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Examining the Neuroprotective Properties of 3-Dimethoxybenzylidene-Anabasine (DMXB-A) in a Third Trimester Chronic Ethanol Exposure Model in Rats

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EXAMINING THE NEUROPROTECTIVE PROPERTIES OF 3-DIMETHOXYBENZYLIDENE-ANABASINE (DMXB-A) IN A THIRD TRIMESTER CHRONIC ETHANOL EXPOSURE MODEL IN RATS

THESIS

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in the college of Arts and Science for Psychology at the University of Kentucky

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ABSTRACT OF THESIS:

EXAMINING THE NEUROPROTECTIVE PROPERTIES OF 3-DIMETHOXYBENZYLIDENE-ANABASINE (DMXB-A) IN A THIRD TRIMESTER CHRONIC ETHANOL EXPOSURE MODEL IN RATS

Excitotoxicity caused by ethanol (ETOH) withdrawal (EWD) is highly detrimental to the developing brain. Targeting this excitotoxicity has been shown to be a promising approach for improving outcome following developmental ETOH exposure. Activation of nicotinic acetylcholine receptors (nAChR), in the central nervous system can be protective against EWD. We examined the ability of DMXB-A, a α_7 nAChR agonist, to reduce neurotoxicity caused by EWD in the hippocampus. To test this, an organotypic hippocampal slice culture was used. Slices were exposed to ETOH (100mM) or control medium. After 10 days, the slices were treated with DMXB-A (1, 3, or 10uM) during EWD. After 24 hours of EWD cell damage in the CA1, CA3, and dentate gyrus of the hippocampus was analyzed. The combination of EWD and NMDA produced increased toxicity compared to controls in the CA1 region, DMXB-A attenuated this effect, suggesting that DMXB-A was protective against EWD neurotoxicity in vitro. These findings are exciting because this drug is currently in clinical trials for a variety of CNS conditions and so has significant translational potential. Further research is necessary to better understand the extent of its neuroprotective properties and to determine its ability to reduce behavioral deficits following prenatal ethanol exposure.

KEYWORDS: DMXB-A, α-7 nAChR, FAS/FASD, OHSC

Logan James Fields

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Table of Contents:

LIST OF TABLES

LIST OF FIGURES

Chapter 1:

Introduction

Ethanol Use, Abuse, and Outcomes

Background

Alcohol is one of the most commonly used drugs in the western world, with an estimated 87% of individuals (18 years of age or older) reporting that they had consumed alcohol at some point in their lifetime, 71% in the last year, and 56 % in the last month (NIAAA, 2014). Though ethanol consumption alone is most often not problematic, approximately 25% of the population (18 years or older) have reported binge drinking (defined as 3 drinks for women and 4 drinks for men in two hours) in the last month and about 7% reporting that they had engaged in heavy drinking (5 or more drinks in one occasion on 5 days in a given month) (NIAAA, 2014). These drinking patterns sometimes lead to alcohol use disorders which can be problematic for the individual and those around them. Ethanol consumption during pregnancy in women who have an alcohol use disorder can have many negative effects on the newborn. The effects of ethanol consumption during pregnancy on infant development have been reported for many years. Some of the earliest examples of ethanol consumption during pregnancy can be seen in ancient Greek and Roman beliefs, stating that being intoxicated at the time of reproduction would lead to the birth of a "damaged child" (Calhoun & Warren, 2007). Clinical descriptions of the association between maternal drinking and the development of learning/attention disorders and physical deformations have been reported for several decades. The first published report was in 1968 by a French physician and his colleagues who described a set of characteristics in children who had been exposed to ethanol in utero (Lemoine et al., 1968). In the United States, the syndrome was first identified by Jones and Smith, who described a pattern of malformations in the offspring of mothers who had consumed ethanol during pregnancy. This research led to the observation that infants who were exposed to ethanol in utero could have a characteristic set of abnormalities which has come to be called Fetal Alcohol Syndrome (FAS) (Jones & Smith, 1973). In order to be diagnosed with the disorder the child has to have (a)

evidence of a characteristic pattern of facial anomalies which can include short palpebral fissures and abnormalities in the premaxillary zone (i.e. flat upper lip, flattened philtrum, and flat midface), (b) evidence of pre- and/or postnatal growth retardation (born small and fail to thrive i.e. low birth weight), and (c) evidence of CNS damage (microcephaly, mental retardation, or neurobehavioral deficits) (Stratton, 1996). Because prenatal ethanol effects vary in severity, with FAS being the most extreme case, a spectrum was developed to aid in the description of children who were only marginally affected by ethanol's effects during gestation (Hoyme, et al. 2004). These guidelines that encompass fetal ethanol effects without the full diagnosis of FAS are known as Fetal Alcohol Spectrum Disorders (FASDs). These guidelines state that an infant exposed to ethanol during pregnancy does not have to demonstrate all of the characteristic symptoms of FAS to be considered to have an alcohol-related disorder (Jones, 2011). This aids families of children affected by ethanol exposure by providing special services to those affected (Jones, 2011). Due to better understanding of the disorder and its diagnosis, estimates of the population affected by FAS/FASD have increased. Estimates taken in the United States suggest that the prevalence of FAS is about 0.5-2.0 cases for every 1000 births (National Institutes of Health., 2013). Recent studies have also shown that misdiagnoses in children in foster/adoptive care is also very prevalent, where 80% of children who met the criteria for diagnosis in the fetal alcohol spectrum had previously not been diagnosed as having been exposed to prenatal alcohol (Chasnoff et al., 2015). Although exact numbers on the prevalence of FASDs in the population are unknown, using conservative estimates from 2012, there may be as many as three times as many cases of FASDs as FAS (Centers for Disease Control, 2012). Healthcare and other indirect costs associated with the disorder have put the likely annual cost of FASDs in the US as high as $3.6 billion (Olson, et. al., 2009). Drinking during pregnancy is the leading preventable cause of mental retardation in modern civilization, and because this has remained an issue the need for treatment is vital.

