Impaired Pavlovian Conditioning and Altered Hippocampal *N*-methyl-D-aspartate Receptor Subunit Composition in a Rat Model of Fetal Alcohol Spectrum Disorder

Research Thesis

Presented in partial fulfillment of the requirements for graduation *with research distinction* in Neuroscience in the undergraduate colleges of The Ohio State University

by

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Abstract

Fetal Alcohol Spectrum Disorders (FASD), resulting from gestational alcohol consumption, is marked by physical and mental abnormalities, including persistent impairments in learning and memory. In this study, rat pups were intragastrically intubated with alcohol (5E rats; 5g/kg/day) or sham intubated across postnatal days 4-9. Adult 5E rats were significantly impaired in trace fear conditioning (TFC), a Pavlovian conditioning paradigm in which the conditioned stimulus (tone) and the unconditioned stimulus (foot shock) are separated by a stimulus-free interval of time. TFC requires hippocampal and prefrontal cortex *N*-methyl-D-aspartate receptor (NMDAR) activation. NMDARs contain four subunits: two mandatory NR1 subunits and two regulatory NR2 subunits. When activated, NR2B-containing NMDARs gate more calcium than NR2A-containing NMDARs. Calcium acts as a second messenger in a molecular signaling cascade that contributes to the induction and maintenance of long-term potentiation (LTP), a learning-mediated enhancement of neural signaling, on which successful TFC is reliant. Thus, NR2B subunits are proposed to enhance learning-dependent LTP more so than NR2A, and ethanol-induced alterations to NMDAR subunit composition could disrupt LTP maintenance and long-term memory storage. In support, whole cell lysate Western blotting revealed an elevated NR2A/NR2B ratio in dorsal hippocampus, but not in ventral hippocampus or medial prefrontal cortex, of adult 5E rats. Utilizing subcellular fractionation, Western blotting showed a significant reduction in synaptic and extrasynaptic NR2B subunits in dorsal hippocampus of 5E rats, which is proposed to impede the synaptic plasticity required for successful TFC. Results are expected to provide new and valuable knowledge regarding the etiology of FASD, and may lead to the use of novel pharmacological therapies targeting NMDARs to ameliorate cognitive deficits in FASD individuals.

Introduction

Fetal Alcohol Spectrum Disorders (FASD) is an umbrella term that encompasses a continuum of physical and mental deficits occurring as a consequence of maternal alcohol consumption during pregnancy (Fryer, 2012; Lucas et al., 2014; Mattson et al., 2011; Pruett et al., 2013). FASD is characterized by progressive neuronal death and long-lasting impairments in learning, memory, and executive function (Ikonomidou et al., 2000; Niccols, 2007; Streissguth, 2007). In humans, prenatal alcohol exposure remains the primary cause of preventable mental retardation (May et al., 2009). Animal models of FASD have been used extensively to explore and understand the changes that the central nervous system (CNS) undergoes as a result of alcohol exposure during early brain development. In the Lindquist lab, rat pups are administered ethanol in a binge-like manner over postnatal days (PD) 4-9—a period analogous to the third-trimester "brain growth spurt" in humans (Bayer et al., 1993)—to assess the long-term deleterious behavioral and biochemical effects of early exposure to alcohol. This project included two experimental groups: a 5g/kg/day of ethanol (5E) treatment group, and a sham intubation (SI) control.

Ethanol exposure during early development damages a number of CNS structures, including the hippocampus and prefrontal cortex (Livy et al., 2003; Mihalick et al., 2001). Additionally, various animal behavior studies have demonstrated impaired forebrain-dependent learning and memory later in life as a consequence of perinatal exposure to alcohol (DuPont et al., 2014; Goodfellow & Lindquist, 2014; Hunt et al., 2009; Lindquist, 2013; Murawski & Stanton, 2010). Replicating previous results (DuPont et al., 2014), 5E rats in the current thesis displayed learning deficits in trace fear conditioning (TFC)—an associative learning paradigm in which the neutral conditioned stimulus (CS; tone) and the aversive unconditioned stimulus (US;

foot shock) are separated by a stimulus-free period of time, referred to as the trace interval. Successful TFC is dependent on forebrain *N*-methyl-D-aspartate receptor (NMDAR) activation (Gilmartin & Helmstetter, 2010; Wanisch et al., 2005).

NMDARs are postsynaptic receptors, which consist of four subunits (i.e., tetramers): two mandatory NR1 subunits and two regulatory NR2 subunits. The NR2 subunit is differentially expressed in the brain. In the forebrain, the hippocampus and medial prefrontal cortex in particular, NR2A and NR2B subunits predominate, whereas NR2C and NR2D are mainly found within cerebellar and subcortical regions of the brain (Monyer et al., 1994). The NR2 subunit determines receptor kinetics—the degree of ion entry, including calcium—by controlling the duration of channel opening (Erreger et al., 2005). When activated, NR2A-containing NMDARs have a higher probability of opening and close faster, gating less calcium. Conversely, NR2B-containing NMDARs have a lower probability of opening, but close slowly, gating more calcium relative to NR2A. In early normal development, NR2B subunit expression is more prevalent than NR2A. However, during the first weeks of a rat's life, NR2 subunit composition undergoes a gradual developmental switch, with an increase in NR2A subunit expression and a decrease in NR2B subunit expression (Monyer et al., 1994).

Exposure to alcohol suppresses NMDAR activity, which facilitates insertion of more NMDARs to maintain a baseline level of cellular activity (Hendricson et al., 2007; Kalluri et al., 1998). During subsequent alcohol withdrawal, the increased NMDAR numbers and activity allow excessive levels of calcium to flow into the postsynaptic terminal, promoting excitotoxicity and cell death (Davidson et al., 1995). Perinatal alcohol exposure in pre-weanling rats was previously shown to alter NMDAR subunit composition marked by significant upregulation of NR2A subunits and/or a diminished expression of NR2B subunits (Brady et al.,

2013; Hughes et al., 1998; Nixon et al., 2002, 2004). Such alterations are thought to be neuroprotective because it prevents excitotoxicity by limiting calcium entry through a presumptive reduction in dihomomeric NR2B-containing NMDARs.

In a non-pathological model of NMDAR activation, calcium signals act downstream of the receptor, activating particular molecular pathways, which can lead to the induction of long-term potentiation (LTP)—a biological mechanism of learning, where signal transduction between two neurons is selectively enhanced by strengthening their synaptic connections (Bellinger et al., 2002). This renders NR2B-containing NMDARs crucial for learning-dependent synaptic plasticity and long-term memory storage (Cui et al., 2011; Müller et al., 2013). Moreover, the Bienenstock, Cooper, and Munro (BCM) theory of synaptic plasticity (1982) posits that LTP and LTD—the latter being the selective weakening of neuronal connections termed long-term depression—are induced on a sliding threshold (i.e., less LTP at the expense of more LTD). The threshold can be altered by NR2 subunit composition and calcium kinetics (Shouval et al., 2002), where an increased NR2A/NR2B ratio results in a higher LTP induction threshold (Yashiro & Philpot, 2008), hindering the protein cascades necessary for successful LTP maintenance and long-term memory storage (DuPont et al., 2014). Subsequent studies, with which I provided assistance, included the aim to relate putative ethanol-mediated aberrations in NR2 subunit composition to TFC deficits observed in the 5E rats. The expression of NR2 subunits in the hippocampus and the prefrontal cortex—regions critical for TFC—were assessed using whole cell lysate Western blotting. The NR2A/NR2B subunit ratio was found to be significantly elevated in just the dorsal hippocampus of 5E rats, relative to control.

Deficits in learning-dependent synaptic plasticity can likely be attributed to functional transmembrane NMDARs (Cao et al., 2011). Present results from whole cell lysate Western

blotting not only contain transmembrane proteins, but also contain intracellular subunit stores (i.e., proteins that have not been expressed on the cell membrane). In adult synapses, synaptic NR2B subunits are typically contained within triheteromeric NMDARs (NR1/NR2A/NR2B) (Hansen et al., 2014; Rauner & Köhr, 2011; Tovar et al., 2013). It has been previously thought that NR2B-containing NMDARs, not NR2A-containing NMDARs, were responsible for LTP induction (Massey et al., 2004). However, recent research suggests that both subunits may be crucial for LTP induction (Li et al., 2001; Müller et al., 2013; Zhou, Ding, et al., 2013). Interestingly, Kollen and colleagues (2008) found that the degree to which NMDARs contribute to synaptic efficacy was not only related to their subunit composition, but also to their respective locations. Synaptic NR2A- and NR2B-containing NMDARs play a critical role in synaptic plasticity (Cui et al., 2011; Li et al., 2001). However, the research regarding the degree to which NR2B-containing extrasynaptic NMDARs contribute to LTP and LTD remains inconclusive. Morishita et al. (2007) demonstrated that NR2B-containing NMDARs are not necessary to trigger LTD, for example, but later research revealed otherwise (Kollen et al., 2008; Liu et al., 2013). Thus, both NR2A- and NR2B-containing NMDARs could potentially contribute to synaptic plasticity, either LTP or LTD.

