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THE EFFECTS OF ALCOHOL AND HYPOXIA EXPOSURE IN THE DEVELOPING BRAIN

Megan L. Carter
University of Kentucky, megancarter84@gmail.com

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Megan L. Carter, Student

Dr. Susan Barron, Major Professor

Dr. Mark Fillmore, Director of Graduate Studies

THE EFFECTS OF ALCOHOL AND HYPOXIA EXPOSURE IN THE DEVELOPING BRAIN

DISSERTATION

A dissertation has been submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Arts and Sciences at the University of Kentucky

By
Megan L. Carter

Lexington, Kentucky

Director: Dr. Susan Barron, Professor of Psychology

Lexington, Kentucky

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ABSTRACT OF DISSERTATION

THE EFFECTS OF ALCOHOL AND HYPOXIA EXPOSURE IN THE DEVELOPING BRAIN

Exposure to alcohol (ethanol (ETOH)) during fetal development results in a variety of cognitive/behavioral deficits. The effects of fetal ETOH exposure vary across individuals and numerous studies have shown that ETOH's interaction with other variables can affect outcome in offspring. These studies investigated the effects of developmental ETOH and hypoxia. It was hypothesized that a history of ETOH during development alters the response to a subsequent hypoxic challenge, such as that which may occur during parturition; this results in central nervous system (CNS) damage and behavioral deficits.

The first study determined if developmental ETOH and hypoxia exposure produced behavioral deficits in a 3rd trimester rodent model. Prior research indicated that chronic ETOH exposure followed by a brief hypoxic challenge produced multiplicative cytotoxicity in an in vitro organotypic hippocampal slice culture (OHSC) model. In vivo, ETOH exposure followed by hypoxia increased locomotor activity in an open field task, compared to controls or subjects exposed to either insult alone. Additionally, ETOH + hypoxia produced acquisition deficits in male subjects in a water maze task.

The second study further investigated ETOH + hypoxic cell damage in the OHSC model. The results from these experiments showed that cytotoxicity increased above control levels over 24 hours in the sensitive CA1 and CA3 regions of the hippocampus. Results also demonstrated that at a relatively "low" concentration of ETOH still sensitized the developing brain to a hypoxic episode. Finally, these data indicate that ETOH withdrawal was necessary to produce an interaction between these two insults.

The third study employed the novel compound, JR220, to determine to role of polyamines in ETOH + hypoxia cytotoxicity in OHSCs. JR220 acts on the polyamine site of NMDAr and previous studies indicate that it is protective against ETOH withdrawal damage. The results from this study suggested that polyamine site blockade was protective against cell damage associated with exposure to ETOH and acute hypoxia.

Collectively, these results indicate that exposure to ETOH during development sensitizes the brain to a brief hypoxic challenge. These results could help explain why some children appear to be more affected by fetal ETOH exposure than others. Both the in vitro and in vivo models developed in this dissertation can be used to further explore the effects of ETOH and hypoxia during fetal development and can be used to screen potential pharmacotherapies.

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

THE EFFECTS OF ALCOHOL AND HYPOXIA EXPOSURE IN THE DEVELOPING BRAIN

By

Megan L. Carter

Susan Barron
Director of Dissertation

Mark Fillmore
Director of Graduate Studies

October 21, 2015
Date

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INTRODUCTION- Overview of the Dissertation

1.1 General Introduction

1.1.1. Background on developmental ETOH: Fetal alcohol syndrome (FAS) (Mattson et al.) was first documented in the United States in 1973 (Jones & Smith, 1973) when doctors described a specific pattern of abnormalities in children born to alcoholic mothers. Since then, a range of behavioral and intellectual deficits resulting from in utero ETOH exposure, collectively referred to as fetal alcohol spectrum disorders (FASDs), have been identified. This term includes the most severe diagnosis of FAS, partial FAS (pFAS), alcohol related birth defects (ARBD), and alcohol related neurodevelopmental disorder (ARND) (May et al., 2014). Recent estimates in the United States and Western Europe suggest that FASDs may affect up to 2-5% of children (May et al., 2009) with an annual cost of approximately $3.6 billion (Olson, Oti, Gelo, & Beck, 2009). Drinking during pregnancy is the leading preventable cause of mental retardation in the western world. Despite knowledge of its teratogenic effects, ETOH consumption during pregnancy remains unacceptably high, with an estimated 12% of U.S. women continuing to drink while pregnant (CDC, online resource).

Full FAS diagnosis requires evidence of facial anomalies, pre and postnatal growth deficiencies, and central nervous system (CNS) abnormalities (Stokowski, 2004). Children with a FASD do not necessarily have the characteristic facial features of full FAS, but they do display neurological impairments including; low IQ, problems with executive function/planning, motor coordination, spatial learning, working memory, cognitive flexibility, social functioning, attention, and hyperactivity (Jones, 2011; Mattson

& Riley, 1998). The consequences associated with FASDs are life-long (Streissguth et al., 2004) and are a significant problem for the individual and society.

1.1.2. Influential factors in fetal ETOH: ETOH-related deficits are heterogeneous and many variables can influence the outcome of fetal ETOH exposure. A major determining factor is the nature of the ETOH exposure: e.g., the timing, amount, and pattern of maternal ETOH consumption. While exposure at any stage of development has the potential to be teratogenic, there is evidence that the CNS is especially sensitive to ETOH during the 3rd trimester of human fetal development (Alfonso-Loeches & Guerri, 2011; Maier, Chen, Miller, & West, 1997). The 3rd trimester, referred to as the brain growth spurt, is characterized by extensive cell growth and differentiation, synaptogenesis, and formation of neural connections (Kleiber, Mantha, Stringer, & Singh, 2013): ETOH can interfere with any or all of these processes, leading to CNS dysfunction.

The pattern of consumption is also important when considering ETOH's effects and current research indicates that binge drinking is particularly dangerous. Binge drinking can lead to higher blood ETOH content and extended exposure periods for the fetus (Paintner, Williams, & Burd, 2012). Higher blood ETOH is associated with elevated CNS toxicity (Pierce & West, 1986; Rasmussen, Erickson, Reef, & Ross, 2009) and even a single administration of a high dose of ETOH can cause damage (Goodlett & Eilers, 1997; Goodlett, Marcussen, & West, 1990). The binge pattern of consumption is also associated with multiple withdrawals, which is linked to increased damage/deficits (Livy, Miller, Maier, & West, 2003). Indeed, most mothers of FASD children report significant binge drinking (Esper & Furtado, 2014). Continuous exposure to low or moderate (i.e., between low/mild and extreme) doses of ETOH can also cause damage

(Patten, Fontaine, & Christie, 2014) but the total amount of ETOH consumed may not be as important, in terms of overall effects, as the pattern drinking.

One puzzling aspect of developmental ETOH exposure is that even under similar exposure conditions, the outcome can dramatically differ between individuals. The effects of ETOH are often dependent on other variables such as genetic background, nutritional status, poly drug use, and hypoxia (Abel & Hannigan, 1995a; Pruett, Waterman, & Caughey, 2013). For example, certain alleles of maternal alcohol dehydrogenase and insufficient levels of dietary antioxidants have been linked to poorer outcome following fetal ETOH exposure (for review see Pruett et al., 2013). The effects of ETOH in combination with hypoxia are particularly interesting because hypoxia it is a common event during gestation.

Hypoxia, a decrease in oxygen levels, can occur periodically during normal fetal development and during parturition. Hypoxia occurs in approximately 5-25 per 1000 live births with 15% of these cases being moderate or severe (Low, 2004) and the outcome can be similar to that seen in children with prenatal ETOH exposure (e.g., learning/memory deficits, hyperactivity) (Dilenge, Majnemer, & Shevell, 2001). The likelihood of hypoxia increases in the presence of ETOH via various mechanisms. For example, ETOH has a dose dependent effect on vasoconstriction of the placenta (Burd, Roberts, Olson, & Odendaal, 2007). ETOH can also cause umbilical cord spasms, abnormal uterine vascular function, and dysregulation of molecules that modulate blood flow (Bosco & Diaz, 2012; Burd et al., 2007; Subramanian et al., 2014). Additionally, ETOH is associated with labor complications, subsequently increasing the possibility of hypoxic events during parturition.

In the near-term fetus/newborn, the CNS is well equipped to handle mild or moderate hypoxic challenges, so these challenges are generally considered benign (Shalak & Perlman, 2004). However, hypoxia could be especially harmful if the integrity of the CNS has already been compromised by other physiological challenges such as infection or inflammation (Rees, Harding, & Walker, 2008), ETOH alters the brain's ability to protect itself during periods of decreased oxygenation. 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 (M. M. 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 consequences of hypoxia.

1.1.3. Animal models of developmental insults: Understanding the processes underlying neurobehavioral disorders (for example, those caused by either ETOH or hypoxia) requires the use of experimental animal models. These models allow for control over possible confounding variables and aspects of exposure including environment, dose or duration, and timing, which is not possible in the human population. Animal models include the use of rodents, zebrafish, sheep, pigs, and some in non-human primates. The majority of studies have used rodent models, which have considerable face validity. For fetal ETOH, numerous rodent models exist that target varying periods of brain development (i.e., first, second, or third trimester or any combination of the three). Rodent models (and other models as well) also differ in their method and pattern of ETOH administration, for example, chronic low exposure via

inhalation vs. acute exposure to high doses of ETOH via oral intubation (for review see Patten et al., 2014). Likewise, hypoxia models vary in terms of the pattern (e.g. complete oxygen deprivation vs. decreased oxygen, or acute vs. chronic) and developmental timing of exposure (Roohey, Raju, & Moustogiannis, 1997).

As stated earlier, the 3rd trimester of CNS development (the brain growth spurt) is a time of heightened susceptibility to the behavioral effects of certain insults including ETOH and hypoxia. In rodents, this growth occurs during the first two postnatal weeks (Dobbing & Sands, 1979) and many animal models investigate the effects of ETOH or hypoxia exposure during this period. In neonatal rats, ETOH is generally administered via oral intragastric intubation (e.g., Idrus, McGough, Riley, & Thomas, 2014; Lewis et al., 2012) while hypoxia can be induced by placement in chambers with little to no oxygen, with or without prior carotid artery ligation, which is a model of hypoxia/ischemia (e.g., Roohey et al., 1997; Vannucci & Vannucci, 2005). ETOH administration during this period produces deficits in a variety of behavioral paradigms that assess motor coordination (Idrus, McGough, Riley, & Thomas, 2011; Lewis, Wellmann, & Barron, 2007; Thomas, Idrus, Monk, & Dominguez, 2010), activity (Smith et al., 2012; Thomas, Biane, O'Bryan, O'Neill, & Dominguez, 2007), and learning and memory (Hunt, Jacobson, & Torok, 2009; Thomas, Abou, & Dominguez, 2009; Thomas et al., 2010; Tiwari, Arora, & Chopra, 2012). Similar to ETOH, hypoxia exposure during this sensitive period 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) These cognitive/behavioral impairments mimic those seen in clinical populations (Patten et al., 2014). Data from these models have helped to elucidate the structural and behavioral effects associated with either ETOH or hypoxia exposure during a critical period of CNS development.

1.1.4. Effects of ETOH and hypoxia on the central nervous system: The use of animal models, along with various imaging techniques, has led to a better understanding of the effects of both ETOH and hypoxia on the central nervous system. The neurodevelopmental deficits are associated with various structural and functional abnormalities observed in the brain.

ETOH: In children with FAS, the corpus callosum can be especially small, and in extreme cases, there can be a complete agenesis of this (Riley & McGee, 2005). In humans and/or animals, size reduction has also been observed in the basal ganglia, cerebellum, cerebrum, and hippocampus (Archibald et al., 2001; Dudek, Skocic, Sheard, & Rovet, 2014; Mattson et al., 1994; O'Leary-Moore, Parnell, Lipinski, & Sulik, 2011). Importantly, ETOH is toxic to both neurons and glia (the major cell types found in the CNS). The effects on glia include impaired myelination/ decreased white matter (Fryer et al., 2009; Spottiswoode et al., 2011) as well as abnormal astroglia morphology and function (Guerri, Pascual, & Renau-Piqueras, 2001), all of which in turn, can affect neuronal development and function.

Hypoxia: Again, much like in instances of fetal ETOH exposure, neurons in the cerebellum, cortex, and hippocampus are particularly sensitive to the damaging effects of hypoxia (Rees et al., 2008). Hypoxia can also injure neurons in the prefrontal cortex and striatum, although this may be specific to events late in fetal development (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, and enlargement of ventricles, among other deficiencies (Rees et. al., 2008).

1.1.5. Mechanisms of damage: Both ETOH and hypoxia produce toxicity via numerous mechanisms. One of the major of the major mechanisms of damage for both

insults is excitotoxicity. This section will focus on excitotoxicity and interrelated processes.

Excitotoxicity occurs during pathological cell stimulation that is often the result of overactiviation of the *N*-methyl-d-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA), and kainate ionotropic glutamate receptors. This leads to excessive intracellular Ca^{2+} that is ultimately toxic to the cell. NMDA, AMPA, and kainite receptors are widely distributed in the CNS and are essential for synaptic plasticity and fast excitatory neurotransmission under normal physiological conditions. The developing brain and specifically the hippocampus, is especially sensitive to excitotoxic insults due to the NMDAr subunit expression found in this region. The NR2B subunit is highly expressed relative to other NMDAr subunits in the developing brain. NR2B subunits have multiple recognition sites for allosteric ligands (such as polyamines) and are more prone to overexcitation than other subunits (for review see Littleton et al., 2001; Mony, Kew, Gunthorpe, & Paoletti, 2009; Sanz-Clemente, Nicoll, & Roche, 2013). Additionally, the developing hippocampus preferentially expresses AMPA receptors that have relatively high Ca^{2+} permeability (Bassani, Valnegri, Beretta, & Passafaro, 2009; Y. Dong et al., 2012).

ETOH and hypoxia produce excitotoxic conditions via different mechanisms. Excitotoxicity occurs during withdrawal from ETOH. Under chronic ETOH exposure, the CNS undergoes compensatory changes, including upregulation of NMDA receptors, in an attempt to maintain normal excitatory transmission (Follesa & Ticku, 1995; Hoffman, 1995; Hu & Ticku, 1995; Snell et al., 1996). Once ETOH is removed from the system (during ETOH withdrawal (EWD)), NMDA receptors are left hypersensitive to glutamate (Kumari & Ticku, 2000). This results in receptor overactivation 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 cascade.

Hypoxia produces toxic increases in extracellular glutamate through impaired glutamate transporter function (Camacho & Massieu, 2006; Swanson, Farrell, & Simon, 1995), release of glutamate from the synapse, and glutamate leakage from damaged cells. Similar to EWD, damaged cells discharge their glutamate, contributing to excessive levels of the neurotransmitter in the synapse. 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 (Y. Dong et al., 2012). Finally, acidosis, which occurs during anaerobic metabolism, makes the cell susceptible to overstimulation (Zhao, Cai, Yang, He, & Shen, 2011).

Both ETOH and hypoxia damage produce an accumulation of polyamines that can potentiate the activity of NMDAr. In certain regions, including the sensitive hippocampus, polyamine expression is elevated as a result of perinatal ETOH exposure (Davidson & Wilce, 1998; Gibson et al., 2003; Wellmann et al., unpublished data). Free radicals produced from both ETOH and hypoxia exposures 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 potentiate excitotoxicity.