Outcomes of FASDs

Fetal Alcohol Spectrum Disorders are difficult to diagnose because the morphological changes commonly found in more severe forms of FAS/FASDs may not

be present in all patients, sometimes leading to missed diagnoses or no diagnoses at all. Treatment for these disorders is also difficult because no medication currently exists for either FAS/FASDs. Because of this, physicians commonly use medication used to treat behavioral disorders like attention deficit/hyperactivity disorders (ADD/ADHD). Children living with FAS/FASDs typically have some behavioral/learning deficits along with social/economic difficulties later in life (Bishop et al., 2007). Other characteristic deficiencies in children with FAS and occasionally FASDs include prenatal and postnatal growth problems (Mattson & Riley, 1998; Mattson et al., 1998 ; Riley et al., 2011). Because there is now an accepted spectrum of severity with FASDs, ranging from minor to severe, diagnoses of the disorder can be challenging. This is especially so in children displaying minimal facial malformations, which is commonly associated with less exposure to ethanol during gestation (Mattson et al., 2010). However, these less severe cases do demonstrate deficits common to more severe FAS, including problems with executive functioning, motor coordination, spatial learning, attention, and hyperactivity (Jones, 2011; Mattson & Riley, 1998). Some cognitive and behavioral assessments have been able to aid in diagnoses of children who had a history of ethanol exposure in utero, as they showed impairments in working memory, planning, and cognitive flexibility which are characteristic of FASD (Mattson et al., 2010). However, this is made difficult because these cognitive deficits can oftentimes resemble characteristic symptoms of ADHD. As many as 94% of children exposed to relatively large amounts of prenatal alcohol could be diagnosed with attention deficit hyperactivity disorder (Peadon & Elliott, 2010). Children with FAS/FASDs can also have impaired social functioning. This problem can cause children with the disorder to experience negative life situations including problems at home, school, and eventually in the workplace (Kully-Martens et al., 2012). These issues associated with FASD present a multitude of problems for the individual, their families and society.

Binge Drinking: Possible Mediator of FASD's

Drinking alcohol during pregnancy is associated with many problems. After a mother consumes alcohol it crosses the placental barrier and within minutes of the onset of a drinking episode, the fetal alcohol content can equal that of the maternal blood

(Paintner, Williams, & Burd, 2012). Additionally, amniotic fluid retains a peak alcohol concentration for a longer period of time due to the decreased ability of the fetus to either metabolize or transfer the ethanol back to the mother, who can rapidly metabolize the chemical, resulting in a prolonged exposure period (Nava-Ocampo et al., 2004). Research on quantity, timing, and pattern of consumption of alcohol during pregnancy and its effects on the fetus in animal models has been studied extensively to help create models of fetal ethanol exposures effects (for review see Painter, 2012). However, research suggests that certain patterns of consumption may be more harmful than others and that exposure at different time points during development differentially affect certain regions of the brain and other organ systems.

A characteristic pattern of consumption for many alcoholics is binge drinking (defined above). In an animal model looking at prenatal development in the rat, binge drinking led to greater elevations in blood alcohol content leading to higher peak periods of ethanol exposure for the developing fetus (Livy et al., 2003). With binge drinking, there is often a binge pattern of drinking followed by periods of withdrawal. This pattern has been linked to more severe deficits in populations of children with FASDs (Paintner et al., 2012). The amount of alcohol consumed may not be as important, in terms of overall detrimental effects, as the pattern of drinking by the mother.

Alcohols Effects on the Developing Brain

The developing fetus is in a constant state of growth and change. Such changes encompass the development of many structures including the brain and nervous system. These systems are highly susceptible to the neurotoxic effects of ethanol during their development (Rodier, 1994). Some of ethanol's effects on the CNS include: disruption in cellular metabolism, an increase in hypoxic events, the creation of free radicals, an inhibition of DNA methylation, and membrane fluidization (For review see: Alfonso-Loeches & Guerri 2011; Berman & Hannigan 2000; Eckardt et al., 2006; Goodlett & Horn, 2001 & 2005; Jones 2011, Olney, J. 2004; West et al., 1994). Each of these are detrimental to the survival of a developing cellular network. One critical period of growth is the third trimester of pregnancy; it is a time of rapid division and migration of neural cells into their constituent networks. This period is often referred to as the brain growth

spurt (Dobbing & Sands 1979). Exposure to alcohol during this time can be especially detrimental, affecting neuronal migration, synaptogenesis, myelination, dendritic aborization, histone methylation and other aspects of brain maturation and differentiation (For review see: Goodlett et al., 2005; Guo et al., 2011; Painter et al., 2012). Regions of the brain that undergo extensive development during this time, e.g., cerebellum and hippocampus, have been shown to be particularly sensitive to ethanol exposure during the third trimester (Alfonso-Loeches & Guerri, 2011), demonstrating the importance of timing.