In our third trimester equivalent binge-like drinking rat FASD model, 5E rats displayed impaired TFC, a forebrain-dependent task, suggesting putative NMDAR hypofunction. Whole cell lysate Western blotting revealed an elevated NR2A/NR2B ratio in dorsal hippocampus only, presumably due to neurocompensatory changes the developing brain undergoes as a consequence of early postnatal exposure to alcohol. Using subcellular fractionation and Western blotting optimized for transmembrane proteins, my senior thesis project aimed to quantify synaptic and extrasynaptic NMDAR subunit expression in dorsal hippocampus (dHc), ventral hippocampus

(vHc), and medial prefrontal cortex (mPFC). We hypothesized that a diminished NR2B subunit expression in the dHc of adult 5E rats would produce an altered NR2A/NR2B subunit ratio, which would then hinder the NMDAR-gated downstream molecular cascades responsible for LTP maintenance and the long-term consolidation of new memories. We further predicted that NR2B subunits would be preferentially decreased in synaptic but not extrasynaptic membrane fractions, accounting for the behavioral deficits seen in trace fear conditioned 5E adult rats.

Methods and Materials

Subjects and Neonatal Treatment

Male and female Long-Evans breeder rats were housed in the Psychology Department vivarium at the Ohio State University. One male and one female were pair housed for one week, after which female rats were checked for parturition twice daily beginning three weeks after separation. On PD 3, litters were culled to 10-12, and rat pups were paw marked using nontoxic black ink for identification purposes. Rats were then pseudo-randomly assigned to one of two treatment groups: 5g/kg/day of ethanol (5E) or sham-intubated (SI). No more than one male and female subject were selected per treatment group per litter to control for potential litter effects.

5E treatment group subjects were administered a milk solution containing 11.33% ethanol via intragastric intubation twice daily across PD 4-9 (0.02778 mL/gram of body weight). The administration also included a third milk-alone solution to ensure proper nutrition and prevent drastic weight loss (Lindquist, 2013). The SI pups were intubated, but without any alcohol or milk administration. In a previous study, we employed an unintubated control (UC) group to evaluate the potential effects of stress associated with the intubation process during early life, and observed no significant difference in learning between UC and SI rats (DuPont et

al., 2014). Therefore, to conserve animals and resources, a UC group was not included in the current study.

Prior to the last intubation on PD4, tail clips were performed to obtain blood samples using heparinized capillary tubes. Blood samples were centrifuged and resulting plasma samples were collected and stored in -80℃ for subsequent blood alcohol concentration (BAC) analysis. An Analox GL5 Analyzer (Analox Instruments, Lunenberg, MA) was used to measure BAC levels. In order to track possible ethanol-induced changes in body weight across development, rat offspring were weighed on PD 10, 15, 21, 30, 45, and 60. Rats were weaned on PD 21, and same-sex housed with littermates until PD 60, after which subjects were singly housed through the end of the experiment. All measures were taken to minimize pain and suffering, and all procedures were in strict compliance with the Ohio State University's animal care guidelines.

Estrus Cycle Tracking

The estrous cycle of female rats were initially tracked between PD 61-65. Samples were obtained between 0900 and 1100. A sterile cotton swab was soaked in a 0.9% saline solution and rolled gently into the vagina. Samples were applied to subbed slides which were stained with toluidine blue and examined under a light microscope. Estrus cycle was tracked based on cell morphology as illustrated by Shors (1998). Subsequent swabs were done daily as described above until females reached proestrus. Females in proestrus were behaviorally conditioned and tested with a male littermate whenever possible. Genitals of male littermates were swabbed to control for stress associated with the swabbing procedure.

Apparatus

Standard conditioning boxes (Coulbourn Instruments, Allentown, PA), encased within noise reduction chambers, consisted of two stainless steel walls, two Plexiglas walls, and a grid

floor with 0.5 cm stainless steel bars spaced 1.5 cm apart. An animal shock generator (model 82400; Lafayette Instruments, Lafayette, IN) and neon grid scrambler (model 58020; Lafayette Instruments) connected to the gridbars delivered the US (foot shock). Rats were trained and tested in two separate contexts, which included different transport procedures. In context 1, the conditioning chamber was lit with a 15 W bulb. The outer room was well-lit and quiet. Rats were transported in their home cages in a stacked manner, two at a time, by the experimenter to the behavior testing room. Chambers were cleaned and scented with a 20% vinegar solution prior to conditioning and testing. In context 2, the conditioning chamber was dark and the outside room was lit with only a red overhead light. A 60 dB non-aversive white noise was provided by a fan inside the chamber. The gridbars were covered with an opaque gray Plexiglas floor, and a small magnet was placed on one stainless steel wall, with a removable pink geometric figure adhered to the door of the conditioning box. Animals were wheeled into the testing room on a metal cart, two at a time, with their home cages covered by towels. Prior to testing, conditioning chambers were cleaned and scented using Windex®.

Behavioral Procedures

On training day, rats were transported to the testing room in their home cages and placed into context 1. After a 240 ± 30 s baseline period, rats were presented 10 CS-US trials with an inter-trial interval (ITI) of 240 ± 30 s. During each trial, a 15 s, 2.8 kHz, 75 dB tone (CS) was presented, followed by a 30 s trace interval, after which a 1 s, 0.8 mA foot shock (US) was administered (Figure 1). At the end of the session, rats were returned to the vivarium. Approximately 24 and 48 h later, rats were measured for freezing behavior to the context and tone in counterbalanced order. During the context test, rats were placed back into the training chamber (i.e., context 1). After a 120 s baseline period, freezing behavior was measured over the

600 s test period. Rats remained in the chamber for an additional 120 s post-test period, after which they were returned to the vivarium. During the tone test, rats were placed into context 2 and after a 135 ± 15 s baseline period were presented with five 15 s tones, separated by a 90 ± 10 s following the end of each trial. Freezing behavior was recorded during each tone as well as in the 30 s period (i.e., trace interval) following CS offset. Rats were returned to the vivarium 120 s after the last trial. The tone test was conducted in a novel context (i.e., context 2) in order to minimize contextual cues and ensure that observed freezing was specific to the tone CS.

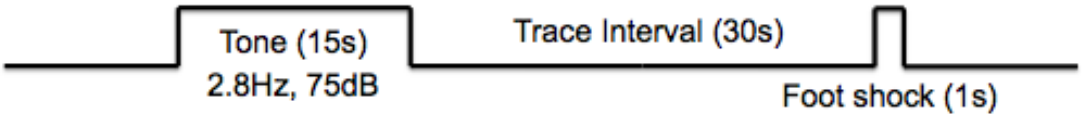

Figure 1. Trace fear conditioning paradigm with the separation of the tone CS and foot shock US presentations by a trace interval of time.

Whole Cell Lysate Sample Extraction and Preparation

Animals were sacrificed at least 3 days after the completion of behavior testing without regard for the estrous cycle. Rats were deeply anesthetized with isoflurane and decapitated. Brains were quickly removed and placed in cold brain matrix containing 1 mm razor blade slots, where rostral cortical tissue was sliced, followed by mPFC dissection. The brain was then placed on ice where the hippocampus was removed and separated into dorsal and ventral sections and then immediately stored at -80ºC until further processing. Tissue was homogenized in boiling hot lysis buffer, consisting of 1% sodium dodecyl sulfate, 1% 1 M Tris stock (pH 7.4), and 1% 100 mM sodium orthovanadate, and centrifuged at 29,000 x g at 15°C for 10 min. The supernatant was aspirated, and protein concentrations were determined via Bradford assay. Samples were diluted to 1.33 µg/µL to contain 20 µg protein, and subsequently combined 1:1 with loading

buffer and heated at 95°C for 5 min. They were placed on ice for 1 min and centrifuged at 8000 rpm at room temperature for 1 min.

Subcellular Fractionation

The goal of the current thesis was to assess synaptic and extrasynaptic NMDAR subunit expression in our rat model of FASD. To this end, I carried out a modified subcellular fractionation protocol (Goebel-Goody et al., 2009) and optimized the existing Western blotting protocol for synaptic and extrasynaptic NMDARs. Brain tissue was microdissected and stored as outlined in the whole cell lysate sample extraction and preparation section. Unlike rats in the whole cell lysate experiment, rats in this current study did not undergo behavior testing. Females in proestrus were pair sacrificed with a male littermate whenever possible. Subsequent steps were performed at 4℃ as outlined in Samudio-Ruiz et al. (2010) (modified).

