EFFECT OF NEONATAL ALCOHOL EXPOSURE ON C-FOS EXPRESSION IN HIPPOCAMPAL CA3 FOLLOWINGEXPLORATION OF NOVEL CONTEXT IN ADOLESCENCE

by

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ABSTRACT

In humans, developmental alcohol exposure (AE) results in a myriad of deficits across several domains of human functioning that persist across the lifespan of the afflicted individual. This issue warrants thorough study, as prenatal AE affects millions of children across the globe every year. The present study uses a rodent model of fetal alcohol spectrum disorders (FASD) to examine the effect of third-trimester equivalent (post-natal day 4-9) alcohol exposure on the density of cells c-Fos as an indirect measure of neuronal activity, in adolescent rats following exploration in a novel context. Rats were exposed to one of three dosing conditions: Two AE groups, exposed in a single binge dose (4.0 g/kg/day or 5.25 g/kg/day), and a sham intubated (SI) group to control for the stress of the alcohol administration procedure. Novel context exposure has been shown to generate c-Fos expression in the hippocampus, at levels correlated with acquisition of a fear-response in a conditioning task in that context (Radulovic, Kammermeier, & Spiess, 1998). Prior experiments have shown hippocampal c-Fos expression and contextual fear conditioning is reduced in animals exposed to alcohol neonatally (Murawski, Klintsova & Stanton, 2012). In CA1, following exploration of a novel context, both total pyramidal cell count and the number of cells expressing c-Fos was decreased in AE animals relative to controls (Murawski et al., 2012). The present study assessed the effect of neonatal AE the density of c-Fos+ cells/mm2 of CA3 using optical densitometry, examining the same tissue assessed by Murawski and colleagues (2012). The present study found a trending increase in c-Fos+ density in dorsal CA3 in the higher AE group (5.25

g/kg/day) compared to SI controls. Differences between groups in total and ventral CA3 c-Fos+ were insignificant. This data may provide useful insight into the pathology of hippocampal circuitry as a result of neonatal AE.

Chapter 1

INTRODUCTION

1.1 Fetal alcohol spectrum disorders

Over the last 40 years, researchers have established that alcohol has major teratogenic properties; research has shown *in utero* exposure to alcohol may confer negative developmental effects to the affected fetus, with lasting cognitive, behavioral, and morphological manifestations. Fetal alcohol syndrome (FAS) refers to the most severe form of abnormal development resulting from gestational alcohol exposure, characterized by stereotyped craniofacial dysmorphologies, microcephaly, low brain weight and volume, and simplified cortical structure (Riley, Infante, & Warren, 2011). Since the advent of the term, a much larger pool of deficits beyond this most serious set of maladies have been identified as consequences of prenatal exposure to alcohol. These span many areas of phenotypic expression, including cognitive and behavioral manifestations. In order to accommodate these less robust yet still impactful negative effects, the non-diagnostic umbrella term "fetal alcohol spectrum disorders" (FASD) was suggested, encompassing all of the effects attributed to maternal alcohol consumption (Sokol, Delaney-Black, & Nordstrom, 2003).

Even with the coining of the term FAS in the 1970s (Jones, Smith, & Hanson, 1976), over 3,500 publications on the topic, and efforts to raise public awareness about the consequences of maternal alcohol consumption, FASD still remains one of the leading causes of preventable cognitive disability in the United States (Riley et al., 2011). Studies spanning from 1993 to 2002 by the CDC estimated the prevalence of

FASD to be between 0.02% and 0.15% of all live births (Riley et al., 2011). However, a different study utilizing in-school studies in the U.S. puts the figures significantly higher, with a 0.2-0.7% prevalence of FAS, and the prevalence of FASD in young school children in the U.S. and Western Europe at 2-5% (May et al., 2009). The prevalence of FASD has a higher rate of incidence than seen in Down syndrome, Cerebral Palsy, Sudden Infant Death Syndrome, Cystic Fibrosis, and Spina Bifida combined (NOFAS, 2004). In a study conducted by the United States National Birth Defects Prevention Study, of 4,088 women who bore infants with birth defects, 30.3% of mothers reported alcohol consumption at some time during pregnancy, with 8.3% reporting binge drinking (Ethen et al., 2009). These studies taken together illustrate a picture suggesting that maternal alcohol consumption presents a very large problem, conferring preventable birth defects to tens of thousands of newborns annually in the Western world.

Looking beyond the first few years of life, detrimental effects associated with gestational alcohol exposure persist both for the afflicted individuals and society as a whole. From a monetary perspective, annual costs of FAS in the U.S. have been estimated as high as $3.6 billion (Olson et al., 2009), and annual cost per individual with FASD in Canada has been reported to be $21,642 (Stade et al., 2009). A myriad of deficits in neurobehavioral functioning that result from FASD persist in those affected throughout their lifetime (Matson & Riley 1998). Broadly, these deficits include, but are not limited to: overall IQ, learning and memory, language, attention, reaction time, visuospatial abilities, executive functioning, fine and gross motor skills, and social and adaptive functioning (Riley & McGee 2005). These deficits often lead to the development of secondary disabilities and trouble maintaining independence in

society (Clarke & Gibbard 2003). For example, Coggins and colleagues showed that understanding of complex language and figures-of-speech is markedly deficient in teens with FAS, making it difficult them to effectively communicate with their peers or much of society (Coggins, Friet, & Morgan, 1998). Further, studies comparing the social skills of children with prenatal alcohol exposure to children without prenatal alcohol exposure matched for age and IQ showed profound deficits in the children with exposure, suggesting there exists neurocognitive deficits in social proficiency in these alcohol exposed (AE) children, which are independent of those that impact raw intellect (Roebuck, Mattson, & Riley, 1999; Mattson & Riley, 2000). Along with many others, the studies mentioned illustrate the pervasive nature of FASD throughout the lifespan in nearly all domains of human functioning required to independently subsist in current society.

1.2 Effects of neonatal alcohol exposure on hippocampal neuroanatomy and hippocampal dependent tasks

Numerous studies have revealed alcohol-related deficits across different modalities of human functioning (Clarke & Gibbard 2003; Coggins et al., 1998; Roebuck et al., 1999; Mattson & Riley 2000; Riley & McGee 2005). In order to attempt to understand these impairments and eventually help restore functioning to those with FASD, recent research has focused on developmental AE induced alterations in the brain. Due to obvious ethical constraints, the scope and impact of FASD research that can be done on human brains is quite limited, mainly to imaging techniques and post-mortem studies. In order to circumvent these limitations, scientists have turned to animal models, often utilizing the well-studied nervous system of the rat to model FASD.

The severity and manifestation of teratogenic effects caused by developmental AE varies greatly, depending upon the timing and degree of exposure, both in humans and in rats (Bonthius & West, 1990; Cartwright & Smith, 1995; Coles, 1994; Maier & West, 2001; Miller, 1992). Research using animal models of FASD aims to target the effect alcohol exposure has on the brain in humans during analogous stages of neural development. Thus, the time point chosen for alcohol administration depends on the stage of neural development, not necessarily corresponding with gestational time point in humans. Many neural structures, such as the hippocampus, cerebellum, and several cortical areas have been shown to be grossly affected in rats when AE coincides with the brain-growth spurt (Pauli, Wilce, & Bedi, 1995; Pierce, Goodlett, & West, 1989; Klintsova et al., 2007); a period of mass neuronal differentiation, maturation, and synaptogenesis corresponding with the end of the third trimester in humans and the first two postnatal weeks in rats (Dobbing & Sands, 1979). Rats exposed to alcohol in binge-like fashion during this time period have exhibited significant loss of cerebellar Purkinje and granule cells, CA1 pyramidal cells, and reductions in dendritic spine density in the medial prefrontal cortex (Whitcher & Klintsova, 2008). During this time period, peak blood-alcohol content (BAC) is negatively correlated with total brain weight at the end of treatment, suggesting the deleterious effects of AE are dose-dependent (Maier & West, 2001).

