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EXAMINING THE INTERACTION OF NEONATAL ALCOHOL AND HYPOXIA IN VITRO

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EXAMINING THE INTERACTION OF NEONATAL ALCOHOL AND HYPOXIA *IN VITRO*

THESIS

A thesis submitted in partial fulfillment of the
requirements for the degree of Master of Science in the
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By

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Lexington, Kentucky

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ABSTRACT OF THESIS

EXAMINING THE INTERACTION OF NEONATAL ALCOHOL AND HYPOXIA *IN VITRO*

Exposure to ethanol (ETOH) during fetal development results in a range of cognitive/behavioral deficits. There are differences in sensitivity to the effects of ETOH that could be explained by other factors, such as hypoxia. Similar mechanisms of damage underlie both ETOH, more specifically ETOH withdrawal, and hypoxia. Based on this overlap, it was hypothesized that sub threshold levels of these insults may interact to produce increased damage in sensitive brain regions. This study used a rodent organotypic hippocampal slice culture model to investigate the interaction of hypoxia and ETOH withdrawal and to determine possible developmental differences in the sensitivity to these insults. The combination of ETOH and hypoxia produced greater damage in the CA1 and CA3 hippocampal regions, as measured by propidium iodide uptake. Differences in outcome were noted between on postnatal (PND) 2 and PND 8 tissue. ETOH alone caused damage as measured by the neuronal marker NeuN, suggesting the ETOH/hypoxia interaction involves different cell types and that caution should be taken when determining appropriate levels of exposure. This data could explain why some offspring appear more sensitive to ETOH and/or hypoxic challenges during early life.

KEYWORDS: Fetal ethanol, Ethanol withdrawal, Hypoxia, Oxygen Glucose Deprivation, Hippocampal slice culture

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Dedicated to my parents

TABLE OF CONTENTS

LIST OF FIGURES

Chapter 1: Introduction

In this thesis, I examined the hypothesis that fetal alcohol exposure and perinatal hypoxia interact to produce synergistic brain damage. I first provide background on fetal alcohol exposure and hypoxia and how these insults produce a range of deficits in the developing central nervous system. This will be followed by a review of the mechanisms through which these two challenges may interact and finally data investigating this possible interaction will be presented. The interaction of fetal alcohol exposure and hypoxia is of particular interest because it may help to explain some of the variability in outcome that is observed in clinical populations.

Fetal Alcohol Spectrum Disorders

Background

For centuries, it has been known that alcohol consumption during pregnancy can lead to developmental anomalies in children although a specific syndrome associated with prenatal alcohol exposure was not described until 1968 in France (Lemoine, Harousseau, Borteyru, & Menuet, 2003)(1968). This was independently documented in the United States in 1973 (Jones & Smith, 1973) when doctors recognized a pattern of specific abnormalities in the children of alcoholic mothers which became known as Fetal Alcohol Syndrome (FAS). While FAS is one of the most severe diagnoses resulting from alcohol exposure during fetal development, there are a range of disorders that fall under the term Fetal Alcohol Spectrum Disorders (FASDs). Recent estimates taken in the

United States and Western Europe suggest that FASDs may affect up to 2-5% of children (May et al., 2009) and with this high prevalence rate, it is not surprising that the annual cost of FASDs in the US may be as high as $3.6 billion (Olson, et. al., 2009). Maternal drinking during pregnancy is the leading preventable cause of mental retardation in the western world and despite preventative efforts and knowledge of its effects, alcohol consumption during pregnancy remains unacceptably high.

Fetal Alcohol Exposure

Alcohol consumption during pregnancy is dangerous because alcohol readily crosses the placental barrier and within minutes of the onset of a drinking episode, the fetal alcohol content is equal to that of the maternal blood (Paintner, Williams, & Burd, 2012). Adding to this, the fluid of the fetal compartment retains a peak alcohol concentration for a longer period of time due to the inability of the fetus to metabolize the alcohol, resulting in a prolonged exposure period (Paintner et al., 2012). While the exact amount, timing, and pattern of alcohol consumption necessary to cause damage to the fetus is unknown, there is evidence that certain patterns may be more harmful than others and that exposure at different time points during development could differentially affect certain regions of the brain and other organ systems.

Binge drinking, a pattern of consumption often observed in alcoholics, can be especially damaging. Binge drinking leads to greater elevations in blood alcohol content and extended exposure periods for a developing fetus. This

pattern is also associated with multiple alcohol withdrawals, which is linked to increased deficits (Livy, Miller, Maier, & West, 2003). Even if a mother only drinks heavily on the weekends, the cumulative exposure time for the fetus could be as much as 2,200 hours with peak concentrations approaching .40 percent (Paintner et al., 2012). The total amount of alcohol consumed may not be as important, in terms of overall detrimental effects, as the pattern drinking.

The body's organs, including the brain, are probably the most susceptible to the teratogenic effects of alcohol during their development. The central nervous system (CNS) continues to develop throughout prenatal and early postnatal life (Rodier, 1994), leaving it especially sensitive to fetal alcohol exposure. The third trimester of pregnancy is a time of rapid CNS proliferation often referred to as the brain growth spurt. Alcohol exposure during this time can be especially detrimental, affecting neuronal migration, synaptogenesis, mylenation, dendritic aborization and other aspects of brain maturation. Regions of the brain that undergo extensive development during this time, e.g., cerebellum and hippocampus, have been shown to be selectively damaged by exposure during the third trimester (Alfonso-Loeches & Guerri, 2011), highlighting the importance of exposure and timing.

Clinical Outcomes

Historically, children with FAS were identified by discriminating facial features that include small palpebral fissures, flat midface, indistinct philtrum, and thin upper lip. Other associated features include ear anomalies, low nasal

bridge, micrognathia, and epicanthal folds (Stokowski, 2004). Beyond the classic facial features, children with FAS have prenatal and postnatal growth deficiencies and some range of CNS dysfunction (Mattson & Riley, 1998; Mattson, Riley, Gramling, Delis, & Jones, 1998; Riley, Infante, & Warren, 2011). Individuals without the full FAS diagnosis, those with a FASD, can display a range of neurobehavioral deficits while lacking the classic physical characteristics of FAS. These neurological impairments include; lowered IQ, problems with executive function, motor coordination, spatial learning, attention, and hyperactivity (Jones, 2011; Mattson & Riley, 1998). The majority of children with a FASD have below average overall intelligence and the most severe cases of fetal alcohol exposure can result in mental retardation (Mattson et al., 2010). Beyond IQ, neurobehavioral assessments that measure abilities such as executive functioning and spatial reasoning are able to identify alcohol exposed children versus non-alcohol exposed children with exposed children demonstrating impaired working memory, planning, and cognitive flexibility compared to matched controls (Mattson et al., 2010). Fetal alcohol exposure often leads to attention and hyperactivity problems and it is possible that up to 94% of children exposed to large doses of alcohol are diagnosed with attention deficit hyperactivity disorders (Peadon & Elliott, 2010). FASD children may also have impaired social functioning and, with reduced social abilities, they can experience problems at home, in school, and various other settings; leaving them at increased risk to act inappropriately and experience rejection (Kully-Martens, Denys, Treit, Tamana, & Rasmussen, 2012). The cognitive, behavioral, and

social consequences associated with FASDs are life-long (Streissguth et al., 2004) and present a significant problem for the individual and society.

Changes in the Brain

The cognitive and behavioral deficits observed in individuals with a FASD can be linked to several brain structure abnormalities. Through the use of MRI, it is possible to observe structural changes in the brains of children exposed to alcohol (ethanol (ETOH)) *in utero* and several areas appear to be particularly affected. In children with FAS, the size of the corpus callosum can be decreased, and in extreme cases, there can be a complete agenesis of this (Riley & McGee, 2005). Size reduction has also been observed in the basal ganglia (Mattson et al., 1994), cerebellum, and cerebrum (Archibald et al., 2001). Understanding of the brain dysmorphology and the behavioral deficits induced by fetal ETOH exposure has been improved through the use of animal models.

Results from both human and animal studies suggest that normal myelination is impaired by ETOH. Following high levels of ETOH exposure, white matter injury is observed (Fryer et al., 2009; Sowell et al., 2008) but not with lower levels (Kenna et al., 2011). Using diffusion tensor imaging (DTI), several studies show decreases in white matter in areas such as the cerebellum, cerebellar peduncles (Spottiswoode et al., 2011) posterior cingulate, temporal lobe, and areas of the frontal, occipital, and parietal lobes (Fryer et al., 2009). Decreases in myelination lead to impaired neuronal signaling and given the

number of regions affected by this, it is not surprising that children with a FASD can have a display a range of CNS dysfunction.

Of particular interest for this proposal are the effects of fetal ETOH exposure on the hippocampus. While gross structural abnormalities are not apparent in MRIs of those with FASD, animal and behavioral data suggest that there is damage at the cellular and molecular level. Behavioral data from humans and animals reveal deficits in learning and memory (Berman & Hannigan, 2000; Lewis et al., 2012); both of which are thought to be hippocampal dependent functions. Cellular studies examining the direct effects of ETOH on cellular survival in the developing hippocampus show that ETOH, or more specifically ETOH withdrawal, can cause damage to cells in various regions of the hippocampus (Barron, Mulholland, Littleton, & Prendergast, 2008; Wilkins et al., 2006). This evidence of hippocampal damage offers further insight into the widespread the effects of fetal ETOH exposure on the CNS.

Animal Models

Animal models of FAS have been developed in attempt to understand the deficits observed in the clinical population following fetal ETOH. These models allow for control over possible confounding variables and aspects of exposure including environment, dose of ETOH, and exposure timing, which is not possible in the human population. The third trimester brain growth spurt observed in humans overlaps with the first postnatal week of rat CNS development (Dobbing & Sands, 1979). To test the effects of ETOH during this time of CNS growth,

some rat models use a neonatal exposure paradigm, exposing rat pups to ETOH during the first postnatal week (Riley, Barron, Melcer, & Gonzalez, 1993; Wellmann, Lewis, & Barron, 2010) Exposure during this period produces deficits in a variety of behavioral paradigms that assess motor coordination (Idrus, McGough, Riley, & Thomas, 2011; Lewis, Wellmann, & Barron, 2007; J. D. Thomas, Idrus, Monk, & Dominguez, 2010), activity (Smith et al., 2012; J. D. Thomas, Biane, O'Bryan, O'Neill, & Dominguez, 2007), and learning and memory (Hunt, Jacobson, & Torok, 2009; J. D. Thomas, Abou, & Dominguez, 2009; J. D. Thomas et al., 2010; Tiwari, Arora, & Chopra, 2012). The impairments observed in these paradigms overlap with those seen in the FASD clinical population. Data from these models have helped to elucidate the structural and behavioral effects associated with ETOH exposure during a critical period of CNS development.

General Hypothesis

It is rare that developmental insults, such as fetal ETOH exposure, occur in the absence of other challenges. For example, prenatal ETOH is often associated with exposure to smoking and poor nutrition. Hypoxic events, such as those that occur during the perinatal period, are potentially damaging to the developing fetus. Interactions with other insults, such as perinatal hypoxia, can influence the severity of fetal ETOH related damages and may help explain some of the variability observed in the fetal alcohol population.

Hypoxia

Background

Hypoxia is a common occurrence during the perinatal period, particularly during parturition. Hypoxia refers to a depletion of oxygen levels and can be produced through asphyxia or ischemia and for the purposes of this paper; the term hypoxia will be used to refer to any decrease in oxygen flow to the CNS. The causes of perinatal hypoxia can include ruptured uterus, cord compressions, and contraction induced pressure on the infant (Fahey & King, 2005; Mistovich, Krost, & Limmer, 2006)

The occurrence of recognized hypoxia at birth is approximately 5-25 per 1000 live births with approximately 15% of these cases being moderate or severe (Low, 2004). Perinatal hypoxia is commonly recognized as a cause of CNS damage and can result in long term neurological morbidity. Severe hypoxic episodes can lead to major deficits including cerebral palsy, mental retardation, epilepsy, or fetal/neonatal mortality. In the near-term fetus or newborn, the CNS is well equipped to handle mild or moderate hypoxic challenges, so these challenges are generally considered benign (Shalak & Perlman, 2004). However, these events could result in a range of cognitive and behavioral deficits especially if the integrity of the CNS has already been compromised by other physiological challenges (e.g. infection or perhaps prenatal ETOH exposure) (Rees, Harding, & Walker, 2008). Identification of high risk infants and developments of therapeutic interventions that can reduce these damages of ongoing CNS injury are critical for improving the outcome of many children.

Changes in the Brain

Much like ETOH, the degree of CNS damage following a hypoxic challenge depends on the timing, nature, and duration of the episode. Neurons in the cerebellum, cortex and hippocampus are particularly sensitive to the damaging effects of acute hypoxic insults that occur early in fetal development (Rees et al., 2008) while hypoxia occurring later in development, in the near term infant, seems to preferentially affect neurons in the prefrontal cortex and striatum (Tolcos et al., 2003). Chronic hypoxia during CNS development, which can be caused by placental insufficiency, leads to reduced brain weight, decreased myelination, impaired synaptogenesis, impaired dopaminergic activity, and enlargement of ventricles, among other deficiencies (Rees et. al., 2008). Given the wide range of possible CNS damage, it is not surprising that a variety of behavioral and cognitive deficits are associated with hypoxia.

Clinical Outcomes

Children that have experienced severe hypoxic episodes, similar to those exposed to ETOH, can suffer significant cognitive, behavioral, and social delays. These children are less intelligent than their peers and perform significantly worse on neuropsychological tests (van Handel, Swaab, de Vries, & Jongmans, 2007). Children with moderate hypoxic damage have impaired attention and concentration, hyperactivity, executive function, and visual-spatial abilities (van Handel et. al., 2007). These findings indicate that even moderate hypoxia at birth

can cause significant developmental problems and, as with fetal alcohol exposure, treatment should be considered for any children at risk.

Animal Models

Many animal models have been developed to explore the effects of hypoxia on the developing brain. These models vary in terms of the type (e.g. complete oxygen deprivation or decreased oxygen) and timing (e.g. at birth or several days following birth) of hypoxic exposure, mimicking different possibilities of the human condition. Similar to neonatal ETOH exposure in rodents, hypoxia exposure during critical periods of brain development causes hyperactivity (Decker et al., 2003; Juarez, Gratton, & Flores, 2008) and impairments in learning and memory (Boksa, Krishnamurthy, & Brooks, 1995; Ikeda et al., 2004; Karalis et al., 2011) overlapping what is often seen in the clinical population. Animal models such as these may be useful for assessing interactions between fetal ETOH and hypoxia, given the considerable similarities in cognitive and behavioral outcomes.