Extracted tissue samples were homogenized in a lysis buffer containing 20 mM Tris (pH 7.4), 1 mM EDTA, 320 mM sucrose, 20 mM sodium pyrophosphate, 10 mM sodium fluoride, 20 mM β-glycerophosphate, and 0.2 mM sodium orthovanadate. Homogenate was centrifuged twice (1000 × g for 10 min)—the supernatant was aspirated and the pellet was resuspended in 150 μL homogenization buffer before the second centrifugation. The resulting supernatant was saved after both steps, and was centrifuged (15,000 × g for 30 min), aspirated, then discarded. The remaining pellet was combined with ice-cold deionized water containing protease inhibitor cocktail (1:1000) (Thermo Scientific, Rockford, IL) and was briefly homogenized, before adding 3.75 μL HEPES-NaOH buffer (pH 7.4) (final concentration 7.5 mM). Vortexed samples were then incubated on ice for 30 min before centrifugation (22,000 × g for 20 min). The supernatant was discarded and obtained pellets were resuspended in 900 μL homogenization buffer before homogenization, followed by topping samples with 450 μL Triton X-100 buffer (without Triton)

containing 10 mM Tris (pH 7.4), 5 mM NaF, 1 mM EDTA, 0.5 mM EGTA, 0.2 mM sodium orthovanadate, and 20 µL protease inhibitor cocktail, and 840 µL 1X Triton buffer containing 1% Triton X-100. Samples were vortexed and incubated on ice for another 30 min. Samples were then centrifuged (100,000 × g for 60 min). The resulting supernatant contained the extrasynaptic fraction, whereas the pellet was the synaptic fraction.

To solubilize synaptic fractions, 75 µL homogenization buffer containing 1% SDS (w/v) was added to the samples. Samples were thoroughly mixed, heated at 90-100℃ for 5 min, placed on ice for 1 min, then centrifuged (8,000 rpm for 1 min). Extrasynaptic fractions were precipitated by adding 4X of sample volume of ice-cold acetone to the samples. Samples were then briefly vortexed and incubated at -20℃ for 4 hours. Sample were subsequently centrifuged (15,000 × g for 10 min) and the acetone-containing supernatant was removed. To solubilize extrasynaptic fractions, 100 µL homogenization buffer containing 1% SDS (w/v) was added before mixing. Samples were heated at 90-100℃ for 5 min, placed on ice for 1 min, then centrifuged (8,000 rpm for 1 min). Both synaptic and extrasynaptic fractions were prepared for Western blotting as outlined in the whole cell lysate sample extraction and preparation section.

Western Blotting with Whole Cell Lysate and Fractioned Samples

Bradford protein assay, sample preparation, and Western blotting were followed as established in current lab protocols. Bradford protein assay was used to determine protein concentrations in fractioned samples, and samples were diluted to 1.33 µg/µL. Samples were prepared using a loading buffer containing Laemmli sample buffer (Bio-Rad, Hercules, CA) and β-mercaptoethanol (BME) (Bio-Rad, Hercules, CA) to contain a final protein concentration of 5 µg per well for synaptic fractions, and 8 µg per well for extrasynaptic fractions (20µg for whole cell lysate). Prepared samples were loaded into a 12-well (10-well for whole cell lysate) 7.5%

TGX gel (Bio-Rad, Hercules, CA) and placed into an electrophoresis chamber at 150 V for roughly 70 min. Gel-bound proteins were then transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA) at 100 V for 60 min. After transfer, the membrane was cut using a razor blade at 130 kD to separate NR2 (180 kD) from NR1 (120 kD) and the loading control, Actin (42 kD) (GAPDH for whole cell lysate (38 kD)). Membrane portions were blocked individually in a prepared TBST solution containing 5% nonfat dry milk (w/v) (Bio-Rad, Hercules, CA) for 60 min at room temperature to prevent non-specific antibody binding. Membranes containing synaptic and extrasynaptic fractions were then incubated separately overnight at 4℃.

Primary-antibody-containing blocking solution for whole cell lysate samples consisted of the following: anti-NR2A (1:3000, PhosphoSolutions, Aurora, CO), anti-NR2B (1:1500, Millipore, Billerica, MA), or a cocktail of anti-NR1 (1:3000, BD Biosciences, San Jose, CA) and anti-GAPDH (1:5000, Novus, Littleton, CO). Primary-antibody-containing blocking solution for synaptic fractions consisted of the following: anti-NR2A (1:2000 PhosphoSolutions, Aurora, CO), anti-NR2B (1:1500 Millipore, Billerica, MA), or a cocktail of anti-NR1 (1:1250 BD Biosciences, San Jose, CA), anti-PSD-95 (1:1000 Cell Signaling Technology, Inc., Danvers, MA), and anti-actin (1:1500 PhosphoSolutions, Aurora, CO). Primary-antibody-containing blocking solution for extrasynaptic fractions consisted of the following: anti-NR2A (1:200 PhosphoSolutions, Aurora, CO), anti-NR2B (1:400 Millipore, Billerica, MA), or a cocktail of anti-NR1 (1:250 BD Biosciences, San Jose, CA), anti-PSD-95 (1:1000 Cell Signaling Technology, Inc., Danvers, MA), and anti-actin (1:1500 PhosphoSolutions, Aurora, CO).

On the following day, membranes for whole cell lysate samples were incubated in a secondary-antibody-containing blocking solution containing either goat anti-mouse (1:2000, BD Biosciences, San Jose, CA) for NR1 and GAPDH, or goat anti-rabbit for NR2A and NR2B

(1:5000 Pierce, Rockford, IL) for 60 min at room temperature. Membranes for synaptic and extrasynaptic fractions were rinsed in TBST (4 × 7 min) before incubation in a secondary-antibody-containing blocking solution: goat anti-mouse (1:2000, BD Biosciences, San Jose, CA) and goat anti-rabbit (1:5000, Pierce, Rockford, IL) cocktail for NR1/Actin/PSD-95, or goat anti-rabbit for NR2A and NR2B (1:5000 Pierce, Rockford, IL) for 60 min at room temperature. Membranes were rinsed again in TBST (4 × 7 min), and subsequently immersed in Clarity Western blotting substrate reagent (Bio-Rad, Hercules, CA) for 5 min. Imaging was done using a FluorChem M system (ProteinSimple, San Jose, CA), and blots were analyzed using AlphaView software (ProteinSimple, San Jose, CA). Statistical analyses were done using a one-way and repeated measures analysis of variance (ANOVA). Significance was denoted with a p-value of less than 0.05.

Results

Blood Alcohol Concentration (BAC) levels and Body Weight

The mean (± SE) peak BAC was 347.5 ± 9.9 mg/dl in 5E female rats and 353.8 ± 8.0 mg/dl in 5E male rats. Body weights of SI (n = 9 males, 11 females) and 5E rats (n = 10 males, 10 females) were measured across PD 4-9, 10, 15, 21, 30, 45, and 60 and analyzed via 2 (Treatment) x 2 (Sex) x 6 (Day) repeated measures ANOVA (Table 1). Collectively, 5E rats were smaller than SI rats across PD 4-9 (Treatment, $F(1, 33) = 29.66$, $p < 0.001$; Day, $F(5, 165) = 696.77$, $p < 0.001$; Treatment x Day, $F(5, 165) = 31.32$, $p < 0.001$). Across PD 10-60, males weighed significantly more than females with no difference in treatment group (Sex, $F(1, 33) = 90.52$, $p < 0.001$; Day, $F(5, 165) = 3627.72$, $p < 0.001$; Sex x Day, $F(5, 165) = 176.60$, $p < 0.001$). Bonferroni-corrected one-way ANOVAs, requiring (at 6 contrasts) $p < 0.0083$ for

significance (maintaining a family-wise $\alpha = 0.05$) indicated that 5E rats weighed less than SI rats across PD 5-9, and males weighed significantly more than females on PD 30, 45, and 60.

	PD4	PD5*	PD6*	PD7*	PD8*	PD9*	PD10	PD15	PD21	PD30+	PD45+	PD60+
SI M (n=9)	10.40 ± 0.14	12.11 ± 0.21	13.72 ± 0.24	15.37 ± 0.30	17.24 ± 0.38	19.02 ± 0.41	21.10 ± 0.43	30.47 ± 0.72	48.40 ± 1.49	102.76 ± 2.33	216.31 ± 5.01	319.22 ± 6.57
SI F (n=11)	10.27 ± 0.25	12.04 ± 0.29	13.57 ± 0.29	15.22 ± 0.32	17.00 ± 0.38	18.77 ± 0.52	20.56 ± 0.48	29.16 ± 0.68	47.40 ± 1.39	91.84± 1.30	160.80 ± 3.03	208.51 ± 4.24
5E M (n=10)	10.14 ± 0.37	10.21 ± 0.37	11.24 ± 0.45	12.40 ± 0.60	13.94 ± 0.65	15.70 ± 0.68	17.54 ± 0.81	26.73 ± 1.03	43.48 ± 2.21	93.73 ± 3.71	204.27 ± 5.22	308.00 ± 7.46
5E F (n=10)	9.74 ± 0.47	10.01 ± 0.55	11.00 ± 0.60	12.22 ± 0.73	13.66 ± 0.89	15.28 ± 1.04	17.16 ± 1.09	27.69 ± 1.16	44.14 ± 1.99	87.10 ± 3.44	158.18 ± 3.44	213.14 ± 7.28

Table 1. Mean (± SE) body weight across lifespan by neonatal treatment group and sex. 5E rats weighed significantly less than SI rats across PD 5-9. Male rats weighed significantly more than female rats across PD 30-60. * Denotes significant treatment group effect. + Denotes significant sex effect

Trace Fear Conditioning

A total of 35 rats were used for the behavior study. The manuscript for which this thesis is a part will examine sex effects based on neonatal ethanol exposure and TFC. The specifics of that data do not pertain to the current results, thus freezing behavior was collapsed across sex for all behavioral analyses. The 5E rats froze significantly less to the tone ($F(1, 33) = 15.477$, $p < 0.01$), the trace interval ($F(1, 33) = 8.317$, $p < 0.01$), and the context ($F(1, 33) = 8.550$, $p < 0.01$) (Figure 2).