There are several molecular processes intimately related to excitotoxicity including inflammation, oxidative stress, and mitochondrial membrane permeability. These mechanisms can operate separately or in combination with each other and/or excitotoxicity. Inflammation is a fundamental physiological response to pathogenic stimuli (e.g., infection or trauma) that acts to remove pathogens/debris and repair damaged tissue. Microglia, the resident immune cells in the CNS, are activated under numerous pathological conditions. These actions are neuroprotective at first but can become neurodestructive if glia are continuously activated. In the neonatal brain, both ETOH (e.g., Drew, Johnson, Douglas, Phelan, & Kane, 2015) and hypoxia (for review see Hagberg et al., 2015; McRae, Gilland, Bona, & Hagberg, 1995) activate microglia to a pro-inflammatory state. Activated glia produce pro-inflammatory molecules including cytokines and chemokines that potentiate microglia activation. Additionally, cytokines contribute to the excitotoxic process; for example, TNF-alpha blocks glutamate uptake (Zou & Crews, 2005) and enhances glutamate release (Takeuchi et al., 2006). Cytokines also induce glial enzymes including iNOS, which leads to the production of nitric oxide (NO). Among other actions, NO causes further release of glutamate from astrocytes and neurons, which can potentiate excitotoxicity (G. C. Brown & Bal-Price, 2003). During the inflammatory process, there is an increase in the production of reactive oxygen species (ROS). When there is an imbalance between ROS and the cell's antioxidant defense systems, oxidative damage occurs. ROS interact with important cellular components including proteins, lipids, and DNA resulting in disrupted functioning and cell death signaling (Halliwell et al., 1992). Under certain conditions, ROS and other reactive molecules also further microglia activation. Oxidative stress/damage is observed in studies of both neonatal ETOH and neonatal hypoxia (for review see Brocardo, Gil-Mohapel, & Christie, 2011; Gill & Perez-Polo, 2008).

Finally, shifts in the mitochondrial membrane potential have recently been described as an important mediating factor in excitotoxicity (Abramov & Duchen, 2008; Nicholls & Budd, 2000). In response to conditions including high concentrations of mitochondrial Ca^{2+} and oxidative stress, a mitochondrial permeability transition pore is opened. This can lead to dysregulation of intracellular Ca^{2+} concentrations, production of free radicals, the release of pro-apoptotic factors into the cytosol, and necrosis (for review see G. C. Brown & Bal-Price, 2003; X. X. Dong, Wang, & Qin, 2009; Luo, 2015).

1.1.6. Pharmacological interventions: Currently, there are no specific neuroprotective treatments for infants and children affected by fetal ETOH exposure (although newborns may be treated with benzodiazepines to reduce physical withdrawal symptoms). Similarly, in cases of perinatal hypoxia, newborns are typically treated with anti-epileptics to control the resulting seizures (Shetty, 2015) or hypothermia to reduce potential CNS damage (for review see Lai & Yang, 2011). Numerous biological interventions to treat or prevent the underlying neuropathology of either ETOH or hypoxia have been investigated. These include (but are not limited to) anti-inflammatory compounds, antioxidants, dietary supplements, and various neurotransmitter receptor modulators (e.g., Barron et al., 2012; Joya, Garcia-Algar, Salat-Batlle, Pujades, & Vall, 2015; for review see Juul & Ferriero, 2014; for review see Martinez & Egea, 2007; Monk, Leslie, & Thomas, 2012; Volbracht, van Beek, Zhu, Blomgren, & Leist, 2006; Wozniak et al., 2015; Zhang et al., 2014). Excitotoxicity is a major mechanism of damage for both fetal ETOH and hypoxia, thus many interventions focus on reducing this via modulation of ionotropic glutamate receptors, especially the NMDA receptor. Acute treatment with NMDA receptor modulators is protective in models of both perinatal ETOH (Idrus et al., 2014; Lewis et al., 2012; Thomas, Weinert, Sharif, & Riley, 1997; Wellmann, Lewis, & Barron, 2010) and hypoxia (Feng, Piletz, & Leblanc, 2002; Kass & Lipton, 1982).

1.2. Scope of Dissertation

1.2.1 *Aim of dissertation*: The main purpose of this dissertation was to develop in vitro and in vivo rodent models to investigate the effects of exposure to ETOH and hypoxia during CNS development.

1.2.2. *Rationale*: Drinking during pregnancy can result in a range of cognitive and behavioral deficits in the exposed offspring. The effects of fetal ETOH vary across individuals and are dependent on factors including the dose, timing, and duration of exposures. However, even under similar exposure conditions, some children may be affected while others are not. One explanation of this phenomenon is that ETOH exposure interacts with other variable such as maternal smoking or under nutrition (Abel & Hannigan, 1995). Hypoxia, a common occurrence during fetal development, is a factor that could contribute to morbidity following ETOH exposure. The presence of ETOH can increase the likelihood of hypoxic events via mechanisms including umbilical cord constriction and reduced oxygen levels in the maternal blood supply (Bosco & Diaz, 2012; Burd et al., 2007; Subramanian et al., 2014). Similar to ETOH, fetal hypoxia can result in abnormal CNS development and function and both insults share common mechanisms of damage including excitotoxicity.

Examining a potential interaction of fetal ETOH and hypoxia is especially intriguing given the potential for their co-occurrence and the overlaps in mechanisms of damage. An interaction of this nature could explain why some children appear to be more affected by in utero ETOH exposure and others are more resistant. By developing models to investigate fetal ETOH in combination with hypoxia exposures, research can be conducted to characterize potential CNS damage and the resulting behavioral deficits and possible therapies can be identified.

1.2.3 Hypothesis: Exposure to ETOH during fetal development sensitizes the brain to subsequent hypoxic challenges and this occurs, in part, via excitotoxic mechanisms. Modulation of the NMDA receptor should improve outcome in both cellular and behavioral assays.

This proposal is composed of a general introduction (Chapter 1), 3 separate manuscripts (Chapters 2, 3, and 4), and a general discussion (Chapter 5).

Chapter 2

Title: PRE-EXPOSURE TO ETHANOL SENSITIZES THE DEVELOPING BRAIN TO A MILD HYPOXIC CHALLENGE

2.1. Abstract

BACKGROUND: Exposure to ethanol (ETOH) during fetal development results in a variety of cognitive/behavioral deficits. Understanding why some infants are more affected than others is a question that needs further study. One hypothesis is that ETOH exposure during early development reduces the ability of the developing brain to compensate for subsequent challenges. This study investigated the hypothesis that developmental ETOH exposure increases the consequences of a subsequent mild hypoxic challenge. This question was addressed using both in an *in vitro* and an *in vivo* rodent model.

METHODS: In Experiment 1, a neonatal rat organotypic hippocampal slice culture (OHSC) model was used to investigate the effects of chronic ETOH exposure and ETOH withdrawal (EWD) on a subsequent oxygen glucose deprivation [OGD]) of varying durations (as a model for hypoxia). Cell damage was examined using propidium iodide (PI) uptake. In Experiment 2, this question was examined using a "3rd trimester" ETOH exposure rodent model. Subjects received ETOH from postnatal days (PND) 1 – 7 and experienced a brief hypoxic challenge on PND 8 (ETOH/hypoxia), These offspring were tested in open field (PND 20-21), spatial learning and 24 hr retention in a water maze on two consecutive days between PND 40 - 45.

RESULTS: Experiment 1: The combination of EWD and 30 min OGD exposure produced greater damage in the CA1 and CA3 hippocampal sub-regions compared to controls or either insult alone. Experiment 2: ETOH/hypoxia offspring displayed

hyperactivity and increased activity in the center of the open field chamber compared to all other treatment groups. This occurred at doses of ETOH and hypoxia that alone did not result in behavioral changes compared to control subjects. Male ETOH/hypoxia exposed offspring also showed acquisition deficits in the spatial learning task although 24 hr retention was not impaired.

CONCLUSIONS: These data suggest that chronic ETOH exposure to a low dose of ETOH, followed by a brief hypoxic challenge, resulted in damage to neonatal hippocampal slices *in vitro* and altered outcome in certain hippocampal-dependent behavioral tasks in vivo. These effects were not observed when either insult occurred alone. The observed interactions of ETOH/EWD and hypoxia may have important clinical implications as it may help explain differences in outcome in individuals with prenatal ETOH or hypoxic histories. Furthermore, these results may provide us with a new way to think about how to target toxicity related to fetal ETOH exposure in select populations.

2.2. Introduction

Drinking during pregnancy continues to be a significant public health problem. It is estimated that the resulting Fetal Alcohol Spectrum Disorders (FASDs) affect 2-5% of children in the Western World (May et al., 2009), with an annual cost of $3.6 billion annually in the United States (Olson, et. al., 2009). Individuals with a FASD can display a range of neurobehavioral consequences including; lowered IQ, problems with executive function, hyperactivity, motor coordination, social functioning, spatial learning, and attentional deficits (Jones, 2011; Mattson & Riley, 1998). 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.

While it is well known that ETOH consumption during pregnancy can lead to developmental abnormalities, the amount, timing, and pattern of consumption necessary to cause damage to the fetus is less well understood. Even under similar ETOH exposure conditions, there can be considerable variation in outcome across individuals (Ouko et al., 2009; Ungerer, Knezovich, & Ramsay, 2013). One hypothesis suggests that a previous history of prenatal ETOH exposure reduces the ability of an organism to cope with other environmental or biological challenges (Abel & Hannigan, 1995b; Abel & Sokol, 1986). Examples of additional challenges that might potentiate the effects of prenatal ETOH exposure include smoking during pregnancy (Batel, Pessione, Maitre, & Rueff, 1995; Bien & Burge, 1990; Burling & Ziff, 1988), genetic differences in susceptibility, poorer nutrition associated with alcoholism and/or hypoxic episodes in utero (Gloria et al., 1997; McClain & Su, 1983). Any of these could result in poorer outcome when they occur in combination with developmental ETOH exposure.

In this study, the effects of developmental ETOH exposure and EWD on the response to a brief hypoxic challenge were examined. Perinatal hypoxia is recognized as a cause of CNS damage and can result in long term consequences; severe hypoxic episodes lead to major deficits including cerebral palsy, mental retardation, epilepsy, or fetal/neonatal mortality (van Handel, Swaab, de Vries, & Jongmans, 2007). Mild or moderate hypoxic episodes experienced by the near-term fetus or newborn are generally considered benign because the CNS has a variety of compensatory mechanisms to protect against these challenges (Shalak & Perlman, 2004). However, mild hypoxia could have a more serious consequence if the integrity of the CNS has already been compromised by other physiological challenges (e.g. infection) (Rees et al., 2008). The relationship between ETOH and perinatal hypoxia is further complicated because ETOH can increase the risk of hypoxia during gestation through several

mechanisms including reduced oxygen supply via umbilical cord constriction and vasoconstriction of the placenta (Bosco & Diaz, 2012; Burd et al., 2007). Studies using a sheep model of prenatal ETOH exposure have shown that the fetus is typically able to increase blood flow to areas such as the cerebellum in response to decreased oxygen (Gleason et al., 1997) but ETOH attenuates this response (Mayock et al., 2007).

The likelihood of a potentiated response to hypoxia during EWD is further supported given the overlap in mechanisms of damage, including excitotoxicity via overactivation of *N*-methyl-d-aspartate receptors (NMDAr) and oxidative stress (Choi & Rothman, 1990; Hoffmann, Coppejans, Vercauteren, & Adriaensen, 1994). During chronic ETOH exposure, such as that which can occur in instances of fetal ETOH exposure, the brain undergoes compensatory changes in an attempt to maintain homeostatic functioning, including upregulation of NMDAr (Hu, Follesa, & Ticku, 1996; Hu & Ticku, 1995; Kalluri, Mehta, & Ticku, 1998) and NMDAr potentiation via raised polyamine levels (Davidson & Wilce, 1998; Gibson et al., 2003). However, once ETOH is removed from the system (EWD), the CNS is left vulnerable to excitotoxic damage. Similarly, hypoxia contributes to excitotoxic conditions through impaired glutamate reuptake and release of glutamate into the synapse (Choi & Rothman, 1990; Rothman & Olney, 1986; Swanson et al., 1995), and an accumulation of polyamines (Longo et al., 1993) that further potentiate the activity of NMDAr. Excess Ca^{2+} can accumulate in cells and activate damaging proteases, endonucleases, and phospholipases (Favaron et al., 1990; Mark et al., 2001; Mills & Kater, 1990). Furthermore, both EWD and hypoxia can lead to increased production of free radicals which can damages cells through interactions with vital proteins and lipids (Blomgren & Hagberg, 2006; Guerri, 1998; Lievre et al., 2000; Mishra, Zanelli, Ohnishi, & Delivoria-Papadopoulos, 2000; West, Chen, & Pantazis, 1994). This overlap in mechanisms of damage contributes to the

rationale for the hypothesis that previous ETOH exposure may sensitize the developing brain to a hypoxic challenge.

The current study employed an in vitro organotypic neonatal hippocampal slice culture (OHSC) (Experiment 1) and an in vivo "3rd trimester" neonatal exposure model (Experiment 2) to assess the consequences of moderate neonatal ETOH exposure followed by a brief hypoxic challenge. The developing hippocampus is an ideal structure for this study as it is implicated in many of the cognitive and behavioral outcomes following fetal ETOH exposure including hyperactivity and impaired learning and memory (Berman & Hannigan, 2000; Lewis et al., 2012; Mattson, Schoenfeld, & Riley, 2001). OHSC is a complex in vitro model that maintains neurons and glia and preserves much of the complex circuitry of an intact hippocampus (Stoppini, Buchs, & Muller, 1991). It has been used previously in studies of ETOH/EWD (Barron, Mulholland, Littleton, & Prendergast, 2008; Wilkins et al., 2006) and hypoxia (Bonde, Noraberg, Noer, & Zimmer, 2005; Noraberg et al., 2005). Similarly, the behavioral paradigms in this study were chosen because they have been shown to be sensitive to either neonatal ETOH or hypoxia (Almli et al., 2000; Idrus et al., 2014; Lewis et al., 2007; Monk et al., 2012; Pereira et al., 2007; Smith et al., 2012; Wellmann et al., 2010).

2.3. Materials and Methods

Animals

Adult parent Sprague Dawley rats were obtained from Harlan Labs (Indianapolis, IN). Offspring were generated in the University of Kentucky Psychology Department's breeding colony. Females were placed individually with a male overnight and the presence of a seminal plug the following morning was used as an indicator of copulation. Pregnant females were moved to a temperature and humidity controlled nursery for the duration of gestation. On PND1, litters were culled to 10 pups, keeping 5 male and 5

female when possible. 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), and the University of Kentucky's Institutional Animal Care and Use Committee.

Experiment 1:

Hippocampal Slice Culture Preparation

The method for the organotypic hippocampal slice preparation (OHSC) was a minor modification of Stoppini and colleagues (1991). Brains from PND8 rat pups were transferred to a petri dish containing ice-cold dissecting medium [Minimum Essential Medium (Gibco BRL, Gaithersburg, MD), 25mM HEPES (ATCC, Manassas, VA), 200mM L-glutamine (Invitrogen, Carlsbad, CA), 50µM streptomycin/penicillin (ATCC, Manassas, VA)]. For each animal, both left and right hippocampi were dissected out and coronally sectioned into 200µm slices using a McIllwain tissue chopper (Campden Instruments Ltd., Lafayette, ID). Slices were selected using a dissecting microscope and plated on teflon membrane inserts (Millicell-CM 0.4µm; Millipore, Marlborough, MA, USA), with 3 slices/insert. Inserts were maintained in 6 well culture plates containing 1mL culture media [dissecting media, 36mM 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 incubated (37°C, 5%CO2, 95%air) for 5 days in vitro (DIV) (Y. Tang et al., , 2006) allowing slices to adhere to the membrane before the first media change. There were 3-4 replications per treatment condition.