Outcome Summary

Fetal ETOH and perinatal hypoxia have significant overlap in some of the behavioral outcomes seen both in animal models and in the human population, such as impaired intelligence, learning and memory deficits, and problems with hyperactivity. The similarities in outcome may be partially related to the fact that these events can co-occur, both affect a variety of brain structures, and share some mechanisms of damage.

Fetal Alcohol Exposure and Hypoxia

Alcohol use during pregnancy increases the likelihood of hypoxic events for the fetus with reports of increased labor complications and perinatal asphyxia. ETOH causes vasoconstriction of the placenta; an effect that is dose dependent and lasts as long as alcohol is in the system (Burd, Roberts, Olson, & Odendaal, 2007). It can also cause umbilical cord spasms and dysregulation of molecules that modulate blood flow; for review see (Bosco & Diaz, 2012; Burd et al., 2007) all of which can result in decreased oxygen delivery to the fetus.

ETOH also alters the brain's ability to protect itself from hypoxic damage. In adult alcoholics, vascular damage increases the risk for hypoxic events such as stroke and ischemia. With this lowered level of oxygen, there is an increase cerebral blood flow in an attempt to maintain a steady rate of oxygen and prevent damage (Brown, Wade, & Marshall, 1985). In sheep models of prenatal ETOH exposure, a normally developing fetus is able to increase blood flow to areas such as the cerebellum in response to decreases in oxygen levels (Gleason, Iida, Hotchkiss, Northington, & Traystman, 1997). ETOH exposure attenuates this compensatory increase in blood flow to the brain (Mayock, Ness, Mondares, & Gleason, 2007), potentiating the damaging consequences of hypoxia.

While ETOH exerts some of its damage by increasing the likelihood of hypoxic events, both insults share mechanisms of damage at the cellular/molecular level and, when both occur together, there could be an exacerbation of injury.

Shared Mechanisms

Both ETOH and hypoxia damage cells through many different mechanisms that are beyond the scope of this proposal. Of interest to this proposal are the shared mechanisms of damage, excitotoxicity and oxygen free radical formation/oxidative stress. Excitotoxicity is cell damage or death that occurs when the cell is overstimulated, often resulting from overactiviation of the *N*-methyl-d-aspartate (NMDA) receptor. Oxidative stress occurs when there are excess free radicals that can damage important cellular components. Both ETOH, more specifically ETOH withdrawal, and hypoxia can induce excitotoxic and oxidative damage in the developing brain.

NMDA receptors

The NMDA receptor is a tetrameric ionotropic glutamate receptor found in the CNS; it facilitates excitatory synaptic transmission and is important in neuronal processes such as long term potentiation (Collingridge & Bliss, 1987). NMDA receptor channels are permeable to Ca^{2+}, Na+, and K+, but are blocked at resting membrane potential by Mg+. Depolarization removes this blockade and when glutamate binds to the receptor, Ca^{2+} flows into the cell. There are also binding sites for Zn^{2+} and gylcine, a co-agonist, and polyamines (allosteric modulators; putrescine, spermine, and spermidine). These molecules can increase or decrease receptor response to glutamate, altering the amount of Ca^{2+} entering the cell; for review see (Rock & Macdonald, 1995). While normal receptor functioning is critical for synaptic plasticity and learning/ memory

formation, abnormal activity is implicated in a variety of neurological dysfunctions and disorders; for review see (R. J. Thomas, 1995).

ETOH, NMDA receptors, Excitotoxicity

Acutely, ETOH has a net inhibitory effect on the CNS. This is achieved primarily via two broad mechanisms: enhanced inhibitory transmission and diminished excitatory transmission. ETOH interacts with GABA-A receptors to increase the effects of GABA, the main inhibitory neurotransmitter in the CNS. ETOH decreases excitatory transmission through antagonistic interactions with glutamate receptors, including NMDA receptors. In the presence of chronic ETOH, the CNS goes through compensatory changes in an attempt to maintain homeostatic excitatory transmission. One of these changes is upregulation of the NMDA receptor which occurs through increases in the number of receptors (Hoffman, 1995; Hu & Ticku, 1995) changes in receptor subunit composition (Follesa & Ticku, 1995; Snell et al., 1996) which can make them more sensitive to glutamate/ overexcitation and increases in polyamine levels (Gibson et al., 2003); for review see (Littleton et al., 2001). More specifically, in *in vivo* models of fetal alcohol exposure, changes in the NMDA receptor are dependent on dose and timing (Nixon, Hughes, Amsel, & Leslie, 2002, 2004). In the hippocampus, perinatal ETOH exposure produces increases in NR2A expression during ETOH withdrawal while no changes are seen with the NR2B subunit (Nixon et al., 2004).

While ETOH is still present, compensatory changes can be beneficial, but once ETOH is removed, they contribute to withdrawal symptoms and excitotoxic cell damage or death. When the inhibitory effects of alcohol are removed (ETOH withdrawal) NMDA receptors are left hypersensitive to glutamate; this results in receptor over activation and excessive Ca^{2+} influx into the cell. Excess Ca^{2+} in the cytoplasm triggers damaging signaling pathways and interferes with cell structure and function; this eventually leads to cell damage and death. Damaged cells release glutamate into the extracellular space, further contributing to the excitotoxic process; for review see (Kumari & Ticku, 2000).

Hypoxia, NMDA receptors, Excitotoxicity

Oxygen deprivation causes cell damage through similar excitotoxic mechanisms. Hypoxia produces toxic increases in extracellular glutamate through impaired glutamate transporter function; for review see (Camacho & Massieu, 2006; Swanson, Farrell, & Simon, 1995), release of glutamate from the synapse, and glutamate leakage from damaged cells. Under hypoxic conditions, there is a switch from normal aerobic respiration to anaerobic metabolism which causes rapid energy (ATP) depletion. Without adequate ATP, glutamate transporters lose normal functioning and glutamate cannot be taken out of the synapse. Excess Ca^{2+} in the cell and free radical inhibition of mitochondrial components promotes glutamate release into the synapse (Dong et al., 2012). Finally, as during ETOH withdrawal, damaged cells discharge their glutamate.

Anaerobic metabolism leads to a buildup of lactic acid, producing a state of acidosis, an increase in the acidity of tissue or fluid. In low pH conditions, GABA transmission is impaired and certain ion channels become more responsive to glutamate activation (Zhao, Cai, Yang, He, & Shen, 2011), creating an imbalance that leaves cells sensitive to overstimulation. Acidosis is thus another mechanism through which hypoxia produces excitotoxicity.

ETOH, Hypoxia, and Polyamines

Both ETOH and hypoxia enhance excitotoxic damage because they produce an accumulation of polyamines that potentiate the activity of NMDA receptors. Free radicals produced from these insults induce the activity of ornithine decarboxylase (ODC), the rate limiting enzyme in polyamine synthesis. Polyamine metabolism produces free radicals that can further increase ODC activity (Saito, Packianathan, & Longo, 1997); promoting the production of more polyamines that further contribute to excitotoxicity.

ETOH, Hypoxia, and Oxidative Stress

Finally, both ETOH withdrawal and hypoxia can lead to increased production of free radicals. ETOH metabolism produces free radicals. Both insults lead to excess intracellular Ca^{2+} which indirectly increases free radical production; for review see (R. J. Thomas, 1995). In a normally functioning cell, free radical levels are managed through scavenger molecules. As a result of certain challenges, including ETOH exposure and hypoxia, the scavengers are saturated and excess free radical molecules are free to interact with important

lipids, proteins, and molecules in the cell, resulting is disrupted cellular functioning and cell death signaling (Halliwell, 1992).

Hypothesis

Given the overlap in mechanisms of damage, ETOH withdrawal could exacerbate hypoxic damage when both occur at sub-threshold levels, producing synergistic damage in sensitive areas including the hippocampus. The proposed studies will investigate the possibility of an interaction between these two challenges using an organotypic hippocampal slice culture model.

Organotypic Hippocampal Slice Culture Models

The organotypic hippocampal slice culture model was developed in 1991 by Stoppini and colleagues (Stoppini, Buchs, & Muller, 1991) and has since been used as an investigative tool for neuroprotection, neurogensis, and neurotoxicity. This model allows for the maintenance of neurons and glia and maintains some of the complex circuitry of an intact hippocampus. These models can be used to measure cell death or damage or to analyze electrical potentials inside and outside of the cell. Slices are sensitive to a variety of insults including ETOH and hypoxia; for review see (Noraberg et al., 2005).

Hippocampal slice cultures models are particularly interesting for studying excitotoxic damage; the hippocampus has a high density of NMDA receptors, a key factor in excitotoxic cell death. The hippocampus is also implicated in some of the cognitive and behavioral deficits observed following fetal alcohol exposure or perinatal hypoxia, such as impaired learning and memory.

Chapter 2: Methods

Experiment 1:

This study was designed to address whether mild levels of ETOH withdrawal and hypoxia exposure interacted to produce cellular damage in our hippocampal slice culture model. The study first looked at the effects of varying levels of hypoxia in combination with ETOH withdrawal and then investigated possible developmental differences in sensitivity to these insults.

Hippocampal Slice Culture Preparation

On postnatal (PND) 8 or PND 2 Sprague Dawley rat pups (3 male and 3 female, belonging to the same litter) born in the University of Kentucky Psychology Department's breeding facility were sacrificed for brain removal. Brains were directly transferred to a petri dish containing ice cold dissecting medium [Minimum Essential Medium (Gibco BRL, Gaithersburg, MD), 25 mM HEPES (ATCC, Manassas, VA), 200 mM L-glutamine (Invitrogen, Carlsbad, CA), 50 µM streptomycin/penicillin (ATCC, Manassas, VA)]. For each animal, both hippocampi were dissected out and coronally sectioned into 200 µm slices using a McIllwain Tissue Chopper (Campden Instruments Ltd., Lafayette, ID). Slices were selected using a dissecting microscope and then plated on teflon membrane inserts (Millicell-CM 0.4 µm; Millipore, Marlborough, MA, USA), with 3 slices on each insert. The inserts were maintained in 6 well culture plates containing 1mL culture media [dissecting media, 36 mM glucose, 25% Hanks' balanced salt solution (HBSS; Gibco BRL, Gaithersburg, MD), 25% heat-inactivated horse serum (HIHS; Sigma, St. Louis, MO)] in each well. Plates

were maintained undisturbed in an incubator (37°C/5%CO2/95%air) for 5 days in vitro (DIV), to allow slices to adhere to the membrane before the first media change. There were 3-4 replications per treatment condition. The care of animals was carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1996), as well as the University of Kentucky's Institutional Animal Care and Use Committee.

ETOH and Hypoxia Treatments

On DIV 5, inserts were transferred to 100 mM ETOH media or control media, with inserts from each subject being evenly distributed between the two treatment groups. While this is a high dose of ETOH (similar to a blood alcohol content of .48), alcoholics/ binge drinkers can often reach extremely high blood alcohol levels (Paintner et al., 2012). It is also important to note that rapid evaporation occurs over the course of ETOH exposure *in vitro* (Prendergast et al., 2004). Plates were placed in propylene containers with 50 mL of control or 100mM ETOH containing distilled water on the bottom. These containers were placed in plastic freezer bags to limit ETOH evaporation, filled with an air mixture and incubated until DIV 10 when inserts were transferred to fresh media, maintaining the same treatment groups. On DIV 15, control and ETOH inserts were further divided into oxygen glucose deprivation (OGD) (a common method for inducing hypoxic conditions *in vitro*) or control groups, giving a total of 4 treatment conditions; control/control, control/ETOH, OGD/control, and OGD/ETOH. ETOH slices were removed from ETOH at this time, beginning the

process of EWD. Inserts were then transferred to either glucose free culture media [D-glucose free modified eagle medium (Gibco BRL, Gaithersburg, MD) 25 mM HEPES (ATCC, Manassas, VA), 200 mM L-glutamine (Invitrogen, Carlsbad, CA), 50 µM streptomycin/penicillin (ATCC, Manassas, VA)] (OGD) or regular culture media (control) and then placed in incubation chambers in which they were flushed with either anaerobic gas (5% CO2, 95% N2) (OGD) or control air (5% CO2, 21% O2, 74% N2) at 25 L/min for 4 min. Based on previous data, this time period is sufficient to flush the chambers (Mulholland et al., 2005). Chambers were quickly sealed after the 4 min and placed in the incubator for the treatment duration (15, 30, or 60 min for PND 8; only 30 min for PND 2). Following the treatment, all inserts were placed in 1mL of culture media containing the fluorescent marker propidium iodide (PI; 3.74 µM, SigmaAldrich) (Exp 1) or plain culture media (Exp 2) and incubated for 24 hours.

Fluorescence Imaging

PI binds to the DNA of damaged cells and when excited at 488nm light, emits a red fluorescent signal that can be captured using fluorescent microscopy. Slice images were captured using SPOT Advanced version 4.0.9 software (W. Nuhsbaum Inc., McHenry, IL, USA) connected to an inverted Leica DMIRB microscope (W. Nuhsbaum Inc.). PI uptake in the CA1, CA3, and DG cell layers was measured using ImageJ version 1.46 software (National Institute of Health (NIH)). Background signal was subtracted from the cell layer fluorescence and this value was used for statistical analysis.

To supplement results found with fluorescent intensity in the 3 cell layers, the total area of PI fluorescence above a low intensity threshold of 30 was measured, using whole slice images converted to 8 bit images using Image J version 1.46 software (NIH). This method of PI quantification was adapted from Loetscher et al. (2009) in which pixels above a predetermined intensity threshold of 75 were counted; 30 was used as the threshold in this experiment to include pixels that emit low levels of PI fluorescent signal. One issue with measuring cell death by simply looking at average intensity of each region is that it is still unclear exactly what information the intensity is conveying in terms of cell damage. With the imaging techniques used in this study, it is impossible to look at individual cell damage, instead this technique captures the fluorescent signal of the entire 200 micron- thick slice. There is a spectrum of PI intensities in these images; there is not an all or none dichotomy. While possible explanations exist, such as a more intense pixel represents a higher density of cells expressing PI fluorescence, our understanding remains limited, rendering it important to supplement fluorescence intensity measurements. It is important to include low levels of PI intensity in the analysis of total area given that the difference between a pixel with high intensity versus a pixel with low intensity is unclear. This analysis was performed only for the culture preps exposed to 30 min OGD and ETOH.