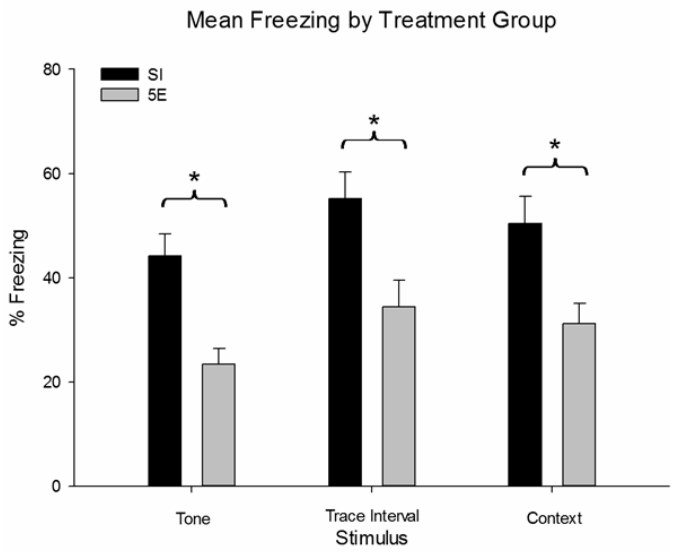

Figure 2. Retention test freezing behavior (mean ± SE) by neonatal treatment group: 5E rats froze significantly less during the tone presentation, the trace interval, and the context test

Whole Cell Lysate Western Blotting

A total of 13 rats from the behavior study were used for Western blotting analysis (n = 6-7 per group). The optical density (OD) values of individual NMDAR subunit expression were first normalized to OD values of GAPDH expression. There were no significant changes in NR1, NR2A, or NR2B subunit expression in dHc, vHc, and mPFC (figure not shown). The ratio of NR2A/2B was also analyzed based on changes in individual subunit expression (NR2A OD/NR2B OD). Relative to controls, 5E rats showed a significant increase in the NR2A/2B ratio in dHc ($F(1, 11) = 4.860$, $p = 0.05$), but not in vHc or mPFC (Figure 3).

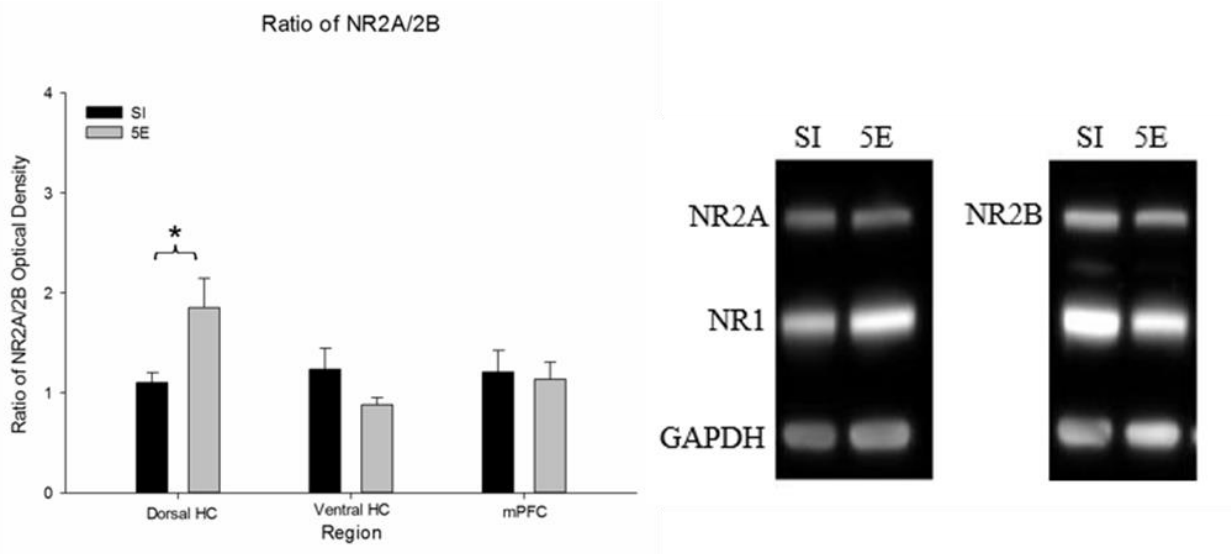

Figure 3. The NR2A/NR2B subunit ratio by brain region across treatment group in whole cell lysate samples: 5E rats showed a significant increase in the NR2A/NR2B ratio in dorsal hippocampus (dHc) compared to controls. *Denotes significant neonatal treatment group effect ($p < 0.05$) (left). Representative Western blot images of NMDAR subunit expression and GAPDH (loading control) by treatment group (right).

Western Blotting with Fractioned Samples

A total of 15 rats were used for synaptic and extrasynaptic Western blotting analysis (n = 7-8 per treatment group). The optical density (OD) values of individual synaptic and extrasynaptic NMDAR subunit expression were first normalized to OD values of Actin

expression (NMDAR subunit OD / Actin OD). We used actin as our loading control, instead of GAPDH, to address GAPDH signal reliability issues initially encountered in fractioned samples.

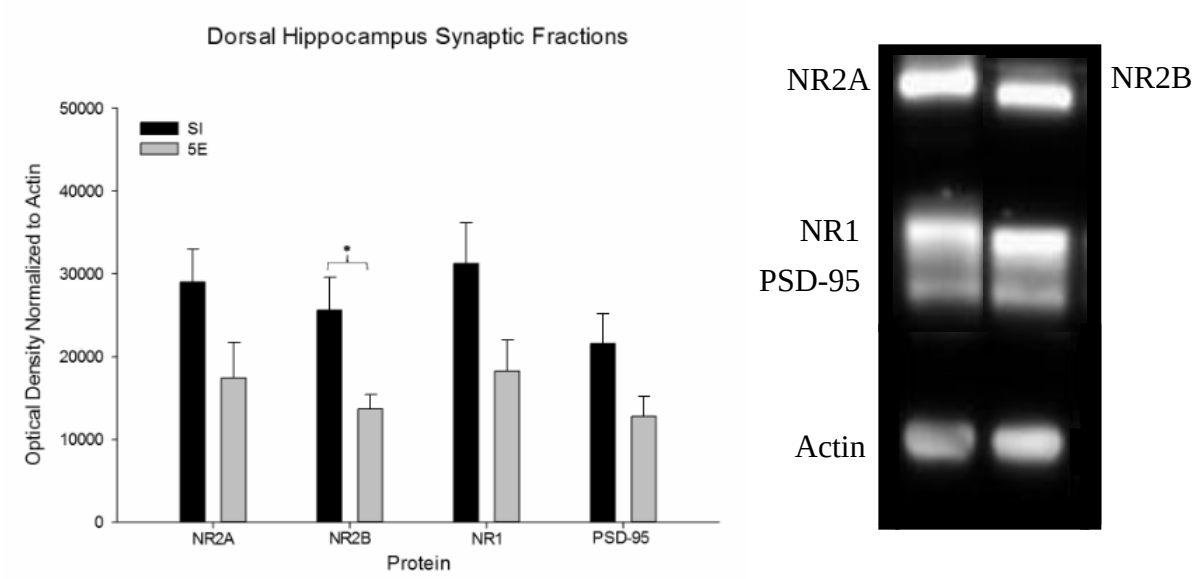

Figure 4. Synaptic fraction Western blotting results (mean ±SE) by neonatal treatment group in dorsal hippocampus (dHc): 5E rats showed significant reductions in NR2B subunits (left). Representative Western blot images of synaptic NMDAR subunit expression and Actin (loading control) (right) taken from one SI rat. Presence of PSD-95 expression indicated successful isolation of synaptic NMDAR subunits. *Denotes significant neonatal treatment group effect ($p < 0.05$).

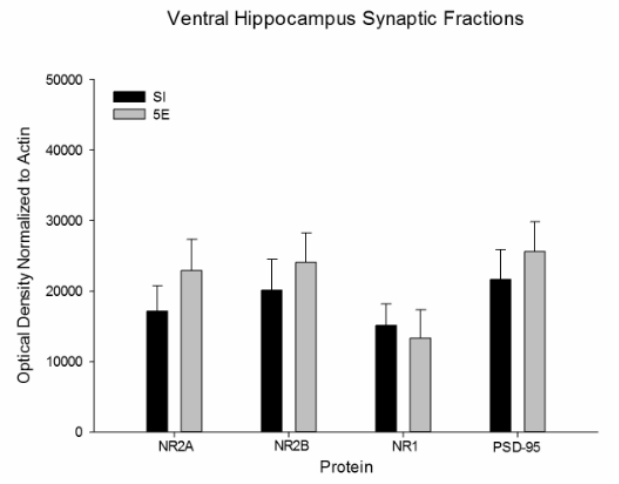

Figure 5. Synaptic fraction Western blotting results (mean ±SE) by neonatal treatment group in ventral hippocampus (vHc): no significant treatment group effects were noted.