ETOH and Hypoxia Treatments

On DIV5, inserts were transferred to 100mM ETOH or control media, with inserts from each subject being evenly distributed between the two treatment groups. Plates were placed in propylene containers with 50mL of distilled water, with or without 100mM

ETOH, on the bottom of the tray. Previous studies using this paradigm showed that although the initial concentration of ETOH is 100mM, there is a gradual decline over the 5 days with a final concentration of 45mM ETOH on day 5, yielding an average of 65mM ETOH (Prendergast et al., 2004). Containers were placed in plastic freezer bags to reduce ETOH evaporation, filled with an air mixture (5%CO2, 21%O2, 74%N2) and incubated until DIV10 when inserts were transferred to fresh media, maintaining the same treatment groups. On DIV15, control and ETOH slices were further divided into 2 groups; oxygen glucose deprivation (OGD) (to induce hypoxia) or control air group, giving a total of 4 treatment conditions; control, ETOH, OGD, and ETOH/OGD. Inserts were transferred to either glucose-free culture media [D-glucose free modified eagle medium (Gibco BRL, Gaithersburg, MD) 25mM HEPES (ATCC, Manassas, VA), 200mM L-glutamine (Invitrogen, Carlsbad, CA), 50µM streptomycin/penicillin (ATCC, Manassas, VA)] (OGD) or control media and then placed in incubation chambers that were flushed with anaerobic gas (5%CO2, 95%N2) (OGD) or control air (5%CO2, 21%O2, 74%N2) at 25L/min for 4 min. The 4 min was used because it is sufficient to flush out air and fill the chambers with the anaerobic gas (Mulholland et al., 2005). Chambers were then sealed and placed in the incubator for the treatment duration (15, 30, or 60 min). Following the treatment, all inserts were placed in 1mL of culture media containing propidium iodide (a non-specific marker of cell damage) (PI; 3.74µM, Sigma Aldrich). Cytotoxicity as measured by PI uptake correlates with other reliable measures of cell death (e.g., Wilkins et al., 2006; Zimmer, Kristensen, Jakobsen, & Noraberg, 2000).

Fluorescence Imaging

PI fluorescence was measured 24 hrs after initiation of EWD and OGD. Fluorescence was visualized with SPOT Advanced version 4.0.9 software for Windows (W. Nuhsbaum Inc., McHenry, IL) using a 5X objective on a Leica DMIRB microscope (W. Nuhsbaum Inc.) fitted for fluorescence detection (mercury-arc lamp) and connected

to a computer via a SPOT 7.2 color mosaic camera (W. Nuhsbaum Inc.). PI has a maximum excitation wavelength of 536nm and a band-pass filter was used that excites the wavelengths between 515 and 560nm. Emission of PI in the visual range is 620nm. The intensity of PI fluorescence, in arbitrary optical units, was determined in the primary hippocampal cell layers of the DG, CA3, and CA1 using Image J 1.29x (National Institutes of Health, Bethesda, MD).

Experiment 2:

In Vivo Experiments

Pups were assigned to one of the following neonatal treatment groups: ETOH (4.5 g/kg), intubated control (IC), or non-treated control (Abel & Dintcheff). A milk-based diet (with or without the addition of ETOH) (West et al., 1984) was administered via oral intragastric intubation. On PND1-7 (once daily), pups were weighed, numbered, and intubated. On PND8, 21 hours following the final intubation (during EWD), pups were placed in in incubator chambers (Modular Incubator Chamber-101; Billups-Rothenburg). Chambers were placed in a warm water bath (75°C) to maintain body temperature and were flushed with 5%CO2, 21%O2, 74%N2 (control air) or 100%N2 (to induce hypoxia), for 8.5 min. The ETOH dose was chosen based on pilot studies in which similar doses showed no effect on the behavioral outcomes used in this study. Hypoxia duration was also based on pilot studies because it produced minimal mortality and did not produce measurable behavioral impairments. After 8.5 min, pups recovered on a heating pad before being returned to the dam. A maximum of one male and one female from a litter were included in each cell of the experimental design to preclude potential litter effects.

Blood ETOH Concentrations (BECs)

A subset of pups, not used for behavioral testing, was intubated as described above. On PND7, approximately 20uL of tail blood was taken from each pup at no more than 3 of the following time points: 30, 60, 120, 240, 480, and 600 min after the final

ETOH intubation (n= 10-14/time-point). Plasma was separated and analyzed using an Analox AM1 Analyzer (Analox Instruments) to determine peak BECs.

Open Field Testing

On PND20 and 21, litters were separated from the dam and groups of 2 subjects were taken into a dimly lit test room. Following 10 min habituation, pups were placed into separate 58cm-diameter circular chambers where locomotor activity was tracked for 30 min using a San Diego Instruments Polytracker (SMART program; Panlab, S.L). The dependent measures for the 2 days of testing were total distance travelled and distance travelled in the center of the chamber (defined as a 14.5cm-diameter imaginary inner circle), broken down into 5 min time blocks or as a total for the 30 min testing period. Center distance is thought to measure inhibitory control and/or anxiety because rats typically display thigmotaxis. Upon completion of testing, pups were weaned and housed with 2-3 same-sex conspecifics with ad libitum access to food and water.

Water Maze Testing

Testing was conducted on two consecutive days between PND 40-45. This Hebb style water maze was adapted from von Euler, Bendel, Bueters, Sandin, and von Euler (2006). A distinct advantage of this water maze task relative to others (e.g., the Morris Water Maze) is that acquisition occurs within a single test session, making it possible to easily discern acquisition from retention deficits. The maze has multiple arms and requires a series of three correct choices to reach the hidden platform. On testing days, the maze was filled with room temperature water tinted with black non-toxic tempura paint to hide the platform and allow for motion tracking (SMART program; Panlab, S.L.) Subjects were given a maximum of twenty, 60 sec trials/day to swim from the same start position to the submerged platform. Upon success, the rat remained on the platform for 5 sec, concluding the trial. The rat was then placed in a cage under a

heat lamp for a 30 sec intertrial interval. If the subject did not reach the platform at the end of 60 sec, the experimenter guided it to the platform. Trials were repeated until criterion, defined as 2 consecutive trials with no errors, was reached. Twenty-four hours following acquisition, retention was tested using the same procedures. The dependent measure was the number of trials to criterion during acquisition and retention.

Statistical Analyses: Data were analyzed using the Statistical Package for the Social Sciences (SPSS) Version 20 Software (IBM Corporation, 2011). Experiment 1: An overall 2 x 3 x 2 x 3 repeated measures (RM) analysis of variance was performed including the variables; ETOH, OGD duration, SEX, and hippocampal region (within-subject). Replicate was used as a covariate to control for differences across litters/culture preparations. When no main effect or interaction with SEX was observed, further analyses were conducted collapsed across this factor for ease of interpretation. Significant interactions were examined using post hoc pair-wise comparisons with Tukey-Kramer correction for family-wise error. Data were converted to percent control for graphic presentation.

Experiment 2: An ANOVA was used for all behavioral studies using a 3 x 2 x 2 factorial design with neonatal TREATMENT, HYPOXIA, and SEX with RM where warranted. For ease of interpretation, IC and NTC groups were collapsed into a single control group because there were no differences between them on the dependent measures. In cases where there was an interaction with SEX and other variables, ANOVAs were conducted on each sex separately to better understand the interaction. As described above, if there were no effects of SEX, the data was collapsed and all significant interactions were broken down using Tukey-Kramer correction.

2.4. Results

Experiment 1: OGD in Combination with EWD in OHSC

Results from Experiment 1 are presented in figure 1. The overall 2 x 4 x 2 x 3 (ETOH, OGD, SEX, REGION) revealed numerous significant interactions with OGD duration and REGION including REGION x ETOH x OGD DURATION, F (2,173) =3.91, p <.05, which led to separate analyses for each time point (15, 30, and 60 min) and region (DG, CA3, CA1).

Consistent with our hypothesis, 30 min OGD during EWD increased PI uptake in the CA1 and CA3 regions, relative to all other groups (figure 1a). The ETOH x OGD x SEX ANOVA revealed 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. There were no significant effects of EWD, OGD, or their combination in the DG.

This pattern was not observed with other durations of OGD treatment. Following 60 min OGD, there was an apparent ceiling effect; this level of OGD itself was neurotoxic and EWD did not potentiate toxicity (figure 1b). The ANOVA revealed a main effect of OGD in the CA1, F(1,72) =92.966, p <.001 and CA3, F(1,72) =13.057, p =.001 with no effects in the DG. There were no significant treatment effects or interaction in any region with EWD after 15 min OGD (figure 1c).

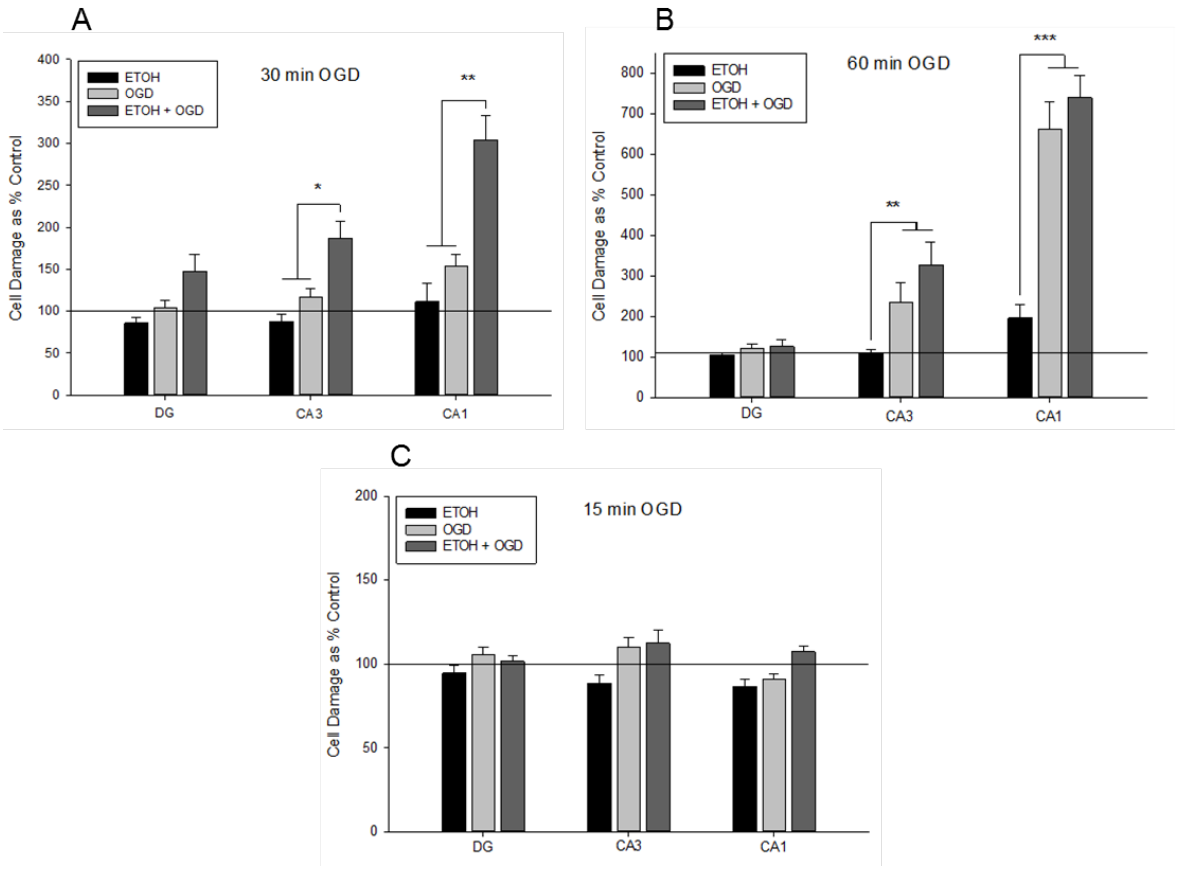


Figure 2.1. PI uptake ± S.E.M. expressed as percent control in the DG, CA3, and CA1 regions of the PND8 hippocampus, 24 hours following initiation of EWD and OGD treatment. A) At 30 min OGD, the ETOH/OGD slices had greater PI uptake compared to all other conditions in the CA3 ($p < .05$) and the CA1 ($p < .01$). B) At 60 min OGD, 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. C) At 15 min OGD, There were no significant treatment effects.

Experiment 2: Open Field & Water Maze

Bodyweights: Administration of 4.5g/kg ETOH on PND 1–7 reduced bodyweight compared to controls after the second day of ETOH treatment. A RM ANOVA [NEONATAL TREATMENT (ETOH, IC, or NTC) x SEX x PND] revealed a significant NEONATAL TREATMENT x PND interaction, $F(2,93) = 12.80$, $p < .001$ such that ETOH exposed animals weighed less than the IC and NTC animals on PND3-8 (Table 1). By PND 20, the start of open field-testing, there were no significant differences in weight as a function of neonatal treatment (Table 2). BECs: BEC peaked at 240 min, with an average of 224.9±10.6 mg/dL.

Locomotor activity: ETOH/hypoxia offspring displayed increased locomotor activity on day 1 of testing relative to controls or either treatment alone and increased activity relative to ETOH or hypoxia subjects on day 2 (figure 2). The RM ANOVA on distance travelled across the 2 days of testing revealed a significant ETOH x HYPOXIA interaction, $F(1,79) = 5.91$, $p = .017$.

Center activity: A DAY x ETOH x HYPOXIA x SEX RM ANOVA was conducted for distance travelled in the center of the open field. There was a significant DAY x ETOH x SEX interaction, $F(1,79) = 5.73$, $p = .019$. To better understand this data, separate analyses were run for both days. On day 1, there was also a HYPOXIA x SEX, $F(1,79) = 3.85$, $p = .053$ and an ETOH x HYPOXIA interaction $F(1,79) = 3.60$, $p = .061$ that approached significance. Separate analyses were then run for both sexes to better understand these interactions. Neonatal ETOH exposed males traveled a greater distance in the center relative to non-ETOH exposed males; main effect of ETOH, $F(1, 45) = 5.03$, $p = .019$. In contrast, among females, post-hoc comparisons revealed that ETOH/ hypoxia treated females displayed significantly greater activity compared to ETOH or control females (see figure 3a). On day 2, a different pattern in activity

emerged. There was a significant ETOH x SEX interaction, $F(1,79) = 6.93$, $p = .010$. Both groups of females that had been exposed to ETOH neonatally traveled more in the center on the 2nd day of testing relative to the non-ETOH exposed groups; main effect of ETOH, $F(1,34) = 6.56$, $p = .015$. There were no group differences in males (see figure 3b).

