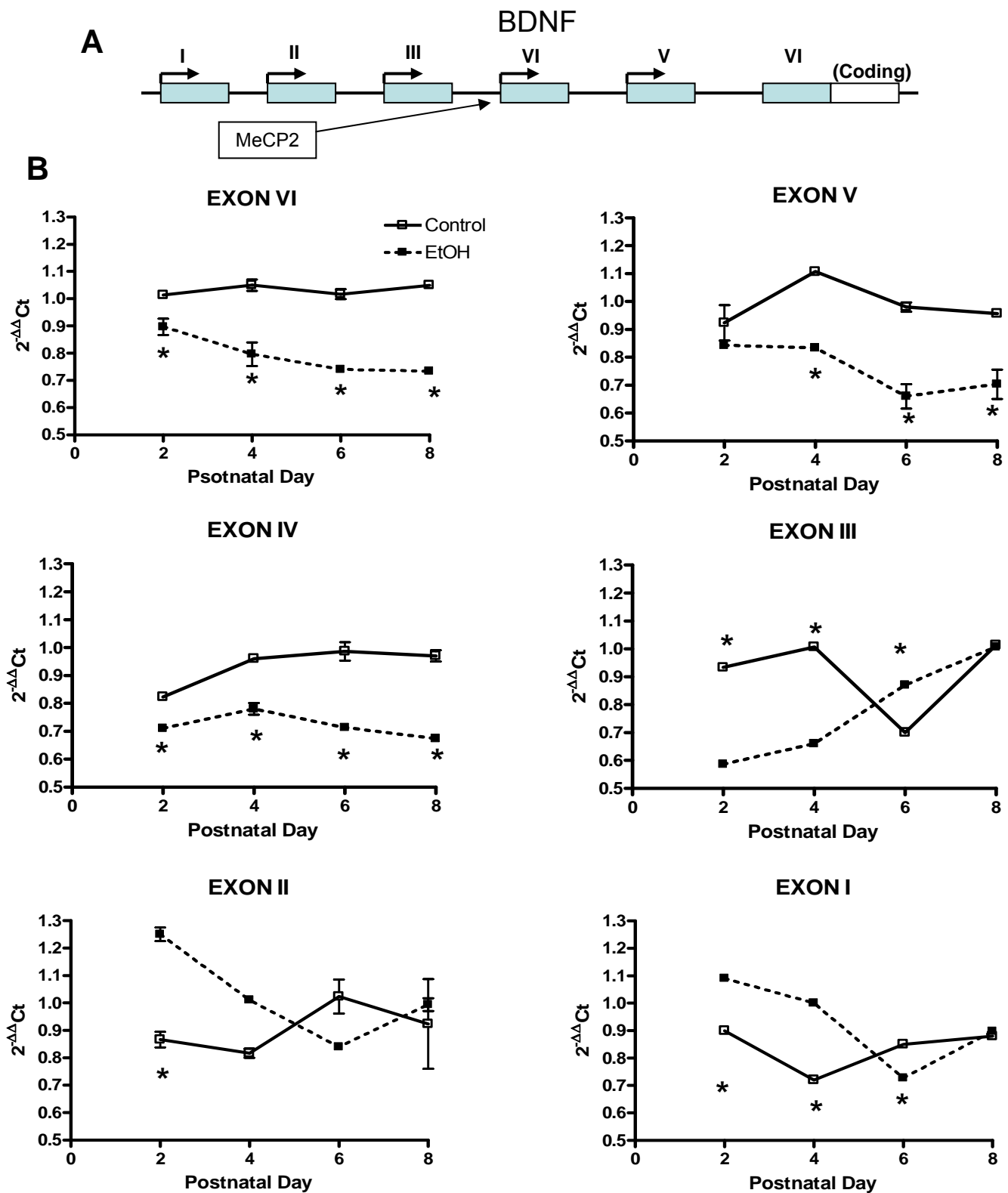


Figure 8.2.6. The effects of vapor EtOH exposure on BDNF mRNA levels from the hippocampus of P2-8 rats. (A) BDNF gene showing mRNA exons tested. Note MeCP2 acts as a negative regulator of transcription at exon IV. (B) Results of PCR assays performed in the Caldwell Lab for exons I-VI. Data were analyzed by two-way ANOVA, followed by Bonferroni post hoc tests. $n=3$ for each condition, and $*=p<0.05$

8.2.4. Conclusions

Acute EtOH exposure significantly decreased the pMeCP2/total MeCP2 ratio, whereas the positive control condition with NMDA produced a significant increase in the ratio. It appears as if the effect of EtOH was due to an increase in total MeCP2 levels; however, this did not reach significance. We then addressed this using a different method, immunohistochemistry. There was no change in total protein MeCP2 protein levels with either EtOH or NMDA treatment, thus indicating possible artifacts from western blotting techniques or slice experiment protocols.