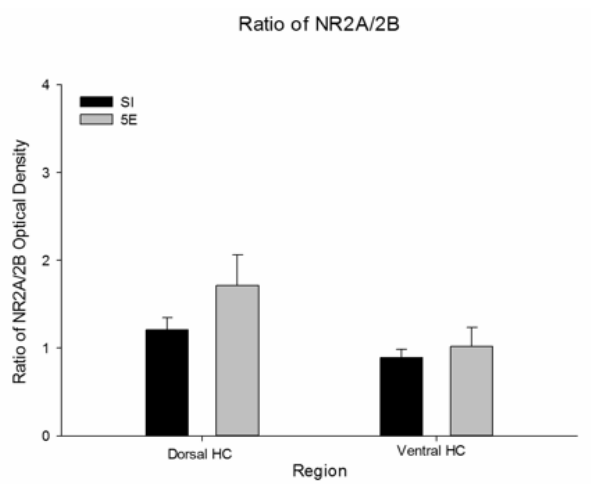

Figure 6. The NR2A/NR2B subunit ratio in synaptic fractions by brain region across neonatal treatment groups: no significant treatment group effect was noted.

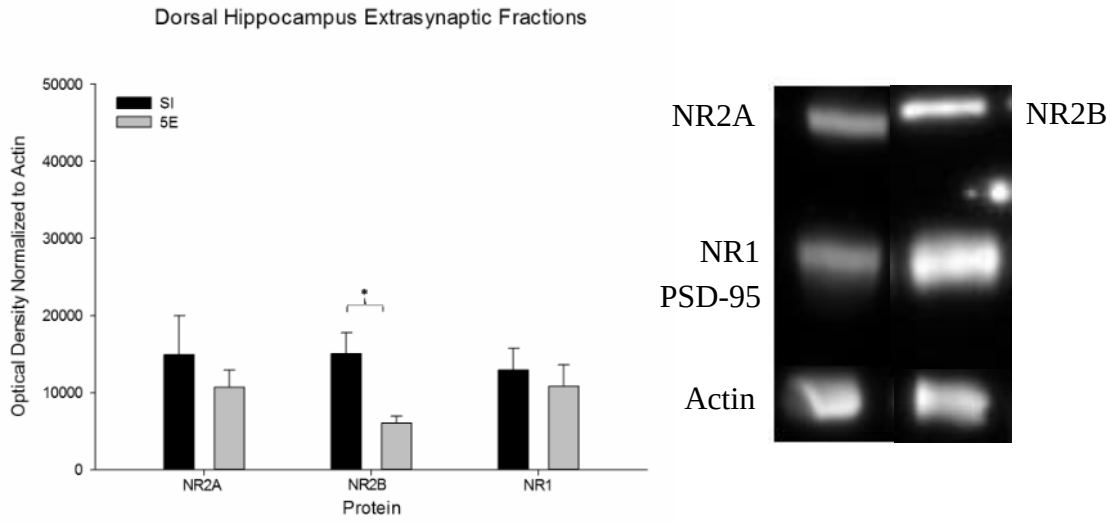

Figure 7. Extrasynaptic fraction Western blotting results (mean ±SE) by neonatal treatment group in dorsal hippocampus (dHc): 5E rats showed diminished NR2B subunit expression (left). Representative Western blot images of extrasynaptic NMDAR subunit expression and Actin (loading control) taken from one SI rat (right). Absence of PSD-95 expression indicated successful isolation of extrasynaptic NMDAR subunits. *Denotes significant neonatal treatment group effect ($p < 0.05$).

In dHc synaptic fractions (n = 6-7 per group), ANOVA by treatment group indicated a significant reduction in NR2B expression ($F(1, 11) = 6.675$, $p < 0.05$) but not NR2A expression ($F(1, 11) = 3.940$, $p = 0.07$) (Figure 4). Diminished expression in both NR1 ($F(1, 11) = 4.201$, $p = 0.06$) and PSD-95 ($F(1, 11) = 3.788$, $p = 0.08$) approached but did not reach statistical significance in 5E rats relative to controls (Figure 4). No significant treatment group effects were found in synaptic vHc fractions (Figure 5), though consistency between whole-cell and synaptic fraction results do provide validity for the different measures of protein concentrations (Figures 3 & 5). The ratio of NR2A/2B was also analyzed based on changes in individual subunit expression (NR2A OD/NR2B OD). Results revealed no significant difference in the NR2A/NR2B ratio in either dHc or vHc by treatment group (Figure 6). In dHc extrasynaptic fractions (n = 5 per group), ANOVA by treatment group revealed a significant decrease in NR2B expression ($F(1, 8) = 9.552$, $p < 0.05$) in 5E rats compared to controls (Figure 7) and a trend towards an increased NR2A/2B ratio ($F(1, 8) = 4.099$, $p = 0.08$) (Figure 8). It must be noted that

synaptic samples from mPFC, and extrasynaptic samples from both vHc and mPFC were not analzyed in this study. This was attributed to insufficient protein concentrations for the sample preparation phase and/or unreliable signals with the primary antibody concentrations outlined in the Western Blotting section concerning fractioned samples.

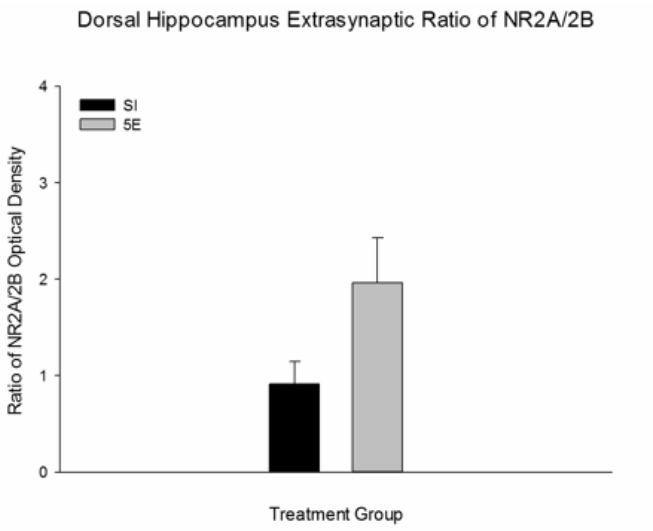

Figure 8. The NR2A/NR2B subunit expression ratio in dorsal hippocampus extrasynaptic fractions across treatment group: no significant treatment group effects were noted

Discussion

A wealth of previous research has shown that alcohol administration in rodents during or immediately after gestation can wreak havoc on the developing central nervous system (CNS), which includes diminished dendritic spine density, impaired neurogenesis, progressive cell loss, and corollary deficits in learning and memory tasks (Hamilton et al., 2011; Hamilton et al., 2010; Klintsova et al., 2007; Whitcher & Klintsova, 2008). In addition, early-life exposure to alcohol during the third trimester equivalent period in rodents can disrupt NMDAR-mediated synaptogenesis and neuronal migratory patterns (Gambrill & Barria, 2011; Georgiev et al., 2008; Komuro & Rackic, 1993), leading to abnormal forebrain development and cognitive function, including impairments in learning and memory.

Using a third trimester binge-like drinking rat FASD model, we demonstrated that 5E rats were behaviorally impaired in TFC—specifically, the 5E rats froze significantly less during the context and tone tests. For the latter, freezing was reduced during the tone CS and the subsequent

30 s trace interval. In addition, analysis of NMDAR subunit composition using whole cell lysate Western blotting revealed an increased NR2A/NR2B ratio in dHc, but not vHc or mPFC, of 5E rats, relative to controls. Western blotting analysis using a subcellular fractionation protocol further showed a reduction in both synaptic and extrasynaptic transmembrane NR2B subunits in dHc of 5E rats. Considering the critical role of dHc and mPFC NR2B subunits in successful TFC (Gao et al., 2010; Gilmartin et al., 2013), the reduction in synaptic dHc NR2B subunit expression (Figure 3) is proposed to disrupt the long-term consolidation of new fear memories. Interestingly, and contrary to our hypothesis, the expression of extrasynaptic NR2B subunits in dHc of 5E rats was also significantly diminished (Figure 5), though their exact contribution to TFC remains unclear.

The role of the prefrontal cortex and the hippocampus in trace fear conditioning

Successful TFC relies on a distributed neural network, which includes the medial prefrontal cortex and the hippocampus (Raybuck & Lattal, 2014). The mPFC, which regulates attentional processes and working memory, is believed to be critical for successful TFC (Wang et al., 2013). For instance, mPFC neurons are known to fire across the trace interval during TFC, similar to working memory, bridging the temporally discontinuous CS and US signals (Gilmartin & McEchron, 2005). The homolog to the rodent mPFC in humans is the dorsolateral prefrontal cortex, which is also involved in tasks requiring working memory, like n-back tests and human TFC (Barbey et al., 2013; Jansma et al., 2000). Lesions of the mPFC or mPFC NMDAR antagonism have been shown to impair TFC acquisition and expression (Gilmartin & Helmstetter, 2010; Gilmartin et al., 2013). Similar impairment in TFC expression were found following mPFC inactivation after one month of conditioning (Quinn et al., 2008), suggesting the mPFC is required for the long-term storage of the trace fear memory.