Experiment 2:

Experiment 2 used immunohistochemistry in the OHSC model to further examine the previously found interaction between hypoxia and ETOH withdrawal. The specific nature of cellular damage was investigated using neuronal nuclear

protein (NeuN) staining to assess the effects of these insults on neurons. NeuN, now identified as Fox-3 (Kim, Adelstein, & Kawamoto, 2009), is expressed specifically in the nuclei of postmitotic neurons and is an accepted marker for both developing and mature neurons.

Immunohistochemistry

OHSC preparation and ETOH and OGD treatment followed the same methods described in Exp 1. Three replicates were used for Exp 2.

Tissue slices were fixed using 10% formalin (Buffered Formalde-Fresh Low Odor 10% Formalin, Fisher Scientific), 24 hours following 30 min OGD treatment. Inserts were placed directly from the culture media into formalin plates with 1mL on top, 1mL on bottom and allowed to sit for 30 min. Inserts were then washed twice with 1 X phosphate buffer solution (PBS) and refrigerated at 4°C in 1 X PBS for no more than 7 days.

On day 1 of immunohistochemistry, inserts were exposed to 1 mL of buffer (200 mL non-sterile 1 X PBS; 200 µL Triton; 10 mg BSA) on the top and bottom for 45 minutes to allow for the breakdown of cell membranes. Inserts were then transferred to plates containing 1 mL 1 X PBS. The primary antibody (NeuN) was diluted in (1:200) with the buffer; 1mL of this solution was placed on top of each insert and plates were refrigerated for 24 hours. On day 2, inserts were washed twice with 1 X PBS. A 1:200 dilution of the secondary antibody (Fit-C), used to measure fluorescence, will be made and 1mL will be placed on the top of each insert for 24 hours. On day 3, plates were washed twice with 1 X PBS and immediately imaged, using the same procedures as described for PI imaging.

As with PI, NeuN immunoreactivity was measured in the DG, CA3, and CA1 cell layers. To supplement this data, total area of NeuN fluorescence above a threshold of 15 was measured, using the same methods described for Exp 1.

Statistical Analysis

All statistical analyses were conducted using IBM Statistical Package for the Social Sciences (SPSS) Version 20 Software (IBM Corporation, 2011). For each hippocampal region and for total area of fluorescence (for both PI and NeuN staining), an initial 2 x 2 x 2 (SEX x ETOH x OGD) analysis of variance (ANOVA) was conducted. To assess differences in sensitivity as a function of age, the age of the pups that the cultures were derived from was included in an additional analysis as a factor in an analysis with the 30 min OGD time point (Exp 1 only). This time point was chosen because it had previously been shown to be sensitive to ETOH/OGD in the PND 8 tissue. Replicate was used as a covariate in the analyses this analysis to control for differences across litters/ culture preparations. If no main effects or interactions with sex were found, further analysis were conducted collapsed across this factor. Significant interactions were investigated using post hoc pair-wise comparisons with Tukey's LSD correction for family-wise error. The data was converted to percent control for graphic presentation.

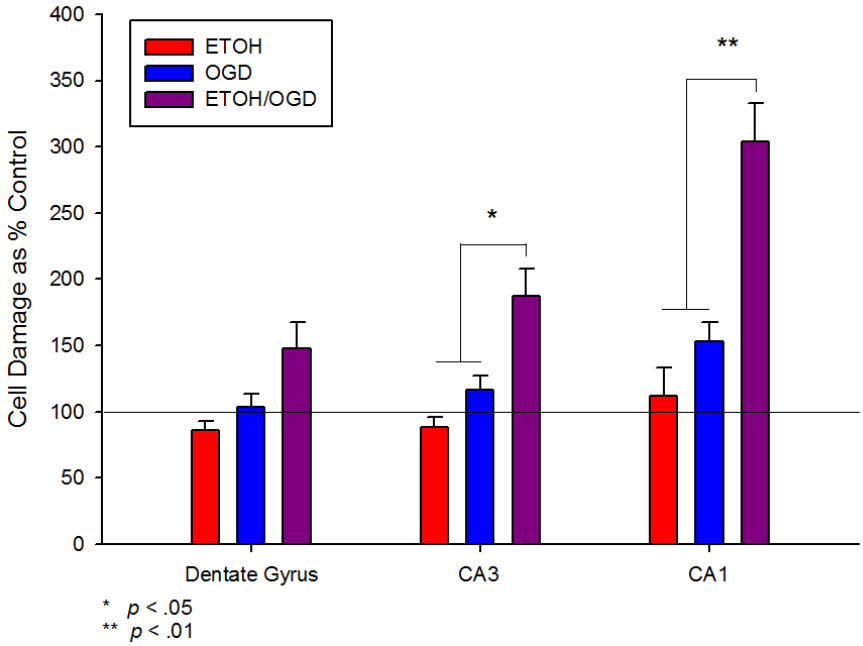
Chapter 3: Results

Experiment 1

Varying durations of OGD in Combination with ETOH withdrawal

The first part of this experiment assessed the effects of varying durations of OGD using PND 8 rat pups. Consistent with our hypothesis, 30 min OGD combined with ETOH withdrawal produced synergistic toxicity in the CA1 and CA3 regions as demonstrated by a significant ETOH x OGD interaction in the CA1, F (1, 70) = 5.771, $p < .05$ and CA3, F (1, 70) =6.247, $p < .05$. Post hoc comparisons for each region revealed that this interaction was driven by the ETOH/OGD condition which had greater PI fluorescence compared to all other groups (Figure 3.1). There was also a main effect of ETOH, F (1, 70) = 12.652, $p = .001$ and a main effect of OGD, F (1, 70) = 24.060, $p <.001$ in the CA1; both ETOH and OGD increased fluorescence. In the CA3, there was a main effect of OGD, F (1, 70) = 11.472, $p = .001$, with OGD exposed slices having greater fluorescence. There were no significant effects in the DG.

Figure 3.1. PI uptake expressed as percent control in the DG, CA3, and CA1 regions of the PND 8 hippocampus, 24 hours following initiation of ETOH withdrawal and 30 min OGD treatment. ETOH/OGD differed significantly from all other conditions in the CA3 ($p < .05$) and the CA1 ($p < .01$).



This pattern was not observed with other durations of OGD treatment. It was clear that 60 min OGD had a ceiling effect; that is – this level of OGD itself was toxic and previous ETOH exposure did not potentiate damage beyond that produced by the OGD. There was a main effect of OGD in both the CA1, F (1, 72) = 92.966, $p < .001$ and CA3, F (1, 72) =13.057, $p = .001$ and no effects of treatment in the DG (Figure 3.2). There were no significant treatment effects in any region with EWD after 15 min OGD (Figure 3.3).

Figure 3.2. PI uptake expressed as percent control in the DG, CA3, and CA1 regions of the PND 8 hippocampus, 24 hours following initiation of ETOH withdrawal and 60 min OGD treatment. OGD treated slices differed significantly from non-OGD slices in the CA3 ($p < .01$) and CA1 ($p < .001$); this effect was not potentiated by ETOH.

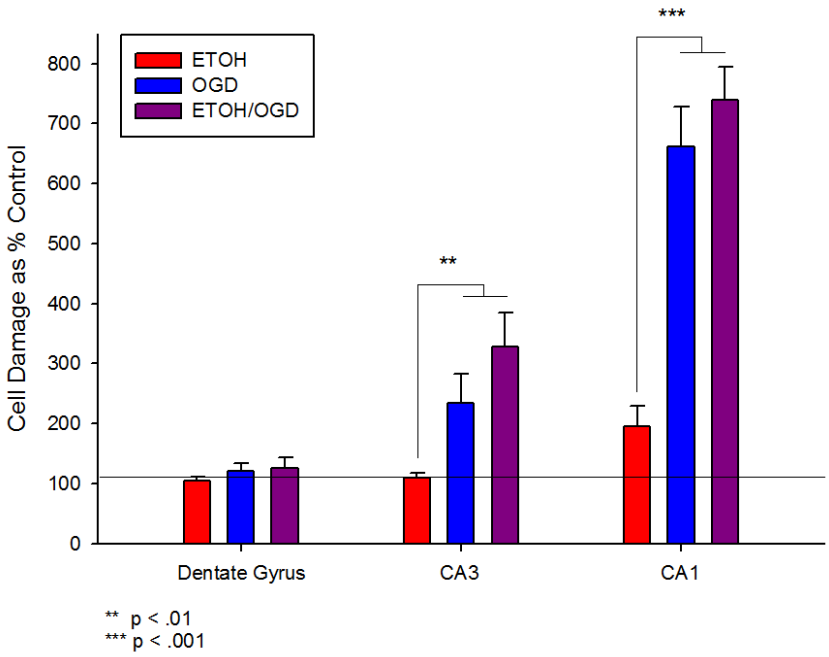
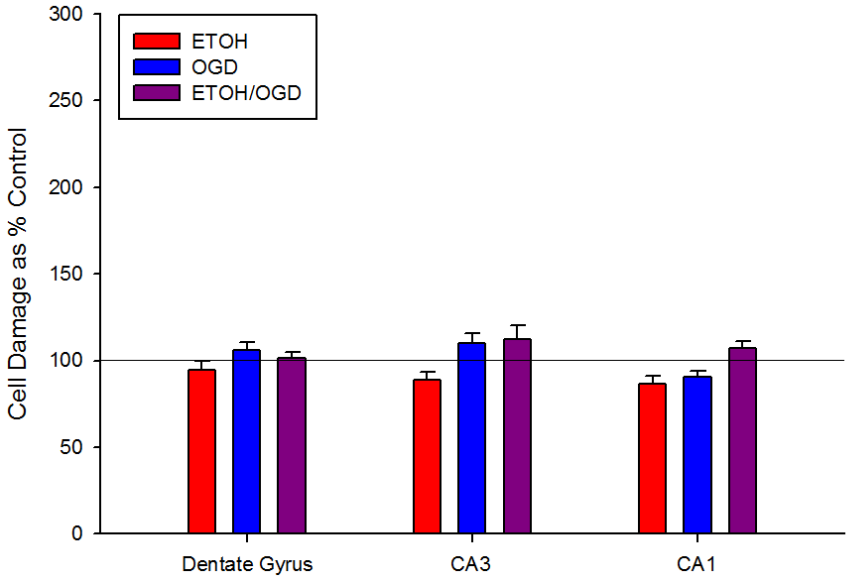


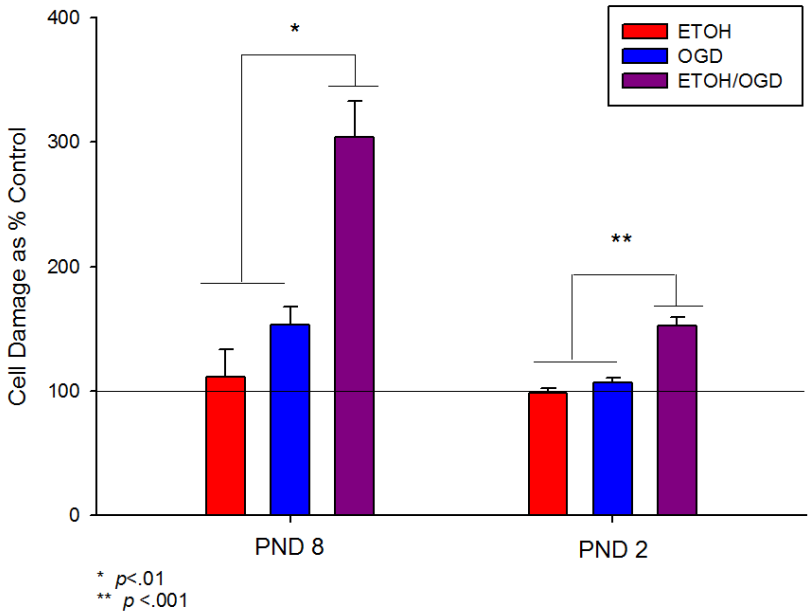
Figure 3.3. PI uptake expressed as percent control in the DG, CA3, and CA1 regions of the PND 8 hippocampus, 24 hours following initiation of ETOH withdrawal and 15 min OGD treatment. There were no significant treatment effects.



Developmental Differences

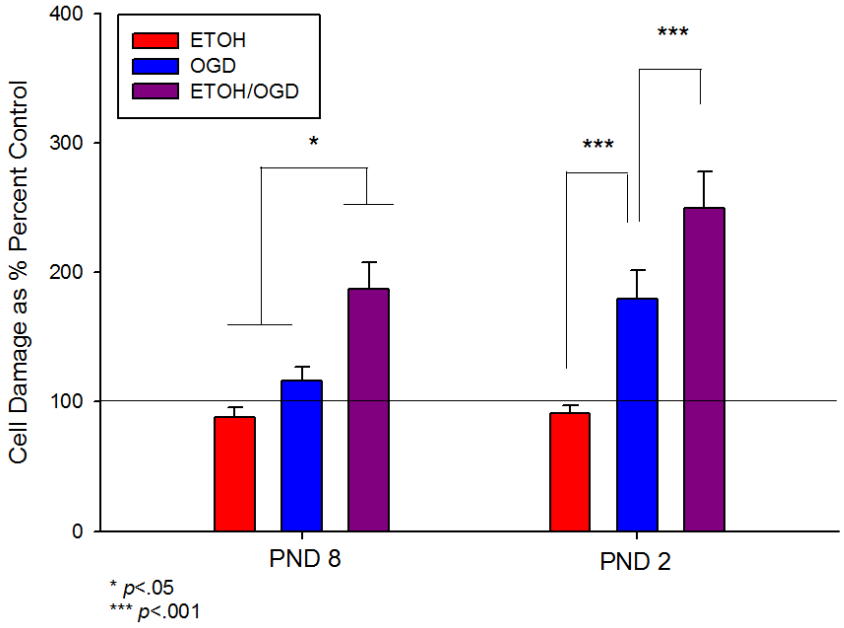
In the CA1 region, there were clear developmental differences in the response to ETOH/OGD between the PND 2 and PND 8 hippocampal tissue. In tissue derived from PND 8 pups, ETOH/OGD produced significantly greater damage than that from PND 2 pups; this was shown by the significant ETOH x OGD x AGE interaction, F (1, 270) = 4.002, $p < .05$. The combination of ETOH and OGD produced greater toxicity compared to all other treatment groups for both ages, with this effect being more pronounced in PND 8 tissue (Figure 3.4).