To further address this issue, we switched to an *in vivo* EtOH exposure paradigm. Total MeCP2 levels were not different between control and EtOH groups on any of the days tested (P2-8) coinciding with the immunohistochemistry data, and further suggesting that there was no change in MeCP2 levels with either acute or *in vivo* EtOH exposure.

This EtOH *in vivo* exposure paradigm also produced a decrease in BDNF mRNA levels for the coding exon (VI) on all days tested. This was also seen for exon IV and exon V (with the exception on P2 for exon V). This is interesting in light of the fact that MeCP2, as well as other factors including CREB, regulate the promoter of this exon IV. Future studies are needed to determine if EtOH affects phosphorylation of MeCP2, and its role in the regulation of BDNF mRNA levels.

8.3. The effects of electrode separation on the presynaptic volley and fEPSP in field recordings in P7-9 rat pups.

8.3.1. Purpose of the experiment

In recordings from neonatal animals, we found it difficult to separate out the presynaptic volley from the field response. This experiment series address two issues: (1) does increased distance result in increased separation between the volley and fEPSP response (as it does in more mature animals), and (2) input-output curve data showing the effect of recording distances on fEPSP amplitude.

8.3.2 Methods

Recordings of AMPAR-mediated fEPSPs were performed as described in chapter 4. Briefly, the ACSF contained blockers of NMDA- and GABA_A receptors (AP5 50 μ M, and gabazine 10 μ M). Recordings were performed by placing the stimulating electrode in the vicinity of the Schaffer collaterals, and first placing the recording electrode ~400 μ m away (distance 2) and then ~200 μ m away (distance 1). Input-output curves were collected at both recording locations. The reference to calculate the separation distance was the diameter of the outer pole of the stimulating electrode (125 μ m)

8.3.3 Results

Figure 8.3.1A-B shows that with increasing distances between the stimulation and recording electrodes, there is a decrease in the amplitude in responses. Separation of the presynaptic volley with increasing

recording/stimulating electrode separation could not be assessed because the volley became undetectable at the ~400 μm separation distance. In addition, the input-output curves showed a decrease in fEPSP amplitude at this distance (Fig. 8.3.1C; $n=3$; $p<0.05$ by two-way ANOVA).

8.3.4 Conclusions

There should be better separation between the volley and fEPSP with increased distances between the recording/stimulating electrodes; however, the small amplitude of the volley and fEPSP response at greater inter-electrode distances made it difficult to assess this possibility. Moreover, the small fEPSP amplitude at greater separation distances would make it difficult to perform pharmacological experiments with agents that inhibit these responses, such as in the case of EtOH.