Changes in the brain

Children with an FASD show characteristic disabilities that can be related back to changes in the CNS. Through the advancement of neuroimaging techniques it has become possible to study brains of children with an FASD. Using magnetic resonance imaging (MRI) there are several distinct brain regions that are more typically affected by ethanol exposure than others in both FAS and FASDs. Some structures affected have shown decreases in white matter compared to typically developing peers. Archibald and colleagues (2001) found in 12 children diagnosed with FAS that cerebellar gray and white matter was significantly reduced when compared to patients without FAS diagnoses. In children with FASDs there were differences in fiber tracts like the corpus callosum compared to control participants (Fryer et al., 2009). Other regions affected by prenatal ethanol exposure include the posterior cingulate, temporal lobe, and specific areas of the frontal, occipital, and parietal lobes in both FAS and FASD (Riley & McGee, 2005; Mattson et al., 1994; Spottiswoode et al., 2011). Furthermore, moderate to heavy exposure to ethanol has been shown to cause a decrease in myelination and thus cellular signaling in both human studies and animal models (Fryer et al., 2009; Sowell et al., 2008). Further understanding of the effects of ethanol exposure has led to more accurate development of diagnostic tests related to behavior and cognition that have been used in both human FASDs and animal models.

Of particular interest for this proposal are the effects of fetal ETOH (ethanol) exposure on the hippocampus. Imaging studies using young children diagnosed with FAS have not shown dramatic changes structurally to the hippocampus (Archibald et al., 2001;

Riley & McGee, 2005), but because so many of the behavioral deficits associated with FASDs are learning and memory oriented, it suggests that this structure may be partially responsible for the deficits. Although the changes related to this structure may not be apparent at the macro level it may be the case that cellular and molecular functioning in this structure may be altered by ethanol exposure during development. The CA1 region of the hippocampus appears to be especially sensitive to ethanol's effects during late prenatal and early postnatal development. Tran & Kelly (2003) reported there was cell loss in the CA1 region of the hippocampus when ethanol was administered during the 3rd trimester equivalent in rodents. Livy and colleagues (2003) found similar results when ethanol was administered during the 3rd trimester equivalent in the CA1. However, they also found a significant reduction in cell numbers and density in the CA3 region of the hippocampus. Previous studies using in-vitro hippocampal cultures have shown that ETOH exposure followed by withdrawal in the hippocampus drastically reduces survival of the cells in several regions of the hippocampus (Barron et al., 2008; de Fibre, 2003 & 2005, Li et al., 2000 & 2002; Prendergast et al., 2000 & 2004; Wilkins et al., 2006). This research has stimulated study into the cellular mechanisms related to ETOH exposure and withdrawal in the hippocampus and how novel compounds may mitigate this damage.

Modeling FAS/FASDs in Animals

The use and development of animal models in studying FASDs is a critical component and powerful tool in the process of understanding the disorder (for review see, Patten et al., 2014). Some of the most important components to consider are the species used, timing of alcohol administration (e.g. 1st, 2nd, or 3rd trimester), the level of intoxication and the administration pattern. Rodents (mice, rats, guinea pigs) are the most common species used in research accounting for over 50% of studies (Clancy et al., 2007), and are the choice of animal for the current proposal. This proposal uses a rodent model of ethanol exposure because of the similarities between human and rodent CNS development (Dobbing & Sands 1979). Dose is also a very important consideration when modeling ETOH exposure; the quantity used in an animal model is extremely important in determining whether you are trying to model more or less severe ethanol effects. A wide range of blood alcohol levels have been used ranging from 100-400mg/dl; this

equates roughly to moderate to heavy binge-like ethanol exposure (Patten, 2014). The route of administration is also important when considering an animal model because of the various benefits and drawbacks. The most commonly used in modeling fetal ethanol exposure includes voluntary drinking and intubation method (Patten, 2014). Both of these methods can be used in adult pregnant female rats (modeling 1st and 2nd trimester) and neonates (modeling 3rd trimester) (Patten, 2014). Intubation is more typically used for controlled dosing, because voluntary consumption is highly subject to individual differences. However, both higher and more moderate BACs resulting from these routes of administration are correlated to increased neurotoxicity and decreased white matter in a dose-dependent fashion (Patten, 2014).

The developing mammalian nervous system is particularly sensitive to the effects of teratogenic compounds. During development, the burgeoning nervous system goes through massive amounts of cell generation, migration and proliferation, and finally pruning and cell death, processes which occur throughout life but at a slower pace (de Graaf-Peters & Hadders-Algra, 2006; Patten et al., 2014). This process occurs at different rates and times during gestation, with some regions going through rapid changes later in development. Uniquely, small animals such as rodents go through this development at different times than do humans. In humans, gestation occurs over three characteristic trimesters (3, 3-month blocks) all of which occur prenatally. During these trimesters the brain is going through rapid development, with the first two trimesters characterized by the formation of the neural tube, gastrulation, some cell proliferation and migration. The third trimester in humans is commonly referred to as the "brain growth spurt," and is a time where the brain is undergoing rapid growth (Dobbing & Sands 1979). In rodents, gestation typically occurs within 18-23 days compared to around 280 days in humans. Extensive research investigating the differences between human and animal CNS development has produced maps known as "trimester equivalents" in rodents using anatomical comparisons and differences in cellular number (both glial and neural) to compare the development of the two species. The first trimester equivalent in rodents is roughly during gestational days (GD) 1-10, with GD 10-20 roughly corresponding to the 2nd trimester, and the third trimester mostly occurring after birth during post natal days (PND) 1-10 (Clancy et al., 2007). However, these times are not concrete and represent a