In addition to the mPFC, the involvement of the hippocampus has been extensively documented. However, the precise role the hippocampus plays in TFC remains unclear. In animal studies utilizing hippocampal lesions, TFC was shown to be disrupted (Bangasser et al., 2006; Burman et al., 2006; McEchron et al., 1998). The specific role of hippocampal subareas engaged in TFC, such as dHc and vHc, remains less clear, however (Cox et al., 2013; Czerniawski et al., 2009; Esclassan et al., 2009; Trivedi & Coover, 2004; Yoon & Otto, 2007). The involvement of vHc in TFC has been documented in a number of studies, where lesioning of the vHc resulted in impaired TFC (Cox et al., 2013; Czerniawski et al., 2009; Gilmartin et al., 2012; Yoon & Otto, 2007), likely due to its role in relaying information between the dHc, mPFC, and the amygdala.

Similarly, the contribution of dHc to TFC has been elucidated in various electrolytic and chemical lesioning studies, where damage of the dHc was demonstrated to hinder acquisition of TFC (Burman et al., 2006; Chowdhury et al., 2005; Quinn et al., 2002). Furthermore, NMDAR antagonism in dHc was also shown to impair TFC acquisition and expression (Esclassan et al., 2009; Raybuck & Lattal, 2011). It is important to mention that delay fear conditioning, in which the CS and US overlap and co-terminate, remained intact even with damage or inactivation of the dHc (Burman et al., 2006; Chowdhury et al., 2005; Esclassan et al., 2009; Quinn et al., 2002; Raybuck & Lattal, 2011). Consistent with earlier research (DuPont et al., 2014; Guimarãis et al., 2011), we previously reported that the dHc is required for long trace intervals (15 s and 30 s) but not short trace intervals (5 s) during TFC (DuPont et al., 2014). These findings support the idea that dHc is required for TFC, specifically when the CS and US signals are separated by a long trace interval (i.e., > 5-10 s).

As noted above, successful TFC is critically dependent on NR2B-containing NMDAR synaptic plasticity in both the mPFC and, relevant to the current thesis, the dHc (Gilmartin & Helmstetter, 2010; Guimarãis et al., 2011; Quinn et al., 2005; Wanisch et al., 2005). Previous research has demonstrated that pre- and/or postnatal ethanol exposure in pre-weanling rats can significantly elevate NR2A subunit expression and/or diminish NR2B subunit expression (Hughes et al., 1998; Nixon et al., 2002, 2004). The decrease in NR2B subunits, relative to NR2A, is suggested to impede the consolidation of new short-term memories (Cui et al., 2013; Suvarna et al., 2005). To our knowledge, however, no prior rat studies have examined NMDAR subunit expression in adult rats exposed to neonatal ethanol.

N-methyl-D-aspartate receptors (NMDARs)

The NMDAR is an ionotropic postsynaptic receptor involved in learning and memory. It requires glutamate binding and postsynaptic membrane depolarization to open, enabling the receptors to act as coincidence detectors for presynaptic and postsynaptic activity. The structure of the NMDAR contains an extracellular N-terminal end and an intracellular C-terminal end, which allows the receptor to interact with various cytosolic proteins (Paoletti & Neyton, 2007). Each NMDAR is composed of four subunits: two ubiquitous NR1 subunits and two regulatory NR2 subunits. The NR2 subunits expressed in the forebrain are predominantly either NR2A or NR2B (Monyer et al., 1994). Furthermore, the NR2 subunit composition confers distinct kinetics to the NMDAR.

Within the complex of the NMDAR, the NR2A subunit has a higher opening probability and a shorter opening duration compared to the NR2B subunit, making it close faster, thus, gating less calcium into the cell (Erreger et al., 2005). This makes the NR2B subunit, within the NMDAR, critical for forebrain-dependent learning. (Gilmartin et al., 2013; Muller et al., 2013;

Wang et al., 2006; Zhou et al., 2007). The influx of calcium activates downstream molecules that eventually remodel the synapse to increase its efficacy (Cull-Candy & Leszkiewicz, 2004). Such modifications, also known as synaptic plasticity, play a key role in associative learning, and allow for sustained neural firing during the trace interval in the prefrontal cortex. More specifically, NR2B-containing NMDARs—which can integrate asynchronous inputs with its long open channel time (Wang et al., 2013)—are posited to provide for sustained firing (as in working memory) in various neural substrates, including the amygdala, hippocampus, perirhinal cortex, and the prefrontal cortex (Chiba, 2000; Gruart et al., 2006; Muller et al., 2013; Power et al., 1997).

Compared to synaptic NMDARs, extrasynaptic NMDARs exhibit a lesser affinity to the amino acid neurotransmitter glutamate. Limited glutamate release by low frequency stimulation was documented to preferentially activate synaptic NMDARs (Ivanov et al., 2006). Moreover, studies that took advantage of glutamate transporter inhibitors and high frequency stimulation found that activation of extrasynaptic NMDARs relied on the availability of spilled over glutamate (Grebenyuk et al., 2004; Harris & Pettit, 2008; Lozovaya et al., 2004; Milnerwood et al., 2010; Tzingounis & Wadiche, 2007). These results taken together signify that extrasynaptic NMDARs, by virtue of their more distant location and possible structural differences, require higher levels of glutamate release and at the same time display a lower affinity for released glutamate than synaptic NMDARs.

Some of the structural differences that may contribute to the variable affinity for glutamate can be attributed to the abundance of postsynaptic density (PSD) proteins at the synapse relative to the extrasynapse (Gladding & Raymond, 2011). Additionally, more structural and scaffolding proteins and effectors that anchor and cluster NMDARs are embedded in the

synapse (Gladding & Raymond, 2011). In support, PSD-95 expression was evident in synaptic, but not in extrasynaptic Western blotting representative blots (Figures 3 and 7). Furthermore, NMDAR subunit composition can dictate the affinity of the receptor to glutamate. Both synaptic and extrasynaptic NMDARs require glutamate binding to open, but they exhibit different binding affinities for endogenous coagonists. Specifically, synaptic NMDARs bind D-serine more readily whereas extrasynaptic NMDARs bind glycine more readily (Papouin et al., 2012). Differences in endogenous coagonist binding suggests that the subunit structure between synaptic and extrasynaptic NMDARs may differ slightly, hence potentially affecting the receptor's affinity to various ligands and resulting calcium kinetics (Zhou et al., 2014).

Composition profile of NMDARs during normal and abnormal postnatal development

In normal early brain development, the majority of NMDARs are composed of the NR2B subunit. Over the first few weeks of a rat's life, a gradual development change in NMDAR subunit composition occurs, with an increase in NR2A subunit expression and a decrease NR2B subunit expression (Monyer et al., 1994). However, when alcohol is present during early brain development, glutamatergic activity is suppressed, which facilitates more NMDAR insertion to maintain baseline activity (Kalluri et al., 1998). During alcohol withdrawal, the increased calcium flow into the cell due to NMDAR upregulation leads to excitotoxicity (Hoffman & Tabakoff, 1994; Manev et al., 1989). As a result, the developing brain exposed to alcohol is proposed to undergo a period of neuro-compensation, in which the NMDAR subunit composition—which regulates the amount of calcium influx—is modified by upregulating the NR2A subunit, assuming they replace existing NR2B subunits, and/or downregulating NR2B subunits (von Engelhardt et al., 2009). Such modification produces an exaggerated NR2A/NR2B ratio, which is thought to be neuroprotective because it limits the size and duration of the

calcium pulse, hence, potentially protecting the cells against ethanol-induced cell death. Such changes occur in response to acute alcohol exposure, though current results suggest such changes in NMDAR subunits in neonate rats can persist through adulthood. It is also important to highlight that most NMDARs in the adult hippocampus are thought to be triheteromeric (NR1/NR2A/NR2B) and not dihomomeric (NR1/NR2A-B) (Rauner & Köhr, 2011).

The NR2A/NR2B ratio and learning-dependent synaptic plasticity

For several decades the importance of NMDARs to synaptic plasticity has been well established. An interesting model from the 1980s suggests that the induction of LTP/LTD lies on a sliding threshold, which can be modified via prior experience of the synapse, among other factors (Bienenstock et al., 1982). In terms of current work, the BCM theory of synaptic plasticity states that the LTP/LTD sliding threshold can also be modified based on NMDAR subunit composition and calcium kinetics (Abraham et al., 2001; Abraham, 2008; Castellani et al., 2001; Shouval et al., 2002). Elevating the NR2A/NR2B ratio, as occurs in 5E rats, would be predicted to diminish the induction or maintenance of learning-dependent LTP (Yashiro & Philpot, 2008).