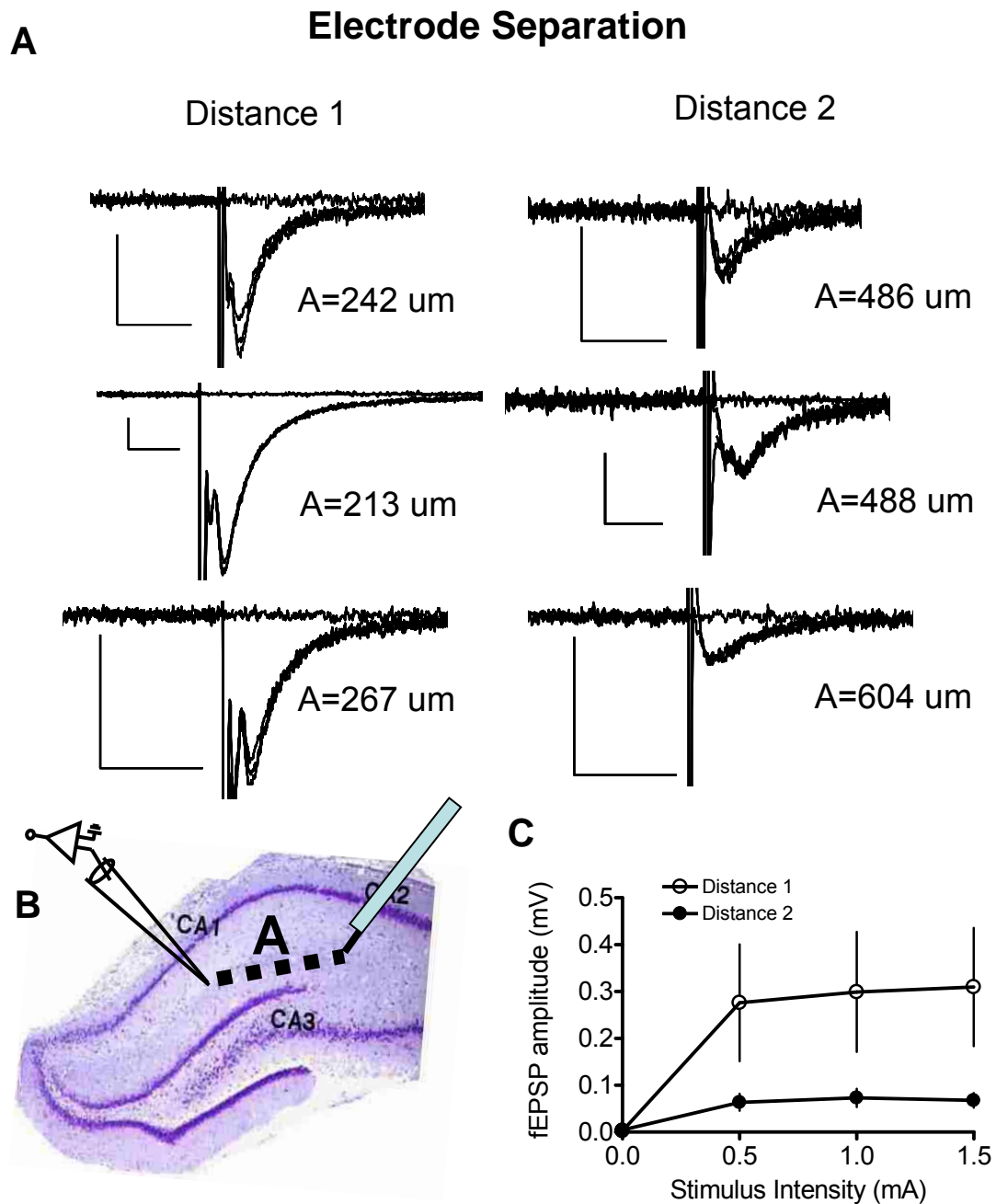


Figure 8.3.1. Effects of electrode separation on fEPSP responses in the CA1 hippocampus of P7-9 rat pups. (A) Pairs of recordings of AMPAR-mediated fEPSPs obtained with 2 different electrode separation distances in presence of NMDA and GABA_A receptor blockers (AP5: 50 μ M and gabazine 10 μ M) using increasing stimulus intensities. (B) Representation of measured distance between stimulation and recording electrode. (C) Input-output curve data for the different recording distances. (Stimulus duration 75 μ s; scale bar is 0.1 mV/20 ms)

8.4. BS³ crosslinking pilot study addressing AMPAR (GluR1-4) trafficking with the adenylyl cyclase activator, forskolin in the microdissected CA1 hippocampus from P5 rat pups.

8.4.1 Purpose of the experiment

This was a pilot study to determine the feasibility of studying changes in the surface expression of AMPARs in neonatal rat pups. Studies have shown that LTP mechanisms involve increased surface expression of AMPARs, a process that depends on PKA activity (Yasuda et al., 2003; Grosshans et al., 2002).

8.4.2 Methods

Brain slices (400 μm) were prepared as previously described in chapter 4. After a recovery period, half of the slices were incubated in forskolin (50 μM) for 5 min (32°C for all incubations) followed by a 20 min chase in standard ACSF. The other halves were incubated in ACSF for 25 min. Then, slices were placed into air-tight scintillation vials containing cold oxygenated ACSF with or without BS³ (1 mg/ml), a membrane impermeable crosslinking agent. These were then incubated at 4°C to stop any further trafficking for 45 min with gentle shaking. Then slices were washed 4x in cold oxygenated ACSF containing 20 mM Tris (pH 7.5). The CA1 regions from brain slices were microdissected, sonicated in 75 μL homogenization buffer buffer (20 mM HEPES, 0.5% Triton-X, 0.5 M NaCl, 10 mM NaF, 2 mM NaOrthovandronate, 0.2 mM EDTA, 1 mM DTT), then frozen in dry ice-EtOH cooled isopentane. Samples were run using western blotting

protocols as previously described in Appendix 8.2.2. Data were normalized to actin.

8.4.3 Results

BS³ is a cell-impermeable crosslinking agent that results in a smear (surface expressed AMPARs crosslinked to other proteins) on the top of the western blot, and a clear band of non-crosslinked AMPARs (internal pool). We used this technique to determine the forskolin-induced change in the internal pool normalized to total AMPAR levels in non-BS³ treated slices. Comparisons between control and forskolin treated conditions are shown in figure 8.4. Forskolin caused a significant decrease in the GluR1 internal pool, suggesting that it increased surface expression of this subunit (fig. 8.4B). However, it did not affect the internal pool for other GluR subunits.