Due to the small molecular size and hydrophobic properties of alcohol, it can easily cross the placental and blood-brain barriers through simple diffusion across the membranes. Alcohol transferred across the placenta accumulates densely in the hippocampus (Ho, Fritchie, Idanpaan-Heikkila, & McIsaac, 1972), making this structure among the most impacted during the brain growth spurt. In rats, binge-like

alcohol exposure during this brain growth spurt results in the most severe neurological deficits, likely because it causes a higher peak BAC than exposure paradigms with daily dosage broken into several administrations throughout the day (Maier & West, 2001). These findings have spurred much research into the effects of third-trimester equivalent AE on the hippocampus. Some of the specific hippocampal deficits observed in the literature as a result of third trimester equivalent AE reduction in mature hippocampal cell number (Klintsova et al., 2007), volumetric reductions in both CA1 (Cornu ammonis) and CA3 subfields of the hippocampus (Livy, Miller, Maier, & West, 2003), loss of CA1 pyramidal cell number and decrease in their density (Tran & Kelly, 2003), and long-term potentiation (LTP) inhibition in CA1 (Puglia & Valenzuela, 2010). These neuroanatomical deficits give rise to deficits observed in several hippocampal dependent behavioral tasks in AE rats. These tasks include place learning (Goodlett & Johnson, 1997), spatial acquisition in the Morris water maze (Goodlett & Peterson, 1995; Tomlinson, Wilce, & Bedi, 1998) as well as several versions of fear-conditioning (Murawski & Stanton, 2010; Hunt, Jacobson, & Torok, 2009; Tran & Thomas, 2007). The presence of pathology in hippocampal anatomy in combination with deficits in tasks that have been shown to require hippocampal activity suggest in developmentally AE animals suggest that many of the behavioral abnormalities that result from neonatal AE are due to damage of the hippocampus.

1.3 Role of c-Fos as an indirect marker for neuronal activity

Upon cell stimulation, the first wave of genes activated are known as Immediate Early Genes (IEG). To be considered an IEG, a gene must be rapidly activated, expressing a protein product without impediment from protein synthesis

inhibitors (Herrera & Robertson, 1996). One particularly well-characterized IEG is c-Fos. C-Fos is a proto-oncogene that heterodimerizes with members of the jun family of IEGs. These heterodimers in turn bind to AP-1 binding sites on DNA, repressing or activating transcription of genes containing AP-1 sites depending on which jun family member c-Fos binds to and the local cellular environment (Sheng & Greenberg, 1990). This paradigm of extracellular stimuli modulated IEG regulatory specificity allows extracellular signals to mediate of some aspects of neuronal plasticity. This occurs through differential expression of late-response genes encoding proteins, influencing neuronal phenotype.

The combination of rapid expression following neuronal stimulation and low basal expression levels make measurement of c-Fos a viable indirect marker of neuronal activity (Herrera & Robertson, 1996). C-Fos is considered an indirect marker of neuronal activity, as its expression is a result of neuronal stimulation, not a necessary component of neuronal activation. The current study uses c-Fos expression to examine neuronal activation in hippocampal subfield CA3 following exposure to and exploration of a novel context.

1.4 Induction of IEGs following novel context exposure

Induction of c-Fos and other IEGs has been shown to occur in response to a variety of novel stimuli (Tischmeyer & Grimm, 1999). Specifically, literature exists providing evidence that c-Fos is expressed following spatial novelty in the rodent brain (Handa, Nunley, & Bollnow, 1993; Kerr, Beck, & Handa, 1996; Papa, Pellicano, Welzl, & Sadile, 2003; Zhu, Mccabe, Aggleton, & Brown, 1997). Expression of c-Fos mRNA and protein above basal levels following exposure to novelty has been documented in many brain areas, including the anterior pituitary gland, portions of the

thalamus, the prefrontal, visual, cingulate, and parietal cortices, and the hippocampus (Handa et al., 1993; Keilmann & Herdegen, 1997; Montag-Sallaz, Welzl, Kuhl, Montag, & Schachner, 1998; Montero, 1997). The present study examines c-Fos expression in CA3 of the hippocampus following novelty of spatial and locational context. The hippocampus has been shown to increase c-Fos expression to a greater degree following exposure to a novel environment than to other novel stimuli such as novel objects, and research suggests that exposure to novel context elicits c-Fos expression in the hippocampus more so than does exposure to familiar context (Handa et al., 1993; Murawski et al., 2012; Zhu et al., 1997). It is therefore reasonable to assert that induction of c-Fos in the hippocampus may play a substantial role in processing or encoding of a novel context. The present study seeks to assess c-Fos expression following novel context exposure in CA3 with and without neonatal AE.

1.5 Study Aims

The current study is examining the effect of binge-like AE during PD4-9 on CA3 c-Fos expression following 5 minutes exploration in a novel context in rats. The tissue used for the study is the same analyzed for CA1 c-Fos expression in a previous study (Murawski et al., 2012). Examining the differences in neuronal activation of CA3 in AE versus SI animals may give insight into what role, if any, CA3 has in encoding representation of a novel context. In addition to the insights the results may provide regarding the role of CA3 neuronal activation following AE, contrasting these results with those obtained from the same tissue assessing CA1 neuronal activity will provide information on how activity levels of CA3 and CA1 following novel context exposure respond to neonatal AE. Considering that along with CA1, c-Fos+ cell counts in CA3 of non-AE animals were higher following exposure and exploration of

a novel context (Murawski et al., 2012), it would be unsurprising if similar results were found following AE for CA3 c-Fos+ cell counts as were found in CA1 in Murawski and colleagues' analysis.

A second aim of the study is to examine the dorsal and ventral portions of CA3 individually following exploration of a novel context, as the results of previous experiments suggest the DH and VH may have differing roles in encoding of contextual novelty (Anagnostaras, Gale, & Fanselow, 2002; Maren, 1999; Maren & Fanselow, 1997; Matus-Amat, Higgins, Barrientos, & Rudy, 2004; Richmond et al., 1999). If one region of the CA3 is affected more than the other, it may also provide some insight into the importance of the DH and VH in the processing and encoding of a novel context. It is difficult to hypothesize whether the effect of AE will differ between dorsal and ventral CA3, as their previous literature examining the effects of lesioning DH and VH didn't differentiate between which subfields were lesioned (Barrientos, O'Reilly, & Rudy, 2002; Matus-Amat et al., 2004; Matus-Amat, Higgins, Spruger, Wright-Hradesty, & Rudy, 2007; Rudy & Reilly, 1999; Schiffino, Murawski, Rosen, and Stanton, 2011).

Chapter 2

METHODS

2.1 Animal subjects

The animals used in the study were male Long-Evans rats (n = 28) derived from 19 litters. The animals were bred at the animal housing colony of the Office of Laboratory Animal Medicine at the University of Delaware. All pups were born on gestational day 22, which was considered postnatal day (PD) 0. On PD3 males from separate litters were fostered into male-only experimental litters in order to achieve nine pups per litter, eliminating the possibility of differences in maternal care due to the presence female pups. Pups were given non-toxic subcutaneous injections of black-ink of a combination of paws for identification. A given cross-fostered litter used no more than 4 original litters to make the experimental litters. Dams were given *ad libitum* access to food and water, and were kept on a 12:12 light dark cycle. On PD4, pups were randomly assigned to one of two treatment groups: alcohol-exposed (AE) or sham-intubated (see section 2.2 Alcohol Exposure). On PD21, pups were weaned and divided into housing groups of 4 or 5 until PD29, at which point subjects were moved to individual cages for the remainder of the study.

2.2 Alcohol exposure

Pups from each litter were assigned as follows: 3 pups were assigned to each of the 3 dosing conditions: 4.00g/kg/day, 5.25g/kg/day, or the sham-intubated (SI) control group. Pups were intragastrically intubated with the amount of alcohol dictated

by their weight and dosing condition. SI pups were intubated but no liquid was given through the intubation tube, as a control for the stress caused by the intubation process. The intragastric intubation paradigm consists of passing small, flexible plastic tubing lubricated with corn oil down the esophagus into the stomach of the pup. Alcohol was administered in a single binge exposure as part of a milk formula solution, with alcohol by volume (ABV) concentrations of the formula were 18.19% or 23.94% for the 4.00g/kg/d and 5.25g/kg/d groups, respectively. Once daily, from PD4-9, pups were briefly removed from their mothers and put into clear Lexan containers atop a heating pad to provide warmth while separated from their mothers. Pups in the AE treatment groups were given intubations of milk 2 and 4 hours after the alcohol intubation, while SI pups received sham intubations.