Figure 3.4. PI uptake expressed as percent control in the CA1 region for PND 8 and PND 2 tissue, 24 hours following ETOH withdrawal and 30 min OGD treatment. The interactive effect of ETOH and OGD is greater in PND 8 slices.



The developmental differences observed in the CA3 differed from those seen in the CA1. There was a main effect of age, F (1, 270) = 18.314, $p < .001$, such that PND 2 tissue had greater fluorescence overall. There was an ETOH x OGD interaction, F (1, 270) = 4.028, $p < .05$, but this did not interact with age. A separate analysis for PND 2 slices showed that there was an ETOH x OGD interaction in these tissue, F (1, 200) = 4.460, $p < .05$, consistent with the interaction seen PND 8 tissue. Again, the combination of ETOH and OGD produced greater damage relative to all other treatment groups. Unlike tissue from PND 8 pups, OGD alone also produced damage above control levels for PND 2 slices in this region, $p < .001$ (Figure 3.5).

Figure 3.5. PI uptake expressed as percent control in the CA3 region for PND 8 and PND 2 tissue, 24 hours following ETOH withdrawal and 30 min OGD treatment. ETOH/OGD produced toxicity above all other treatment groups in both PND 8 and PND 2 tissue while PND 2 tissue had greater fluorescence overall compared to PND 8. OGD alone was sufficient to increase toxicity above control levels in PND 2 tissue.



Similar to the effect seen in the CA3, there was a main effect of age in the DG, F (1, 270) = 4.644, p < .05, with PND 2 slices having greater PI fluorescence. Analysis of PND 2 slices showed an ETOH x SEX interaction, F (1, 200) = 4.358, p < .05. Female slices had *less* PI fluorescence when exposed to ETOH compared to non-ETOH slices, while in males, greater fluorescence was observed in ETOH treated slices.

Area Analysis

Consistent with analysis of PI fluorescent intensity in the 3 regions, analysis of total area of PI fluorescence revealed that there was an interaction of ETOH and OGD in PND 8, F (1, 212) = 12.444, p = .001 (Figure 3.6). In this tissue, the combination of ETOH and OGD resulted in increased area of PI signal. In PND 8 tissue there was also a main effect of ETOH, F (1, 212) = 22.370, p < .001 and a main effect of OGD, F (1, 212) = 48.643, p < .001; each insult increased total area of PI signal. In PND 2 tissue there was a main effect of ETOH, F (1, 200) = 9.233, p < .05, main effect of OGD, F (1, 200) = 9.323, p< .05, with each insult increasing the total area of PI fluorescence. There was also main effect of SEX, F (1, 200) = 9.438, p < .05 in PND 2 tissue such that female slices showed less total area of fluorescence compared to males. The ETOH x OGD was not significant at PND 2, but the ETOH x OGD x SEX interaction approached significance, F (1, 200) = 3.413, p = .066 (Figure 3.7).

Figure 3.6. Total area of PI fluorescence in PND 8 tissue expressed as percent control, 24 hours following ETOH withdrawal and 30 min OGD treatment. ETOH/OGD produced an increase in total area of PI signal above all other treatment groups. OGD alone also produced an increase in area of PI signal.

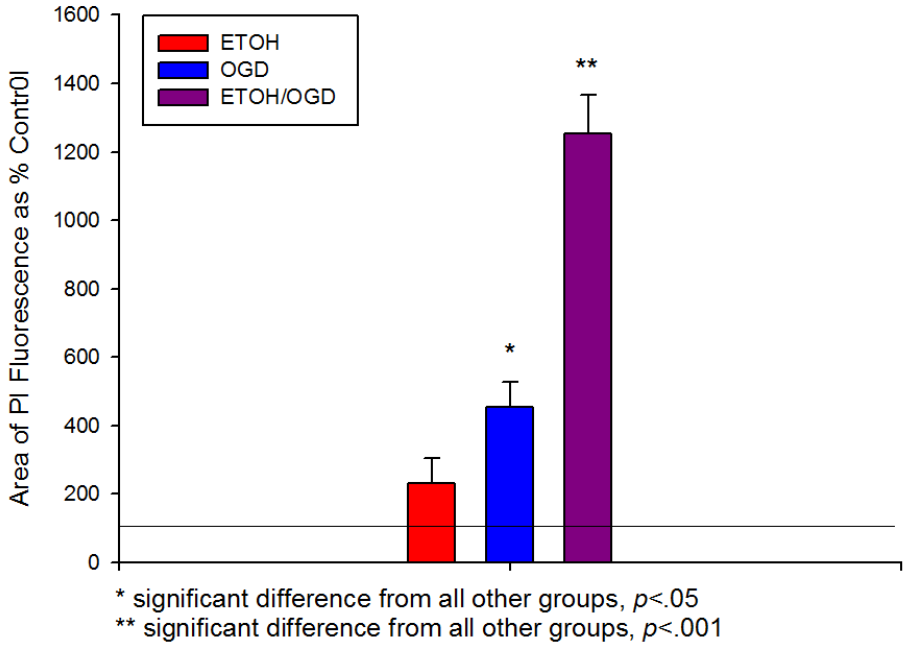
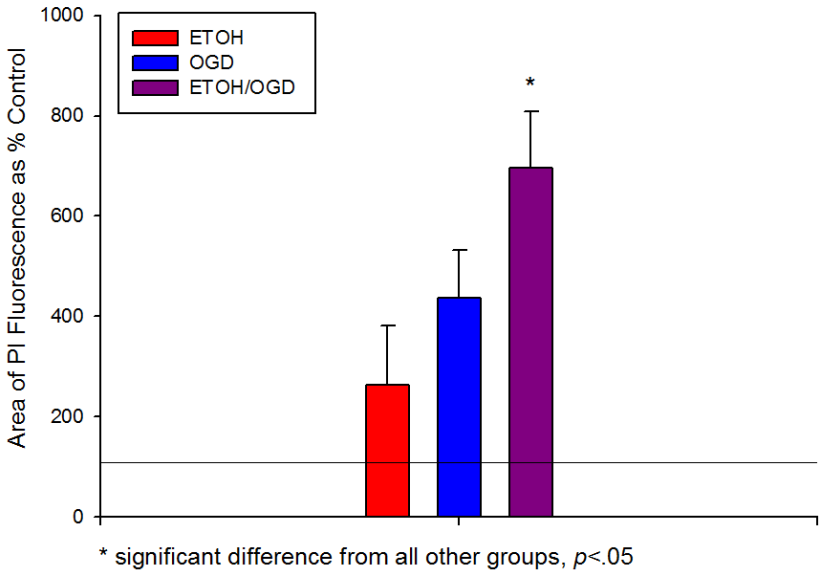


Figure 3.7. Total area of PI fluorescence in PND 2 tissue expressed as percent control, 24 hours following ETOH withdrawal and 30 min OGD treatment. ETOH/OGD produced an increase in total area of PI signal above all other treatment groups although there was not a significant interaction.

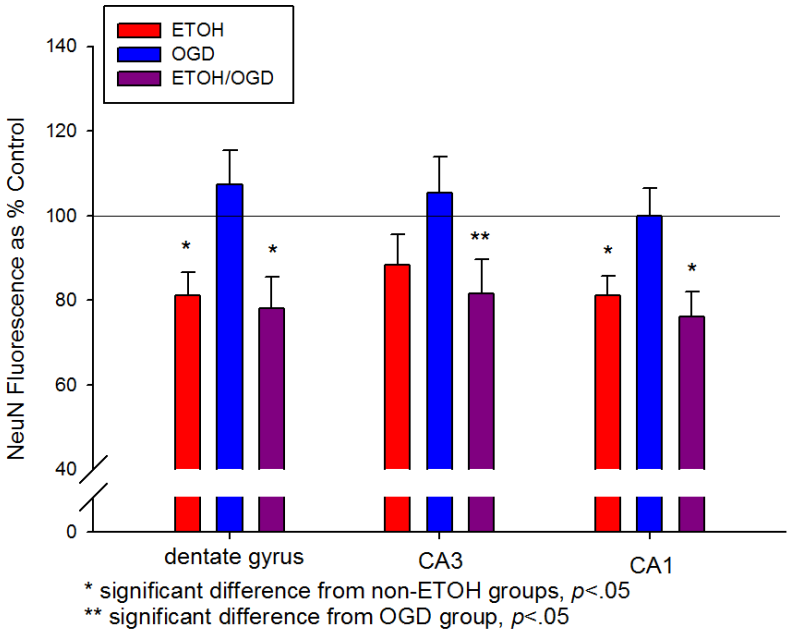


Experiment 2 NeuN immunoreactivity

Exposure to ETOH resulted in reduced NeuN immunoreactivity in the CA1, F (1, 233) = 17.492, $p < .001$, CA3, F (1, 233) = 5.255, $p<.05$, and DG, F (1, 233) = 13.099, $p < .001$, regardless of OGD exposure. Unlike the results observed with PI, there was no interaction of ETOH with OGD and there was no effect of OGD in any regions (Figure 3.8).

A main effect of sex was observed in both the CA1, F (1, 233) = 4.626, $p < .05$ and CA3, F (1, 233) = 11.985, $p = .001$, such that tissue derived from females pups showed less NeuN fluorescence compared to that from males.

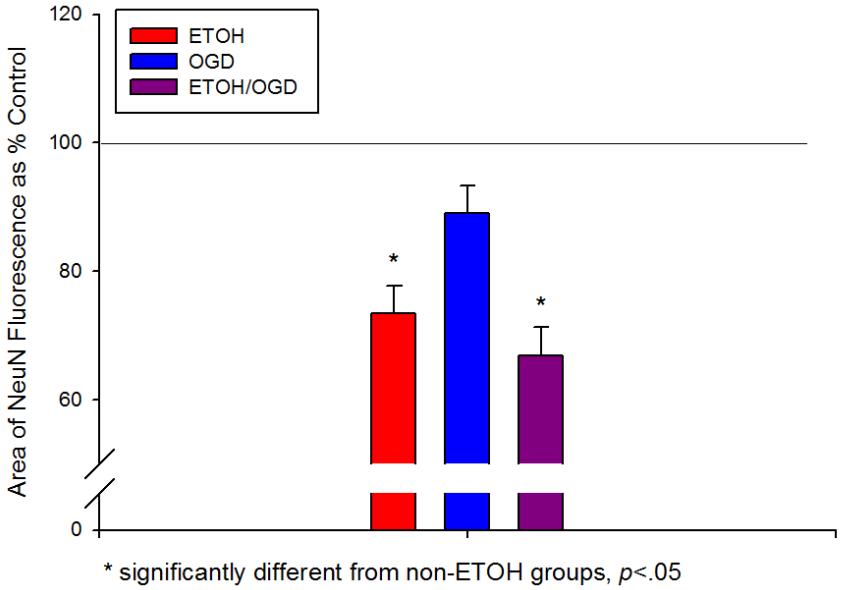
Figure 3.8. NeuN fluorescence expressed as percent control in the DG, CA3, and CA1 regions of the PND 8 hippocampus, 24 hours following initiation of ETOH withdrawal and 30 min OGD treatment. ETOH treatment resulted in a decrease in NeuN signal in the DG and CA, regardless of OGD treatment while the combination of ETOH and OGD decreased NeuN signal in the CA3.



Area Analysis

Analysis of total area of NeuN fluorescence supported data found in the regional analysis. There was a significant effect of ETOH, F (1, 233) = 25.527, p<.001, with ETOH slices showing less total area of immunoreactivity compared to non-ETOH treated slices (Figure 3.9). There was also a main effect of SEX, *F* (1,233) = 8.274, *p* <.05; female tissue had less area of NeuN signal compared to males.

Figure 3.9. Total area of NeuN fluorescence in PND 8 tissue expressed as percent control, 24 hours following ETOH withdrawal and 30 min OGD treatment. ETOH treatment produced a decrease in total area of NeuN signal compared to non-ETOH treated tissue.



Chapter 4: Discussion

Experiment 1

The results from this experiment indicated that mild levels of ETOH withdrawal and hypoxia interacted to produce synergistic damage in the developing hippocampus. This effect was observed in both PND 8 and PND 2 tissue, although the CA1 and CA3 regions of the hippocampus appeared to be differentially affected. In PND 8 tissue, the CA1 was more sensitive to the combination of ETOH withdrawal and OGD while in PND 2 tissue, the CA3 showed greater damage. In younger slices, OGD alone was sufficient to produce damage above control levels in the CA3; this was surprising considering that younger brains are generally thought to be more resilient to hypoxic damage; for review see (S. J. Vannucci & Hagberg, 2004) although overall, more damage was observed in older slices. This interaction could involve several different mechanisms and presents possible clinical implications.

Mechanisms Experiment 1

As mentioned in the introduction, there are multiple, overlapping mechanisms involved in the detrimental effects of ETOH and of hypoxia that could result in multiplicative damage when the two challenges are combined. The present study examined cell damage 24 hours following hypoxic insult and the initiation of ETOH withdrawal; glutamate induced excitotoxicity and the production of free radicals following both ETOH (Mayer et al., 2002; Vallett et al., 1997) and hypoxia [(Ahlgren, Henjum, Ottersen, & Runden-Pran, 2011); for review see (Johnston, 2001)] occur during this 24 hour time period. While the

mechanism was not investigated, the timing and region specificity of PI uptake suggest the possible role of excitotoxicity.