loosely organized trajectory that has considerable overlap throughout development. Utilization of this information has informed researchers in producing models of diseases and disorders that occur during these times. Investigating the effect of ethanol on the developing fetus has produced several ethanol exposure methods that utilize either a prenatal (exposure during gestation) or neonatal (exposure shortly after birth) paradigm (Berman & Hannigan 2000). In the prenatal exposure paradigm, the pregnant dam is exposed to ethanol either through diet or by intragastric intubation (Berman & Hannigan, 2000; Kelly & Streissguth 2000; Thomas et al., 2009). Prenatal exposure to ethanol has been shown to lead to deficits in social behavior (e.g. play behavior, social recognition, and active interaction), activity (hyperactivity and impulse control), and learning/memory, which is associated with deficits found in FAS/FASDs in humans (Berman & Hannigan 2000; Driscoll et al., 1990; Kelly & Steissguth, 2000). Other behavioral characteristics associated with prenatal ethanol exposure include feeding difficulties, low birth and brain weight, poor reflex development and motor function, and increased activity. All of these have correlates with clinical populations of children with FAS (Driscoll et al., 1990; Thomas et al., 2009).

Another model is used to focus more specifically on the effects of "3rd trimester" exposure, in which rat pups are exposed to ethanol during the first weeks of postnatal life (Goodlett & Johnson 1997; Goodlett & Peterson 1995; Kelly & Tran 1997; Riley et al., 1993; Wellmann et al., 2010). With this model, the offspring receives ethanol either through a temporary or indwelling intragastric feeding tube. For the model using oral intubation the dose of ethanol typically ranges between 2 and 6 grams per kilogram (g/kg) of ethanol a day, with higher doses (6 g/kg) producing BACs of more than 200 milligrams per deciliter (mg/dl) (Patten et al., 2014). An advantage of this model and other intubation models is the relative ease with which the experimenter can control the dose of ethanol received, resulting in fairly predictable BACs. Also, intragastric intubations do not require surgical implantation of feeding tubes. Previous research has shown that ETOH exposure using this method produces deficits in a variety of behavioral paradigms that assess motor coordination (Idrus et al., 2011; Lewis et al., 2007; Thomas et al., 2004), activity (Smith et al., 2012; Thomas et al., 2007), and learning and memory (Hunt et al., 2009; Thomas et al., 2009; Tiwari et al., 2012). This evidence has helped

demonstrate the validity of the model because the deficits found closely parallel the deficits commonly seen in clinical populations of FASDs (Kelly et al., 2000; Lewis et al., 2007; Riley et al., 1993; Thomas et al., 2004; Tran et al., 2000; Wellman, 2010).

Summary:

The development of FAS/FASDs through the exposure to ethanol during prenatal development remains an important social and clinical problem. There is no simple solution for this disorder because of the various constraints discussed above and the complicated nature of the disorder itself. As a result, research continues in the development of better understanding some of the mechanisms that underlie the development and consequences of FAS. This basic research is essential and may provide potential relief for a disorder that currently has no clear solutions.

A Possible Treatment

Treating FAS

Currently, there are no effective clinically-approved pharmacotherapies for the treatment of toxicity induced by ethanol withdrawal in the fetus or newborns that were exposed to ethanol during pregnancy. Therefore, it is clear that development of pharmacotherapies aimed at reducing the damage caused by ETOH exposure are needed. One type of pharmacotherapy for FAS/FASDs can either focus on reversal/prevention, such that treatment is administered during gestation or shortly after birth when the diagnosis of the disorder has not yet occurred (Riley et al., 2011). Classically, therapies aimed at treating children with FAS/FASDs have been focused on providing supportive and protective environments for optimal outcomes (Pruett et al., 2013). Medication for the management of these cognitive symptoms are most often the same drugs used for the management of disorders like ADHD, a comorbid diagnosis commonly made in children with an FASD (Pruett et al., 2013). This treatment strategy has had mixed results in children with FASDs where symptoms of inattention were not reduced (Doig et al., 2007). Another study looking at a variety of treatments found that various medications - whether they are stimulants, SSRIs, or mood stabilizers - have some degree of success in patients with FAS, but no single medication could treat all the symptoms associated with