On the first day of alcohol administration only (PD4), 2 hours after alcohol administration, small tail clips were taken from the pups, and 20 μL blood samples were taken using heparinized capillary tubes. Blood samples from AE pups on PD4 were centrifuged. Plasma was extracted from centrifuged blood and stored at -20°C. Plasma samples were then analyzed an Analox GL5 Analyzer (Analox Instruments, Luneburg, MA, USA).

2.3 Novel context exposure

On PD31 rats were taken from their home-cage, weighed, and then placed into an (11×11×18 cm^3) clear Lexan transport cage with orange construction paper surrounding all four sides. They were transported in groups of four to a room adjacent to the room in which the novel context exposure took place. Each container was sterilized with 5% ammonium solution before the subjects were placed into them to eliminate any odors that could affect c-Fos expression. The subjects were then each

placed into one of four identical clear (16.5×21.1×21.6 cm^3) Plexiglas chambers within a fume hood. The chambers had a stainless steel bar floor 11.5 cm from the top of the chamber, composed of nine 0.5cm diameter grid bars positioned 1.25cm apart. The floors and sides of adjacent chambers were opaque. Subjects were placed in the novel context, allowed to explore freely for five minutes before removal and return to their home cage.

2.4 Tissue preparation

Rats were sacrificed 2 hours after exposure to the novel context, animals were deeply anesthetized through an i.p. sodium pentobarbital injection and transcardially perfused with 50 ml of heparinized 0.1 M phosphate-buffered saline (PBS) at pH 7.2, followed by 200 ml of cold 4% paraformaldehyde in 0.1M PBS. Once perfused, brains were removed, postfixed overnight in 4% paraformaldehyde in 0.1M PBS, and then transferred to a 30% sucrose, 4% paraformaldehyde solution the following day, and again a week later. Entire dorsal-ventral axes of the brains were sectioned horizontally into 40 μm serial sections using a cryostat. Sections were collected in order and then stored at -20°C in cryoprotectant solution (30% sucrose and ethylene glycol in 0.1 M PBS) until immunohistochemical staining.

2.5 C-Fos immunohistochemistry

One section of tissue was chosen at random from the first 16, and every 16th section from that section on was used for immunohistochemical staining for c-Fos in order to obtain a systematic random set of sections. The pseudorandomly selected sections were then pre-incubated in 1% hydrogen peroxide solution for 20 minutes, put into Tris-buffered saline (TBS) washing solution, and then transferred into a

blocking solution consisting of 10% normal goat serum (NGS) and 0.5% Triton X-100 in 0.1M TBS. The sections then underwent incubation in the primary anti-Fos antibody (rabbit polyclonal, raised against a peptide corresponding to amino acids 3-16 of human Fos, Santa Cruz Biotechnology, Santa Cruz, CA; 1:500 concentration) for 36 hours. The sections then were washed three times 10 minutes each in TBS, and then incubated in corresponding secondary antibody solution (Vector, anti-rabbit, 1:1000 concentration) for 1 hour and placed into Avidin-Biotin Complex (ABC) solution for an additional hour (VectaStain ABC kit, Vector Laboratories). All antibodies and ABC solutions were diluted in 10% NGS and 0.1M TBS. Immunoreactions were developed in diaminobenzidine (DAB) peroxidase substrate (Sigma) for 5-20 minutes. Control sections were put through the same protocol, but in the absence of the primary antibody. After extensive rinsing in TBS, free-floating sections were mounted maintaining order onto gelatin-coated slides and cover-slipped. All immunohistochemical methods have been described by Murawski and colleagues (2012).

2.6 Image acquisition

Slide-mounted horizontal sections stained for c-Fos were examined using light microscopy. Boundaries of CA3 were defined according to The Rat Brain in Stereotaxic Coordinates (Paxinos & Watson, 1998). Two pictures of the CA3 were taken through a 20x lens (200x magnification), centered at ¼ and ¾ of the way along the band of CA3 to mitigate the possibility any bias towards picking portions of CA3 with higher or lower density of c-Fos+ cells. All pictures were taken at the same brightness level and camera settings using StereoInvestigator software (Micro Bright Field Inc., Williston, VT, USA).

2.7 Optical densitometry

Images of CA3 were analyzed using ImageJ software to determine the number of activated (c-Fos+) cells per unit area (mm^2) of examined CA3 through ratio of pixel luminescence of individual stained cells to that of the background. The CA3 cell layer was traced in the image to restrict the total area considered to that of the CA3. Images were converted to 16-bit grayscale, ten units brightness were subtracted, and the sharpen tool of the software was used. These steps were included in the procedure in order to enhance to signal to noise ratio of c-Fos+ cells/non-activated cells. The experimenter then sets a threshold for pixel luminescence at which all activated cells appear, but no inactivated cells are counted. The program then applies the threshold, producing a new image only containing the cells with pixel luminescence below the threshold. Light intensities from the microscope were held constant for all sections, leaving only the uncontrollable intrinsic variability of basal pixel luminescence in staining as the only influence on light effect. Due to this variability, all the thresholds were set by the experimenter, who is blind to the experimental condition of the animal. The program is set so that particles less than 5 pixels are not included in the analysis, to prevent inorganic debris from artificially inflating the cell count. The ImageJ software analyzes the particles remaining in the image after thresholding, and gives an output of number of cells and the total area.

Sections were qualified as either dorsal or ventral based on the presence of the medial portion of the DG. Moving ventrally down the dorsoventral axis, sections were considered dorsal if the medial DG persisted, and ventral if it was absent. This transition occurred at approximately -4.5 mm Bregma (Paxinos & Watson, 1998).

2.8 Statistical Analyses

Graph pad prism 6 was used for all statistical analyses. A one-way anova was run on the data with no main effect of neonatal treatment found. A Tukey's post hoc test was conducted to examine for differences within specific treatment groups.

Chapter 3

RESULTS

3.1 Weights and blood alcohol concentrations

Animals were weighed on the first (PD4) and last (PD9) day of AE, and on the first day of training (PD31). Table 1 shows the average weights for each dosing condition at each date the animals were weighed. These figures are the averages of all 78 animals that were AE, of which a subset of 28 were used in this study. All dosing condition groups gained a significant amount of weight throughout the dosing period, as well as up to the date of testing (Table 1). The following statistical analysis was performed and reported by Murawski and colleagues (2012). A 3 (dosing condition)×2 (days) ANOVA on PD4 and PD9 body weights revealed significant main effects of dosing condition [$F(2,75)=9.8$, $p<0.01$] and days [$F(1,75)=1158.8$, $p<0.01$] with a dosing condition×days interaction [$F(2,75)=64.3$, $p<0.01$]. A Newman–Keuls *post hoc* test showed that although body weights did not differ among groups at PD4 (*p's*>0.66), at PD9 Group 5.25 g/kg/day body weights were significantly lower than either the SI group or the 4.00 g/kg/day group (*p's*<0.01), which did not differ from one another ($p>0.60$). Analysis of PD31 body weights revealed significant differences between dosing conditions ($p<0.02$), with the 5.25 g/kg/day group weighing significantly less than the 4.00 g/kg/day group ($p<0.01$; 86.6 g±3.3 vs. 100.5 g±2.4). Neither AE group differed significantly from the SI group (*p's*>0.06; 95.1 g±3.9).

The significant reductions in body weight seen in the AE groups is not surprising as it has been reported in several other studies; these studies have found

these lower-weight AE animals to exhibit deficits in tasks that are hippocampal dependent, but not in control tasks (Hunt, Jacobson, & Toros, 2009; Murawski & Stanton, 2010; Thomas & Tran, 2012). This suggests that the reduced body weight in AE rats does not cause global cognitive impairments, and that the deficits seen in hippocampal-dependent tasks following neonatal alcohol exposure are mediated by mechanisms independent of these body weight reductions. Further, there is no evidence that body weight reductions would affect activation of hippocampal neurons or c-Fos expression, and is unlikely to confound interpretation of the results of the present study.