The degree to which LTP expression is maintained is believed to occur as a result of the downstream signaling cascades activated following NMDAR-gated calcium flux. Thus, a reduction in NR2B expression may be productive against excitotoxicity during early life exposure to ethanol, but putative NMDAR hypofunction based on diminished NR2B expression, coupled with general cell loss, could thwart the recruitment of synaptic plasticity molecules, which would hinder learning-dependent synaptic plasticity. Similarly, selective antagonism of extrasynaptic NR2B subunits was demonstrated to amplify LTD expression using a low frequency stimulation protocol (Kollen et al., 2008; Liu et al., 2013). The reduction of

extrasynaptic NR2B subunit expression may mediate an exaggerated LTD expression. More research needs to be done to conclusively determine whether behavioral deficits are attributable to LTP dysfunction and/or LTD amplification. At a minimum, however, current whole-cell immunoblotting results indicate the 5E adult rats express aberrant levels of NR2 subunits, offering strong evidence that the neurotoxic effects of alcohol on NMDAR composition and function persist through adulthood, or, at least, re-emerge in adult subjects.

The NR2A and NR2B subunits mediate different downstream calcium signaling cascades specific to the induction of LTP. Calcium entering the cell through NMDAR-activation has a high affinity for alpha-calcium/calmodulin-dependent protein kinase II (CaMKIIα). The protein complex then binds to the free-floating cytosolic c-terminal domain of the NR2B subunit, which allows the NMDAR to be open for a longer duration, thus prolonging the influx of calcium (O'Leary et al., 2011; Zhou et al., 2007). Furthermore, interactions with the NR2B subunit allows CaMKIIα autophosphorylation, which recruits downstream synaptic-plasticity related molecules, such as extracellular-signal-regulated kinase 1/2 (ERK 1/2) and mitogen-activated protein kinases (MAPK), even in the absence of calcium (Atkins et al., 1998; DuPont et al., 2014; Samudio-Ruiz et al., 2009; Sessoms-Sikes et al., 2005; Thomas & Huganir, 2004; Zhou et al., 2009). The downstream molecular cascade facilitates the maintenance of LTP by inserting α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors and recruiting cytoskeletal components, such as activity-regulated cytoskeleton-associated protein (Arc/Arg3.1) (Bloomer et al., 2007; Messaoudi et al., 2007; Shepherd et al., 2006; Yang et al., 2008). Taken together, this makes the NR2B-containing NMDARs critical for learning-dependent synaptic plasticity and long-term memory storage (El Gaamouch et al., 2012; Halt et al., 2012).

Whole cell lysate Western blotting results, which included intracellular subunit stores, revealed alterations in the NR2A/NR2B ratio in dHc of adult 5E rats (Figure 2), following exposure to alcohol across PD 4-9. A subcellular fractionation protocol, coupled with an acetone precipitation and detergent extraction protocol, was employed to assess transmembrane NMDAR subunit composition. The objective of this project was to relate functional changes in NR2 subunit expression to the deficits in TFC observed in the 5E rats. We demonstrated significant reductions in synaptic (PSD-95 associated) and extrasynaptic NR2B protein expression in dHc of 5E rats. Our results are consistent with the hypothesis that postnatal ethanol disrupts synaptic NMDAR composition and forebrain development resulting, in adult rats, in impaired synaptic plasticity and TFC. Possible roles for extrasynaptic NMDARs in the acquisition of TFC are unclear, though they do play other critical roles in early development (discussed below).

Synaptic and extrasynaptic NMDAR distribution and postnatal developmental expression pattern

During early brain development, a greater number of NMDARs are believed to occupy the extrasynapse in immature neurons (Li et al., 2002). As the brain undergoes rapid development during early postnatal life, NMDAR populations can begin to vary in the degree to which they occupy the synapse versus the extrasynapse of mature neurons (Gladding & Raymond, 2011; Petralia, 2012; Petralia et al., 2010). In mature neurons, it was found that the more stable NMDARs—those that do not laterally diffuse or reversibly associate with synaptic scaffolding proteins—occupy the synapse as opposed to the extrasynapse (Chen et al., 2007). Harris and Pettit (2007) utilized an immunofluorescence protocol in dissected hippocampal slices obtained from PD 14-21 rats and found that a considerable percentage of dendritic NMDARs were localized in extrasynaptic regions of pyramidal neurons in hippocampal area CA1. It was previously thought that more NR2B-containing NMDARs gradually occupy extrasynaptic

regions, while NR2A-containing NMDARs occupy primarily synaptic regions as a function of postnatal brain development (Groc et al., 2009; Monyer et al., 1994; Thomas et al., 2006; Tovar & Westbrook, 1999). However, no notable difference were found between populations of functional hippocampal NR2B-containing NMDARs between synaptic and extrasynaptic regions (Harris & Pettit, 2007). Furthermore, in mature neurons, the distribution of NR2A and NR2B subunits was also found to be similar in the extrasynapse (Petralia et al., 2010). Interestingly, in vitro studies suggest that the distinct circular clustering pattern of extrasynaptic NR2B-containing NMDARs may be indicative of previous presence of synapses (Petralia et al., 2010; Sans et al., 2000; Storey et al., 2011), presumably eliminated during synaptic pruning. However, these results are not definitive as similar outcomes may or may not arise with vivo models.

The role of synaptic and extrasynaptic NMDAR coactivation in neuronal survival and death

A number of studies have highlighted the contribution of synaptic NMDARs in learning-mediated synaptic plasticity, neuronal survival, and cell death. Activation of synaptic NMDARs mediates cell survival by activating pro-survival gene transcription effectors, like cyclic-AMP response element binding protein (CREB), recruiting antioxidants to protect against oxidative insult, and turning off apoptotic gene expression, such as forkheard box protein O (FoxO) and p53 (Gladding & Raymond, 2011; Hardingham & Bading, 2010). Both synaptic NR2A- and NR2B-containing NMDARs contribute to the aforementioned events; however, it is suggested that they do so through different molecular cascades (Liu et al., 2007; von Engelhardt et al., 2007; Yashiro & Philpot, 2008; Zhou, Ding, et al., 2013). In pathological models, excessive influx of calcium into the cell was shown to induce neuronal death, but it was later demonstrated the calcium-influx activation of pro-apoptotic pathways contributed to neuronal death, and not the load of calcium entry (Manev et al., 1989; Sattler et al., 1998; Tymianski et al., 1993). In

cases of brain trauma, for instance, blocking synaptic NMDARs can attenuate neuronal death (Sattler et al., 2000; Wroge et al., 2012; Young et al., 2010). Importantly, similar effects have been noted following postnatal ethanol exposure—e.g., Young et al. (2010) that MK-801 (a NMDAR open-channel blocker) administration during the alcohol withdrawal period ameliorated cell death within the cerebellum.

Various in vitro studies have attempted to elucidate the role of extrasynaptic NMDARs, which appear to differ as a function of development. During early development, extrasynaptic NMDARs are thought to be important for the development and maturation of neurons during synaptogenesis, differentiation of neurons, neuronal migration, and survival of neuroblasts (Georgiev et al., 2008; Komuro & Rackic, 1993; Sin et al., 2002; Wang et al., 2011). In mature neurons—such as in adult rats in the current study—functional extrasynaptic NMDARs contribute to the modulation of excitatory postsynaptic potential (EPSP) via calcium-induced calcium flux glutamate spillover (Faber & Korn, 1988). Therefore, most, if not all of extrasynaptic NMDAR activation occurs in tandem with synaptic NMDAR activation. Astrocyte glutamate release can also serve as a way to monitor overall neuronal activity by primarily binding to proximal NR2B-containing NMDARs (Bergersen & Gundersen, 2009; Fellin et al., 2004; Hamilton & Attwell, 2010). However, physiological properties conferred to extrasynaptic receptors also depend on the number of activated extrasynaptic NMDARs, their subunit composition, and the collection of signaling proteins with which they interact (Hardingham & Bading, 2010).

It is widely accepted that activation of extrasynaptic NMDARs contribute to neuronal death to a greater degree than activation of synaptic NMDARs by shutting off CREB and ERK1/2 signaling, activating FoxO gene expression, and activating inflammatory mediators,

such as neuronal cyclooxygenase (COX)-2 and lipid peroxidation (Bordji et al., 2010; Gladding & Raymond, 2011; Hardingham & Bading, 2010; Stark & Bazan, 2011). Nonetheless, there is a lack of understanding regarding the role of extrasynaptic involvement in vivo because it is difficult to ascertain whether extrasynaptic NMDAR activation by itself contributes to cell death (Gladding & Raymond, 2011).