the disorder (Coe et al., 2001). Previous cellular and behavioral work in animals has outlined the importance of ethanol withdrawal after exposure as being a potentially treatable source of cellular toxicity related to FAS/FASDs. Neonates exposed to chronic levels of ETOH often experience many periods of withdrawal during and after birth because of binge-like exposure to ETOH during gestation. Withdrawal from ethanol in the neonate is severely damaging to the CNS and produces many deficits demonstrated both in cellular (de Fiebre et al., 2003; Li et al., 2004; Prendergast et al., 2000 & 2004) and in-vivo models (Barron et al., 2008; Goodlett & Horn 2001; Thomas et al., 1997 a. and b.). The development of a pharmacotherapy aimed at reducing the damage associated with this ethanol withdrawal (EWD) is appealing because it is potentially a one-time treatment that would protect the fetus or newborn after ethanol has left the system, either during parturition or when a mother is seeking help for a use disorder. Most research has focused on targets that are involved directly with the damaging effects of ethanol, and because ethanol's effects on the developing CNS are numerous, many compounds have been studied for their potentially therapeutic effect. A few examples of compounds used prenatally to treat the teratogenic effects of ethanol follow. Using in-vitro models, researchers have examined ethanol's ability to inhibit L1, an immunoglobulin responsible for cell adhesion and neuronal migration. It is thought that if ethanol's action on L1 could be reversed pharmacologically that it would reduce ethanol's damaging effects on the fetus (Wilkemeyer et al., 2000). Other compounds like ascorbic acid (vitamin C) have also been investigated in Xenopus embryos and was able reduce damage produced by reactive oxygen species produced by chronic ethanol exposure (Peng et al., 2005). Other compounds like buspirone and ipsapirone, both serotonergic agonists, have also been used to prevent ethanol damage to serotonergic neurons during pregnancy (Pruett et al., 2013). There is also much work focusing on reducing cell damage via the glutamatergic system via direct action or modulation at the n-methyl-d-aspartate (NMDA) receptor. For example, CP-101-606 (a novel NR2B NMDA receptor antagonist) and MK-801 (a noncompetitive antagonist) were able to attenuate behavioral deficits caused by ethanol exposure (Lewis et al., 2011; Thomas et al., 1997). Although much of the work using various compounds that utilize different neuronal systems have had varying degrees of success in reducing behavioral deficits and cellular damage related to ethanol exposure,

none of them have yet been approved for clinical treatment of this problem. Because of this, research continues in searching for compounds that have potential in treating ethanol withdrawal-related toxicity.

A Promising Candidate

The Cholinergic System

Because no clinically-approved pharmacotherapy currently exists to treat/reduce/reverse the damage caused by ethanol/EWD, much research has been conducted on various drugs that have been shown to have neuroprotective properties (as described above). One promising avenue is using drugs that target cholinergic receptors, which is the focus of this proposal.

The cholinergic system is one of the most widespread and commonly studied systems in the nervous system. Its functions are varied by region, and span both the central and peripheral nervous systems. In the CNS, the cholinergic system is thought to be a facilitator of processes related to wakefulness and attention, and is thought to play a critical role in both learning and memory (Thomas, 2003). Ethanol's effects on the cholinergic system are widespread, with exposure to high doses of ethanol during the 2nd week of gestation producing a loss of immunoreactivity to choline-acetyltransferase (ChAT, an enzyme that signals the presence of acetylcholine-containing cells) in the medial septal nuclei, caudate, and putamen, but not a loss in the number of cells present (Heaton et al., 1996; Nagahara & Handa 1999 a.). These results suggest that cholinergic function was impaired in the first weeks of prenatal development, but research looking further into development showed that eventually these deficits decreased and returned to control levels (Nagahara & Handa 1999 a.). Also, physostigmine, an acetylcholinesterase inhibitor that increases the amount of available ACh (acetylcholine), reduced hyperactivity in rats following prenatal ETOH compared to controls (Nagahara & Handa 1999 b.). In addition, when exposed to ethanol during the last two weeks of gestation, nicotine did not produce the typical alterations in locomotor behavior or an increase in memory performance when compared to control animals (Nagahara & Handa 1999 b.).

Ethanol is also thought to affect both cholinergic receptor systems (muscarinic and nicotinic) within the developing CNS. For the muscarinic system, studies have found that during postnatal development there is an up-regulation of muscarinic ACh receptors in the hippocampal formation in response to ethanol exposure (Nio et al., 1991). Also in these same studies, scopolamine (a muscarinic antagonist) impaired fetal ETOH-exposed rats in an alternation based task. Finally, when exposed to ethanol during early postnatal development, PND 4 and 8 (via intragastric intubation), rats showed an increase in striatal muscarinic receptor affinity (Light et al., 1989). Sex differences were again observed; ETOH-exposed female rats had fewer cholinergic receptors in the striatum and cerebellum (Light et al., 1989). This evidence suggests that exposure to ethanol during development alters the function of the muscarinic cholinergic system and may affect males and females differently.

The Nicotinic Receptor System

The nicotinic receptor system is a component of the cholinergic system and plays many crucial roles in both the peripheral and central nervous systems. The receptor is named so because of its high affinity for nicotine (a psychoactive chemical commonly found in tobacco). These receptors were the first membrane receptor proteins identified in the 1970s (Dani, 2001). They fall into a class of ligand-gated ion channels that respond endogenously to acetylcholine or exogenously to nicotine (Dani, 2001; Pauly et al., 2004). The function and location of the receptor in either the CNS or peripheral nervous system (PNS) is dependent upon the type and arrangement of its subunits. The nicotinic receptor is an ion-channel that is made of five membrane-spanning regions; each of the five constituent regions is composed of different or similar subunits (Wu & Lukas, 2011). The subunits that form nicotinic receptors in neural tissue include alpha (2-9) and beta (2-4); these subunits can be arranged in many different conformations that are either heteromeric (alpha2-alpha6 and beta2-beta4), or homomeric (alpha7-alpha9) (Dani, 2001).

Deficits in nicotinic receptors have been implicated in several neurological disorders including Alzheimer's disease, Parkinson's diseases, and Down's syndrome (Levin, 2002). Along these lines, agonists targeting the nicotinic receptor system have

been shown to be neuroprotective. Pre-treatment with nicotine, carbachol (mixed nicotinic/muscarinic agonist), and nornicotine has been shown to be neuroprotective against the excitotoxic effects of excessive glutamatergic activity in cortical cell cultures (Ferchmin et al., 2003; Marin et al., 1994; Akaike et al., 1994). Nicotine treatment can also reduce cellular toxicity induced by ischemic damage both in-vivo and in-vitro in gerbils and rats (Pauly et al., 2004). Nicotine can also reduce the damage caused by beta-amyloid plaques, related to the development of Alzheimer's disease (Shimohama et al., 2001).