Average BACs of each condition were obtained using blood samples taken after the first day of AE (PD4). The average BACs differed significantly between the 4.00 g/kg/day group (342.4±10.9 mg/dl) and the 5.25 g/kg/day group (435.8±13.0 mg/dl) [$F(1,49)=30.5$, $p<0.01$] (Murawski et al., 2012).

Table 1 Animal weights (g, (±SE)) and BACs (mg/dl, (±SE)) of SI, 4.00g/kg/day, 5.25 g/kg/day dosing conditions on PD4, PD9, and PD31. *indicates significant difference (*P=0.05*) from SI group.

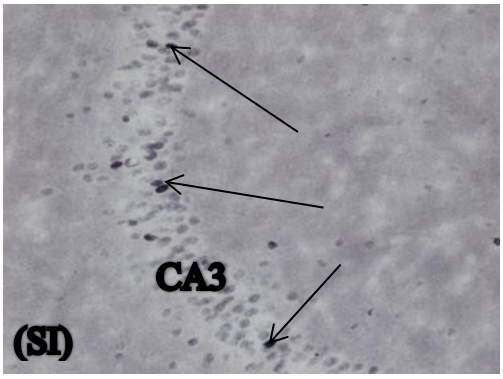
Dosing condition	Body Weight (g)			BAC (mg/dl)
	PD4	PD9	PD31	
SI	10.7 (±.4)	18.7 (±.6)	95.1 (±3.9)	-
4.00 g/kg/day	11.3 (±.2)	19.0 (±.5)	100.5 (±2.4)	342.4 (±10.9)
5.25 g/kg/day	11.1 (±.3)	14.3 (±.5)*	86.6 (±3.3)*	435.8 (±13.0)

3.2 Total c-Fos+ cell density in CA3

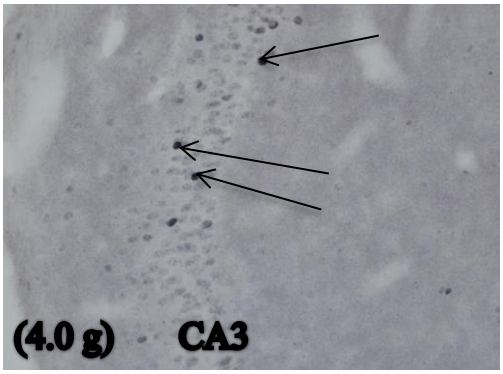
Immunohistochemical staining using an antibody against c-Fos was performed on every 16th section throughout entirety of hippocampus, providing ~8 sections per

animal for analysis. C-Fos+ cells were identified through thresholding to a level of darkness that the researcher determined constituted activation using ImageJ software. Quantification of c-Fos+ cells was used as an indirect measure of neuronal activity following exposure to a novel context at PD31. A one-way ANOVA examining c-Fos+ cell density (c-Fos+ cells/mm^2) across the dosing conditions found no significant differences [$F(2,24)=0.7821$, $P>0.49$] (Figure 2) between dosing conditions.

A.



B.



C.

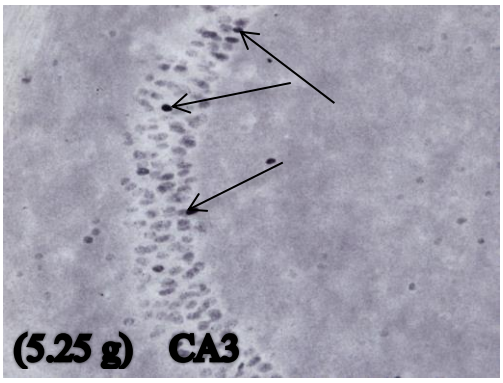


Figure 1. Representative images of CA3 immunohistochemically stained for c-Fos from animals in (A) SI, (B) 4.0 g/kg/day, and (C) 5.25 g/kg/day dosing conditions. C-Fos+ cells are darkly stained and indicated on each picture by the arrows. The area used to calculate the density of c-Fos+ cells is indicated (CA3), the densely packed band labelled in the images. All three images have similar numbers of a c-Fos+ cells and similar CA3 areas, as no significant differences were found in c-Fos+ cell density between the three conditions. Images were taken at a magnification of 200x.

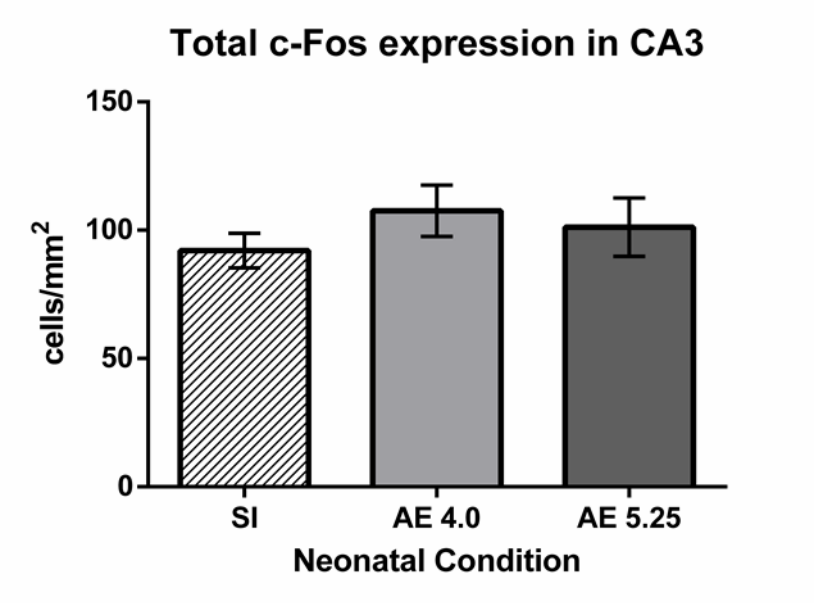


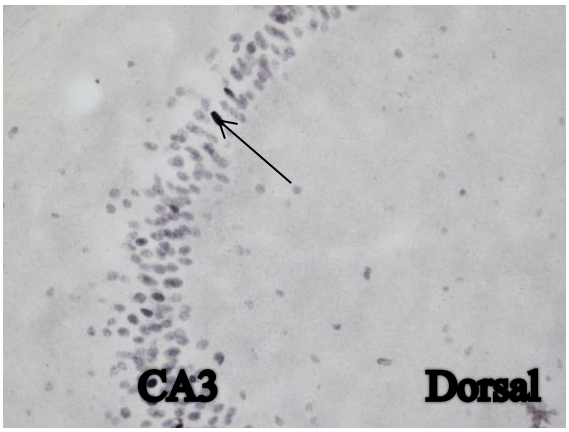
Figure 2. Mean densities of CA3 c-Fos+ cells (±SE) following 5 minutes exploration of a novel context in dosing conditions SI, 4.0 g/kg/day, and 5.25 g/kg/day. No significant differences between dosage conditions were found (p>.05). Data from one animal was omitted because it was an outlier (>2 SD) (n=27; SI=10, 4.0 g/kg/day=10, 5.25 g/kg/day=7).Values for total number of c-Fos+ cells and area are listed in Table 2 (Appendix A).

3.3 C-Fos+ cell density in dorsal CA3

The relationship of c-Fos+ cell density and dosing condition was also examined when the CA3 dorsal and ventral regions were considered separately, to assess if one area may be more susceptible to the effects of neonatal AE than the other. There is a noticeably different pattern in cellular arrangement and size between dorsal and ventral CA3, with ventral CA3 having cells with smaller radii and in greater number and density than in the dorsal CA3 (Figure 3). A one-way ANOVA examining c-Fos+ cell density (c-Fos+ cells/mm^2) across the dosing conditions found a trending effect of dosing condition on number of c-Fos+ cells [F(2,25)=2.635, p>0.0915],

with the highest AE treatment group (5.25 g/kg/day) having the highest c-Fos+ cell density (Figure 4). A Tukey post-hoc test confirmed trending difference between the SI and the 5.25 g/kg/day dosing conditions (p=.083). Though not significant, this increase in cell number suggests there may be some effect of AE on c-Fos expression in dorsal CA3 following novel context exposure.

A.



B.