Excitotoxicity/ Ionotropic Glutamate Receptors

Both ETOH withdrawal; for review see (Lovinger, 1993) and OGD; for review see (Johnston, 2001) produce cell damage through excitotoxic pathways. The NMDA receptor is an important mediator of excitotoxic cell damage (R. J. Thomas, 1995; Waxman & Lynch, 2005) and antagonism of this receptor following ETOH withdrawal (Lewis et al., 2012; Mayer et al., 2002; Stepanyan et al., 2008) and hypoxia (Albers, Goldberg, & Choi, 1989a) separately, can be neuroprotective. It is well established that the CA1 region of the hippocampus is particularly susceptible to excitotoxic challenges compared to the other regions (Butler et al., 2010; Davolio & Greenamyre, 1995; Vornov, Park, & Thomas, 1998), consistent with the results in PND 8 tissue from this study. Several variations in NMDA receptor expression influence the sensitivity of the CA1. In the CA1 of hippocampi derived from PND 8 rats, there is a higher density of NMDA receptors and higher expression of NR2B subunits compared to the CA3 and dentate (Butler et al., 2010). Receptor channels containing NR2B have a more selective Ca^{2+} permeability, have a higher affinity for glutamate and glycine, and have longer open times all of which are beneficial under normal developmental conditions, but may increase a cell's sensitivity to excitotoxic challenges (Nagy, Kolok, Dezso, Boros, & Szombathelyi, 2003). NR1 subunits containing a splice variant associated with a lack of spermine potentiation (Durand, Bennett, & Zukin, 1993) have higher expression in the CA3 and dentate

compared to the CA1, while a different splice variant, which is associated with increased sensitivity to NMDA and ischemic insult, has greater expression in the CA1 (Coultrap, Nixon, Alvestad, Valenzuela, & Browning, 2005). Given these differences in subunit expression, it is not surprising that the CA1 showed the greatest damage following ETOH exposure and OGD.

Although the pharmacology of receptors containing the NR2B subunit makes them sensitive to overexcitation transition to NR2A expression during hippocampal development is temporally linked to increased excitotoxic sensitivity (Brewer et al., 2007). The NR2A subunit has a greater current peak compared to NR2B receptors (Cheng, Fass, & Reynolds, 1999) and it is present during synapse formation and may be involved in a positive feedback loop that potentiates glutamate release (Norris et al., 2006). The transition from predominate NR2B expression to NR2A expression begins on PND 7 in the neonatal rat (Wenzel, Fritschy, Mohler, & Benke, 1997) and may contribute to the overall greater damage following ETOH and OGD in slices taken from PND 8 pups

The results in PND 2 tissue indicating that the CA3 experienced greater damage in response to ETOH/OGD compared to the CA1 were unexpected, although, several possible explanations exist. Kainate and AMPA receptor subunit expression varies across development and hippocampal region. In the CA3 of younger rat brains (PND 4), there is a greater relative presence of Ca^{2+} permeable subunits compared to the CA1; this is in contrast to the PND 7 brain in which expression in the CA1 and CA3 is the same (Pellegrini-Giampietro,

Bennett, & Zukin, 1992). If the younger brain has more Ca^{2+} permeable receptors in the CA3 vs CA1, the CA3 could be more susceptible to excitotoxic insults such as ETOH withdrawal and OGD. Another contributing factor, in OHSC models, may be that the younger hippocampus has fewer functional synaptic connections between the CA3 and CA1 (Muller, Buchs, & Stoppini, 1993). During ETOH withdrawal, CA1 toxicity can be reduced by interrupting the projections coming from the CA3 (Prendergast et al., 2004). With a relative lack of stimulation from the CA3, the CA1 in younger tissue has less excitatory input to potentiate excitotoxic damage and thus may show less damage compared to older hippocampal slices.

Oxidative Stress

Oxidative stress is closely connected to excitotoxicty/ excessive activation of iontropic glutamate receptors [for review see (Michaelis, 1998)], plays a role in the cascade of excitotoxic damage (Johnston, 2001), and is another potential mechanism through which ETOH/ETOH withdrawal and hypoxia could interact. As stated previously, free radicals (key factors in oxidative damage) are formed during both ETOH exposure/withdrawal (Vallett et al., 1997) and hypoxia (Gill & Perez-Polo, 2008; Noraberg et al., 2005). In the neonate, during ETOH exposure, there are excess levels of free radicals as a result of ETOH metabolism and of ETOH's interference with antioxidant defenses; for review see (Goodlett & Horn, 2001) and in adult models, oxidative stress during ETOH exposure is implicated in neurodegeneration (Crews & Nixon, 2009). High concentrations of intracellular Ca^{2+}, such as that occurring during ETOH

withdrawal and hypoxia, promotes free radical production through a number of pathways including enzyme activation and disruption of mitochondrial functioning; for review see (Gill & Perez-Polo, 2008). The developing brain is particularly susceptible to oxidative damage and free radical oxidation of lipids, proteins, DNA, and other cellular components can result in lasting cytostructural changes (Halliwell, 1992).

In hippocampal slices, oxidative insults produce damage in the CA1 and CA3; for review see (Noraberg et al., 2005). There is evidence that the CA1 is particularly sensitive to oxidative damage (Vornov et al., 1998; Won et al., 1999) which is consistent with results from PND 8 slices in this study. Depending on the severity of the insult, cellular injury in the hippocampus can occur within 24 hours or over the course of several days (Vornov et al., 1998) and this injury occurs in both neurons and glia (Won et al., 1999). It has been suggested that free radicals play a role in both immediate and delayed neuronal damage following hypoxia (Won et al., 2001) which could factor into the differences between finding in Exp 1 and Exp 2, which is further discussed below.

Experiment 2

Data from Exp 2, looking specifically at neuronal damage, offered a different perspective on the effects of ETOH and OGD on the developing hippocampus. Unlike findings with PI, NeuN staining revealed only a main detrimental effect of ETOH exposure, regardless of OGD treatment. Several factors could be contributing the differences in the results with PI and those with

NeuN, including the timing of fluorescent measurement and cell types being assessed. Discrepancies in the results highlight the importance of understanding the timeline of cell damage and using multiple markers/ understanding the subtleties that underlie each stain.

The results of this study are consistent with a previous OHSC study, using 100 mM ETOH exposure over 10 days, indicating a decrease in NeuN immunoreactivity in the CA1 24 hours following initiation of ETOH withdrawal (Wilkins et al., 2006). The lack of an OGD effect or interaction between ETOH and OGD as measured by NeuN fluorescence is likely due to the fact that assessment was made 24 hours following the insults. Previous hippocampal slice culture studies show neuronal damage that is measureable over the course of two to four days following initial hypoxic/ ischemic insult (Xu et al., 2002); for review see (Cimarosti & Henley, 2008). It is important to note that reduced immunoreactivity in the DG, CA3, and CA1 following ETOH withdrawal occurs at 3 days post-insult (Wilkins et al., 2006), thus, in order to capture an effect of OGD and a possible interaction with ETOH, future experiments should examine NeuN fluorescence at additional time points, including 72 hours following ETOH withdrawal and OGD.

Mechanisms Experiment 2

Delayed neuronal damage, particularly in sensitive brain areas such as the hippocampus, is one of the hallmarks of hypoxia/ischemia (Nitatori et al., 1995; Pulsinelli, Brierley, & Plum, 1982). In hippocampal slices, as time following

OGD insult increases, blockage of NMDA receptors with the antagonist MK-801 becomes increasingly less protective (Ahlgren et al., 2011). This suggests that there is early excitotoxic injury as a result of NMDA receptor activation, followed by damage mediated through other mechanisms. Briefly, cell death following an acute hypoxic challenge begins with energy depletion and neuronal release of glutamate coupled with impaired glutamate uptake by glia, resulting in initial excitotoxicity. Delayed neuronal damage begins 8 – 48 hours after the challenge, and involves an accumulation of mitochondrial injury and release of pro-apoptotic molecules that signal for cell death; for review see (Rees, Harding, & Walker, 2011); differences in results from Exp 1 and Exp 2 could be explained in part by the mechanism of the damage and the time point that these measures were assessed.

While delayed neuronal damage following OGD may explain why an interaction was not found 24 hours following OGD with NeuN, it does not account for the interactive damage observed with PI. PI is a nonspecific marker of cell damage that is incorporated into the DNA of any cell type with a compromised membrane and the interaction found in this study likely involves not only neurons but glia as well. White matter damage is classically associated with hypoxia/ischemia in infants and there is evidence that glial cells, specifically the oligiodendrocytes that make up myelin, experience rapid cell death after hypoxic insult; for review see (Matute et al., 2001; Rees et al., 2011). Unlike neurons, these cells express AMPA and kainite but not NMDA receptors and their subunit composition make them especially sensitive to excessive glutamate activation

(Tekkok, Ye, & Ransom, 2007); for review see (Matute et al., 2001). There is also evidence that astroglia are damaged prior to neurons following hypoxia and that they experience early functional changes that contribute to delayed neuronal damage (Ouyang, Volobouéva, Xu, & Giffard, 2007). In PND 8 hippocampal cultures, ETOH withdrawal produces a loss in astroglia after 24 hours, specifically in the CA1 and DG (Wilkins et al., 2006). These effects of ETOH withdrawal and OGD on glia suggest that the interaction found with PI in the present study is likely a result of the combination of neuronal and glial damage and future research should include a glial marker.

General Discussion

The purpose of this study was to determine if subthreshold levels of ETOH exposure and hypoxia (OGD) interact to produce increases in cellular damage. Experiment 1 is the first study to demonstrate that there is an interaction between ETOH and hypoxia exposures that by themselves, do not produce damage as measured by PI uptake in the developing hippocampus. Such an interaction may explain why there is large variability in outcome following fetal alcohol exposure in the clinical population. For example, a child exposed to only subthreshold levels of alcohol during development may not experience overt deficits. However, a child exposed to the same levels of alcohol during development that is then subjected to a hypoxic challenge could experience CNS damage that results in cognitive and behavioral impairments.

According to Abel & Hannigan (1995), various environmental and biological factors that accompany alcohol exposure may explain why the FAS phenotype exists in only a portion of exposed offspring. Maternal pattern of consumption, smoking, culture/ socioeconomic status, and genetics all have an impact on the effects of alcohol (Abel & Hannigan, 1995; Abel & Sokol, 1986). Much of the cellular/molecular damage and subsequent impairments following fetal alcohol exposure could be the result of the co-occurrence of alcohol exposure and hypoxia (Abel & Hannigan, 1995). In other words, hypoxia could be a contributing factor to the severity of FASDs. Future *in vivo* examination of the interaction between ETOH withdrawal and hypoxia will provide valuable information on the possible deficits caused by the combination of these insults.

Future Models

OHSCs have been used extensively as a model of hypoxia/ischemia and as models for fetal alcohol exposure, with results that are similar to those seen *in vivo*; for review see (Noraberg et al., 2005). Given this overlap, it is reasonable to assume that the interaction observed in this study may also be present in an *in vivo* model of fetal ETOH exposure and hypoxia. Studies on the effects of both fetal ETOH and on hypoxia/ischemia have utilized neonatal rat models. In rodents, the first week of neonatal CNS development is thought to overlap with the 3rd trimester human brain growth spurt (Dobbing & Sands, 1979), a time when the CNS is especially sensitive to toxic insults. ETOH exposure during this period can lead to impairments similar to those seen in children exposed to alcohol during gestation (Lewis et al., 2007; Lewis et al., 2012; Rubin et al., 2009;

Wellmann et al., 2010). Similarly, hypoxic/ischemic challenges during this first week of neonatal development produces deficits that mirror those in the clinical population (Balduini, De Angelis, Mazzoni, & Cimino, 2000; Cai et al., 2009; Fan et al., 2005; Ikeda et al., 2002; Tang & Nakazawa, 2005).

Clinically, the risk of hypoxic episodes increases during parturition (R. C. Vannucci, 1990). In a fetus that has been exposed to alcohol, this is also the time when withdrawal is likely to begin. In the current study, hypoxic exposure occurred at the initiation of ETOH withdrawal, mimicking events that could occur in the human population and indicating that this is a potentially critical period of CNS injury. An *in vivo* model of 3rd trimester ETOH exposure with an acute hypoxic challenge during ETOH withdrawal could potentially capture the interaction. Targeting cell damage during withdrawal has previously been shown to protect against a variety behavioral deficits following neonatal ETOH exposure (Lewis et al., 2012; Rubin et al., 2009) and, in the same respect, certain therapies, when administered shortly following hypoxic/ischemia, reduce subsequent impairments (Cai et al., 2009) and brain damage (Hattori, Morin, Schwartz, Fujikawa, & Wasterlain, 1989). Agents that offer neuroprotection against the combination of ETOH/OGD in hippocampal slices could easily be assessed in an *in vivo* model.

Potential Targets/Mechanisms

Preventing cellular damage that occurs during ETOH withdrawal and following hypoxia could protect against subsequent cognitive and behavioral

deficits. Pharmacological agents that modulate NMDA receptor activity may be especially efficacious, given the potential role these receptors have in the interactive damage observed in this study. Compounds that have inhibitory effects on NMDA receptors reduce deficits following fetal alcohol exposure (Lewis et al., 2012). There is mixed data regarding efficacy of NMDA receptor antagonism following hypoxia/ischemia; [for review see (Albers, Goldberg, & Choi, 1989b; Mcculloch, 1992)] which may be due, in part, to the important role of glial cell types which express other ionotropic glutamate receptors (McDonald, Althomsons, Hyrc, Choi, & Goldberg, 1998; Tekkok & Goldberg, 2001; Tekkok et al., 2007). Reducing excitotoxicity following the combination of ETOH withdrawal and hypoxia may thus require modulation of more than one receptor type.

Limitations

As with any experiment, this study has several limitations that could influence interpretation of results. First, fluorescent measurement is only semiquantative method of cell damage (PI) or neuronal viability (NeuN) analysis. To supplement fluorescent intensity data, area analysis was performed. Measurements of the area of PI fluorescence for each slice above a relatively low threshold (Loetscher et al., 2009) show that the combination of ETOH and OGD produces a marked increase total area of the slice expressing PI above a baseline level of intensity in both PND 8 and PND 2 tissue, providing further support for the hypothesis. Similar analysis done for Exp 2, showed that total area of NeuN fluorescence was reduced by ETOH treatment, coinciding with reduced fluorescent intensity in ETOH exposed slices.

The results from Exp 2 present an important implication and possible limitation to the current study; the severity of the 100 mM dose of ETOH is dependent on the marker being used. ETOH reduced NeuN immunoreactivity below control levels, indicating significant damage as a result of ETOH exposure alone. This suggests that 100mM may not be a mild/ subthreshold dose of ETOH and that using a lower concentration of ETOH and multiple markers should be considered for future studies.