Spatial learning and retention: ETOH/hypoxia exposed males took more trials to acquire the water maze task on day 1 of testing relative to all other treatment groups. The repeated measures ANOVA revealed a significant HYPOXIA x SEX x DAY interaction, $F(1,85) = 6.98$, $p = .010$. For males, the RM ANOVA revealed a significant ETOH x HYPOXIA interaction, $F(1,47) = 6.24$, $p = .016$. There were no differences between treatments groups on 24hr retention (figure 4) and all groups improved from day 1 to day 2. There were no significant treatment effects on acquisition or retention in females.

Table 2.1. Mean neonatal body weights (g) ± S.E.M. for PND 1-8. Exposure to 4.5 g/kg/ day ETOH produced significant differences in body weight compared to control subjects on PND 3-8.

Treatment Group	PND 1	PND 2	PND 3	PND 4	PND 5	PND 6	PND 7	PND 8
ETOH								
Male	7.18 ± 0.15	7.54 ± 0.14	8.86 ± 0.14	10.12 ± 0.21	11.83 ± 0.24	13.88 ± 0.25	15.90 ± 0.25	18.11 ± 0.34
Female	6.69 ± 0.17	7.52 ± 0.12	8.61 ± 0.15	9.83 ± 0.21	10.97 ± 0.61	13.64 ± 0.20	15.64 ± 0.26	17.58 ± 0.34
Intubated Control								
Male	7.06 ± 0.21	8.31 ± 0.15	9.80 ± 0.20	11.35 ± 0.25	13.43 ± 0.27	15.91 ± 0.33	18.17 ± 0.40	20.54 ± 0.41
Female	6.49 ± 0.15	7.77 ± 0.20	9.32 ± 0.25	10.74 ± 0.30	12.54 ± 0.34	13.88 ± 0.41	17.24 ± 0.41	19.72 ± 0.41
NTC								
Male	7.02 ± 0.24	8.25 ± 0.28	9.51 ± 0.42	10.99 ± 0.52	12.96 ± 0.50	15.65 ± 0.67	18.03 ± 0.77	20.50 ± 0.76
Female	6.67 ± 0.31	7.80 ± 0.31	9.20 ± 0.35	10.33 ± 0.51	12.47 ± 0.50	14.83 ± 0.70	17.14 ± 0.82	19.26 ± 0.87

*ETOH exposed subjects weighed significantly less than non-ETOH subjects, $p < .006$

Table 2.2. Mean bodyweights (g) ± S.E.M. during behavioral tests. There were no significant effects of neonatal treatment on bodyweights on PND 20 or 40.

Treatment Group	PND 20	PND 40
ETOH		
Male	56.96 ± 1.01	194.00 ± 6.24
Female	53.93 ± 1.21	160.14 ± 8.96
ETOH + Hypoxia		
Male	56.22 ± 1.34	194.50 ± 2.99
Female	54.52 ± 0.79	156.22 ± 2.24
Control		
Male	59.05 ± 1.14	202.50 ± 4.16
Female	54.92 ± 1.78	157.61 ± 4.80
Hypoxia		
Male	58.02 ± 0.52	196.41 ± 3.85
Female	56.62 ± 0.83	161.92 ± 5.29

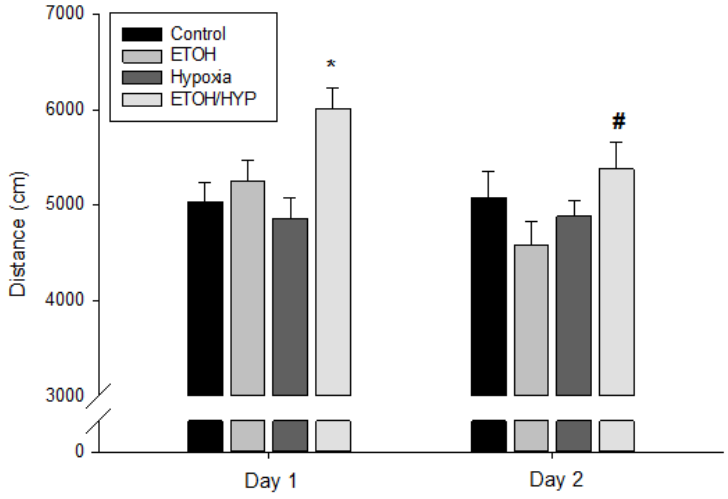


Figure 2.2. Mean total distance traveled across 30 min in the open field ± S.E.M. on day 1 and 2 of testing. On day 1, neonatal treatment of ETOH + hypoxia increased total locomotor activity compared to all other treatment groups. * Indicates significant difference from all other treatment groups, p <.05. On day 2, ETOH + hypoxia treated subjects had increased activity compared to ETOH and hypoxia treatments alone. # Indicates significant difference from ETOH and Hypoxia groups, p <.05.

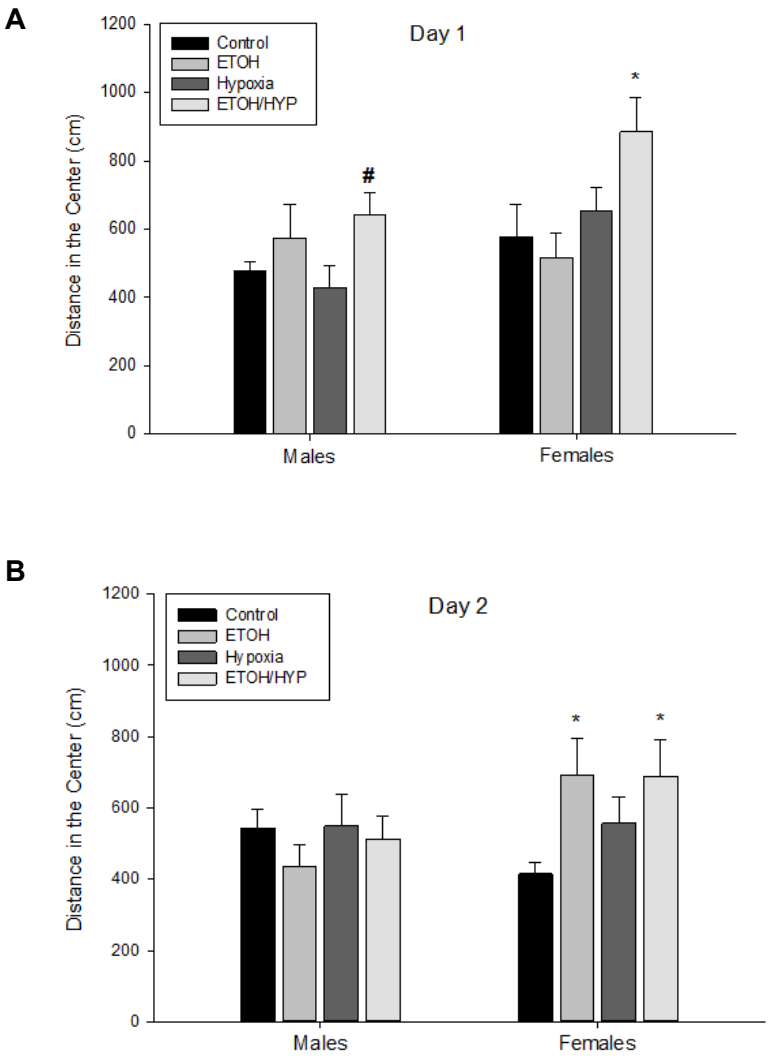


Figure 2.3. Mean activity in the center of the open field ± S.E.M. for females and males over 30 min testing periods. **A)** On day 1, ETOH/hypoxia treated females had greater center distance as compared to control and ETOH females; # indicates significant difference from control and ETOH groups, p <.05. ETOH/hypoxia males had greater center distance compared to hypoxia alone males, * Indicates significant difference from all other treatment groups, p <.05. **B)** On day 2, ETOH treatment alone increased center distance in females, * Indicates significant difference from control and hypoxia groups, p <.05. There were no effects observed in males.

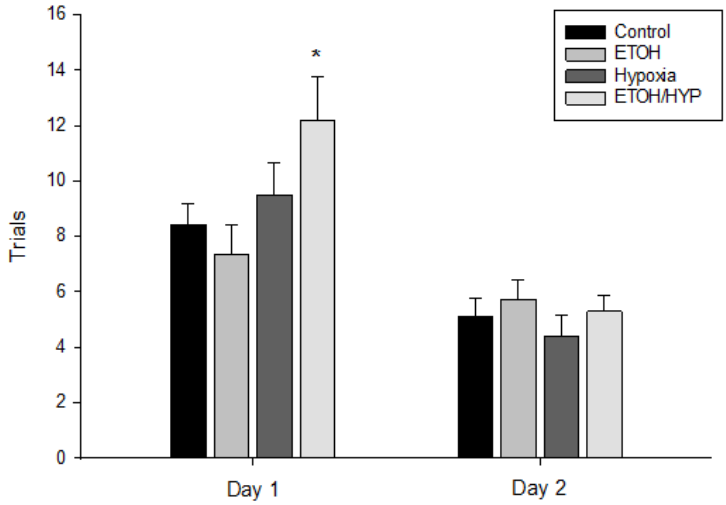


Figure 2.4. Mean total number of trials to criterion ± S.E.M. for male subjects during acquisition (day 1) and retention (day 2) of the water maze task. Neonatal ETOH + hypoxia treatment increased the number of trials necessary to acquire the task above control levels, $p < .05$.

2.5. Discussion

The current study used an in vitro OHSC (Experiment 1) and an in vivo 3rd trimester model (Experiment 2) to determine if exposure to ETOH/EWD during CNS development sensitized the developing brain to a subsequent hypoxic challenge. In Experiment 1, three durations of hypoxia (OGD) were examined following EWD. Consistent with our hypothesis, EWD followed by 30 min OGD increased PI uptake in both the CA1 and CA3 regions while the DG was unaffected. This occurred at an ETOH dose and OGD duration that independently caused no damage relative to controls. The combination of ETOH and hypoxia also produced behavioral deficits (i.e., locomotor activity and spatial learning/memory) with little effect of either challenge alone. The interaction of ETOH/EWD and hypoxia observed both in vivo and in vitro provide support for the hypothesis that subthreshold ETOH exposure during early development can result in adverse outcome if a second mild insult, such as hypoxia, occurs concurrently.

The CA1 hippocampal region was the most sensitive to the combination of EWD and OGD relative to CA3 or DG. This pattern is similar to that reported following EWD in combination with a variety of additional challenges such as exogenous polyamines (Barron et al., 2008; Butler, Berry, Sharrett-Field, Pauly, & Prendergast, 2013), as well as other hippocampal models examining hypoxia (for review see Noraberg et al., 2005). Numerous mechanisms have been proposed to explain these regional differences in sensitivity. During development, there is a greater density of NR1 and NR2B NMDAr subunits in the CA1 region compared to CA3 (Butler et al., 2010). Receptors containing NR2B subunit(s) have properties that enhance synaptic plasticity including increased channel open time/slower deactivation of post-synaptic currents (Kirson & Yaari, 1996; Y. P. Tang et al., 1999) and greater sensitivity to polyamine modulation (Sharma & Reynolds, 1999) compared to other subunits. While this may be beneficial under normal

developmental conditions, these properties potentially increase a cell's sensitivity to excitotoxic challenges. Additionally, an NR1 splice variant, associated with increased sensitivity to NMDA and ischemic insult, has greater expression in the CA1 (Coultrap, Nixon, Alvestad, Valenzuela, & Browning, 2005) and greater Ca^{2+} accumulation and mitochondrial dysfunction occurs in the CA1 compared to the CA3 when challenged (Stanika, Winters, Pivovarova, & Andrews, 2010). While the CA3 and DG are generally more resistant to excitotoxic injury, significant cytotoxicity following ETOH/hypoxia, compared to controls, was still observed in the CA3. The CA3 has qualities that confer protection against overexcitation, including AMPA receptor internalization following OGD (Dennis et al., 2011b) and different NMDAr expression as described above (Butler et al., 2010), but it still contains Ca^{2+} permeable AMPA receptors at levels similar to that in the CA1 (Pellegrinigiampietro, Bennett, & Zukin, 1992). Toxicity is often seen in this region (although to a lesser degree compared to the CA1) following ETOH (e. g., Wilkins et al., 2006) or OGD (e.g., Allard, Paci, Vander Elst, & Ris, 2015). Finally, the DG's relative insensitivity to excitotoxic insults could be explained by differences in cell type (granule vs pyramidal), its response to exogenous NMDA and Ca^{2+} (Butler et al., 2010; Prendergast et al., 2004) and/or mitochondrial enzyme activity (Davolio & Greenamyre, 1995).

In the open field, both male and female offspring exposed to neonatal ETOH/hypoxia were hyperactive on the first day of testing relative to all other treatment groups. This is consistent with previous studies of both neonatal ETOH (at higher concentrations) and hypoxia (Idrus et al., 2014; Lewis et al., 2012; Monk et al., 2012; Smith et al., 2012). In contrast, there were some sex-specific effects on the amount of distance travelled in the center of the open-field chamber. This measure is often used as an indicator of anxiety; less time spent or fewer entries in the center indicating

increased anxiety. However, an alternative explanation is that entering the center is a measure of impulsivity or a failure of inhibitory control since rodents typically avoid novel open spaces. Since the offspring exposed to ETOH/hypoxia were hyperactive, one might expect a similar pattern on entries into the center and this was observed at least for females on the first day in the open field. For males, the results were more complicated to interpret since both ETOH exposed groups (with and without hypoxia) showed a small but statistically significant increase in distance traveled in the center on day 1 relative to controls. These results may suggest that males may be more sensitive to lower doses of ETOH not in overall hyperactivity but at least in ETOH's effects on inhibitory control although again - this was only a slight increase for males on day 1. On day 2, a different pattern emerged for females. ETOH exposed females (with and without hypoxia) displayed increased activity in the center on the 2nd day of open-field testing. There are studies suggesting females may be more sensitive to stress after early ETOH exposure (e.g. Weinberg et al., 2008) and this is one possible explanation for this effect in females on day 2 of testing. Therefore, a greater response to stress may help explain the sex differences observed, at least as measured in entries in the center of the open field. Currently, it is difficult to conclude whether changes in center activity is due to reductions in inhibitory control, increases in general hyperactivity, or changes in anxiety and further work could address this question.

Sex differences were also observed in the water maze task. On this task, ETOH/hypoxia exposed males were impaired in acquisition relative to controls while there was no effect in females. This finding is also supported by previous studies of both neonatal ETOH and hypoxia. Similar to the literature on hyperactivity, exposure to higher doses of neonatal ETOH results in impaired water maze performance (Lewis et al., 2012; Wagner, Zhou, & Goodlett, 2014) and males have shown greater sensitivity to

the effects of both early hypoxia and ETOH exposure on at least some measures of learning and memory (e.g., Goodlett & Peterson, 1995; Smith et al., 2012; Zimmerberg, Sukel, & Stekler, 1991). It is important to note however that there is also evidence that females are more sensitive than males to developmental ETOH exposure in other contexts (An & Zhang, 2013; Barron et al., 2008). Differences among these studies and others highlight the importance of examining both sexes and indicate that sex differences in response to neonatal ETOH or hypoxia may heavily depend on the timing and dose of exposure as well as the outcomes being measured.