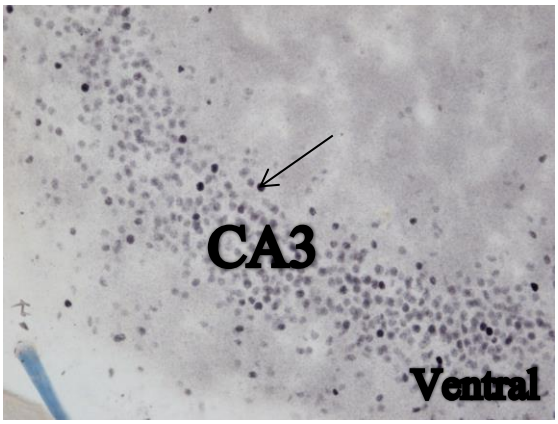
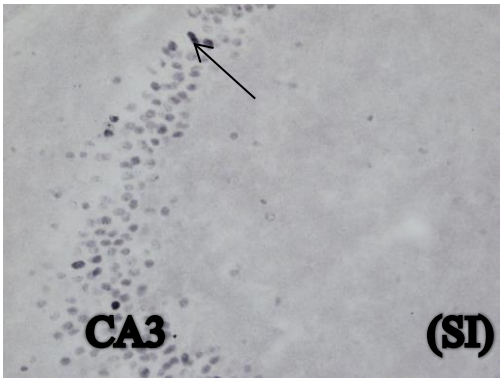
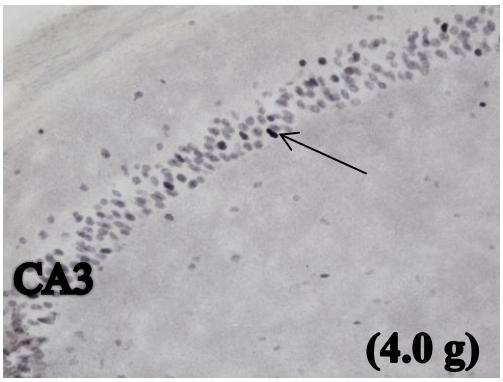


Figure 3. Representative images of (A) dorsal and (B) ventral sections of CA3 from the same animal. Nuclei in the dorsal CA3 have a larger radius than in the ventral CA3. The boundaries of the PCL are less distinct in the ventral CA3, and the area is larger. These images are also representative in terms of c-Fos+ cell density, the ventral CA3 had more c-Fos+ cells across all dosage conditions (Appendices A-C)

A.



B.



C.

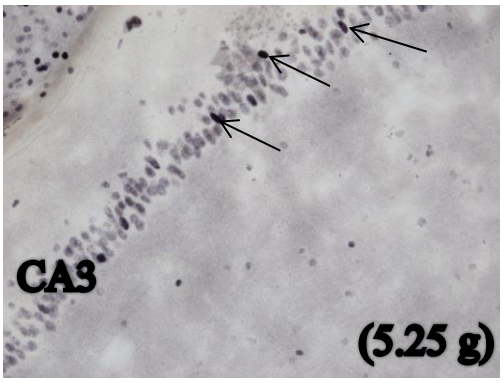


Figure 4. Representative pictures of dorsal CA3 stained for c-Fos in all three dosing conditions: (A) SI, (B) 4.0 g/kg/day, and (C) 5.25 g/kg/day. They all appear somewhat similar, though there is a slightly higher density of c-Fos+ cells in the 5.25 g/kg/day image, as there is a trending increase in c-Fos+ density relative to the SI group. C-Fos+ cells are darkly stained and indicated in each picture by the arrows. The area used to calculate the density of c-Fos+ cells is indicated (CA3).

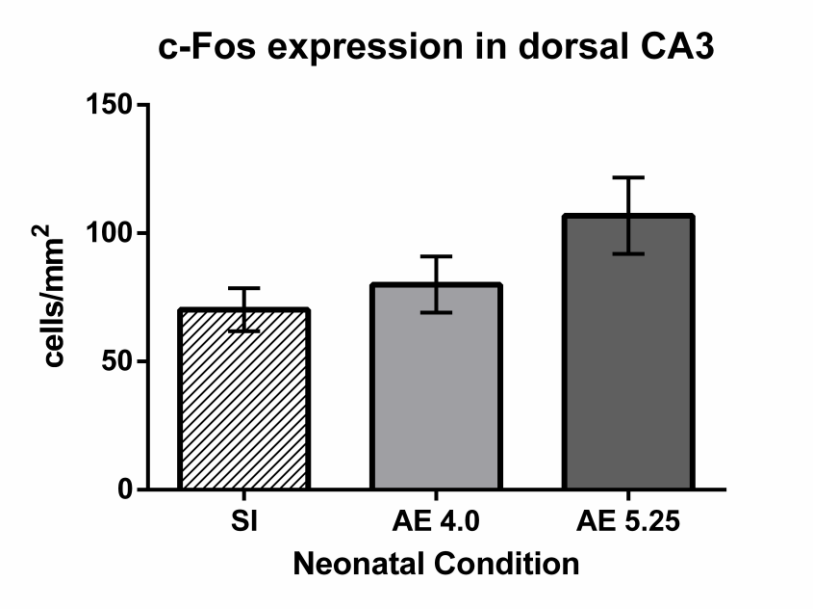
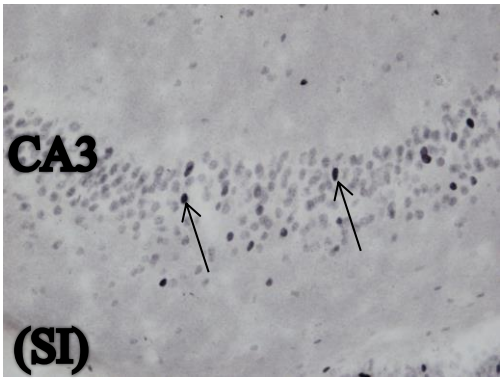


Figure 5. Mean densities of dorsal CA3 c-Fos+ cells (±SE) following 5 minutes exploration of a novel context in dosing conditions SI, 4.0 g/kg/day, and 5.25 g/kg/day (n=28; SI=10, 4.0 g/kg/day=10, 5.25 g/kg/day=8). No significant differences between dosage conditions were found (p>.05). The 5.25 g/kg/day AE group had non-significant but trending higher mean density than the other two dosing conditions (p=.0915). Further analysis using a Tukey post-hoc test revealed a trending yet not significant difference between the SI and the 5.25 g/kg/day dosing conditions (p=.083). Values for total number of c-Fos+ cells and area are listed in Table 2 (Appendix B).

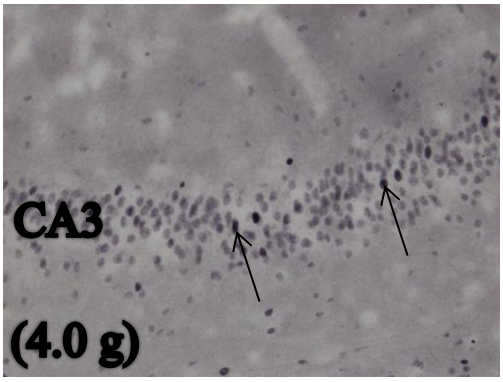
3.4 C-Fos+ cell density in ventral CA3

A one-way ANOVA examining c-Fos+ cell density in the sections containing ventral CA3 (c-Fos+ cells/mm2) found no significant differences [F(2,23)=0.891, p=0.4239] between dosing conditions (Figure 7). Representative sections showing the similarity in c-Fos+ cell density across dosing conditions are displayed in Figure 6.

A.



B.



C.

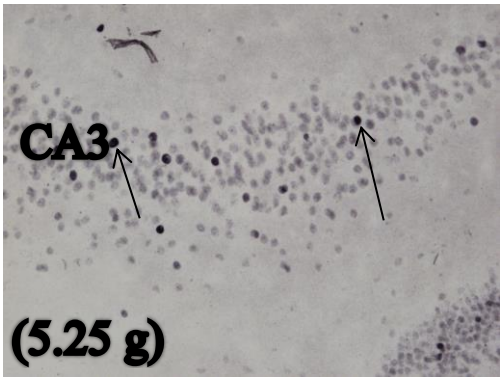


Figure 6. Representative pictures of ventral CA3 stained for c-Fos in all three dosing conditions: (A) SI, (B) 4.0 g/kg/day, and (C) 5.25 g/kg/day. They all appear very similar, as no significant difference in c-Fos+ cell density was found. C-Fos+ cells are darkly stained and indicated on each picture by the arrows. The area used to calculate the density of c-Fos+ cells is indicated (CA3).