Finally, it is important to note that the combination of subthreshold ETOH and OGD producing increases in PI uptake could simply be an additive effect and not a synergistic effect. Synergy is difficult to prove and has underlying implications as to the mechanisms through which the interaction is occurring [for brief review see (Greco, Faessel, & Levasseur, 1996)]. Even if the effect is purely additive, it is very clinically relevant as it can help explain variation seen in children exposed to alcohol during fetal development.

Conclusions

In summary, the present study demonstrated an interactive effect of ETOH withdrawal and hypoxia in the developing hippocampus. This likely involves multiple cell types as well as various mechanisms of damage, as indicated by discrepancies between the two stains used. An interaction of this nature could explain some of the variation in outcome seen in children following fetal alcohol exposure and specifically targeting the mechanisms that underlie these insults could offer protection for children at risk.

References

Abel, E. L., & Hannigan, J. H. (1995). Maternal Risk-Factors in Fetal-Alcohol-Syndrome - Provocative and Permissive Influences. *Neurotoxicol Teratol, 17*(4), 445-462. doi: Doi 10.1016/0892-0362(95)98055-6

Abel, E. L., & Sokol, R. J. (1986). Maternal and fetal characteristics affecting alcohol's teratogenicity. [Research Support, U.S. Gov't, P.H.S. Review]. *Neurobehav Toxicol Teratol, 8*(4), 329-334.

Ahlgren, H., Henjum, K., Ottersen, O. P., & Runden-Pran, E. (2011). Validation of organotypical hippocampal slice cultures as an ex vivo model of brain ischemia: different roles of NMDA receptors in cell death signalling after exposure to NMDA or oxygen and glucose deprivation. *Cell and Tissue Research, 345*(3), 329-341. doi: DOI 10.1007/s00441-011-1218-2

Albers, G. W., Goldberg, M. P., & Choi, D. W. (1989a). N-methyl-D-aspartate antagonists: ready for clinical trial in brain ischemia? [Clinical Trial Review]. *Ann Neurol, 25*(4), 398-403. doi: 10.1002/ana.410250412

Albers, G. W., Goldberg, M. P., & Choi, D. W. (1989b). N-methyl-D-aspartate antagonists: ready for clinical trial in brain ischemia? [Clinical Trial Review]. *Annals of Neurology, 25*(4), 398-403. doi: 10.1002/ana.410250412

Alfonso-Loeches, S., & Guerri, C. (2011). Molecular and behavioral aspects of the actions of alcohol on the adult and developing brain. [Research

Support, Non-U.S. Gov't Review]. *Crit Rev Clin Lab Sci, 48*(1), 19-47. doi: 10.3109/10408363.2011.580567

Archibald, S. L., Fennema-Notestine, C., Gamst, A., Riley, E. P., Mattson, S. N., & Jernigan, T. L. (2001). Brain dysmorphology in individuals with severe prenatal alcohol exposure. [Research Support, U.S. Gov't, Non-P.H.S. Research Support, U.S. Gov't, P.H.S.]. *Dev Med Child Neurol, 43*(3), 148-154.

Balduini, W., De Angelis, V., Mazzoni, E., & Cimino, M. (2000). Long-lasting behavioral alterations following a hypoxic/ischemic brain injury in neonatal rats. *Brain Res, 859*(2), 318-325.

Barron, S., Mulholland, P. J., Littleton, J. M., & Prendergast, M. A. (2008). Age and gender differences in response to neonatal ethanol withdrawal and polyamine challenge in organotypic hippocampal cultures. [Comparative Study Research Support, N.I.H., Extramural]. *Alcohol Clin Exp Res, 32*(6), 929-936. doi: 10.1111/j.1530-0277.2008.00649.x

Berman, R. F., & Hannigan, J. H. (2000). Effects of prenatal alcohol exposure on the hippocampus: spatial behavior, electrophysiology, and neuroanatomy. [Research Support, U.S. Gov't, P.H.S. Review]. *Hippocampus, 10*(1), 94-110. doi: 10.1002/(SICI)1098-1063(2000)10:1<94::AID-HIPO11>3.0.CO;2-T

Boksa, P., Krishnamurthy, A., & Brooks, W. (1995). Effects of a period of asphyxia during birth on spatial learning in the rat. [Research Support,

Non-U.S. Gov't]. *Pediatr Res, 37*(4 Pt 1), 489-496. doi: 10.1203/00006450-199504000-00018

Bosco, C., & Diaz, E. (2012). Placental hypoxia and foetal development versus alcohol exposure in pregnancy. [Research Support, Non-U.S. Gov't Review]. *Alcohol Alcohol, 47*(2), 109-117. doi: 10.1093/alcalc/agr166

Brewer, L. D., Thibault, O., Staton, J., Thibault, V., Rogers, J. T., Garcia-Ramos, G., . . . Porter, N. M. (2007). Increased vulnerability of hippocampal neurons with age in culture: Temporal association with increases in NMDA receptor current, NR2A subunit expression and recruitment of L-type calcium channels. *Brain Res, 1151*, 20-31. doi: DOI 10.1016/j.brainres.2007.03.020

Brown, M. M., Wade, J. P., & Marshall, J. (1985). Fundamental importance of arterial oxygen content in the regulation of cerebral blood flow in man. [Research Support, Non-U.S. Gov't]. *Brain, 108 (Pt 1)*, 81-93.

Burd, L., Roberts, D., Olson, M., & Odendaal, H. (2007). Ethanol and the placenta: A review. [Research Support, N.I.H., Extramural Review]. *J Matern Fetal Neonatal Med, 20*(5), 361-375. doi: 10.1080/14767050701298365

Butler, T. R., Self, R. L., Smith, K. J., Sharrett-Field, L. J., Berry, J. N., Littleton, J. M., . . . Prendergast, M. A. (2010). Selective vulnerability of hippocampal cornu ammonis 1 pyramidal cells to excitotoxic insult is associated with the expression of polyamine-sensitive N-methyl-D-asparate-type

glutamate receptors. [In Vitro Research Support, N.I.H., Extramural]. *Neuroscience, 165*(2), 525-534. doi: 10.1016/j.neuroscience.2009.10.018

Cai, J., Kang, Z., Liu, K., Liu, W., Li, R., Zhang, J. H., . . . Sun, X. (2009). Neuroprotective effects of hydrogen saline in neonatal hypoxia-ischemia rat model. *Brain Res, 1256*, 129-137. doi: 10.1016/j.brainres.2008.11.048

Camacho, A., & Massieu, L. (2006). Role of glutamate transporters in the clearance and release of glutamate during ischemia and its relation to neuronal death. *Archives of Medical Research, 37*(1), 11-18. doi: DOI 10.1016/j.arcmed.2005.05.014

Cheng, C., Fass, D. M., & Reynolds, I. J. (1999). Emergence of excitotoxicity in cultured forebrain neurons coincides with larger glutamate-stimulated [Ca(2+)](i) increases and NMDA receptor mRNA levels. [Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S.]. *Brain Res, 849*(1-2), 97-108.

Cimarosti, H., & Henley, J. M. (2008). Investigating the Mechanisms Underlying Neuronal Death in Ischemia Using In Vitro Oxygen-Glucose Deprivation: Potential Involvement of Protein SUMOylation. *Neuroscientist, 14*(6), 626-636. doi: Doi 10.1177/1073858408322677

Collingridge, G. L., & Bliss, T. V. P. (1987). Nmda Receptors - Their Role in Long-Term Potentiation. *Trends in Neurosciences, 10*(7), 288-293. doi: Doi 10.1016/0166-2236(87)90175-5

Coultrap, S. J., Nixon, K. M., Alvestad, R. M., Valenzuela, C. F., & Browning, M. D. (2005). Differential expression of NMDA receptor subunits and splice

variants among the CA1, CA3 and dentate gyrus of the adult rat. [Comparative Study Research Support, N.I.H., Extramural Research Support, U.S. Gov't, P.H.S.]. *Brain Res Mol Brain Res, 135*(1-2), 104-111. doi: 10.1016/j.molbrainres.2004.12.005

Crews, F. T., & Nixon, K. (2009). Mechanisms of neurodegeneration and regeneration in alcoholism. [Review]. *Alcohol Alcohol, 44*(2), 115-127. doi: 10.1093/alcalc/agn079

Davolio, C., & Greenamyre, J. T. (1995). Selective vulnerability of the CA1 region of hippocampus to the indirect excitotoxic effects of malonic acid. [Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S.]. *Neurosci Lett, 192*(1), 29-32.

Decker, M. J., Hue, G. E., Caudle, W. M., Miller, G. W., Keating, G. L., & Rye, D. B. (2003). Episodic neonatal hypoxia evokes executive dysfunction and regionally specific alterations in markers of dopamine signaling. [Comparative Study Research Support, U.S. Gov't, P.H.S.]. *Neuroscience, 117*(2), 417-425.

Dobbing, J., & Sands, J. (1979). Comparative aspects of the brain growth spurt. *Early Hum Dev, 3*(1), 79-83.

Dong, Y., Zhang, W., Lai, B., Luan, W. J., Zhu, Y. H., Zhao, B. Q., & Zheng, P. (2012). Two free radical pathways mediate chemical hypoxia-induced glutamate release in synaptosomes from the prefrontal cortex. [Research

Support, Non-U.S. Gov't]. *Biochim Biophys Acta, 1823*(2), 493-504. doi: 10.1016/j.bbamcr.2011.10.004

Durand, G. M., Bennett, M. V., & Zukin, R. S. (1993). Splice variants of the N-methyl-D-aspartate receptor NR1 identify domains involved in regulation by polyamines and protein kinase C. [Research Support, U.S. Gov't, P.H.S.]. *Proc Natl Acad Sci U S A, 90*(14), 6731-6735.

Fahey, J., & King, T. L. (2005). Intrauterine asphyxia: clinical implications for providers of intrapartum care. [Review]. *J Midwifery Womens Health, 50*(6), 498-506. doi: 10.1016/j.jmwh.2005.08.007

Fan, L. W., Lin, S., Pang, Y., Lei, M., Zhang, F., Rhodes, P. G., & Cai, Z. (2005). Hypoxia-ischemia induced neurological dysfunction and brain injury in the neonatal rat. [Comparative Study Research Support, N.I.H., Extramural]. *Behav Brain Res, 165*(1), 80-90. doi: 10.1016/j.bbr.2005.06.033

Follesa, P., & Ticku, M. K. (1995). Chronic ethanol treatment differentially regulates NMDA receptor subunit mRNA expression in rat brain. [Research Support, U.S. Gov't, P.H.S.]. *Brain Res Mol Brain Res, 29*(1), 99-106.

Fryer, S. L., Schweinsburg, B. C., Bjorkquist, O. A., Frank, L. R., Mattson, S. N., Spadoni, A. D., & Riley, E. P. (2009). Characterization of white matter microstructure in fetal alcohol spectrum disorders. [Research Support, N.I.H., Extramural]. *Alcohol Clin Exp Res, 33*(3), 514-521. doi: 10.1111/j.1530-0277.2008.00864.x

Gibson, D. A., Harris, B. R., Prendergast, M. A., Hart, S. R., Blanchard, J. A., Holley, R. C., . . . Littleton, J. M. (2003). Polyamines contribute to ethanol withdrawal-induced neurotoxicity in rat hippocampal slice cultures through interactions with the NMDA receptor. *Alcoholism-Clinical and Experimental Research, 27*(7), 1099-1106. doi: Doi 10.1097/01.Alc.0000075824.10502.Dd

Gill, M. B., & Perez-Polo, J. R. (2008). Hypoxia ischemia-mediated cell death in neonatal rat brain. [Review]. *Neurochem Res, 33*(12), 2379-2389. doi: 10.1007/s11064-008-9649-1

Gleason, C. A., Iida, H., Hotchkiss, K. J., Northington, F. J., & Traystman, R. J. (1997). Newborn cerebrovascular responses after first trimester moderate maternal ethanol exposure in sheep. [Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S.]. *Pediatr Res, 42*(1), 39-45. doi: 10.1203/00006450-199707000-00007

Goodlett, C. R., & Horn, K. H. (2001). Mechanisms of alcohol-induced damage to the developing nervous system. [Review]. *Alcohol Res Health, 25*(3), 175-184.

Greco, W. R., Faessel, H., & Levasseur, L. (1996). The search for cytotoxic synergy between anticancer agents: A case of Dorothy and the ruby slippers? *Journal of the National Cancer Institute, 88*(11), 699-700. doi: DOI 10.1093/jnci/88.11.699

Halliwell, B. (1992). Reactive oxygen species and the central nervous system. [Review]. *J Neurochem, 59*(5), 1609-1623.

Hattori, H., Morin, A. M., Schwartz, P. H., Fujikawa, D. G., & Wasterlain, C. G. (1989). Posthypoxic treatment with MK-801 reduces hypoxic-ischemic damage in the neonatal rat. [Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, Non-P.H.S. Research Support, U.S. Gov't, P.H.S.]. *Neurology, 39*(5), 713-718.

Hoffman, P. L. (1995). Glutamate receptors in alcohol withdrawal-induced neurotoxicity. [Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S.]. *Metab Brain Dis, 10*(1), 73-79.

Hu, X. J., & Ticku, M. K. (1995). Chronic ethanol treatment upregulates the NMDA receptor function and binding in mammalian cortical neurons. [Research Support, U.S. Gov't, P.H.S.]. *Brain Res Mol Brain Res, 30*(2), 347-356.

Hunt, P. S., Jacobson, S. E., & Torok, E. J. (2009). Deficits in trace fear conditioning in a rat model of fetal alcohol exposure: dose-response and timing effects. [Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't]. *Alcohol, 43*(6), 465-474. doi: 10.1016/j.alcohol.2009.08.004

Idrus, N. M., McGough, N. N., Riley, E. P., & Thomas, J. D. (2011). Administration of memantine during ethanol withdrawal in neonatal rats: effects on long-term ethanol-induced motor incoordination and cerebellar Purkinje cell loss. [Research Support, N.I.H., Extramural Research

Support, Non-U.S. Gov't]. *Alcohol Clin Exp Res, 35*(2), 355-364. doi: 10.1111/j.1530-0277.2010.01351.x

Ikeda, T., Mishima, K., Aoo, N., Egashira, N., Iwasaki, K., Fujiwara, M., & Ikenoue, T. (2004). Combination treatment of neonatal rats with hypoxia-ischemia and endotoxin induces long-lasting memory and learning impairment that is associated with extended cerebral damage. [Comparative Study]. *Am J Obstet Gynecol, 191*(6), 2132-2141. doi: 10.1016/j.ajog.2004.04.039

Ikeda, T., Mishima, K., Yoshikawa, T., Iwasaki, K., Fujiwara, M., Xia, Y. X., & Ikenoue, T. (2002). Dexamethasone prevents long-lasting learning impairment following neonatal hypoxic-ischemic brain insult in rats. *Behav Brain Res, 136*(1), 161-170.