In the nicotinic system, evidence for the role of cholinergic systems in the effects of ethanol exposure stems from research using choline supplementation, an essential nutrient used in the production of acetylcholine that is also thought to activate nicotinic receptors. Perinatal choline supplementation been shown to reduce open field hyperactivity, spatial working memory deficits, eyeblink and trace fear conditioning deficits (Monk, 2012). Thomas and colleagues (2009) have also found that choline supplementation in early development can lessen the severity of reduced birth and brain weight after ethanol exposure (Thomas, 2009). Choline's effect on cholinergic functioning in a model of ethanol exposure is thought to be mediated in part by the nicotinic receptor system, of which choline has been shown to be a direct agonist (Coutcher, 1992; Thomas, 2013). In animal models, nicotine exposure can reduce deficits related to learning and memory as well as an ability to increase cell survival after hypoxic events, brain damage, and excitotoxicity (Hejmadi et al., 2003; Miwa et al., 2011; Levin & Rezvani, 2000; Lloyd, G. 2000; Pauly et al., 2004; Prendergast et al., 2001a & b). Research has also shown that nicotine treatment during ethanol administration can reduce cell death produced by ethanol-related toxicity in cultured granule cells of the cerebellum (Tizabi et al., 2003). However, nicotine is relatively non-specific for various nicotinic receptor subtypes causing activation of both central and peripheral cholinergic systems. This decreases the usefulness of a nicotine-like drug aimed at treating humans. In order to avoid problems associated with the activation of peripheral targets and find a drug that specifically and exclusively activated neuronal nicotinic receptors, other compounds need to be explored.

Alpha-7 Nicotinic Acetylcholine Receptor

Of the nicotinic receptors implicated in nicotine's neuroprotective effects, the alpha 7 nicotinic acetylcholine receptor (α-7 nAChR) is of particular interest because of its high concentration in neuronal tissue throughout the CNS (Dani, 2001). Stimulation of the α-7 nAChR has increased cognition and decreased behavioral deficits in animals (Timmermann et al., 2007; Olincy et al., 2007). This receptor has also been implicated in mediating synaptic plasticity and regulation of immune function (Kem, W. 2000). Activation of the receptor can result in a reduction in cellular toxicity from ethanol withdrawal (de Fiebre et al., 2003 & 2005; Yangxin et al., 2002), neuroinflammation (Concepcion et al., 2008; de Jonge et al., 2007), glutamatergic toxicity (Dajas-Bailador et al., 2000 & Shimohama et al., 1998), and traumatic brain injury (Pauly et al., 2004) .

α-7 nicotinic receptors are some of the most heavily expressed nicotinic receptors in the CNS, and to a lesser extent in the immune system (Dani, 2001). High densities of this receptor are found in the hippocampus, cerebellum, prefrontal cortex, motor cortex, and basal ganglia (Dani, 2001; Concepcion et al., 2008). The expression of the α-7 nAChR is first observed on neuroblasts early in gestation (Miwa et al., 2011). Also, receptor expression steadily increases until birth. In the third trimester, the density of α-7 receptors in the hippocampus is roughly 3x that of an adult (Miwa et al., 2011). The receptor responds endogenously to several compounds; in adults this is more commonly ACh, but in the neonate it is postulated that choline is the primary endogenous compound acting at the receptor (Miwa et al., 2011). Choline, which has a greater presence in neonates than adults, has roughly 3x the affinity on the α-7 than does ACh (Miwa et al., 2011). Activation of hippocampal α7 nAChRs has led to decreases in learning and memory deficits commonly associated with diseases like Alzheimer's (Meyer et al., 1998; Timmermann et al., 2007). In neurons, these receptors are located on the cell at the axon terminal, the pre-terminal (region just before the terminal that can affect several terminals at once), postsynaptic dendritic membrane, and on glial cells (Albuquerque et al., 2009). The α7 nAChR is selectively permeable to calcium, and is responsible for fast postsynaptic transmission, the modulation of release of numerous transmitters, and long-term potentiation in various areas throughout the brain (Hurst et al., 2013; Dani, 2001;