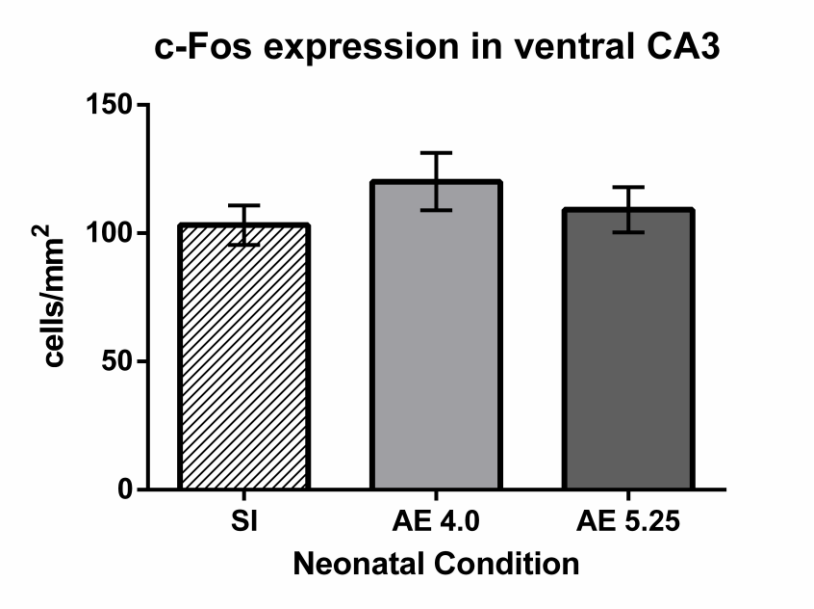


Figure 7. Mean densities of ventral CA3 c-Fos+ cells (±SE) following 5 minutes exploration of a novel context in dosing conditions SI, 4.0 g/kg/day, and 5.25 g/kg/day. Data from two animals in the 5.25g/kg/day group were excluded, as they were outliers (SD >2) (n=26; SI=10, 4.0 g/kg/day=10, 5.25 g/kg/day=6). No significant differences between dosing conditions were found (p>.05). Values for total number of c-Fos+ cells and area are listed in Table 3 (Appendix C).

Chapter 4

DISCUSSION

4.1 Synopsis of results

The present study sought to investigate the effect of developmental AE on c-Fos+ cell density in the CA3 subregion of the hippocampus following exposure to a novel context. There were no significant differences between any of the dosing conditions in c-Fos+ cell density in ventral CA3, dorsal CA3, or the total CA3. These results suggest that other than a possible effect in the dorsal region due to the trending increase observed, neonatal AE does not affect the number of CA3 neurons that express the IEG c-Fos following exploration of a novel context.

Results of the current study were derived from tissue previously examined for c-Fos activation in CA1 by Murawski and colleagues (2012), and it is therefore important to consider the effect of neonatal AE on c-Fos expression in CA3 in the current study, as well as the results of the previous study when interpreting the current results. Previous work has shown that CA3 and CA1 c-Fos activation levels are higher following initial exposure to a novel context, as compared to subsequent exposures to that same context. In a previous study, a rats were given a second exposure in which a brief foot shock was administered followed by immediate removal of the rat from the context, and a third exposure to assess acquisition of association of the foot-shock with the context, lasting five minutes, neither of which induced c-Fos expression close to the degree that the initial exposure to the context did (Murawski et al., 2012). These results suggest that both CA1 and CA3 are active during novel context exposure, and may have a role in encoding the context when novel, possibly establishing a spatial

representation with which to associate the stimulus of the foot-shock in the subsequent exposure. Murawski and colleagues (2012) also showed that AE (4.00 g/kg/day and 5.25 g/kg/day) rats had a dose-dependent reduction in total CA1 pyramidal cell count when compared to the SI control group. Further, rats in the 5.25 g/kg/day AE group had significantly fewer c-Fos+ cells following exploration of a novel context than the SI group. Finally, littermates of the rats whose tissue was used in the current study were evaluated for contextual freezing fear response in the third exposure to the context (24 hours following the exposure in which the animals received a foot-shock), and showed that animals in the both of the AE groups had a significantly reduced freezing response compared to the SI group (Murawski et al., 2012). This suggests the context may not have been properly encoded during the first exposure to the context, a process that involves c-Fos induction.

4.2 Evaluating results in the context of hippocampal circuitry

Previous work showing no impairment in learned freezing as a fear response to control-level cued fear conditioning suggests that no deficit exists in the underlying fear circuit, or the ability to demonstrate fear-learning, as freezing remains intact in neonatally AE rats (Wagner & Hunt 2006; Hunt et al., 2009; Murawski & Stanton, 2010). Moreover, deficits in fear conditioning seen following neonatal AE mimic those seen in hippocampal lesioning studies (Quinn, Oommen, Morrison, & Fanselow, 2002). Thus it seems the hippocampal circuitry is the most pertinent neural structure for study in trying to elucidate the mechanisms driving the deleterious effects neonatal AE that mediate the deficits in novel context encoding.

In contrast to the results of AE on expression of c-Fos in CA1, the same doses of AE had no significant effect on c-Fos+ cell density of the entire CA3 measured as a

whole, or the ventral portion alone, but the highest AE dosing condition (5.25 g/kg/day) showed a trending increase in the dorsal region compared with SI controls. The effect of AE on c-Fos+ cell density in CA3 as a whole may have been overshadowed by the lack of effect in the ventral region, as the ventral region had more c-Fos+ cells and higher c-fos+ cell density than the dorsal region, and thus more of an influence on the figures for the whole CA3.

When considering the results of the present study, it is important to highlight the differential roles of the ventral and dorsal hippocampus. The dorsal two-thirds of the hippocampus receives the majority of input to the hippocampus from cortical areas that process sensory information (Moser & Moser, 1998). Alternatively, the ventral hippocampus has projections to the prefrontal cortex, while the dorsal hippocampus does not (Moser & Moser, 1998). Additionally, the ventral hippocampus is well connected with the amygdala and other subcortical regions whose function ties in closely with the HPA axis, and has been implicated in anxiety-related memory processes (Bannerman et al., 2004). The dorsal hippocampus is much more heavily implicated in spatial learning than the ventral hippocampus, which in light of the trending increase in c-Fos+ cell density of AE (5.25 g/kg/day) rats observed following novel context exploration suggests that there may be increased dorsal CA3 activity in these animals compared to SI controls (Moser & Moser 1998). If this trending difference is large enough to have functional significance in hippocampal information processing, it may be that dorsal CA3 is more active in the higher AE group as a compensatory mechanism to offset reduced activity or cell count of the DG, CA1, or both. Important to note is that the effect of AE on c-Fos+ cell density for the dorsal CA3 may have been more robust if the distinction between dorsal and ventral had

been drawn more ventrally, as studies have found that the primary function changes about two-thirds of the way down the dorsoventral axis of the hippocampus, and the current study drew the distinction about halfway down the axis (Moser & Moser, 1998).

It is useful to take into account hippocampal circuitry as well as hypothesized computational models regarding encoding of spatial contexts when evaluating the results of the present study. When considering the roles of CA3 and CA1 in hippocampal circuitry, there are two pathways of information to consider. The first is the trisynaptic circuit, also known as the indirect pathway, in which information from the entorhinal cortex (EC) is propagated to the DG via the perforant pathway, then to CA3 via the mossy fiber pathway, from which it is then transferred to CA1 through the Schaffer collaterals (Spencer & Kandel, 1962). The second is the direct pathway, a monosynaptic connection in which CA1 receives direct input from the third layer of the entorhinal cortex (Witter, Groenewegen, Lopes, & Lohman, 1989). This discussion will focus on the trisynaptic pathway, as previous literature has suggested encoding of novel context involves or at least activates all three elements of the trisynaptic pathway, while the direct pathway only includes CA1 and the EC (Brown, Hasselmo, & Stern et al., 2014; Vazdarjanova & Guzowski 2004).