8.4.4. Conclusions

It was surprising that forskolin only affected trafficking of GluR1, as the surface expression of GluR2 and/or GluR4-containing AMPARs has been shown to increase in an activity dependent manner. In fact, it is thought that GluR4 (or GluR2 lacking AMPARs) AMPARs are trafficked to the synapse early during stimulation and these are replaced by GluR2 containing AMPARs; however, we did not see significant surface trafficking of the GluR4 AMPAR subunits (Zhu et al., 2000). It is of important note that this was only a pilot study and further investigations are needed to accurately assess this issue.

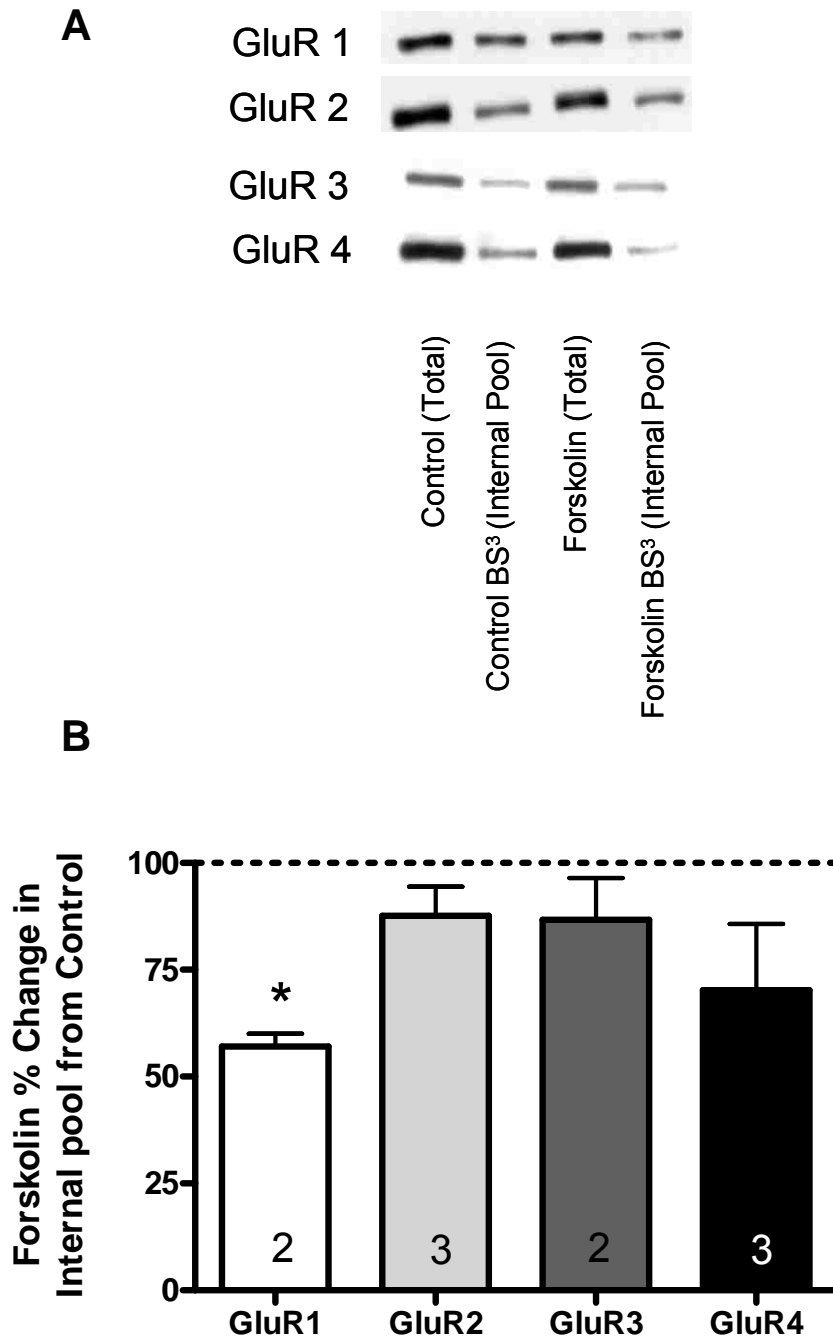


Figure 8.4.1. Pilot experiment addressing the effects of forskolin on GluR trafficking in the microdissected CA1 hippocampal region of P5 rat-pups. (A) Sample western blots for each GluR subunits under control, control BS³ treated, forskolin (50 μ M), and forskolin BS³ treated. (B) Percent change in the internal pool of forskolin treated slices normalized to 100% of control. * $p < 0.05$, by one sample t-test v. 100.

8.5. Paired-pulse ratios of experiments presented in chapter 4, further addressing the acute effects of EtOH in perforated-patch and whole cell experiments exhibiting paired-pulse facilitation in the CA1 hippocampus.