Fabian-Fine et al., 2001). Its permeability to calcium is unique in that the receptor does not require depolarization of the neuron to open (Timmermann et al., 2007). Activation of alpha-7 nAChRs produces a modulatory action which stimulates the release of several neurotransmitters (Timmermann et al., 2007). The α-7 nAChR plays many roles in modulating processes in the brain both pre- and postsynaptically. Postsynaptically, α-7 nAChRs are found on inhibitory GABA'ergic interneurons throughout the hippocampus and receive input from the medial septal nuclei, which plays a role in sensory response within this structure (Miwa et al., 2011). This modulation of inhibitory GABA'ergic interneurons causes the decrease of glutamatergic response in the pyramidal cells of the hippocampus (Miwa et al., 2011). Choline administration during late gestation, during peak α-7 nAChR density, can decrease the number of these receptors and increase excitability and dendritic development in the hippocampus (Miwa et al., 2011). However, choline deficiency, a common occurrence during gestation, is thought to be damaging to the developing CNS. Choline supplementation has been shown to be neuroprotective in several paradigms, including animal models of FAS/FASDs (Miwa et al., 2011; Pauly et al., 2004; Thomas et al., 2004, 2007 & 2009). Also, in late prenatal to early postnatal development, the α-7 nAChR is responsible for the transformation of glutamate neurotransmission from primarily NMDA-type to kainate-aspartate receptors (Miwa et al., 2011). These actions could be important in the α-7 nAChR's effects in mediating the excitotoxic response to ethanol withdrawal. Because ethanol withdrawal is commonly associated with increased activity of glutamatergic receptors that can lead to excitotoxic death, it seems contradictory that possibly stimulating its release through α-7 agonism would decrease excitotoxicity. Several mechanisms could be at work to reduce EWD-induced toxicity. One of the most commonly postulated mechanisms of action for the α-7 nicotinic receptors' effects on ethanol neurotoxicity is the ability of the receptor to reduce calcium toxicity, caused by activation of the NMDA receptor during ethanol withdrawal (de Feibre et al., 2003; Shimohama et al., 1998). For review on NMDA-induced toxicity during ethanol withdrawal (see Tsai et al., 1998; Kalluri et al., 1998; Grant et al., 1990; Eckardt et al., 2006; or Prendergast et al., 2001a. and b.). Nicotine administration during ETOH withdrawal (EWD) has been shown to decrease this calcium-induced excitotoxicity through several possible metabolic mechanisms including the buffering of

internal calcium stores due to calbindin D28K (a calcium sequestering protein) during early development (Prendergast et al., 2001 a.). Prendergast and colleagues (2001 a.) also believe that activation of the α-7 nicotinic receptor could possibly be down-regulating the expression of the NMDA receptor and decreasing withdrawal-induced toxicity Administration of the nicotinic antagonists alpha-bungarotoxin (BTX) or methyllycaconatine (MLA), which block the α-7 nAChR, negates nicotine's protective effects in NMDA-induced toxicity supporting the role of α-7 receptors in neuroprotection (Ferchmin et al., 2003). Another mechanism postulated to lessen EWD-induced toxicity is to reduce the release of reactive oxygen species and cytochrome C (a signal for apoptosis) from the mitochondria (also the primary store for intracellular calcium) caused by α-7 nAChR activation (Li et al., 2000 & 2002). This process is thought to be modulated by the activation of protein kinase C (PKC), which can reduce the release of cytochrome C (Li et al., 2002). Further support for α-7 nAChR's role in reduction of toxicity induced by EWD stems from data showing that α-7 nAChR knockout (KO) mice do not display nicotine-induced neuroprotection (Gahring et al., 2003).

DMXB-A

DMXB-A is a derivative of an anabaseine compound found in marine worms (Olincy & Stevens, 2007). It has a high affinity for the α-7 nAChR, showing about 40% of the efficacy of ACh for the receptor and a much lower affinity for other nicotinic receptors like the α4β2 and type 3 5-HT receptors where it acts as an antagonist (Kem et al., 2006; de Feibre, 2003; Olincy & Stevens 2007). Studies have shown that DMXB-A may act as a noncompetitive partial-agonist with higher doses desensitizing the receptor and lower doses activating them (Kem et al., 2006). It is a lipophilic compound that readily crosses lipid membranes such as the blood brain barrier (BBB) easily (Kem et al., 2006). In the human brain, DMXB-A is less potent than nicotine (Ki=2000) when displacing α-BTX, however in the rat brain DMXB-A is more potent than nicotine at displacing α-BTX at the α-7 nAChR (Ki=650) (Briggs et al., 1997).

Many studies looking at α-7 nAChR's protective properties have used 3-dimethoxybenzylidene-anabasine (DMXB-A) or otherwise known as GTS-21. The drug was the first α-7 nAChR agonist to be used in clinical testing for the treatment of

schizophrenia, Parkinson's disease, and Alzheimer's disease (AD) (Freedman et al., 2014; Kem et al., 2006; Meyer et al., 1997). In phase II clinical studies of patients with schizophrenia, DMXB-A improved attention and memory and sensory inhibition in an animal model of schizophrenia (Freedman et al., 2014; Olincy et al., 2007). DMXB-A was neuroprotective against beta-amyloid toxicity in an animal model of AD (Kem, 2000; Kihara et al., 1997). DMXB-A also improves cognitive function following administration of beta-amyloid (Chen et al., 2010; Meyer et al., 1997). DMXB-A has also shown to be neuroprotective in animal models of global ischemia (Nanri et al., 1998). Data from in-vitro studies also suggest that DMXB-A can dose-dependently reduce cell death following ETOH exposure (de Fiebre et al., 2003; Li et al., 2000 & 2002; Shimohama et al., 1997). Using an embryonic primary hippocampal cell culture model to study the effects of DMXB on ethanol toxicity, De Fiebre and colleagues (2003) found that DMXB effectively increased cell viability after ethanol administration. Also, pre-treatment with DMXB-A at low doses increases cell survival during an ethanol challenge (Li et al., 1999). Most previous studies either used a pre-treatment or a concomitant method for administering DMXB-A with ethanol, but no previous research using a third-trimester chronic ethanol model with post-ethanol exposure of DMXB-A has been examined. Thus, further investigation into the therapeutic potential for DMXB-A is warranted.