A couple of relevant studies will now be considered, followed by discussion of results of the current study in the context of these results, and the trisynaptic pathway. In a particularly interesting study, rats given a single binge-dose (6.0 g/kg) of alcohol intragastrically on PD6 and sacrificed 24 hours later had significantly higher numbers of c-Fos+ cells, apoptotic bodies, and cells expressing NMDA R1 (a marker for cells expressing NMDARs) in CA1, CA3, and the DG (Clements et al., 2012). The

convergence of all of these markers together suggest that during the withdrawal period excitotoxic cell death occurs following this binge-dose, as a result of NMDA-mediated apoptosis. The c-Fos+ increase is due to rebound of glutamatergic transmission during ethanol withdrawal. Also important to note is that this is a .75g/kg higher dose, but for a shorter period of time than the exposure pattern used in the current study. These findings are significant as they suggest that following AE the pups lose significantly more neurons in all three subfields of the trisynaptic pathway than controls, as a result of NMDA-mediated apoptosis (Clements et al., 2012).. The effects of this cell loss on network activation in later life however, are not clear.

The effects this immediate cell loss in each subfield has on encoding of novel context in adolescent rats could be influenced by several factors. First, cell loss could affect the degree of involvement each subfield has in encoding the novel context. It is difficult to speculate on this aspect of the problem, as each subfield's contribution in novel contextual encoding has yet to be fully elucidated. The second factor to consider is the degree to which neurons in each subfield are restored, as the brain is still generating new neurons in all three subfields in development after third trimester equivalent AE. The DG differs from CA1 and CA3 in that it has the capacity to generate new neurons throughout the lifespan, perhaps mitigating the effect of this early insult. The final factor to consider is how much rewiring of information processing neuronal circuits can aid in the repair of the region. One could speculate that CA3 may be better suited to restore function based on its anatomy. The recurrent autoassociative connections may allow CA3 to process information more effectively despite reductions in neuronal count than CA1. The DG may be better suited for effective neuroplastic changes, altering circuitry as a means to mitigate the damage

done from apoptosis, as the flux of new cells can make new connections to CA3 along the mossy fiber pathway. Though only speculative, using the reasoning above, it would seem that CA1 may be at the highest risk for long-term damage to informational processing capabilities, as it does not have neurogenic properties of DG, nor the recurrent connections and direct connections with a neurogenic region of CA3.

The DG has been proposed as a neural mechanism for pattern separation due to its large number of projections in comparison to CA3 and the EC (Amaral, Ishizuka, & Claiborne, 1992). This relatively large amount of projection cells compared to the DG's input or output gives the DG the capacity to encode new stimuli in novel patterns of network activation (Langston et al., 2010). Anatomically, CA3 is well-suited for the computational role of pattern completion, due to its recurrent collateral network (Rudy & Reilly, 2001). In the pattern completion model of memory formation, rapid encoding of conjunctive stimuli are encoded in a context, and the strengthening of synapses between representations of the associated stimuli allow for retrieval of all stimuli within the context upon later exposure to a subset of the original stimuli. Lending further support to the hypothesis of the DG providing sparse input and acting as a pattern separator, a recent study discovered that when mice are exposed to a novel context, new ensembles of DG granule cells are activated, a novel population distinct from those activated by exposure to previous contexts (Deng, Mayford, & Gage, 2013). This study potentially provides a mechanism by which DG could encode novel environments. If DG activity is disrupted as a result of neonatal AE, processing and encoding of novel contexts could be hindered with or without direct negative impact on CA3 activity. However, it is also important to consider the possibility that the large number of projection cells relative to CA3 or the EC may

actually allow the DG to withstand larder amounts of neuronal loss without losing the ability to create sparse representations.

Based on the results of the present study along with current knowledge of the circuitry involved in novel context encoding, the next pertinent question is how CA1 activation could be reduced when activation of CA3, the intermediate subfield of information flow in the trisynaptic pathway, was not affected by AE. One potential explanation is that AE doesn't significantly affect neuronal activation in CA3 upon exposure to a novel context, but that pattern completion during subsequent reactivation during attempted retrieval is compromised due to reductions in NMDA receptor mediated plasticity in CA3. Considering the computational model of CA3 as a pattern completion mechanism, this would prohibit the associations between stimuli in an event, in this case exploration of a novel context, from being formed, resulting in a reduced ability for new associations to be made with the context, and reduced ability for retrieval of the context in later exposures. This could explain the deficits seen in AE rats in fear conditioning studies that are meant to build an association with a shock and a context, and the difficulties observed in encoding contextual novelty (Murawski et al., 2012). Support for this as a viable theory comes from a study in which mice with deletion of the NR1 gene specific to CA3, necessary for NMDA receptor mediated plasticity, were unable to navigate a water maze when a subset of previously learned cues removed (Nakazawa et al., 2002). A similar mechanism could negatively affect the ability of the DG to play it's purported role of pattern separator, with reductions in NMDA receptor-mediated plasticity compromising the stability of activation patterns coding for a given set of stimuli following exposure to a novel environment. Samudio-Ruiz and colleagues showed that adult mice prenatally exposed

to alcohol have reduced levels of ERK1/2, a signaling molecule necessary for activation of signaling cascades that are essential NMDA receptor dependent induction of LTP specifically in the DG (Samudio-Ruiz, Allan, Valenzuela, Perrone-Bizzozero, & Caldwell, 2009). Another possibility is that DG and CA1 may have dysfunction that is causal to the deficits seen in novel context encoding, and the recurrent self-connections of CA3 allow it to remain functional despite teratogenic insult from AE.

Additionally, it is possible that deficits in CA3 morphology or CA3-CA1 communication are responsible for, or contribute to deficits in novel contextual encoding in AE rats. Though it is important to note that AE was at a different developmental time point, Tanaka and colleagues (1991) found decreased number of synaptic junctions in CA3 in rats exposed *in utero* on gestational day 21. This could cause suboptimal communication within CA3, leading to deficits in contextual encoding capabilities. Neuronal populations of CA3 and CA1 exhibit a high degree of overlap when exposed to the same environment twice. In contrast, exposure to a familiar environment and a distinct, novel environment results in significantly less overlap, in both CA1 and CA3, the effect being more pronounced in CA3 (Vazdarjanova & Guzowski 2004). This suggests that CA3 and CA1 may have distinct but complementary roles in processing of novel contextual information. Based on these findings, it is plausible that disruption of the circuitry of one or both subfields through teratogenic insult could result in encoding deficits. Previously discussed potential deficits in NMDA receptor mediated plasticity could underlie deficits in CA3-CA1 information processing (Nakazawa et al., 2002). Due to the complexity and multiple functionality of the trisynaptic pathway, there are many potential mechanisms

one can theorize that may drive the deficits in encoding of novel context in AE animals. It is possible that it is some combination of several of these possibilities in synergy that give rise to reduced contextual encoding ability observed in AE animals. Further work is needed to parse out the specific mechanisms that are most critical to these deficits.