Several potential mechanisms could help explain the sex differences observed in these studies. Sex differences in cell death signaling pathways following neonatal hypoxia/ischemia exist between males and females (for review see Hill & Fitch, 2012). There are also sex differences in response to hypoxic injury such as BDNF signaling (Chavez-Valdez, Martin, Razdan, Gauda, & Northington, 2014) and in baseline autophagy activity during fetal development (Weis et al., 2014). These factors could be protective for females and result in little or no behavioral effects such as what we observed in the water maze task. While developmental ETOH exposure can also modulate similar mechanisms such as BDNF levels (e.g., Balaszczuk, Bender, Pereno, & Beltramino, 2013) to the best of our knowledge, there are no studies suggesting that there are sex differences following ETOH exposure. Still prenatal ETOH exposure can reduce long-term potentiation selectively in adolescent or adult males (Sickmann et al., 2014; Titterness & Christie, 2012), which could especially impact performance in tasks such as the water maze.

These findings support the hypothesis that ETOH sensitizes the developing brain to subsequent hypoxic challenges. Sex differences, however, were only observed in measures of behavioral outcome. This is not unprecedented. Literature from the

hypoxia/ischemia (Smith et al., 2014) and ETOH studies (Patten et al., 2013) have shown sex differences in behavioral outcome but not always in neurochemical/neuroanatomical measures. Findings such as these emphasize the fact that developmental insults can produce sex-dependent behavioral effects via dysregulation of a variety of neural substrates and these sex differences rely on the measures employed. Future in vitro studies using additional time points (e.g. beyond 24 hours post-insult) and additional markers (for example, glial markers) may reveal sex differences in hippocampal slices that were not readily observed using PI and a 24 hour time point. Sex differences in sensitivity to ETOH/hypoxia could also be a result of damage to areas other than the hippocampus, such as the prefrontal cortex, which is also implicated in learning and memory and attention/hyperactivity.

It is important to note that the observed behavioral deficits are not necessarily due solely to the effect of ETOH/EWD and hypoxia exposure on the hippocampus. Many other regions can be significantly affected by ETOH or hypoxia exposure, which in turn, can affect behavior including activity and learning and memory. For example, in a 3rd trimester neonatal ETOH exposure model similar to that used in the current study, hyperactivity in the open field was shown to coincide with changes in dopamine transporter function in the striatum and prefrontal cortex (Smith et al., 2012). From the hypoxia literature, neonatal rats treated with intermittent hypoxia had increased locomotor activity compared to controls and also showed changes in DA systems in the striatum (Decker et al., 2003). Additionally, changes in caspase-3 activation following neonatal ETOH in structures such as the cortex have been associated with water maze deficits (Furumiya & Hashimoto, 2011; Wagner et al., 2014). Similarly, mice treated with acute hypoxia had deficits in the Morris water maze and had decrease in total cerebral volume as compared to controls (Sanches, Arteni, Nicola, Aristimunha, & Netto, 2015).

These examples, along with many others, highlight that numerous changes in neurological structure and function underlie the behavioral effects of developmental ETOH and/or hypoxia.

This study is unique in that it focuses on the consequences of a hypoxic episode that occurs following exposure to ETOH. Clinically, there is increased incidence of hypoxia during parturition (Vannucci, 1990) and for a fetus that has been exposed to ETOH, this is also a time, in theory, when withdrawal may begin or already be in progress. Thus, these models were designed to target a period during parturition in which a fetus previously exposed to ETOH may experience decreases in oxygen levels. The present experiments demonstrated an interactive effect of ETOH/EWD and hypoxia in the developing hippocampus and in behaviors displayed by juveniles. An interaction of this nature could explain some of the variation in outcome seen in children following fetal ETOH exposure and specifically targeting mechanisms that underlie these insults could offer protection for children at risk. These data suggest the need to educate health care professionals on the risks that even "mild" hypoxic events may pose to the ETOH-exposed newborn and future studies in this lab will explore potential therapies to reduce adverse outcomes. If appropriate identification of at-risk newborns is in place, targeting excitotoxicity during EWD and hypoxia would be appealing because potential treatments would be administered at a time when mother and infant are already seeking medical care (i.e., during labor and delivery) and theoretically, only a single drug administration would be necessary to achieve neuroprotection and improve cognitive and behavioral outcomes.

Chapter 3

Title: EXTENDING FINDINGS ON PRE-EXPOSURE TO ETHANOL SENSITIZES THE DEVELOPING BRAIN TO A MILD HYPOXIC CHALLENGE

3.1. Abstract

The effect of fetal ethanol (ETOH) exposure varies across individuals and numerous studies have shown that other developmental factors, such as hypoxia, can influence outcome. Hypoxia is a normal occurrence during fetal development but we have recently demonstrated that previous ETOH exposure can leave the brain sensitive to an otherwise innocuous hypoxic event. To better understand this effect, the current study employed a neonatal rat hippocampal slice culture model (similar to that used in previous experiments) to investigate a number of questions. These questions included: the time course of ETOH and hypoxic damage over 24 hours, the effects of a lower dose ETOH exposure, and the specific role of ETOH withdrawal (EWD). Propidium iodide (PI) uptake was used as a non-specific marker of cell damage in all experiments. In the timeline study, ETOH/EWD + OGD exposed slices showed a steady increase in cytotoxicity from the 4 to 16 hour time point following treatment in the sensitive CA1 hippocampal region. In the CA3 EWD + OGD damage increased from the 4 to 20 hour time point. At a lower concentration of ETOH (50mM), there was an interaction of ETOH/EWD + OGD in the CA1 and CA3, a pattern of PI uptake similar to that seen at the 100mM ETOH exposure in previous studies. Finally, there was still an EWD and OGD interaction when withdrawal was initiated after OGD, however, avoiding EWD altogether resulted in no interactive effect of the two insults. These results provide further support for and characterization of ETOH sensitizing the developing brain to subsequent hypoxia.

3.2. Introduction

It is well known that fetal ETOH exposure has long-term consequences for the developing offspring including lowered IQ, problems with executive function, hyperactivity, motor coordination, social functioning, spatial learning, and attentional deficits (Mattson & Riley, 1998). There is significant variability among affected individuals that, despite extensive research, is not well understood. Factors such as dose and timing of ETOH exposure can influence neurocognitive and behavioral outcomes, but other factors may contribute as well (e.g., Abel & Hannigan, 1995). We have been studying the hypothesis that previous exposure to ETOH reduces the ability of the developing organism to handle additional stressors, specifically hypoxia, thus increasing the likelihood of adverse outcomes.

Hypoxia, a decrease in oxygen levels, occurs throughout normal fetal development and is also likely to occur during parturition (Vannucci, 1990). ETOH exposure can increase the occurrence of hypoxic events in utero (Bosco & Diaz, 2012; Burd et al., 2007), increasing the likelihood that the ETOH-exposed fetus may experience either challenge. Additionally, the combination of fetal ETOH and hypoxia is particularly intriguing given the significant overlap in cellular effects/mechanism of damage. These mechanisms include, among potential others, excitotoxicity, which occurs in instances of both withdrawal from ETOH (EWD) and hypoxia during brain development (e.g., Costa, Soto, Cardoso, Olivera, & Valenzuela, 2000; Mishra, Fritz, & Delivoria-Papadopoulos, 2001). Thus, if EWD and hypoxia occur together, the results could be especially deleterious, particularly in the sensitive developing nervous system.

We have previously demonstrated that the combination of neonatal ETOH + hypoxia produces damage in an organotypic hippocampal slice (OHSC) model and a 3rd

trimester behavioral model, at exposure levels that alone, had little or no effect (Carter et al., submitted to ACER). These models were designed to assess a hypoxic event that might occur during labor and delivery, a time when the at-risk fetus might also begin or already be in withdrawal from ETOH. This is also a period when a mother is likely to be seeking medical attention, which makes it an excellent target for treatments such as pharmacological interventions. Given that these data hold important treatment implications, the interaction of developmental ETOH and hypoxia warranted further investigation.

The current set of experiments used organotypic hippocampal slice cultures (OHSCs) to extend previous results from our lab (listed below as experiments 1, 2, and 3). OHSC was chosen because it is an effective screening paradigm and it has been predictive of in vivo results in previous studies of developmental ETOH (Lewis et al., 2012) and hypoxia (Noraberg et al., 2005). Importantly, it was predictive of certain behavioral effects in our most recent study of ETOH + hypoxia (Carter et al., submitted to ACER).

Experiment 1. The first question addressed was the time-course of damage following EWD and hypoxia. It was hypothesized that toxicity in the CA1 and CA3 (hippocampal regions previously shown to be sensitive to the combination of EWD + hypoxia) would increase over time.

Experiment 2. This experiment investigated the effects of EWD + hypoxia when slices are chronically exposed to 50mM ETOH. Previous studies using 100mM ETOH showed an interaction of EWD and hypoxia in the CA1 and CA3 regions of the hippocampus. It was hypothesized that a more mild ETOH exposure should still produce sensitization to hypoxia in the developing brain.

Experiment 3. This experiment investigated whether or not withdrawal from ETOH is necessary to produce hippocampal toxicity in slices treated with the combination of ETOH and hypoxia. It is hypothesized that the previously observed interactive effects of ETOH and hypoxia were due to excitotoxicity, thus, it was predicted that the presence of continuous ETOH (without withdrawal), ETOH + hypoxia would not result in cytotoxicity.

Overall, results from these cultures support and supplement previous data from this lab but also indicate that the selected hypoxia exposure may not be as benign as previously shown.

3.3. Materials and Methods

The method for the organotypic hippocampal slice preparation (OHSC) was a minor modification of Stoppini and colleagues (1991). Brains from PND8 rat pups were transferred to a petri dish containing ice-cold dissecting medium [Minimum Essential Medium (Gibco BRL, Gaithersburg, MD), 25mM HEPES (ATCC, Manassas, VA), 200 mM L-glutamine (Invitrogen, Carlsbad, CA), 50µM streptomycin/penicillin (ATCC, Manassas, VA)]. For each animal, both left and right hippocampi were dissected out and coronally sectioned into 200µm slices using a McIllwain tissue chopper (Campden Instruments Ltd., Lafayette, ID). Slices were selected using a dissecting microscope and plated teflon membrane inserts (Millicell-CM 0.4µm; Millipore, Marlborough, MA, USA), with 3 slices/insert. Inserts were maintained in 6 well culture plates containing 1mL culture media [dissecting media, 36mM 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 incubated (37°C, 5%CO2, 95%air) for

5 days in vitro (Y. Tang et al.), allowing slices to adhere to the membrane before the first media change.

ETOH and Hypoxia Treatments

On DIV5, inserts were transferred to 100mM ETOH or 50mM ETOH (depending on the experiment) or control media, with inserts from each subject being evenly distributed between the two treatment groups. 100mM ETOH was previously shown to interact with 30 min OGD, while causing no damage on its own. Due to evaporation during incubation, 100mM ETOH actually averages to approximately 65mM (~ 299 mg/dL) ETOH over 5 days in vitro (Prendergast et al., 2004). Evaporation also occurs at 50mM (Butler, Smith, Self, Braden, & Prendergast, 2008) and results in an average concentration over 5 days of approximately 35mM (~ 161 mg/dL). Plates were placed in propylene containers with 50mL of distilled water, with or without the same concentration of ETOH, on the bottom. Containers were placed in plastic freezer bags to reduce ETOH evaporation, filled with an air mixture (5%CO2, 21%O2, 74%N2) and incubated until DIV10 when inserts were transferred to fresh media, maintaining the same treatment groups. On DIV15, control and ETOH slices were further divided into 2 groups; oxygen glucose deprivation (OGD) and control air. OGD slices were placed in glucose-free culture media [D-glucose free modified eagle medium (Gibco BRL, Gaithersburg, MD) 25mM HEPES (ATCC, Manassas, VA), 200mM L-glutamine (Invitrogen, Carlsbad, CA), 50µM streptomycin/penicillin (ATCC, Manassas, VA)] and placed in a chamber that was flushed with anaerobic gas (5%CO2, 95%N2) at 25L/min for 4 min. Alternatively, control slices were placed in regular media and placed in a chamber flushed with control air (5%CO2, 21%O2, 74%N2). Chambers were then sealed and placed in the incubator for 30 min. Following the treatment, all inserts were placed in 1mL of culture media containing propidium iodide (a non-specific marker of cell damage) (PI; 3.74µM, Sigma Aldrich). The specifics for each treatment varied depending on the culture preparation.

Experiment 1: Slices were treated as previously described in Chapter 2: withdrawal from 100mM ETOH was initiated at the beginning of 30 min OGD or control air treatments.

Experiment 2: Slices were used to assess the effects a dose of ETOH lower than that used in the first set of experiments. Slices were treated as described above with 50mM ETOH + 30 min OGD. These slices were removed from ETOH at the beginning of the OGD or control air treatments.

Experiment 3: To assess the role of EWD in ETOH + OGD damage, slices were treated with 100mM + 30 min OGD with ETOH in the media during OGD treatment. After the 30 min exposure, slices were kept in ETOH-free media until imaging at 24 hours. In one culture preparation slices were exposed to 100mM ETOH through imaging (continuous ETOH exposure), avoiding EWD entirely.

Fluorescence Imaging

To assess the effects of EWD, OGD, and EWD + OGD over time (Experiment 1), slices were imaged for PI uptake at 4, 8, 12, 16, 20, and 24 hours following treatments. Slices were kept in an incubator between imaging time points. For experiments 2 and 3, PI fluorescence was measured 24 hours after OGD treatment. Fluorescence was visualized with SPOT Advanced version 4.0.9 software for Windows (W. Nuhsbaum Inc., McHenry, IL) using a 5X objective on a Leica DMIRB microscope (W. Nuhsbaum Inc.) fitted for fluorescence detection (mercury-arc lamp) and connected to a computer via a SPOT 7.2 color mosaic camera (W. Nuhsbaum Inc.). PI has a maximum excitation wavelength of 536nm and a band-pass filter was used that excites the wavelengths between 515 and 560nm. Emission of PI in the visual range is 620nm. The intensity of PI fluorescence, in arbitrary optical units, was determined in the primary hippocampal cell layers of the DG, CA3, and CA1 using Image J 1.29x (National Institutes of Health, Bethesda, MD).

Statistical Analyses

Data were analyzed using the Statistical Package for the Social Sciences (SPSS) Version 20 Software (IBM Corporation, 2011). For the timeline experiment, an ETOH x OGD x SEX x TIME [2 x 2 x 2 x 6] repeated measures (RM) analysis of variance (Hudec et al.) was performed for each region. For the remaining experiments, an ETOH x OGD x SEX x REGION [2 x 3 x 2 x 3] RM ANOVA was used. Replicate was used as a covariate to control for differences across litters/culture preparations. When no main effect or interaction with SEX was observed, further analyses were conducted collapsed across this factor for ease of interpretation. In instances of a significant interaction with region, a separate ANOVA (ETOH x OGD x SEX) was conducted for the CA1, CA3, and DG. Significant interactions were examined using post hoc pair-wise comparisons with Tukey-Kramer correction for family-wise error. Data were converted to percent control for graphic presentation.