8.5.1 Purpose of the experiment

This was a re-analysis of data addressing the effects of EtOH on the paired-pulse ratio in experiments that only demonstrated paired-pulse facilitation, as EtOH could differentially affect the PPR under conditions of paired-pulse facilitation vs. depression.

8.5.2 Methods

Methods for slice preparation, experimental procedures and data collection are as described in chapter 4 (Fig. 6). Data re-analysis consisted of pooling paired-pulse ratios for whole-cell (n=4) and perforated-patch (n=4) experiments. Then a selection criterion was set such that only experiments demonstrating paired-pulse facilitation were selected (i.e. those having a PPR > 1; PPR defined as $fEPSP2 / fEPSP1$) which resulted in the exclusion of 2 recordings from the perforated-patch group. Data was analyzed by one sample t-test from the average of baseline and washout from 100%.

8.5.3 Results

In pooled whole-cell and perforated-patch recordings, 6/8 neurons demonstrated paired-pulse facilitation. In these recordings acute EtOH (80 mM)

application did not affect the paired-pulse ratio (NS by one-sample t-test vs. 100%).

8.5.4 Conclusions

These data indicate that glutamate release is not affected by acute EtOH application when the paired-pulse ratio is limited to the facilitation condition (chapter 4).

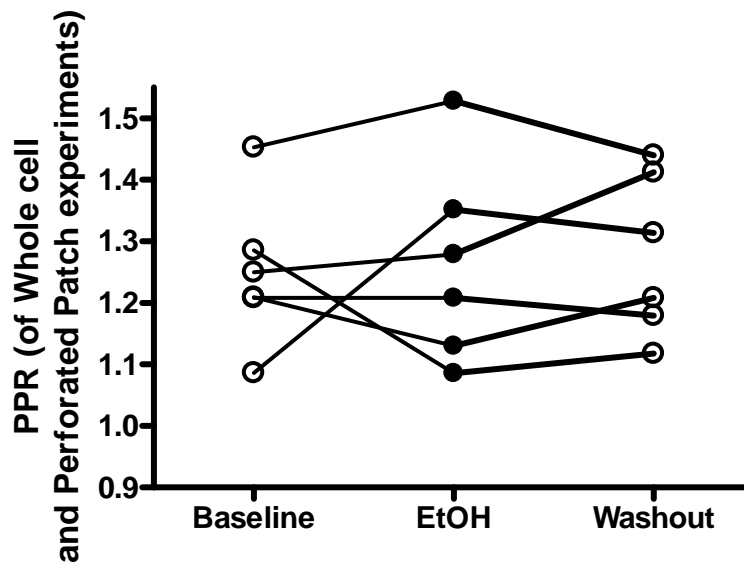


Figure 8.5.1. Whole-cell and perforated-patch experiments addressing the acute effects of EtOH (80 mM) on the paired-pulse ratio in CA1 hippocampal fields or neurons demonstrating paired-pulse facilitation.

8.6. Does contamination of presynaptic volleys by the rising phase of fEPSPs affect volley amplitude and or time-to-peak?

8.6.1 Purpose of the experiment

In a subset of recordings, there was a reduction in the presynaptic volley amplitude after application of EtOH. The question being addressed is: Do EtOH-induced changes in the amplitude of postsynaptic field responses influence the amplitude or the time-to-peak of the fiber volley?

8.6.2 Methods

Methods for slice preparation, experimental procedures and data collection were as described in chapter 4. Recordings of AMPAR-mediated fEPSPs were performed in gabazine (10 μ M), AP5 (50 μ M). fEPSPs were inhibited by NBQX (10 μ M), and the presynaptic volley was inhibited by TTX (0.5 μ M). Data was analyzed by unpaired t-test.

8.6.3 Results

Figure 8.6 shows that NBQX-induced blockade of the fEPSP decreased the fiber volley amplitude by approximately 10% without changing the volley time-to-peak. Partial inhibition of the fEPSP by EtOH, produced a significantly smaller decrease in volley amplitude, with the volley time-to-peak being unaffected as in the case of the NBQX treatment condition.

8.6.4 Conclusions

These data indicate that fEPSPs contaminated the presynaptic volley under our recording conditions. Surprisingly, blockade of the fEPSP did not change the volley time-to-peak as it would have been expected because only part of the rising phase of the fEPSP overlaps with the volley. Instead, the amplitude of the volley decreased when the fEPSP amplitude decreased. The significant difference between the NBQX and EtOH groups indicates that there is a relationship between the magnitude of inhibition of the fEPSP and effects on the fiber volley. The overall conclusion from these experiments is that the fiber-volley that was recorded under our experimental conditions should be addressed as a “contaminated” volley.