Generally, the spillover of glutamate results in the activation of both synaptic and extrasynaptic NMDARs. Thus, it can be postulated that neuronal death requires coactivation of receptors in both locations (Chen et al., 2014; Zhou, Ding, et al., 2013). Many studies have subsequently investigated the functional dichotomy of synaptic and extrasynaptic NMDARs. Papouin and colleagues (2012) noted that antagonism of synaptic NMDARs guarded neurons against excitotoxicity and oxidative insult, whereas inhibition of extrasynaptic NMDARs did not produce a similar neuroprotective effect. As a result, this finding suggests that the mutually opposing role of synaptic and extrasynaptic NMDARs may be simplistic. More recently, several studies have pointed out that activation of both synaptic or extrasynaptic NMDARs was sufficient to activate the ERK 1/2-CREB-BDNF neuronal pro-survival signaling pathway without disrupting cytosolic calcium homeostasis that ultimately causes cell death (Chen et al., 2014; Zhou et al., 2014; Zhou, Ding, et al., 2013; Zhou, Hollern, et al., 2013). However, a prolonged activation of both synaptic and extrasynaptic NMDARs was shown to inhibit the ERK 1/2-CREB-BDNF neuronal pro-survival signaling pathway, thus favoring the activation of pro-apoptotic pathways (Chen et al., 2014; Zhou et al., 2014; Zhou, Ding, et al., 2013; Zhou, Hollern, et al., 2013). Furthermore, the pro-survival gene expression profile, as a result of the coactivation of synaptic and extrasynaptic NMDARs, was similar to the one activated by synaptic NMDARs alone, but not extrasynaptic NMDARs (Zhang et al., 2007; Zhou, Hollern, et al., 2013).

Under normal physiological processes, the biological mechanisms and protein signaling cascades underlying synaptic plasticity and neuronal survivability appear to greatly overlap (Zhou et al., 2014). During early development, when the numbers of extrasynaptic NMDARs are greater than synaptic NMDARs, developing neurons appear to be less susceptible to glutamate-induced neurotoxic insults than mature neurons (Choi & Rothman, 1990; Friedman & Segal, 2010; Li et al., 2002). For example, NMDAR-mediated excitotoxicity remains absent in populations of neurons containing only extrasynaptic NMDARs, such as in retinal ganglion cells (Chen & Diamond, 2002; Ullian et al., 2004; Zhang & Diamond, 2006). This may indicate that biological mechanisms governing cell death are not solely reliant on extrasynaptic NMDARs as previously discussed. This suggests that extrasynaptic NMDARs activation may not be solely sufficient to exert neuronal death, and may be reliant on the activation of synaptic NMDARs as well. Collectively, it can be argued that NMDAR-mediated neuronal death is controlled by both the degree and the duration of the coactivation of synaptic and extrasynaptic NMDAR populations.

The role of synaptic and extrasynaptic NMDARs in synaptic plasticity

A number of studies using pre-weanling rat or adult mice exposed to perinatal ethanol have shown significant reductions in NR2B subunit expression and/or an elevation in NR2A subunit expression (Brady et al., 2013; Hughes et al., 1998; Nixon et al., 2002, 2004; Samudio-Ruiz et al., 2010; Spuhler-Phillips et al., 1997). We have now furthered this prior research by demonstrating changes in NR2B and the resulting NR2A/2B ratio in adult rats exposed to third-trimester equivalent ethanol (Figures 2-7). Results from Western blotting protocol optimized for transmembrane NMDAR subunits further revealed significant reductions in the NR2B subunit in the dHc of 5E rats compared to the controls (Figure 3), though the NR2A/NR2B ratio did not

significantly differ between treatment groups. The synaptic fraction results also showed a slight reduction in NR1 subunit and PSD-95 expression, suggesting the 5E rats may have fewer NMDARs than control rats. Ethanol-induced decreases in hippocampal NR2B subunits impair learning-dependent synaptic plasticity and TFC. Changes in NMDAR subunits may be one of the mechanisms the cell undergoes to protect against further cell death experienced during alcohol withdrawal. How events in early life could be carried forward through maturation, however, is an important question that remains to be fully answered.

During early postnatal brain development, NR2B-containing NMDARs were found to influence proper synaptogenesis and stabilize existing synapses, whereas NR2A-containing NMDARs reduced the number of formed synapses (Gambrill & Barria, 2011). This may be one of the biological correlates explaining the underlying plasticity of the developing brain. During early life exposure to alcohol, when more NR1/NR2B than NR1/NR2A NMDARs are present, NMDARs undergo sustained ethanol-mediated blockade, which in turn causes neurons to upregulate NMDAR expression (Kalluri et al., 1998; Puglia & Valenzuela, 2010). During subsequent alcohol withdrawal, the increased number of NMDARs gates calcium in a manner that is excessive and excitotoxic to the cell (Lee et al., 1994; Spuhler-Phillips et al., 1997; Tymianski et al., 1993).

One of the proposed neuroprotective measures neurons undergo following alcohol exposure is the reduction in trafficking of NR2B-containing NMDARs (Suvarna et al., 2005). The reduction in receptor trafficking was also found to co-occur alongside NR2A-containing NMDAR internalization via H-ras activation and inhibition of Src tyrosine kinases (Suvarna et al., 2005). These proteins play an intricate role NMDAR adhesion to the membrane and controlling channel activity via phosphorylation of the C-terminal domain, and resulting

postsynaptic membrane excitatory response (Ali & Salter, 2001; Thornton et al., 2003). Deletion of H-ras has been shown to increase phosphorylation of the NR2 subunit, allowing the receptor to remain open for longer durations (Thornton et al., 2003). Conversely, inhibition of Src tyrosine kinases expression was demonstrated to mediate NR2A-, and not NR2B-containing NMDAR internalization (Suvarna et al., 2005).

Another proposed neuroprotective mechanism involves the preferential upregulation of NR2A subunit expression. Following blockade of the NMDAR, expression of NR2A subunit, but not NR2B subunit expression was found to be significantly increased (Raeder et al., 2008; von Engelhardt et al., 2009). Vice versa, in disease models involving prolonged release of glutamate, and/or failure of adequate glutamate reuptake, synaptic NR2B-containing NMDARs, to a greater degree than NR2A-containing NMDARs, were found to mediate NMDAR cell death (Fan et al., 2010). Likewise, sustained activation of NR2B-containing NMDARs was also found to facilitate apoptotic pathways (Vacotto et al., 2010). The exact extent to which a higher NR2A/NR2B ratio is neuroprotective remains elusive. However, it appears that repeated alcohol exposure during early life yields significant and persistent changes in the extent and manner that NMDAR subunits are expressed, which could interfere with proper synaptogenesis and forebrain maturation. Such deficits may persist long after alcohol is administered, potentially accounting for deficits in LTP and TFC observed in adult 5E rats in the current thesis.

Conclusions

Using a rat FASD model, we aimed to investigate the deleterious cognitive effects of exposure to alcohol during early brain development. It has been extensively shown using animal studies that exposure to alcohol in early life results in a reduction in the number of neurons, diminished dendritic spine density, and profound learning and memory deficits, as shown using

various Pavlovian conditioning behavioral paradigms (Brown et al., 2008; DuPont et al., 2014; Hamilton et al., 2011; Hamilton et al., 2010; Hunt et al., 2009; Lindquist, 2013; Miki et al., 2003; Murawski et al., 2012; Napper & West, 1993; Pauli et al., 1995; Schreiber & Hunt, 2013; Wagner & Hunt, 2006). TFC is proposed to rely on the dHc and mPFC for initial acquisition and consolidation (lasting several weeks), and the mPFC is required for the trace fear memory's long-term storage (i.e., a month or more after training) (Gerwitz, 2000; McEchron et al., 1998). Furthermore, successful TFC is reliant on the activation of forebrain NMDARs, and specifically on NR2B-containing NMDARs (Gao et al., 2010; Gilmartin & Helmstetter, 2010; State & Fanselow, 2004; Wanisch et al., 2005). The deleterious effects of neonatal exposure to alcohol include, but may not be limited to, the alteration of NMDAR subunit composition and/or the number of functional NMDARs. The almost-significant reduction in NR1 subunits also suggests there may be fewer NMDARs, possibly as a consequence of fewer CA1 neurons (Livy et al., 2003) and diminished spine densities (Mihalick et al., 2001).

In support of our hypothesis, observed deficits in TFC in the 5E rats appears to be due, at least in part, to aberrant NMDAR-dependent plasticity and long-term memory consolidation. Taken together, our 5E rats have now been demonstrated to display impaired TFC and a reduction in synaptic plasticity molecules downstream the NMDAR in dHc, such as ERK1/2 (DuPont et al., 2014). Investigating the NMDAR subunit expression patterns using whole cell lysate Western blotting revealed an elevated NR2A/NR2B ratio in dHc of 5E rats, but these results also consider intracellular subunit stores. The fractionation results indicate transmembrane NR2B subunits are significantly reduced at both the synapse and extrasynapse. Considering the crucial role played by NR2B-specific plasticity, including CaMKII translocation and autophosphorylation, the poor learning in 5E rats is likely due to the loss of NR2B-

containing synaptic receptors. Future research will be required to ascertain the degree to which extrasynaptic NMDARs also contribute to synaptic plasticity and TFC.

Significance

Current findings are expected to help elucidate the underlying causes of the physical and mental deficits in humans suffering from FASD, inform our understanding of the etiology of the disorder in humans, and, ultimately, improve the care and treatment of children and adults with FASD. In addition, results related to aberrant NMDAR composition provides data suggestive of future possible pharmacological treatments in order to ameliorate cognitive impairments in FASD individuals.

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