Johnston, M. V. (2001). Excitotoxicity in neonatal hypoxia. [Comparative Study Research Support, U.S. Gov't, P.H.S.]. *Ment Retard Dev Disabil Res Rev, 7*(4), 229-234. doi: 10.1002/mrdd.1032

Jones, K. L. (2011). The effects of alcohol on fetal development. [Review]. *Birth Defects Res C Embryo Today, 93*(1), 3-11. doi: 10.1002/bdrc.20200

Jones, K. L., & Smith, D. W. (1973). Recognition of the fetal alcohol syndrome in early infancy. *Lancet, 302*(7836), 999-1001.

Juarez, I., Gratton, A., & Flores, G. (2008). Ontogeny of altered dendritic morphology in the rat prefrontal cortex, hippocampus, and nucleus accumbens following Cesarean delivery and birth anoxia. [Research

Support, Non-U.S. Gov't]. *J Comp Neurol, 507*(5), 1734-1747. doi: 10.1002/cne.21651

Karalis, F., Soubasi, V., Georgiou, T., Nakas, C. T., Simeonidou, C., Guiba-Tziampiri, O., & Spandou, E. (2011). Resveratrol ameliorates hypoxia/ischemia-induced behavioral deficits and brain injury in the neonatal rat brain. *Brain Res, 1425*, 98-110. doi: 10.1016/j.brainres.2011.09.044

Kenna, K., De Matteo, R., Hanita, T., Rees, S., Sozo, F., Stokes, V., . . . Harding, R. (2011). Daily ethanol exposure during late ovine pregnancy: physiological effects in the mother and fetus in the apparent absence of overt fetal cerebral dysmorphology. [Research Support, Non-U.S. Gov't]. *Am J Physiol Regul Integr Comp Physiol, 301*(4), R926-936. doi: 10.1152/ajpregu.00711.2010

Kim, K. K., Adelstein, R. S., & Kawamoto, S. (2009). Identification of Neuronal Nuclei (NeuN) as Fox-3, a New Member of the Fox-1 Gene Family of Splicing Factors. *Journal of Biological Chemistry, 284*(45), 31052-31061. doi: DOI 10.1074/jbc.M109.052969

Kully-Martens, K., Denys, K., Treit, S., Tamana, S., & Rasmussen, C. (2012). A review of social skills deficits in individuals with fetal alcohol spectrum disorders and prenatal alcohol exposure: profiles, mechanisms, and interventions. [Review]. *Alcohol Clin Exp Res, 36*(4), 568-576. doi: 10.1111/j.1530-0277.2011.01661.x

Kumari, M., & Ticku, M. K. (2000). Regulation of NMDA receptors by ethanol. [Review]. *Prog Drug Res, 54*, 152-189.

Lemoine, P., Harousseau, H., Borteyru, J. P., & Menuet, J. C. (2003). Children of alcoholic parents - Observed anomalies: Discussion of 127 cases (Reprinted from Quest Med, vol 8, pg 476-482, 1968). *Therapeutic Drug Monitoring, 25*(2), 132-136. doi: Doi 10.1097/00007691-200304000-00002

Lewis, B., Wellmann, K. A., & Barron, S. (2007). Agmatine reduces balance deficits in a rat model of third trimester binge-like ethanol exposure. [Research Support, N.I.H., Extramural]. *Pharmacol Biochem Behav, 88*(1), 114-121. doi: 10.1016/j.pbb.2007.07.012

Lewis, B., Wellmann, K. A., Kehrberg, A. M., Carter, M. L., Baldwin, T., Cohen, M., & Barron, S. (2012). Behavioral deficits and cellular damage following developmental ethanol exposure in rats are attenuated by CP-101,606, an NMDAR antagonist with unique NR2B specificity. [Research Support, N.I.H., Extramural]. *Pharmacol Biochem Behav, 100*(3), 545-553. doi: 10.1016/j.pbb.2011.10.013

Littleton, J. M., Lovinger, D., Liljequist, S., Ticku, R., Matsumoto, I., & Barron, S. (2001). Role of polyamines and NMDA receptors in ethanol dependence and withdrawal. [Review]. *Alcohol Clin Exp Res, 25*(5 Suppl ISBRA), 132S-136S.

Livy, D. J., Miller, E. K., Maier, S. E., & West, J. R. (2003). Fetal alcohol exposure and temporal vulnerability: effects of binge-like alcohol exposure

on the developing rat hippocampus. [Comparative Study Research Support, U.S. Gov't, P.H.S.]. *Neurotoxicol Teratol, 25*(4), 447-458.

Loetscher, P. D., Rossaint, J., Rossaint, R., Weis, J., Fries, M., Fahlenkamp, A., . . . Coburn, M. (2009). Argon: Neuroprotection in in vitro models of cerebral ischemia and traumatic brain injury. *Critical Care, 13*(6). doi: Artn R206 Doi 10.1186/Cc8214

Lovinger, D. M. (1993). Excitotoxicity and Alcohol-Related Brain-Damage. *Alcoholism-Clinical and Experimental Research, 17*(1), 19-27. doi: DOI 10.1111/j.1530-0277.1993.tb00720.x

Low, J. A. (2004). Reflections on the occurrence and significance of antepartum fetal asphyxia. [Review]. *Best Pract Res Clin Obstet Gynaecol, 18*(3), 375-382. doi: 10.1016/j.bpobgyn.2004.02.002

Mattson, S. N., & Riley, E. P. (1998). A review of the neurobehavioral deficits in children with fetal alcohol syndrome or prenatal exposure to alcohol. [Research Support, U.S. Gov't, P.H.S. Review]. *Alcohol Clin Exp Res, 22*(2), 279-294.

Mattson, S. N., Riley, E. P., Gramling, L., Delis, D. C., & Jones, K. L. (1998). Neuropsychological comparison of alcohol-exposed children with or without physical features of fetal alcohol syndrome. [Clinical Trial Comparative Study Research Support, U.S. Gov't, P.H.S.]. *Neuropsychology, 12*(1), 146-153.

Mattson, S. N., Riley, E. P., Jernigan, T. L., Garcia, A., Kaneko, W. M., Ehlers, C. L., & Jones, K. L. (1994). A decrease in the size of the basal ganglia following prenatal alcohol exposure: a preliminary report. [Comparative Study Research Support, U.S. Gov't, Non-P.H.S. Research Support, U.S. Gov't, P.H.S.]. *Neurotoxicol Teratol, 16*(3), 283-289.

Mattson, S. N., Roesch, S. C., Fagerlund, A., Autti-Ramo, I., Jones, K. L., May, P. A., . . . Riley, E. P. (2010). Toward a neurobehavioral profile of fetal alcohol spectrum disorders. [Evaluation Studies Multicenter Study Research Support, N.I.H., Extramural]. *Alcohol Clin Exp Res, 34*(9), 1640-1650. doi: 10.1111/j.1530-0277.2010.01250.x

Matute, C., Alberdi, E., Domercq, M., Perez-Cerda, F., Perez-Samartin, A., & Sanchez-Gomez, M. V. (2001). The link between excitotoxic oligodendroglial death and demyelinating diseases. *Trends in Neurosciences, 24*(4), 224-230. doi: Doi 10.1016/S0166-2236(00)01746-X

May, P. A., Gossage, J. P., Kalberg, W. O., Robinson, L. K., Buckley, D., Manning, M., & Hoyme, H. E. (2009). Prevalence and Epidemiologic Characteristics of Fasd from Various Research Methods with an Emphasis on Recent in-School Studies. *Dev Disabil Res Rev, 15*(3), 176-192. doi: Doi 10.1002/Ddrr.68

Mayer, S., Harris, B. R., Gibson, D. A., Blanchard, J. A., Prendergast, M. A., Holley, R. C., & Littleton, J. (2002). Acamprosate, MK-801, and ifenprodil inhibit neurotoxicity and calcium entry induced by ethanol withdrawal in organotypic slice cultures from neonatal rat hippocampus. [In Vitro

Research Support, Non-U.S. Gov't]. *Alcohol Clin Exp Res, 26*(10), 1468-1478. doi: 10.1097/01.ALC.0000033261.14548.D2

Mayock, D. E., Ness, D., Mondares, R. L., & Gleason, C. A. (2007). Binge alcohol exposure in the second trimester attenuates fetal cerebral blood flow response to hypoxia. [Research Support, N.I.H., Extramural]. *J Appl Physiol, 102*(3), 972-977. doi: 10.1152/japplphysiol.00956.2006

Mcculloch, J. (1992). Excitatory Amino-Acid Antagonists and Their Potential for the Treatment of Ischemic Brain-Damage in Man. *British Journal of Clinical Pharmacology, 34*(2), 106-114.

McDonald, J. W., Althomsons, S. P., Hyrc, K. L., Choi, D. W., & Goldberg, M. P. (1998). Oligodendrocytes from forebrain are highly vulnerable to AMPA/kainate receptor-mediated excitotoxicity. *Nature Medicine, 4*(3), 291-297. doi: Doi 10.1038/Nm0398-291

Michaelis, E. K. (1998). Molecular biology of glutamate receptors in the central nervous system and their role in excitotoxicity, oxidative stress and aging. *Progress in Neurobiology, 54*(4), 369-415. doi: Doi 10.1016/S0301-0082(97)00055-5

Mistovich, J. J., Krost, W. S., & Limmer, D. D. (2006). Beyond the basics: obstetric emergencies. *Emerg Med Serv, 35*(11), 61-65; quiz 66-67.

Mulholland, P. J., Stepanyan, T. D., Self, R. L., Hensley, A. K., Harris, B. R., Kowalski, A., . . . Prendergast, M. A. (2005). Corticosterone and dexamethasone potentiate cytotoxicity associated with oxygen-glucose

deprivation in organotypic cerebellar slice cultures. [In Vitro]. *Neuroscience, 136*(1), 259-267. doi: 10.1016/j.neuroscience.2005.07.043

Muller, D., Buchs, P. A., & Stoppini, L. (1993). Time course of synaptic development in hippocampal organotypic cultures. [Research Support, Non-U.S. Gov't]. *Brain Res Dev Brain Res, 71*(1), 93-100.

Nagy, J., Kolok, S., Dezso, P., Boros, A., & Szombathelyi, Z. (2003). Differential alterations in the expression of NMDA receptor subunits following chronic ethanol treatment in primary cultures of rat cortical and hippocampal neurones. [Comparative Study]. *Neurochem Int, 42*(1), 35-43.

Nitatori, T., Sato, N., Waguri, S., Karasawa, Y., Araki, H., Shibanai, K., . . . Uchiyama, Y. (1995). Delayed Neuronal Death in the Ca1 Pyramidal Cell Layer of the Gerbil Hippocampus Following Transient Ischemia Is Apoptosis. *Journal of Neuroscience, 15*(2), 1001-1011.

Nixon, K., Hughes, P. D., Amsel, A., & Leslie, S. W. (2002). NMDA receptor subunit expression following early postnatal exposure to ethanol. *Developmental Brain Research, 139*(2), 295-299. doi: Pii S0165-3806(02)00515-1 Doi 10.1016/S0165-3806(02)00515-1

Nixon, K., Hughes, P. D., Amsel, A., & Leslie, S. W. (2004). NMDA receptor subunit expression after combined prenatal and postnatal exposure to ethanol. *Alcoholism-Clinical and Experimental Research, 28*(1), 105-112. doi: Doi 10.1097/01.Alc.0000106311.88523.7b

Noraberg, J., Poulsen, F. R., Blaabjerg, M., Kristensen, B. W., Bonde, C., Montero, M., . . . Zimmer, J. (2005). Organotypic hippocampal slice

cultures for studies of brain damage, neuroprotection and neurorepair. [Research Support, Non-U.S. Gov't Review]. *Curr Drug Targets CNS Neurol Disord, 4*(4), 435-452.

Norris, C. M., Blalock, E. M., Thibault, O., Brewer, L. D., Clodfelter, G. V., Porter, N. M., & Landfield, P. W. (2006). Electrophysiological mechanisms of delayed excitotoxicity: positive feedback loop between NMDA receptor current and depolarization-mediated glutamate release. [Research Support, N.I.H., Extramural]. *J Neurophysiol, 96*(5), 2488-2500. doi: 10.1152/jn.00593.2005

Ouyang, Y. B., Voloboueva, L. A., Xu, L. J., & Giffard, R. G. (2007). Selective dysfunction of hippocampal CA1 astrocytes contributes to delayed neuronal damage after transient forebrain ischemia. *Journal of Neuroscience, 27*(16), 4253-4260. doi: Doi 10.1523/Jneurosci.0211-07.2007

Paintner, A., Williams, A. D., & Burd, L. (2012). Fetal alcohol spectrum disorders--implications for child neurology, part 1: prenatal exposure and dosimetry. [Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't Review]. *J Child Neurol, 27*(2), 258-263. doi: 10.1177/0883073811428376

Peadon, E., & Elliott, E. J. (2010). Distinguishing between attention-deficit hyperactivity and fetal alcohol spectrum disorders in children: clinical guidelines. *Neuropsychiatr Dis Treat, 6*, 509-515.

Pellegrini-Giampietro, D. E., Bennett, M. V., & Zukin, R. S. (1992). Are Ca(2+)-permeable kainate/AMPA receptors more abundant in immature brain? *Neurosci Lett, 144*(1-2), 65-69.