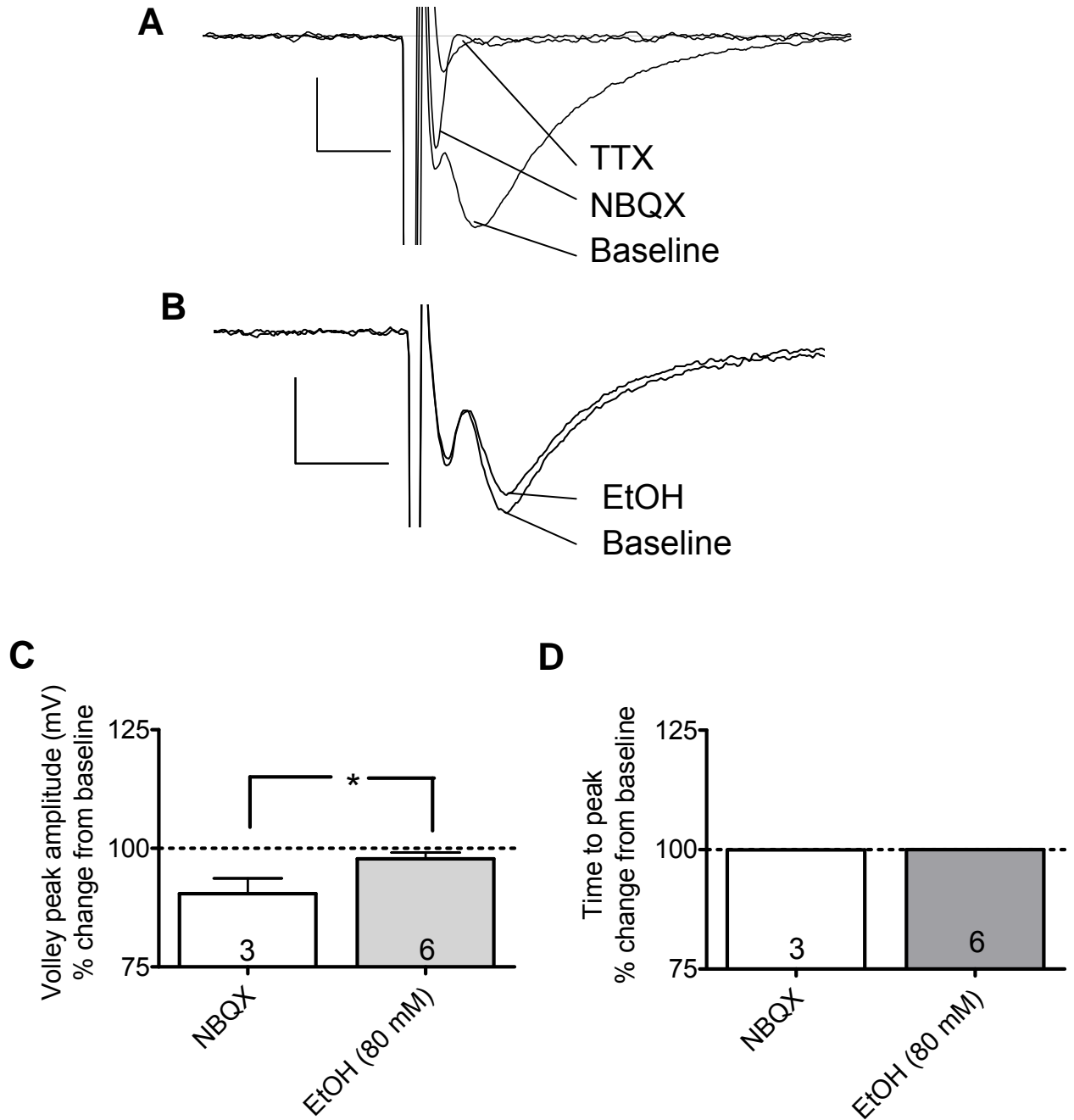


Figure 8.6.1. A decrease in the postsynaptic fEPSP amplitude is associated with a decrease in the amplitude (but not the time-to-peak) of the fiber volley. (A) Sample trace of an AMPAR-mediated fEPSP recording showing the effect of NBQX (10 μ M) and TTX (0.5 μ M). Scale bar 0.2mV/5ms. (B) Sample trace of AMPAR-mediated fEPSP recording showing the effect EtOH (80 mM). Scale bar 0.5mV/5ms. (C) Bar graphs of the normalized the volley amplitude for the NBQX and EtOH groups. (D) Bar graph of the normalized time-to-peak for NBQX and EtOH. * $p < 0.05$ by unpaired t-test.

8.7. Repeated vapor EtOH exposure from P2-P5 does not affect AMPAR GluR1-4 subunit expression by western blotting techniques in the microdissected CA1 hippocampal region of P5 rat pups.