3.4 Results

Experiment 1: Timeline of 100mM EWD + 30 min OGD damage: In the CA1, ETOH/EWD + OGD caused an increase in cell damage relative to all other treatment groups that peaked at 16 hours; there was a significant ETOH x OGD x TIMEPOINT interaction, $F(5,680) = 8.63$, $p < .001$ and a significant ETOH x OGD interaction, $F(1,136) = 16.49$, $p < .001$, among others (see figure 3.1A). In the CA3, treatment with 30 min OGD alone was sufficient to produce cytotoxicity above control levels. There was a significant OGD x TIMEPOINT interaction, $F(5, 680) = 19.06$, $p < .001$, and the ETOH x OGD interaction approached significance ($p = .060$). Based on our *a priori* hypothesis and previous data showing that ETOH/EWD + OGD causes multiplicative damage in the CA3, comparisons at each time point revealed that ETOH/EWD + OGD slices had greater PI uptake compared to all other conditions at 4, 12, 20, and 24 hours

(see figure 3.1B). In DG, there was more cell damage in OGD slices, but this effect did not change over time (not shown). All subjects but 1 was male, so SEX was not included as a factor in any of the analyses.

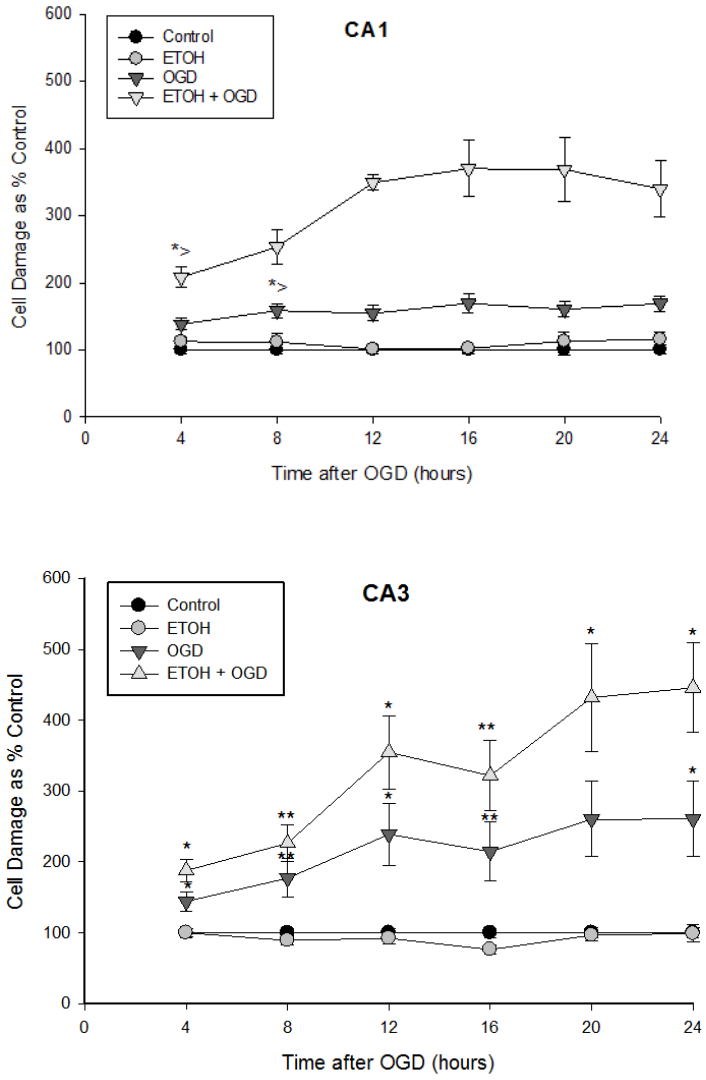


Figure 3.1. Mean PI uptake ± S.E.M. following EWD (100mM) and 30 min OGD over 24 hours in the CA1 and CA3. In the CA1, EWD + OGD caused an increase in cell damage relative to controls that peaked at 16 hours. In the CA3, OGD alone produced damage above control levels. ETOH + OGD still produced significantly greater cytotoxicity at 4,12, 20, and 24 hour time point, compared to all other treatments. * Indicates significant difference from all other treatment groups, p <.05. ** Indicates significant difference from ETOH and Control groups, p <.05.

Experiment 2: 50mM ETOH + 30 min OGD: Even with exposure to a relatively moderate dose of ETOH (50mM vs. 100mM which was used in previous experiments), there was significant cytotoxicity resulting from the combination of ETOH + OGD. The overall 2 x 2 x 2 x 3 revealed a significant REGION x OGD, $F(2,402) = 10.66$, $p < .001$, and ETOH x OGD, $F(1,201) = 9.40$, $p = .002$ interactions. In all 3 regions, there was a significant ETOH x OGD interaction; CA1, $F(1,201) = 9.26$, $p = .003$; CA3, $F(1,201) = 5.06$, $p = .025$; DG, $F(1,201) = 4.55$, $p = .034$. In the CA1 and CA3, ETOH + OGD slices had significantly greater PI uptake compared to all other treatment groups, while in the DG, these slices only differed from ETOH-treated slices. In the CA3, OGD treatment alone also significantly increased PI uptake above control levels (see figure 3.2), indicating that OGD alone was sufficient to cause cell damage in this set of slices.

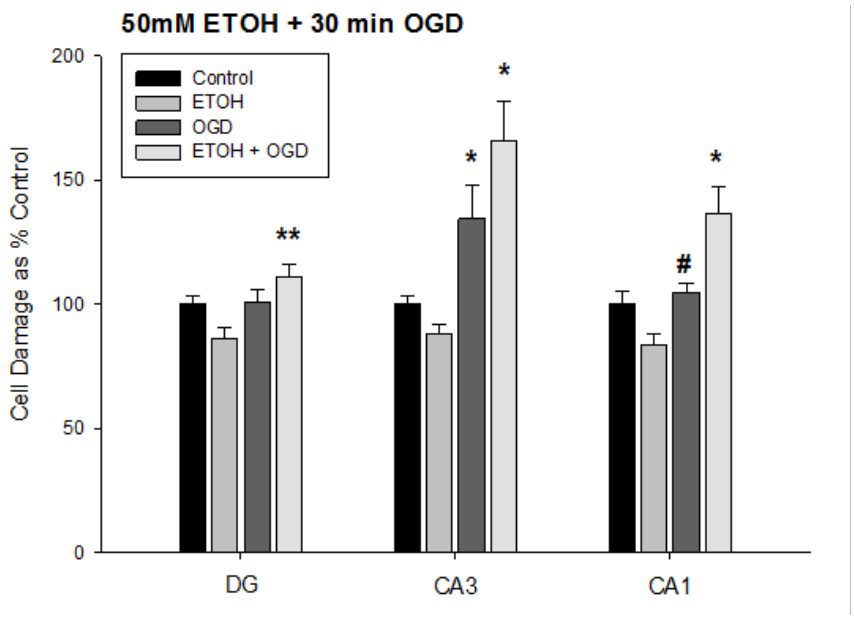


Figure 3.2. Mean PI uptake ± S.E.M. in the CA1, CA3, and DG following EWD (50mM) and 30 min OGD. EWD + OGD produced multiplicative damage in the CA1 and CA3. In the CA3, OGD alone increased PI uptake compared to controls. * Indicates significant difference from all other groups, p <.05. # Indicates significant difference from ETOH slices, p <.05. ** Indicates significant difference from ETOH slices, p <.05.

Experiment 3: 100mM ETOH + 30 min OGD with ETOH on board during OGD treatment: There was still an interactive effect of ETOH + OGD when withdrawal was initiated *after* OGD treatment. The overall 2 x 2 x 2 x 3 revealed a significant REGION x ETOH x OGD interaction F (2,272) =8.89, p =.001 and a significant ETOH x OGD interaction F (1,136) = 4.60, p =.034.

In the CA1, there was a significant ETOH x OGD interaction F (1,136) = 11.68, p =.001; slices treated with ETOH + OGD showed significantly greater PI uptake compared to all other treatment groups. There were no significant treatment effects in the CA3. Interestingly, in the DG, there was a main effect of ETOH, F (1,136) =5.33, p =.022, such that slices exposed to ETOH had lower PI fluorescence compared to those not treated with ETOH. Data from this experiment is shown in figure 3.3.

100mM ETOH + 30 min OGD NO EWD: The 2 x 2 x 3 (ETOH x OGD x REGION) repeated measures ANOVA revealed a significant REGION x OGD interaction, F (2,264) =8.32, p =.001 and a main effect of OGD F (1,132) =12.77, p <.001. All subjects but 1 were female, so SEX was not included as a factor in this analysis

There was a main effect of OGD in the CA1, F (1,132) =24.08, p <.001, and CA3, F (1,132) =7.35, p =.008, such that OGD treatment produced a modest but significant increase in PI uptake. Importantly, ETOH treatment did not potentiate the effect of OGD in any of the hippocampal regions. In the DG, there was a main effect of ETOH, F (1,132) =4.30, p =.040. Slices exposed to ETOH had lower PI fluorescence compared to those not treated with ETOH. While the effect of ETOH in the DG is significant, there may not be biological relevance in these slight differences in PI uptake. Additionally,

data from previous cell culture studies have demonstrated little or no treatment effects in the DG. Data from this experiment is shown in figure 3.4.

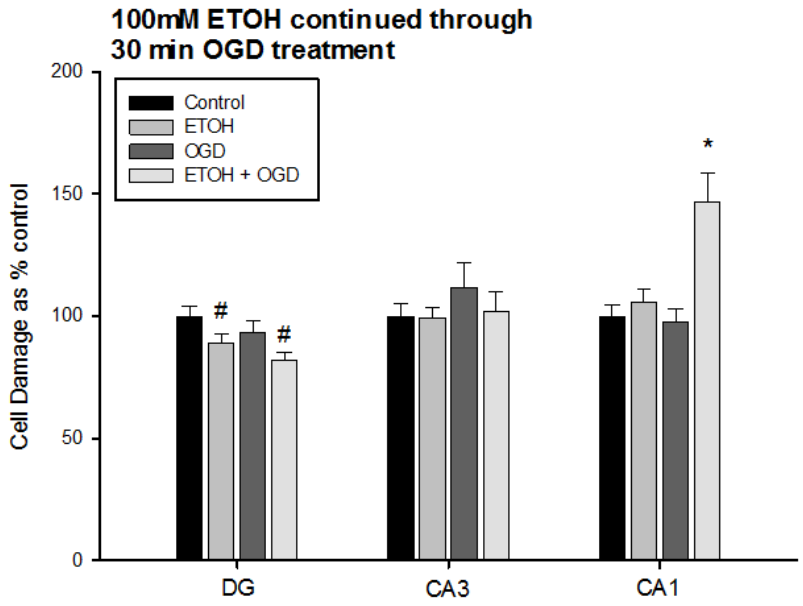


Figure 3.3. Mean PI uptake ± S.E.M. in the CA1, CA3, and DG following EWD (100mM) and 30 min OGD. ETOH was on board during OGD treatment. ETOH/EWD + OGD increased PI uptake in the CA1 compared to the other treatment groups, * p <.05. In the DG, ETOH treatment produced a modest decrease in PI fluorescence, # p <.05.

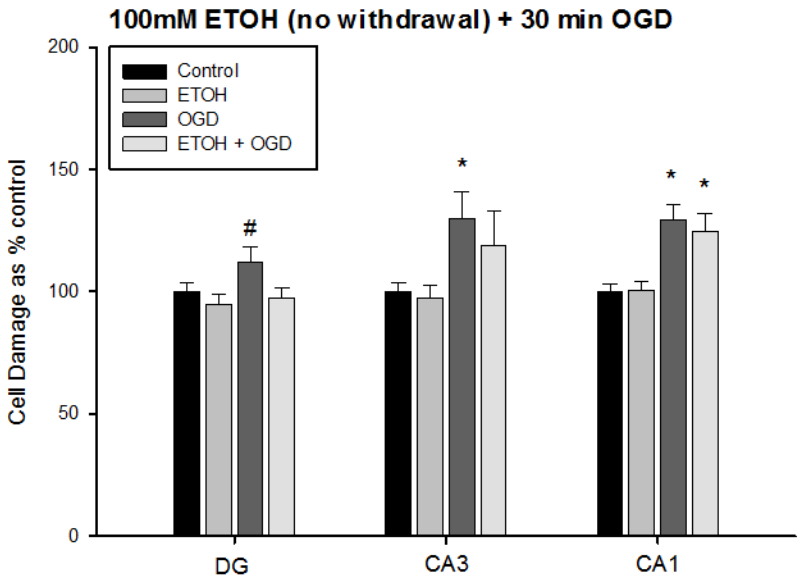


Figure 3.4. Mean PI uptake ± S.E.M. in the CA1, CA3, and DG following continuous exposure to100mM ETOH and 30 min OGD. In all 3 regions, OGD produced slight increases in PI uptake. * Indicates significant difference from other treatment groups, *p* <.05.

3.5 Discussion

The data from the majority of these studies provide further support for our hypothesis that pre-exposure to ETOH sensitizes the developing brain to a subsequent hypoxic challenge. These are the first data showing a timeline of ETOH + OGD damage in hippocampal slices. They demonstrate that in the CA1 (generally the hippocampal region most sensitive to excitotoxicity), damage can be observed as early as 4 hours post insult. Additionally, these data demonstrate that even at half the concentration of ETOH (50mM) used in initial studies, there was a significant effect of ETOH/EWD + OGD in the CA1 and CA3. This suggests that even relatively "low" ETOH exposures can leave the developing brain vulnerable to hypoxia. Finally, postponing EWD (100mM) until after OGD treatment still results in an ETOH + OGD interaction. However, when EWD is avoided altogether, there is no interaction in any hippocampal region indicating that withdrawal may be necessary for the combination of ETOH (withdrawal) and OGD to produce multiplicative damage in the hippocampus.

The data looking at PI uptake following EWD + OGD over time are consistent with previous hippocampal slice culture experiments of ETOH or OGD. In OGD studies, CA1 cytotoxicity (measured by PI uptake) begins as early as 4 hours post-OGD (Holopainen, 2005) and continues through 24 hours over several days. Similarly, at 24 hours, peak EWD toxicity is measured, although this effect is lost at 72 hours (Wilkins et al., 2006). In the CA3, EWD + OGD cytotoxicity did not peak until 20 hours, which could be partially due to the lower sensitivity of this region- although peak fluorescence was similar to that observed in the CA1. Several factors may underlie the observed pattern of PI uptake. For example, the lack of toxicity observed in the DG could be due to regional/cell type differences in mechanisms that are protective against excitotoxicity including higher concentrations of intracellular Ca^{2+} buffers in the granule layer

(Matthews, Schoch, & Dietrich, 2013). Additionally, in the CA1 and CA3, PI uptake may increase and then plateau after the first several hours post-insult as receptors that have been continually activated become desensitized (Cole, ffench-Mullen, & Fisher, 1989). Together, these data indicate that EWD + OGD toxicity begins early post insult and thus potential treatments should be applied early. Indeed, in certain experimental models, NMDA receptor antagonism and other therapeutic interventions are increasingly ineffective against OGD as time passes (Ahlgren, Henjum, Ottersen, & Runden-Pran, 2011). Similarly, pharmacotherapies that target EWD toxicity in the developing brain are effective when ETOH levels in the system approach zero (Idrus et al., 2014; Lewis et al., 2007; Lewis et al., 2012; Wellmann et al., 2010).