Prendergast, M. A., Harris, B. R., Mullholland, P. J., Blanchard, J. A., 2nd, Gibson, D. A., Holley, R. C., & Littleton, J. M. (2004). Hippocampal CA1 region neurodegeneration produced by ethanol withdrawal requires activation of intrinsic polysynaptic hippocampal pathways and function of N-methyl-D-aspartate receptors. [In Vitro

Research Support, U.S. Gov't, P.H.S.]. *Neuroscience, 124*(4), 869-877. doi: 10.1016/j.neuroscience.2003.12.013

Pulsinelli, W. A., Brierley, J. B., & Plum, F. (1982). Temporal Profile of Neuronal Damage in a Model of Transient Forebrain Ischemia. *Annals of Neurology, 11*(5), 491-498. doi: DOI 10.1002/ana.410110509

Rees, S., Harding, R., & Walker, D. (2008). An adverse intrauterine environment: implications for injury and altered development of the brain. [Research Support, Non-U.S. Gov't Review]. *Int J Dev Neurosci, 26*(1), 3-11. doi: 10.1016/j.ijdevneu.2007.08.020

Rees, S., Harding, R., & Walker, D. (2011). The biological basis of injury and neuroprotection in the fetal and neonatal brain. *International Journal of Developmental Neuroscience, 29*(6), 551-563. doi: DOI 10.1016/j.ijdevneu.2011.04.004

Riley, E. P., Barron, S., Melcer, T., & Gonzalez, D. (1993). Alterations in activity following alcohol administration during the third trimester equivalent in P

and NP rats. [Research Support, U.S. Gov't, P.H.S.]. *Alcohol Clin Exp Res, 17*(6), 1240-1246.

Riley, E. P., Infante, M. A., & Warren, K. R. (2011). Fetal alcohol spectrum disorders: an overview. [Research Support, N.I.H., Extramural Review]. *Neuropsychol Rev, 21*(2), 73-80. doi: 10.1007/s11065-011-9166-x

Riley, E. P., & McGee, C. L. (2005). Fetal alcohol spectrum disorders: an overview with emphasis on changes in brain and behavior. [Research Support, N.I.H., Extramural Research Support, U.S. Gov't, P.H.S. Review]. *Exp Biol Med (Maywood), 230*(6), 357-365.

Rock, D. M., & Macdonald, R. L. (1995). Polyamine regulation of N-methyl-D-aspartate receptor channels. [Review]. *Annu Rev Pharmacol Toxicol, 35*, 463-482. doi: 10.1146/annurev.pa.35.040195.002335

Rodier, P. M. (1994). Vulnerable periods and processes during central nervous system development. [Review]. *Environ Health Perspect, 102 Suppl 2*, 121-124.

Rubin, M. A., Wellmann, K. A., Lewis, B., Overgaauw, B. J., Littleton, J. M., & Barron, S. (2009). Difluoromethylornithine (DFMO) reduces deficits in isolation-induced ultrasonic vocalizations and balance following neonatal ethanol exposure in rats. [Research Support, N.I.H., Extramural

Research Support, Non-U.S. Gov't]. *Pharmacol Biochem Behav, 92*(1), 44-50. doi: 10.1016/j.pbb.2008.10.008

Saito, K., Packianathan, S., & Longo, L. D. (1997). Free radical-induced elevation of ornithine decarboxylase activity in developing rat brain slices. *Brain Res, 763*(2), 232-238.

Shalak, L., & Perlman, J. M. (2004). Hypoxic-ischemic brain injury in the term infant-current concepts. [Review]. *Early Hum Dev, 80*(2), 125-141. doi: 10.1016/j.earlhumdev.2004.06.003

Smith, A. M., Wellmann, K. A., Lundblad, T. M., Carter, M. L., Barron, S., & Dwoskin, L. P. (2012). Lobeline attenuates neonatal ethanol-mediated changes in hyperactivity and dopamine transporter function in the prefrontal cortex in rats. [Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't]. *Neuroscience, 206*, 245-254. doi: 10.1016/j.neuroscience.2011.11.018

Snell, L. D., Nunley, K. R., Lickteig, R. L., Browning, M. D., Tabakoff, B., & Hoffman, P. L. (1996). Regional and subunit specific changes in NMDA receptor mRNA and immunoreactivity in mouse brain following chronic ethanol ingestion. [Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S.]. *Brain Res Mol Brain Res, 40*(1), 71-78.

Sowell, E. R., Johnson, A., Kan, E., Lu, L. H., Van Horn, J. D., Toga, A. W., . . . Bookheimer, S. Y. (2008). Mapping white matter integrity and neurobehavioral correlates in children with fetal alcohol spectrum disorders. [Comparative Study Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't]. *J Neurosci, 28*(6), 1313-1319. doi: 10.1523/JNEUROSCI.5067-07.2008

Spottiswoode, B. S., Meintjes, E. M., Anderson, A. W., Molteno, C. D., Stanton, M. E., Dodge, N. C., . . . Jacobson, S. W. (2011). Diffusion tensor imaging of the cerebellum and eyeblink conditioning in fetal alcohol spectrum disorder. [Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't]. *Alcohol Clin Exp Res, 35*(12), 2174-2183. doi: 10.1111/j.1530-0277.2011.01566.x

Stepanyan, T. D., Farook, J. M., Kowalski, A., Kaplan, E., Barron, S., & Littleton, J. M. (2008). Alcohol Withdrawal-Induced Hippocampal Neurotoxicity In Vitro and Seizures In Vivo are Both Reduced by Memantine. *Alcoholism-Clinical and Experimental Research, 32*(12), 2128-2135. doi: DOI 10.1111/j.1530-0277.2008.00801.x

Stokowski, L. A. (2004). Fetal alcohol syndrome: new guidelines for referral and diagnosis. [News]. *Adv Neonatal Care, 4*(6), 324.

Stoppini, L., Buchs, P. A., & Muller, D. (1991). A simple method for organotypic cultures of nervous tissue. [Research Support, Non-U.S. Gov't]. *J Neurosci Methods, 37*(2), 173-182.

Streissguth, A. P., Bookstein, F. L., Barr, H. M., Sampson, P. D., O'Malley, K., & Young, J. K. (2004). Risk factors for adverse life outcomes in fetal alcohol syndrome and fetal alcohol effects. [Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S.]. *J Dev Behav Pediatr, 25*(4), 228-238.

Swanson, R. A., Farrell, K., & Simon, R. P. (1995). Acidosis Causes Failure of Astrocyte Glutamate Uptake during Hypoxia. *Journal of Cerebral Blood Flow and Metabolism, 15*(3), 417-424.

Tang, A. C., & Nakazawa, M. (2005). Neonatal novelty exposure ameliorates anoxia-induced hyperactivity in the open field. [Comparative Study Research Support, N.I.H., Extramural Research Support, U.S. Gov't, P.H.S.]. *Behav Brain Res, 163*(1), 1-9. doi: 10.1016/j.bbr.2005.03.025

Tekkok, S. B., & Goldberg, M. P. (2001). AMPA/Kainate receptor activation mediates hypoxic oligodendrocyte death and axonal injury in cerebral white matter. *Journal of Neuroscience, 21*(12), 4237-4248.

Tekkok, S. B., Ye, Z., & Ransom, B. R. (2007). Excitotoxic mechanisms of ischemic injury in myelinated white matter. *Journal of Cerebral Blood Flow and Metabolism, 27*(9), 1540-1552. doi: DOI 10.1038/sj.jcbfm.9600455

Thomas, J. D., Abou, E. J., & Dominguez, H. D. (2009). Prenatal choline supplementation mitigates the adverse effects of prenatal alcohol exposure on development in rats. [Research Support, N.I.H., Extramural]. *Neurotoxicol Teratol, 31*(5), 303-311. doi: 10.1016/j.ntt.2009.07.002

Thomas, J. D., Biane, J. S., O'Bryan, K. A., O'Neill, T. M., & Dominguez, H. D. (2007). Choline supplementation following third-trimester-equivalent alcohol exposure attenuates behavioral alterations in rats. [Comparative Study Research Support, N.I.H., Extramural]. *Behav Neurosci, 121*(1), 120-130. doi: 10.1037/0735-7044.121.1.120

Thomas, J. D., Idrus, N. M., Monk, B. R., & Dominguez, H. D. (2010). Prenatal choline supplementation mitigates behavioral alterations associated with prenatal alcohol exposure in rats. [Research Support, N.I.H., Extramural]. *Birth Defects Res A Clin Mol Teratol, 88*(10), 827-837. doi: 10.1002/bdra.20713

Thomas, R. J. (1995). Excitatory amino acids in health and disease. [Review]. *J Am Geriatr Soc, 43*(11), 1279-1289.

Tiwari, V., Arora, V., & Chopra, K. (2012). Attenuation of NF-kappabeta mediated apoptotic signaling by tocotrienol ameliorates cognitive deficits in rats postnatally exposed to ethanol. *Neurochem Int, 61*(3), 310-320. doi: 10.1016/j.neuint.2012.05.010

Tolcos, M., Harding, R., Loeliger, M., Breen, S., Cock, M., Duncan, J., & Rees, S. (2003). The fetal brainstem is relatively spared from injury following intrauterine hypoxemia. [Comparative Study Research Support, Non-U.S. Gov't]. *Brain Res Dev Brain Res, 143*(1), 73-81.

Vallett, M., Tabatabaie, T., Briscoe, R. J., Baird, T. J., Beatty, W. W., Floyd, R. A., & Gauvin, D. V. (1997). Free radical production during ethanol intoxication, dependence, and withdrawal. [Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S.]. *Alcohol Clin Exp Res, 21*(2), 275-285.

van Handel, M., Swaab, H., de Vries, L. S., & Jongmans, M. J. (2007). Long-term cognitive and behavioral consequences of neonatal encephalopathy

following perinatal asphyxia: a review. [Research Support, Non-U.S. Gov't Review]. *Eur J Pediatr, 166*(7), 645-654. doi: 10.1007/s00431-007-0437-8

Vannucci, R. C. (1990). Experimental biology of cerebral hypoxia-ischemia: relation to perinatal brain damage. [Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S. Review]. *Pediatr Res, 27*(4 Pt 1), 317-326. doi: 10.1203/00006450-199004000-00001

Vannucci, S. J., & Hagberg, H. (2004). Hypoxia-ischemia in the immature brain. [Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S. Review]. *J Exp Biol, 207*(Pt 18), 3149-3154. doi: 10.1242/jeb.01064

Vornov, J. J., Park, J., & Thomas, A. G. (1998). Regional vulnerability to endogenous and exogenous oxidative stress in organotypic hippocampal culture. [Research Support, U.S. Gov't, P.H.S.]. *Exp Neurol, 149*(1), 109-122. doi: 10.1006/exnr.1997.6673

Waxman, E. A., & Lynch, D. R. (2005). N-methyl-D-aspartate receptor subtypes: multiple roles in excitotoxicity and neurological disease. [Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S. Review]. *Neuroscientist, 11*(1), 37-49. doi: 10.1177/1073858404269012

Wellmann, K., Lewis, B., & Barron, S. (2010). Agmatine reduces ultrasonic vocalization deficits in female rat pups exposed neonatally to ethanol. [Research Support, N.I.H., Extramural]. *Neurotoxicol Teratol, 32*(2), 158-163. doi: 10.1016/j.ntt.2009.11.005

Wenzel, A., Fritschy, J. M., Mohler, H., & Benke, D. (1997). NMDA receptor heterogeneity during postnatal development of the rat brain: differential expression of the NR2A, NR2B, and NR2C subunit proteins. [Research Support, Non-U.S. Gov't]. *J Neurochem, 68*(2), 469-478.

Wilkins, L. H., Jr., Prendergast, M. A., Blanchard, J., Holley, R. C., Chambers, E. R., & Littleton, J. M. (2006). Potential value of changes in cell markers in organotypic hippocampal cultures associated with chronic EtOH exposure and withdrawal: comparison with NMDA-induced changes. [Comparative Study Research Support, N.I.H., Extramural]. *Alcohol Clin Exp Res, 30*(10), 1768-1780. doi: 10.1111/j.1530-0277.2006.00210.x

Won, M. H., Kang, T. C., Jeon, G. S., Lee, J. C., Kim, D. Y., Choi, E. M., . . . Cho, S. S. (1999). Immunohistochemical detection of oxidative DNA damage induced by ischemia-reperfusion insults in gerbil hippocampus in vivo. [Research Support, Non-U.S. Gov't]. *Brain Res, 836*(1-2), 70-78.

Won, M. H., Kang, T. C., Park, S. K., Jeon, G. S., Kim, Y. W., Seo, J. H., . . . Cho, S. S. (2001). The alterations of N-Methyl-D-aspartate receptor expressions and oxidative DNA damage in the CA1 area at the early time after ischemia-reperfusion insult. *Neurosci Lett, 301*(2), 139-142. doi: Doi 10.1016/S0304-3940(01)01625-1

Xu, G. P., Dave, K. R., Vivero, R., Schmidt-Kastner, R., Sick, T. J., & Perez-Pinzon, M. A. (2002). Improvement in neuronal survival after ischemic preconditioning in hippocampal slice cultures. [In Vitro Research Support,

U.S. Gov't, Non-P.H.S. Research Support, U.S. Gov't, P.H.S.]. *Brain Res, 952*(2), 153-158.

Zhao, H., Cai, Y., Yang, Z., He, D., & Shen, B. (2011). Acidosis leads to neurological disorders through overexciting cortical pyramidal neurons. [Research Support, Non-U.S. Gov't]. *Biochem Biophys Res Commun, 415*(2), 224-228. doi: 10.1016/j.bbrc.2011.08.008

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  - Lewis, B., Wellmann, K.A., Kehrberg, A.M.H., **Carter, M.L.**, Baldwin, T., Cohen, M., & Barron, S. Behavioral deficits and cellular damage following developmental ethanol exposure in rats are attenuated by CP-101,606, an NMDAR antagonist with unique

NR2B specificity. *Pharmacology, biochemistry, and behavior*. 2012 Jan;100(3):545-53.

- Wellmann, K.A**.,** Lewis, B., **Carter, M.L**., & Barron, S. Neonatal ethanol exposure alters ultrasonic vocalizations in neonatal and periadolescent rats and agmatine reduces these deficits. *Accepted pending revisions. Developmental Psychobiology*
- Barron, S., Lewis, B., Wellmann, K.A., **Carter, M.L.,** Farook, J., Ring, J., Rogers, D., Holley, R., Crooks, P., & Littleton, J. Polyamine modulation of NMDARs as a mechanism to reduce effects of alcohol dependence. *In press: Recent Patents on CNS drug Discovery: Special Issue on Alcohol*

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