8.7.1 Purpose of the experiment

The purpose of this study was to further confirm the lack of a functional change in AMPAR-mediated fEPSPs described in chapter 6 using biochemical techniques.

8.7.2 Methods

Vapor EtOH exposure and the preparation of brain slices occurred exactly as described in chapter 6. SECs were 45-64 mM on P5 when acute brain slices were prepared. After a recovery period of 90-120 min, the CA1 hippocampal region from brain slices were microdissected out in PBS or ACSF at 4°C, and immediately placed in homogenate buffer, and run using western blotting techniques as described in 8.2.2. Antibodies for GluR1-4 were from Millipore (Billerica, MA) and used at 1:1000 for GluR1,3,4 and 1:2000 for GluR2.

8.7.3 Results

Vapor EtOH exposure paradigm did not affect the expression of GluR1-4 levels in the CA1 hippocampal region (NS by one-sample t-test from 100% of control; n=3)

8.7.4 Conclusions

These data further support a lack of functional change in AMPAR-mediated fEPSP as described in chapter 6.

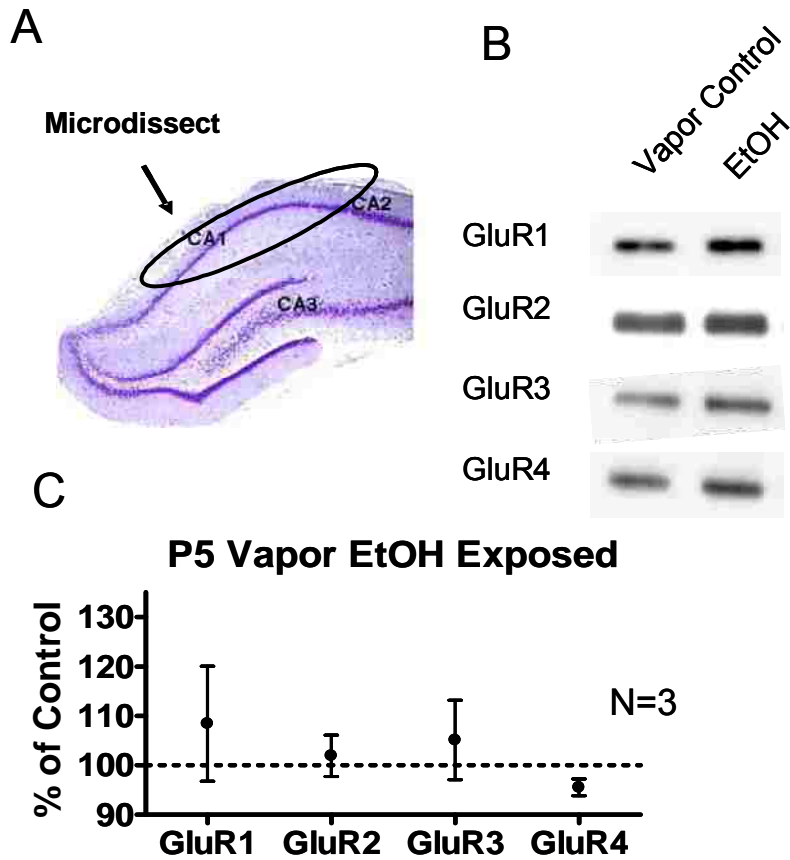


Figure 8.7.1. Repeated EtOH vapor exposure from P2-P5 (BEC 45-64 mM) does not affect AMPAR levels in the microdissected CA1 region of the hippocampus. (A) After EtOH exposure on P5, brain slices were prepared, allowed to recover for 90-120 min, and the CA1 hippocampal region was microdissected. (B) Sample blots showing GluR1-4 bands from control and EtOH exposure groups. (C) Average of 3 litters for each condition showing AMPAR GluR1-4 levels as a % of control.

8.8.1. Reanalysis of NMDAR-mediated fEPSP input-output curves normalizing data to the presynaptic volley demonstrated no difference between control and EtOH exposed animals

8.8.1 Purpose of the experiment

The purpose of this reanalysis was to investigate the presynaptic effects of developmental EtOH exposure, and address uncertainty pertaining to the lack of a change in synaptic strength (NMDAR- and AMPAR-mediated).

8.8.2 Methods

Data was obtained and collected as performed in chapter 6.

8.8.3 Results

There was no significant difference in the input-output curves when the fEPSP amplitude was normalized to the presynaptic volley in brain slices from control and EtOH exposed P7-9 rat pups (Fig. 8.8.1).

8.8.4 Conclusions

These data further support that the long-lasting (at least 1-7 hrs) impairments in synaptic plasticity are not a result of changes in synaptic efficacy for NMDAR-mediated responses. However, due to the difficulty in analyzing the presynaptic volley in AMPAR-mediated experiments, it remains a possibility that input-output curves for AMPARs could be affected. These parameters must be kept in mind when interpreting data pertaining to synaptic strength.