A subset of the data from this set of experiments indicated that withdrawal from chronic exposure to 50mM ETOH was sufficient to sensitize the developing hippocampus to 30 min OGD. Hippocampal studies with 50mM ETOH show that it produces damage in combination with other insults or in instances of repeated withdrawal (Butler et al., 2008; Reynolds, Berry, Sharrett-Field, & Prendergast, 2015), but does not produce cytotoxicity alone as measured by PI uptake or neuron-specific nuclear protein (NeuN) immunofluorescence. This exposure is half of that used in our previous experiments and equates to approximately 161 mg/dL over a 5-day exposure period. Although this is twice the legal limit for ETOH intoxication in the U.S., it is a level that would be easily attained by an alcoholic and/or a binge-drinking woman (Paintner et al., 2012). These data provide evidence that even relatively doses (and a subthreshold dose in this model) (Barron et al., 2008; Collins, Zou, & Neafsey, 1998) of ETOH leave the developing brain vulnerable to hypoxia.

Interestingly, in the three 50mM ETOH culture preparations, the pattern of cell damage shows that the CA3 was *more* sensitive than the CA1 to OGD alone or EWD +

OGD. This is in contrast to the previous data in PND 8 tissue from our lab and also multiple other studies of either EWD or OGD, indicating that the CA3 is less affected by either of these insults (and other excitotoxic challenges) (Cronberg, Jensen, Rytter, & Wieloch, 2005; Dennis et al., 2011a; Lutz, Carter, Fields, Barron, & Littleton, 2015; Stanika et al., 2010; Wilkins et al., 2006). However, there is some data from our lab showing that the CA3 is more affected than the CA1 by EWD + OGD in tissue from PND 2 pups (unpublished data). There are also some instances in slice cultures in which the CA3 shows a unique vulnerability (e.g., Butler et al., 2008) but more information is needed to determine why this effect was observed in these cultures.

One of the most important findings from this set of experiments is that withdrawal from ETOH is likely necessary to produce multiplicative cellular damage in combination with OGD. In agreement with other studies (e.g., Reynolds et al., 2015; Wilkins et al., 2006), continuous ETOH exposure without withdrawal does not result in hippocampal cytotoxicity. Not surprisingly, when EWD begins after OGD treatment, there was still an interaction of EWD and OGD in the sensitive CA1 region. Excitotoxicity begins soon after the onset of tissue oxygen depletion and continues up to hours following the insult (for review see Dirnagl et al., 1999). Additionally, data from the current study show that substantial EWD + OGD- induced PI fluorescence is negligible at 4 hours and increases steadily until 16 hours post-insult. Therefore, starting EWD after OGD is still well within the window of tissue susceptibility. While other mechanisms of damage cannot be ruled out, these findings suggest that excitotoxicity occurring during EWD + OGD plays a major role in the damage observed in hippocampal slices and potentially in our in vivo model.

The current set of experiments supplement the data presented earlier in Chapter 2. However, one notable difference between the data sets is that 30 min OGD does

appear to produce increases in PI fluorescence compared to controls, suggesting this may be more than a mild or sub threshold exposure. Other OGD models show durations of 30 min or even fewer produce significant hippocampal cytotoxicity (Ahlgren et al., 2011; Holopainen, 2005; Wise-Faberowski, Robinson, Rich, & Warner, 2009) so the current findings are not entirely unexpected. For the most part, any effects of 30 min OGD on PI fluorescence in this study were modest and there was still an interaction of EWD and OGD. Importantly, EWD, but not continuous ETOH exposure, potentiated OGD cell damage. Taken together, these data provide a better understanding of the interaction of ETOH and OGD in the developing brain and also suggest the need for further investigation.

Chapter 4

THE EFFECTS NMDA RECEPTOR MODULATION ON CELL DAMAGE FOLLOWING DEVELOPMENTAL ETHANOL AND HYPOXIA EXPOSURE

4.1 Abstract

The effects of fetal ethanol (ETOH) exposure vary across individuals and numerous studies have shown that other developmental variables influence the outcome. Hypoxia, a decrease in oxygen, is a common occurrence during fetal development, and may contribute to morbidity following ETOH exposure. Studies conducted in our laboratory suggest there is a synergistic effect of ETOH withdrawal (EWD) and hypoxia during a time of brain development that overlaps the 3rd trimester brain growth spurt in humans. Damage from these two insults may occur through overlapping mechanisms including overactivation of N-methyl-D-aspartate receptor (NMDAr) (excitotoxicity). A promising alternative to full NMDAr blockade, which can be toxic, is modulation at the polyamine site. The current studies used a 3rd trimester hippocampal slice culture model to test the hypothesis that blocking the polyamine site on NMDAr would protect against cell damage following the combination of developmental EWD and hypoxia. Hippocampal slices were taken from postnatal day (PND) 8 rat pups and exposed to an initial concentration of 100mM ETOH for an average of 65mM ETOH for 10 days. At the initiation of EWD, slices were exposed to either 30 min control air/media *or* oxygen glucose deprivation (OGD) as a model of hypoxia. Following OGD or control air exposure subsets of slices were exposed to JR220, a novel compound that displaces the polyamine spermidine on the NMDAr. Twenty-four hours following the initiation of EWD and OGD, cell damage was assessed using propidium iodide (PI) uptake (a marker for non-specific cell damage). The preliminary results suggest that polyamine

site blockade may be protective against EWD + OGD induced damage as measured by PI uptake.

4.2. Introduction

Ethanol (ETOH) consumption during pregnancy continues to be a major problem, despite preventative efforts and knowledge of its harmful effects. Exposure to ETOH during fetal development results in a variety of cognitive and behavioral deficits, with a range of adverse effects observed in the clinical population. One explanation for the variability among affected individuals is that ETOH exposure interacts with other developmental challenges such as cigarette smoke or hypoxia. Hypoxia is a common occurrence during fetal development, but previous exposure to ETOH can compromise the brain's ability to cope with decreases in oxygen levels (Carter et al., in preparation). More specifically, we have shown that the combination of ETOH withdrawal (EWD) and hypoxia produces hippocampal cytotoxicity and behavioral deficits in 3rd trimester rodent models. This experiment assessed the hypothesis that the interactive effect of EWD and hypoxia is a result of excitotoxicity via overactivation of N-methyl-D-aspartate receptor (NMDAr). Traditional NMDAr antagonists such as MK801 are toxic and thus have limited clinical application. Therefore this study used a novel compound, JR220, which acts on the polyamine site of the NMDAr, to determine if manipulation of the polyamine site on NMDAr reduces EWD-associated and hypoxic damage.

Polyamines (putrescine, spermine, and spermidine) are small compounds that serve a number of important physiological functions. In the CNS, they can influence gene regulation, cell-cell communication, cell growth, and importantly, they act as allosteric modulators of the NMDAr (Pegg & Wang, 2009). Polyamines also interact with other ion channel proteins including α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPArs). Their interactions with AMPArs are especially important because

polyamines permeate this channel and enter the extracellular space in an activity dependent manner (for review see Stromgaard & Mellor, 2004). While the actions of polyamines are critical for normal CNS development and function, excessive levels can be deleterious under certain conditions. Chronic ETOH exposure and hypoxic conditions increase polyamine levels/synthesis via, among other possible mechanisms, increased ornithine decarboxylase activity, the rate-limiting step in their synthesis. (Davidson & Wilce, 1998; Gibson et al., 2003; Longo et al., 1993) and recent work from our lab shows that polyamine levels are elevated in brain structures, including the hippocampus, following neonatal ETOH exposure (Wellmann et al., unpublished data). NR2B expression, which is high during development, increased during chronic ETOH, and prolonged by fetal ETOH exposure, confers receptor sensitivity to polyamine potentiation (Follesa & Ticku, 1995; Prendergast & Mulholland, 2012; Rock & Macdonald, 1995). Compounds that have some action at the polyamine site protect against EWD (Gibson et al., 2003; Lewis et al., 2012) and hypoxia/ischemia (Gilad & Gilad, 2000; Reyes, Reyes, Opitz, Kapin, & Stanton, 1998; Yu, Marcillo, Fairbanks, Wilcox, & Yezierski, 2000).

JR220 was developed as an analog of the aminoguanidine agmatine. Agmatine interacts with several molecular targets including ion channels, nitric oxide synthesis, polyamine sites on NMDArs, and polyamine metabolism (Littleton et al., 2001; Molderings & Haenisch, 2012). While agmatine has been shown to be neuroprotective in various models (Lewis et al., 2007; Wang, Iyo, Miguel-Hidalgo, Regunathan, & Zhu, 2006; Wellmann et al., 2010; Zhu, Wang, & Bissette, 2006), it is a relatively non-specific compound. Compared to agmatine, JR220 is much more selective for inhibition of polyamine potentiation of the NMDAr and it also has 200x the potency of agmatine, with an IC50 of 3.6mM (Barron et al., 2012). Because of its potency and selectivity, JR220 is an exciting prospect both for understanding the mechanism underlying EWD + hypoxic damage and as a potential therapeutic agent.

Recently, JR220 was screened in a number of rodent models of ETOH exposure/withdrawal. These screens indicated that JR220 is protective when administered during EWD (i.e., in organotypic hippocampal slices, handling induced convulsions, and in a 3rd trimester neonatal exposure paradigm). Additionally, JR220 reduces voluntary ETOH consumption in rats and mice (Barron et al., 2012). While JR220 has not yet been tested in models of hypoxia, its parent compound agmatine is protective against hypoxic injury in prenatal and neonatal rat models (Feng et al., 2002; Gilad & Gilad, 2000; Kim et al., 2004). Preliminary results from the current neonatal organotypic hippocampal slice culture study indicate that JR220 may be protective against the combination of developmental ETOH/EWD + hypoxia. Preliminary results also suggest that polyamine enhancement of NMDAr activity is responsible (at least partially) for the observed cytotoxicity.

4.3 Methods

Hippocampal Slice Cultures

The organotypic hippocampal slice culture model used in previous experiments was used to determine if JR220 is protective against EWD + OGD cytotoxicity. Slices were obtained from PND8 male and female rat pups from 4 separate litters. Tissue preparation was performed as described in Chapter 2. On DIV 5 inserts were transferred to 100mM ETOH media or control media and this was done again on DIV 10, maintaining the same treatment groups. On DIV 15, control and ETOH slices were further divided into 30 min oxygen glucose deprivation (OGD) or control air groups, giving the following treatment groups: control, ETOH (more specifically, EWD), OGD, and EWD + OGD. ETOH slices began withdrawal at the start of the OGD or control air treatment. After 30 min OGD or control air treatment, slices were placed in regular culture media containing propidium iodide (PI; a non-specific marker of cell damage; 3.74μM) with or without the addition of JR220 (50, 100, or 125 μM). This yielded a total

of 8 treatment groups: Control (+/- JR220); EWD (+/- JR220); OGD (+/- JR220); EWD + OGD (+/- JR220). There was a total of 36 - 72 slices per treatment group. Fluorescence was measured 24 hours after OGD. The intensity of PI fluorescence, in arbitrary optical units, was determined as described above, in the primary hippocampal cell layers of the DG, CA3, and CA1 using densitometry.

Statistical Analyses

All statistical analyses were conducted using IBM Statistical Package for the Social Sciences (SPSS) Version 20 Software (IBM Corporation, 2011). For each region, a 2 x 2 x 4 x 2 analysis of variance (Hudec et al.) was performed including the variables: ETOH, OGD, JR220, and SEX. Replicate was used as a covariate to control for differences across litters/culture preparations. If there was no main effect or interaction with SEX, further analyses were conducted collapsed across this factor for ease of interpretation. Significant interactions were examined using post hoc pair-wise comparisons with Tukey-Kramer.

4.4 Results

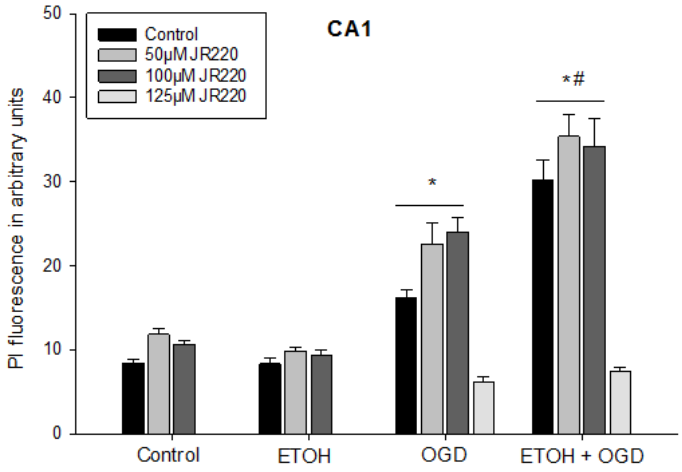
In the CA1, EWD + OGD treatment produced an increase in cell damage relative to all other treatment groups; significant ETOH x OGD interaction, $F(1,305)= 43.21$, $p<.001$. This effect was attenuated by treatment with 125μM JR220 (see figure 4.1 CA1). Plates containing control and ETOH treated slices were lost during incubation due to infection, therefore, control + 125 μM JR220 and ETOH + 125 μM JR220 treatment groups were not available for the data analysis therefore; these results should be interpreted with some caution. OGD alone produced slight increases in PI uptake, which was also attenuated by 125μM JR220 (see figure 4.1 CA1). There was also a

main effect of JR220, $F(1,305)= 4.64$, $p= .001$, such that slices treated with JR220 had less PI uptake compared non-JR220 slices.

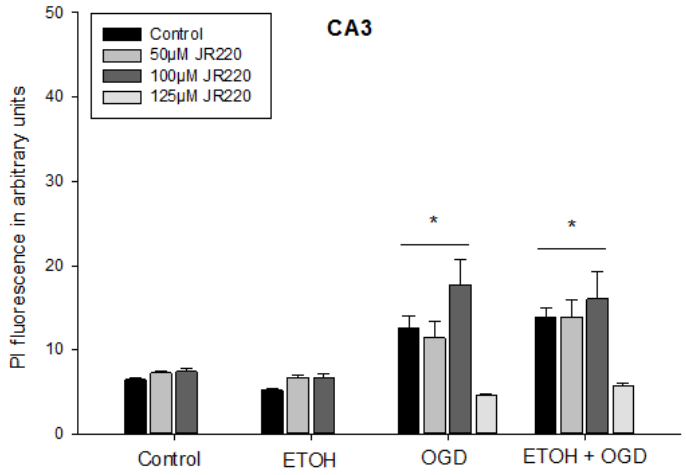
In the CA3, OGD alone produced damage that was not potentiated by EWD; main effect of OGD $F(1,305)= 69.37$, $p<.001$. Treatment with 125μM JR220 reduced PI uptake to control levels (see figure 4.1 CA3). There was also a main effect of JR220, $F(1,305)= 5.84$ such that slices treated with JR220 had less PI uptake compared to non-JR220 slices.

In the DG, there was very little PI uptake for all treatment groups compared to the CA1 and CA3. There were some statistically significant effects, however the biological significance is questionable due to the very slight differences in PI fluorescence. With this in mind, there was a main effect of ETOH, $F(1,305)= 6.16$, $p= .014$ such that ETOH treated slices had less PI uptake compared to non-ETOH slices. There was also a main effect of OGD, $F(1,305)= 10.39$, $p= .001$; generally, OGD treated slices showed slightly greater toxicity as compared to non-OGD slices. Finally, there was a main effect of JR220, $F(1,305)= 20.23$ $p< .001$. Interestingly, at 50 and 100μM, JR220 produced marginal increases in PI uptake while at 125μM it reduced toxicity.