4.3 Alternative explanations of results

There are also alternative explanations for the results of the present study, and those previously obtained regarding CA1 using the same tissue (Murawski et al., 2012). Taking into the account the absence of a significant effects on c-Fos+ cell density in CA3 in the current study, a possible explanation for the lack of acquisition of fear response in littermates of the rats used in this present study, observed upon reintroduction to the context the rats received a foot shock in a prior exposure following neonatal AE, would be that the decrease in total CA1 pyramidal count is sufficient to disrupt encoding of the context during the initial exposure, when the context was novel. In analysis of this same tissue, Murawski and colleagues (2012) found that although the total c-Fos+ count in CA1 was significantly decreased in the 5.25 g/kg/day AE group relative to the SI group, 6% of total pyramidal cells were c-Fos+, across all dosing conditions, suggesting that the number of total activated c-Fos cells in CA1 could be the factor that is negatively affected by neonatal AE. Since the total CA1 c-Fos+ cell count decreased in proportion with the total CA1 pyramidal cell count in the tissue used during the current study, it is difficult to make any concrete conclusions with the data on CA3 c-Fos+ density without having data on the total number of CA3 pyramidal cells in addition to the total number of CA3 c-Fos+ cells. If the area of CA3 tissue analyzed in the present study is correlated with total CA3

pyramidal cell number, the results obtained would be similar to those obtained by Murawski and colleagues (2012) for CA1. That is, if total c-Fos+ cell count and total pyramidal cell count were estimated using unbiased stereology as CA1 was in the study conducted by Murawski and colleagues, it could show a reduction on both measures as their study did, while still showing no change in c-Fos+ cell density per mm^2. Conversely, if CA1 was analyzed using the quantification methodology used in the present study, it is possible that there would be no significant differences between dosing conditions in terms of c-fos+ cell density per mm^2. Therefore, when considering that all three subfields that comprise the trisynaptic circuit are critical in hippocampal computation and the ambiguity in interpretation of the results of the present study and results from the Murawski study (2012) due to differences in cell quantification methodology, an analysis of all three of these areas in both total pyramidal cell (granule cell in the case of DG) count and c-Fos+ count using the same method of unbiased stereology needs to take place to yield results that are less equivocal.

There may be other IEG, such as ZIF268, Arc, or EGgr1 that would be more suitable for measuring neuronal activity. Other methods for more direct measurement of hippocampal activity could also prove useful. For example, Functional Magnetic Resonance Imaging (fMRI) or *in vivo* cell recording could provide new insights into the differences in neural firing patterns between AE animals and SI controls after exposure to a novel context.

4.4 Relevance to FASD in humans

Understanding the computational processes and neuroconnectivity involved in the contextual encoding of novel environment, and the deficits seen in rodent models

of FASD will give insight into analogous deficits in humans with FASD, and possibly inspire ideas for remediation of those deficits. Though the mechanisms and dynamics of contextual encoding of novelty in humans will obviously not be the same as in rodents, information obtained from rodent models will still be of value, as it is reasonable to assume that major motifs of the underlying circuitry and modes of computation have been phylogenetically conserved from the common ancestor of rats and humans, just as general hippocampal macrostructure and mnemonic function have been (Clark & Squire, 2013).

Though the results of this experiment did not do much in the way of shedding light on potential cognitive, behavioral, or pharmacological interventions for humans, further work building off of these studies may. Additionally, there does exist literature with findings that provide support for the efficacy of several behavioral and pharmacological interventions. One such idea for intervention is the administration of either a glutamate antagonist or an NMDAR-blocking agent, in order to mitigate the neuronal loss during alcohol withdrawal resulting from excitotoxic cell death resulting from NMDAR-mediated apoptosis. This would be of most use in cases where a pregnant mother has been known to be drinking while pregnant and has been hospitalized, or in the case a baby born to a mother who admits she has very recently consumed alcohol.

Another pharmacological intervention garnering attention is choline supplementation. Choline supplementation during pregnancy has been shown to attenuate the negative effects that characterize the FASD (Zeisel, 2011). The availability of choline, a methyl group donor, during pregnancy and early development is critical for the methylation of histones and DNA, playing a role in modulation of

gene expression critical to optimal neural function. Alcohol disrupts the metabolism of choline, reducing its availability, causing aberrant epigenetic marking. Increasing cholinergic function has been shown to reverse AE induced deficits in the some fear-conditioning tasks, through injection of the acetylcholinesterase inhibitor physostigmine prior to each phase (Dokovna, Jablonski, & Stanton, 2013). Physostigmine may work through a different mechanism than epigenetics however, such as increased levels of synaptic acetylcholine, as effective changes in gene expression would likely not be seen in such a short time frame.

A third intervention for mitigation of neonatal AE induced deficits is voluntary exercise, especially when followed by environmental complexity. Voluntary exercise, in the form of *ad libitum* wheel access in the rodent model, has been shown to produce an increase in neurogenesis in the rodent models of FASD. Voluntary exercise alone has been shown to improve spatial memory and contextual learning in rats exposed to alcohol both prenatally and postnatally during the second trimester (Sim et al., 2008) and third-trimester equivalent to humans (Christie et al., 2005; Thomas, Sather, & Whinery, 2008). Studies have shown that in rodent models of FASD, voluntary exercise in the form of wheel running significantly increased the proliferation of neurogenic cells in the DG, but had no effect on the survival of these newly generated neurons. (Helfer, Goodlett, Greenough, & Klintsova, 2009; van Praag, Christie, Sejnowski, & Gage, 1999). A later study by Hamilton and colleagues (2012) showed that if housing in a complex environment directly follows this voluntary exercise, the newly generated neurons have a much higher survival rate, and are effectively integrated into the hippocampal circuitry. Voluntary exercise is a very viable intervention in humans, because there are numerous ways it can be accomplished, and

it is essentially free. Though not as easy to qualify environmental complexity in humans as it is in a rodent model, the common denominators that can be extracted from rodent models and applied to human interventions are novelty, utilization of multiple sensory modalities, and facilitation of social interaction. Implementation of voluntary exercise and environmental complexity as a behavioral intervention along with further research into the efficacy of the choline supplementation, NMDAR-blockers, glutamate antagonists, and novel treatments for the effects brought on by the FASD, there is hope for treatments that may greatly improve the quality of life of those living with one of the disorders, and their ability to contribute to society.

4.5 Conclusion and future directions

The current study set out to obtain information on the effect of neonatal AE on c-Fos+ cell density in CA3 of the adolescent rat, following exploration of a novel context. It was expected that AE would cause a decrease in neuronal excitability, and therefore AE rats would have lower densities of c-Fos+ cells in CA3. The results contradicted this hypothesis, as a trending increase c-Fos+ cell density was seen in dorsal CA3 following 5.25 g/kg/day AE when compared SI controls, and no significant effects were found in ventral CA3. There are many potential interpretations of these results, due to the multiple factors that could alter function of the hippocampal circuitry. Much future work remains to be done in elucidating the mechanisms by which neonatal AE disrupts the ability of adolescent rats to effectively encode novel contextual information. There are several promising directions for study such as analysis of all three elements of the trisynaptic circuit in the same tissue following novel context exploration in rats with and without neonatal AE, that if pursued are

likely to provide a clearer picture of the mechanisms that underlie deficits in contextual encoding of novelty observed in AE rats.

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Appendix A

MEAN C-FOS+ CELL COUNT, PYRAMIDAL CELL LAYER AREA, AND C-FOS+ CELL DENSITY (±SEM) IN CA3 BY DOSING CONDITION AT PD31 FOLLLOWIGN NOVEL CONTEXT EXPLORATION

Dosing Condition	**c-Fos+ cell count**	**Area (mm^2)**	**c-Fos+ cells/mm^2**
SI	74.80	0.80539	92.04864 (±6.76)
4.0 g/kg/day	83.50	0.78158	107.57328 (±10.05)
5.25 g/kg/day	80.29	0.82995	101.17817 (±11.38)

Appendix B

MEAN C-FOS+ CELL COUNT, PYRAMIDAL CELL LAYER AREA, AND C-FOS+ CELL DENSITY (±SEM) IN DORSAL CA3 BY DOSING CONDITION AT PD31 FOLLOWING NOVEL CONTEXT EXPLORATION

Dosing Condition	c-Fos+ cell count	Area (mm^2)	c-Fos+ cells/mm^2
SI	19.7	0.26815	70.21661 (±13.22)
4.0 g/kg/day	18.3	0.24588	79.99903 (±10.96)
5.25 g/kg/day	24.125	0.24243	109.29054 (±15.82)

Appendix C

MEAN C-FOS+ CELL COUNT, PYRAMIDAL CELL LAYER AREA, AND C-FOS+ CELL DENSITY (±SEM) IN VENTRAL CA3 BY DOSING CONDITION AT PD31 FOLLOWING NOVEL CONTEXT EXPLORATION

Dosing Condition	c-Fos+ cell count	Area (mm^2)	c-Fos+ cells/mm^2
SI	55.1	0.53724	103.15465 (±7.66)
4.0 g/kg/day	65.2	0.53570	120.08114 (±11.18)
5.25 g/kg/day	57.67	0.55570	109.13209 (±8.81)