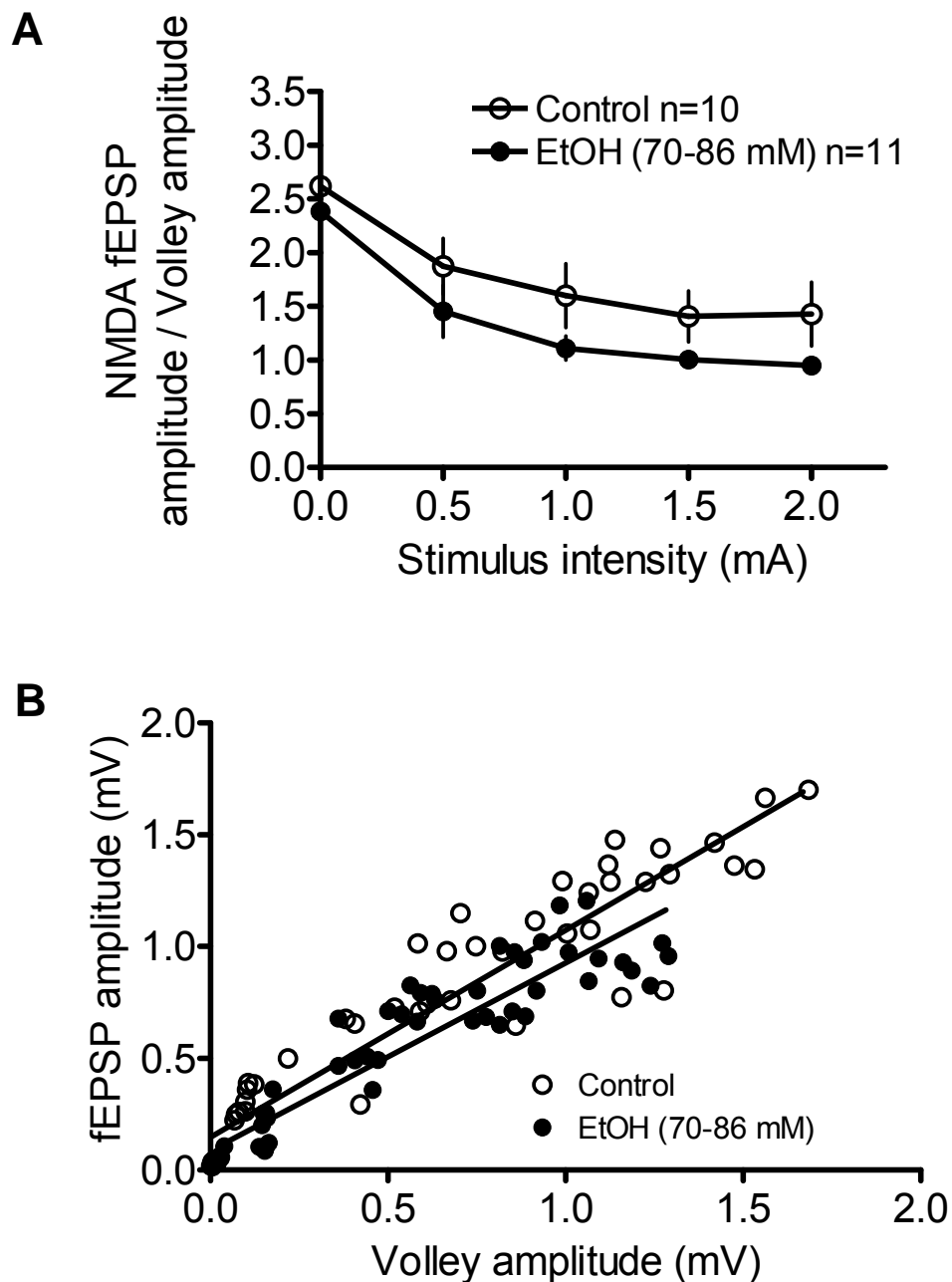


Figure 8.8.1. NMDAR-mediated input-output curves normalized to the presynaptic volley are not different between control and EtOH exposed animals measured in the CA1 hippocampal region in brain slices from P7-9 rat pups. (A) NMDAR-mediated fEPSPs normalized to the presynaptic volley. Data were not significantly different when analyzed by two-way ANOVA. (B) Plot of fEPSP amplitude v. volley amplitude. Linear regression for control ($p < 0.05$; $r^2 = 0.88$) and EtOH ($p < 0.05$; $r^2 = 0.87$) were not significantly different for the slope of the line ($p = 0.19$; $F = 1.67$)

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