A



B



C

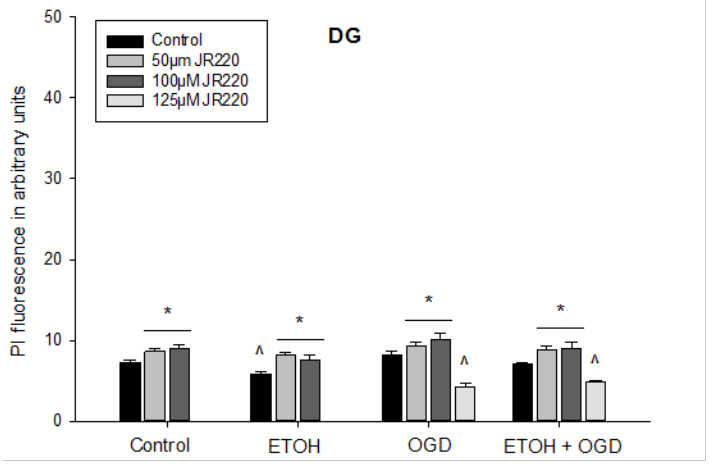


Figure 4.1. Mean PI uptake ± S.E.M. in the CA1, CA3, and DG regions 24 following 30 min OGD and initiation of EWD. **A)** The combination of EWD and OGD produced multiplicative damage in the CA1, which was attenuated by 125μM JR220. **B)** In the CA3, OGD alone produced pronounced damage that was not potentiated by EWD. This effect was attenuated by treatment with 125uM JR220. **C)** In the DG, ETOH treatment appears to decrease PI uptake compared to control slices while treatment with 50 and 100μM JR220 increases PI uptake. However, 125μM reduces PI fluorescence below control levels. * Indicates significantly greater than controls, p <.05; # indicates significantly greater than OGD, p <.05; ^ indicates significantly less than controls, p <.05.

4.5 Discussion

This study investigated the efficacy JR220 in an organotypic hippocampal slice culture model of developmental ETOH/EWD + hypoxia (OGD). Additionally, through the use of JR220, this study sought to understand the role of polyamine modulation of NMDAr in the cytotoxicity resulting from these two insults. Consistent with previous hippocampal slice culture studies, the combination of EWD and 30 min OGD produced multiplicative damage in the sensitive CA1 region as measured by PI uptake. This effect was attenuated by the highest concentration of JR220 (125μM). In the CA3, OGD produced cytotoxicity that was not potentiated by previous ETOH exposure. As in the CA1, 125μM JR220 reduced EWD + OGD-induced PI uptake. In the DG, a different pattern of PI fluorescence was observed. Compared to the CA1 and CA3, there was little PI fluorescence in the DG. Although there were some statistically significant results in this region, the biological relevance of such small differences in PI uptake is questionable. This is consistent with the previous hippocampal slice culture studies discussed in this dissertation, which show few observable treatment effects in the DG. It is important to note that this data should be interpreted with caution, as control slices treated with 125μM JR220 were lost during incubation. Future studies should include these control groups so all the appropriate group comparisons can be made. However, overall, the preliminary data from the CA1 and CA3 indicate that JR220 is protective against combined exposure to ETOH/EWD and OGD as well as OGD exposure alone. The protective effect of JR220 suggests that polyamine enhancement of the NMDAr is a contributing factor to cell damage in this model.

We have previously shown that 50-100μM JR220 is protective against EWD-induced cytotoxicity in the CA1 of hippocampal slices (Wellmann et al., unpublished data). In the current study, 125μM was effective in reducing toxicity, while lower concentrations were

ineffective. This could indicate a larger shift in polyamine levels in combined exposure to EWD and OGD as compared to ETOH treatment alone, which would not be entirely surprising given that separately, both insults increase levels in the developing brain (Davidson & Wilce, 1998; Gibson et al., 2003; Longo et al., 1993). If there were relatively more polyamines in the system, a higher concentration of JR220 could theoretically be required to displace polyamines from their binding sites on NMDAr. Alternatively, it is possible that the combination of EWD and OGD has a unique effect on NMDAr expression or function, making the receptors more sensitive to events such as polyamine potentiation. In developing brain tissue ETOH and hypoxia (separately) produce changes in NMDArs, especially in NR1, NR2A, and NR2B subunits, which are abundant in certain regions including the hippocampus (Butler et al., 2010; Nixon, Hughes, Amsel, & Leslie, 2002, 2004; Wise-Faberowski et al., 2009). These alterations are heavily dependent on the nature of the exposures (e.g., dose, duration, developmental stage) and the experimental model so it is difficult to speculate what receptor changes may be occurring in this exposure model. Future experiments should examine this question from a more mechanistic approach looking at the effects of ETOH and OGD (hypoxia) on NMDAr numbers and sensitivity as well as endogenous polyamine levels to better understand ETOH and OGD's actions.

Further support for the role of polyamines and/or NMDAr in EWD + OGD toxicity is provided by preliminary hippocampal studies using ifenprodil and CP-101, 606 (traxoprodil). Both of these compounds act on the NR2B subunit of the NMDAr (Amico-Ruvio, Paganelli, Myers, & Popescu, 2012; Chazot, Lawrence, & Thompson, 2002). As stated earlier, the NR2B subunit, which is highly expressed in the developing hippocampus, especially in the CA1 region, increases NMDAr sensitivity to polyamine potentiation (Littleton et al., 2001; for review see Mony et al., 2009). In these

experiments, slices were treated with the same ETOH and OGD exposure as in the current study: 100mM chronic ETOH for 10 DIV followed by 30 min OGD exposure at the beginning of EWD. Slices were then exposed to either ifenprodil or CP-101, 606 for 24 hours until imaging for PI uptake. In the CA1 of slices exposed ETOH + OGD, CP-101, 606 significantly reduced cytotoxicity resulting from exposure to the two insults. A similar pattern was observed in slices treated with ifenprodil, although differences did not reach statistical significance (unpublished data).

While these results are preliminary, they are exciting for several reasons. They provide the first evidence that NMDAr modulation is effective in reducing toxicity resulting from exposure to ETOH/EWD and hypoxia. These results are also the first to demonstrate that JR220 is effective not only in models of developmental ETOH and subsequent withdrawal but also hypoxia. Finally, only a single JR220 treatment was necessary to reduce cytotoxicity in the CA1 and CA3. This has important clinical implications as treatment with JR220 or a similar compound could be administered when a mother and newborn are already seeking medical treatment (i.e., during labor and delivery). Future studies should more directly investigate the mechanisms underlying exposure to ETOH/EWD and hypoxia and also examine JR220 our in vivo model of ETOH + hypoxia.

Chapter 5

DISCUSSION

5.1. General Discussion

5.1.1. Summary of Findings: The purpose of these studies was to investigate the hypothesis that exposure to ETOH during development sensitizes the CNS to a hypoxic challenge. Hypoxia, which is a common event during fetal development and parturition, may contribute to morbidity following fetal ETOH exposure and this could explain why some children appear to be more affected than others. Targeting damage that results in a situation such as this could reduce or rescue the effects of ETOH exposure. Initial data from our organotypic hippocampal slice culture model show that 100mM EWD in combination with 30 min OGD (hypoxia) produces multiplicative damage in the CA1 and CA3 regions of the hippocampus.

The following study assessed the behavioral consequences of combined exposure to ETOH/EWD + hypoxia in a 3rd trimester neonatal model. Rats exposed to the combination of ETOH/EWD + a brief hypoxic episode (during EWD) displayed behavioral deficits in the open field and in a water maze task (effects that were somewhat sex-dependent). This occurred at doses of ETOH and hypoxia that had little or no behavioral effects alone.

Additional cell cultures studies (Chapter 3) extended initial findings in hippocampal slices. Results show that EWD + OGD damage slowly increases over the first few hours post-insult in the CA1 and CA3 as measured by PI uptake. In another set of culture preps, the data indicated that a more “mild” exposure to ETOH (50mM vs. the previous 100mM chronic exposure) still results in sensitization to subsequent 30 min OGD in the CA1 and CA3. Data from the last experiment in the 2nd series of studies showed that

EWD appears to be necessary to produce multiplicative cytotoxicity. When slices were exposed to continuous ETOH (avoiding withdrawal), there was no interaction between ETOH and OGD.

The last set of hippocampal slice culture experiments was designed to assess the protective effects of JR220 and also understand the role of NMDAr in the damage underlying exposure to ETOH/EWD and hypoxia. JR220, which acts on the polyamine site of NMDAr, reduced cytotoxicity in slices treated with the combination of EWD and OGD. These data, along with those showing withdrawal is necessary to produce an interaction of ETOH/EWD and hypoxia, are the first to indicate that NMDAr-medicated excitotoxicity is responsible for cell damage. Taken together, the data presented in this dissertation show that exposure to ETOH during CNS development can sensitize the brain to an acute hypoxic challenge and this may be due in part to excitotoxic mechanisms.

5.1.2. *Future Directions*: These studies show the impact of ETOH exposure followed by a brief hypoxic challenge, however, the mechanisms underlying this effect are still unclear. Initial data with JR220 point to NMDAr mediated excitotoxicity, but future studies should involve a more thorough investigation. The role of NMDAr activity could be further examined using compounds whose mechanisms are better understood than JR220; very preliminary data in hippocampal slices with CP-101, 606 and ifenprodil implicate NMDAr overacitvity in cytotoxicity following exposure to EWD and OGD. Further evaluation of excitotoxicity, by examining other cellular events such as Ca^{2+} entry or of the role of other ion channels would contribute to understanding of this mechanism. Also, as stated earlier, looking at changes in NMDAr and in polyamines would aid in the understanding of ETOH/EWD + OGD's actions. Finally, while excitotoxicity appears to be a major factor in cell damage, other mechanisms cannot be

ruled out and are likely to be contributing factors to damage. Additional mechanisms should be examined (e.g., inflammation and oxidative stress) in order to draw conclusions about what cellular events occur as a result of exposure to ETOH/EWD and hypoxia.

Future studies should also better characterize the cell damage following ETOH/EWD + hypoxia. Previous work from our lab in hippocampal slices demonstrated EWD + hypoxia (OGD) produces multiplicative damage as measured by PI fluorescence (a non-specific marker of cell damage), but not neuronal nuclei (NeuN) immunoreactivity, which is specific to neurons (unpublished data). This discrepancy indicates that glia, as well as neurons, are likely injured as a result of ETOH/EWD and/or hypoxia. Thus, staining for astrocytes, oligodendrocytes, and microglia should be considered. In addition to other cell markers, the effects of ETOH/EWD and hypoxia beyond the 24-hour time point should be examined. In both ETOH and hypoxia, cell damage occurs over the course of hours to days (Nitatori et al., 1995; Pulsinelli, Brierley, & Plum, 1982; Wilkins et al., 2006). Finally, all of this should be investigated in other structures (e.g., the cerebellum and prefrontal cortex) as the effects of ETOH/EWD or hypoxia vary across different brain regions. Knowing how ETOH/EWD+ hypoxia affects the various structures and cell types found in the CNS and how this changes over time would allow for the cell damage to be more accurately targeted.

The current data from hippocampal slices could be supplemented by similar measurements performed on in vivo tissue. While hippocampal slices have much of the circuitry of an intact hippocampus, there are inherent differences between the cell culture model and live animal model. Therefore, although the in vitro and in vivo models used in the current study compliment each other, it is difficult to draw certain conclusions such as what changes in the brain are associated with behavioral deficits. In vivo staining (for

example, using NeuN and glia markers) of brain tissue from rodents treated with neonatal ETOH and hypoxia would more accurately depict what changes in the brain underlie observed behavioral deficits.

Finally, further investigation of JR220 should be done to determine its clinical potential. JR220 has already been tested in a 3rd trimester ETOH exposure model similar to that in the current study. A single administration during EWD was shown to be effective in reducing ETOH-related deficits (Wellmann et al., unpublished data). Testing JR220 in the current neonatal model of ETOH + hypoxia would be a natural next step in this series of experiments. Future studies should also characterize pharmacodynamic and pharmacokinetic properties of JR220. It is known that JR220 interacts with NMDAr at the polyamine site, however potential interactions with other receptors or systems in the CNS cannot be excluded. Additionally, the safety of JR220 has yet to be determined. In initial organotypic hippocampal screenings, JR220 did not produce toxicity at concentrations up to 500μM (Barron et al., 2012). Measures of activity in an elevated plus maze indicate that at certain doses, JR220 may have sedative effects (which is not entirely surprising given its effects on NMDAr) (Barron et al., 2012). Eventually, JR220 should be screened in a variety of assays to determine other potential mechanisms as well as its effects on other CNS structures and its safety/acceptable range of doses. The preliminary studies described in Chapter 4 of this dissertation, along with previous data from this lab and its collaborators, indicate that JR220 is a promising candidate for treatment of ETOH and/or hypoxia-related damage.

5.1.3. Clinical Implications/Conclusions: Currently, there are no biological treatments for infants and children affected by a FASD. Instead, treatment is generally found in the form of life long interventions that are catered to specific cognitive or behavioral issues (Bertrand et al., 2005). One reason for the lack of pharmacotherapies is perhaps an

unclear understanding of how ETOH produces neurological damage. ETOH affects the developing brain via diverse mechanisms and the severity of these effects could depend on the presence of additional factors including ETOH's interactions with other insults. This is the first study to establish that exposure to sub threshold levels of ETOH can make the brain susceptible to an acute hypoxic event, which would normally be considered benign under typical developmental conditions. Identification of newborns affected by an event such as this would allow for appropriate interventions to be implemented. While it is unreasonable to predict that all damage from developmental ETOH exposure could be rescued with a single drug treatment, pharmacological intervention with a compound such as JR220 could provide neuroprotection during and critical period of damage. This could potentially reduce or eliminate the need for long-term interventions, which would benefit the individual and society.

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VITA

Megan L. Carter
Experimental Psychology
University of Kentucky College of Arts and Sciences, Lexington, KY

Education

University of Kentucky, Lexington, KY August, 2010- present
Doctoral Candidate Researcher
Current GPA 3.96/4.0
Passed Qualifying Exam November 2013

Master of Science, Psychology, January 2013
Graduate GPA 3.93/4.0
University of Kentucky, Lexington, KY

Bachelor of Arts, Psychology, May 2007
Cum Laude, Honors Program
Major GPA 3.84/4.0
Cumulative GPA 3.51/4.0
University of Kentucky, Lexington, KY

Publications

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. *Recent Patents on CNS drug Discovery: Special Issue on Alcohol.* Aug 2012; 7(2): 129-144

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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*

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Seminars

Carter, M.L. "Alcohol Sensitizes the Developing Brain to Subsequent Hypoxic Challenge." NIDA Training Grant Symposium, April 22, 2014.

Carter, M.L. "Understanding the Interaction of Neonatal Alcohol and Hypoxia *in vitro."* NIDA Training Grant Symposium, March 20, 2013.

Carter, M.L. "Examining the Interaction of Neonatal Alcohol and Hypoxia: The Behavioral Edition." Behavioral Neuroscience and Psychopharmacology Departmental Brown Bag, March 2013.

Carter, M.L. "Examining the Interaction of Neonatal Alcohol and Hypoxia *in vitro*." Behavioral Neuroscience and Psychopharmacology Departmental Brown Bag, March 2012.

Funding/Awards

July 2014 - present: Kentucky Research Challenge Trust Fund pre-doctoral fellow.

July 2012 - July 2014: National Institute on Drug Abuse pre-doctoral trainee on T32 DA 016176.