The exact mechanism with which activation of the α-7 nAChR works to reduce the toxic effects of ethanol exposure and withdrawal remain somewhat unclear. However, as mentioned above, this process is most likely mediated by several different mechanisms that work in unison to reduce toxicity. Studies that have explored the interaction between DMXB-A and ethanol-induced toxicity have shown that the drug prevents ethanol-induced increases in intracellular calcium, the buildup of reactive oxygen species, and the release of cytochrome C (Li et al., 2000). Other studies have suggested that the protective effects of DMXB-A might not be due solely to the activation of the α-7 receptor, but due to action on other nicotinic receptor subtypes as well (de Feibre et al., 2003). Another emerging hypothesis using a neuro-inflammation model, indicates the actions of α-7s expressed on microglia and possibly macrophages could contribute to the neuroprotection in models of cellular stress (Parada et al., 2013). However, no research, to the best of my

knowledge, has examined the effects of DMXB-A on “third trimester” ETOH exposure using either an in vivo or in vitro rodent model.

Chapter 2:

Testing DMXB-A's Effects in an Organotypic Hippocampal Slice Culture Model

Hypothesis

The current hypothesis is that the α-7 nAChR agonist DMXB- A should reduce the amount of cell damage caused by chronic ethanol exposure and withdrawal in a developmental organotypic hippocampal slice culture in a rodent model. Investigation into this is of importance in furthering the understanding of the potential therapeutic efficacy of α-7 agonists on the development of FAS/FASDs.

Methods:

Organotypic Hippocampal Slice Culture: Overview

The organotypic hippocampal slice culture (OHSC) model was developed in 1981 by Gahwiler and colleagues (Gahwiler, 1981) and simplified by Stoppini and colleagues in 1991 (Stoppini et al., 1991). OHSC has since been used as an investigative tool to study both pharmacological and environmental manipulations that could provide neuroprotection, neurogenesis, and/or neurotoxicity. The OHSC model has many benefits relative to simpler in-vitro culture models (like primary cell culture models) that make it a useful model to use when investigating neurotoxicity. First, it allows for the maintenance of neurons and glia and maintains much of the complex circuitry of an intact hippocampus (Noraberg et al., 2005), providing a more valid model in assessing neuroprotection and neurorepair and allowing easy quantification of cell markers, including cell damage. This model also allows investigators to characterize compounds of interest, possibly revealing their mechanism of action. Through much investigation OHSC has shown sensitivity to a variety of insults including ETOH; for review see (Noraberg et al., 2005).

Hippocampal slice culture models are particularly useful for studying excitotoxic damage related to chronic ethanol exposure in the current study because of the susceptibility of the hippocampus to toxicity related to ethanol withdrawal. As stated previously, the hippocampus is also useful in a culture model using DMXB-A because of

the high concentration of α-7 nAChRs which have been implicated in EWD-related neuroprotection in this structure. Also, the hippocampus is heavily implicated in some of the cognitive and behavioral deficits observed following fetal alcohol exposure, such as impaired learning and memory.

Animals

Male and female Sprague-Dawley rat pups were used for this experiment. The animals were obtained through breeding in the rat vivarium in Kastle Hall. All animals were housed in a 12 hour light/dark cycle, and were allowed continuous access to food and water. After each breeding session pregnant females were identified by the presence of a vaginal plug. Pregnant females were removed from the colony and taken to a temperature and humidity controlled nursery for the duration of their pregnancy. After parturition, pups remained with their mother until postnatal day (PND) 1. On PND 1, litters were culled to 10, maintaining 5 male and 5 female pups when possible; allowing for an even distribution of subjects between the experimental groups and sexes. The care of animals was carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1996), as well as the University of Kentucky's Institutional Animal Care and Use Committee

Hippocampal Slice Culture Preparation and Treatments

On PND8, non-treated litters were sacrificed for brain removal. The brains were removed and placed in a tissue culture dish containing dissecting medium [Minimum Essential Medium (Gibco BRL, Gaithersburg, MD), 25 mM HEPES (ATCC, Manassas, VA), 200 mM L-glutamine (Invitrogen, Carlsbad, CA), 50 μM streptomycin/penicillin (ATCC, Manassas, VA)]. The hippocampus from each brain was removed and then sliced coronally at 200 um using a McIllwain Tissue Chopper (Campden Instruments Ltd., Lafayette, ID). Slices were separated and placed on Teflon membrane inserts (Millicell-CM 0.4 μm; Millipore, Marlborough, MA, USA). Each insert holds 3 slices with 6 inserts per culture plate. The wells contained 1mL culture media [dissecting media, 36 mM glucose, 25% Hanks' balanced salt solution (HBSS; Gibco BRL, Gaithersburg, MD), 25% heat-inactivated horse serum (HIHS; Sigma, St. Louis, MO)].

Plates were then placed in an incubator (37°C/5%CO2/95%air) for 5 days in vitro (DIV) for adherence to the Teflon membrane.

On DIV 5, the inserts were split into a 100mM ETOH-treated and control groups. After the groups were divided, the 6 well plates were placed into propylene containers with 50mL of control or 100 mM ETOH (averaging approx. 65mM over 5 days in vitro [Prendergast et al. 2004]) in sterile water with 50uM gentamicin added to avoid chances of bacterial growth. The plates were placed into plastic freezer bags and inflated with an air mixture to prevent ETOH evaporation. This procedure was repeated on DIV 10 to provide the slices with fresh ETOH. On DIV 15 the slices were removed from control or ETOH media and treated with a combination of DMXB-A (at 0, 1, 3 or 10uM) control media, and/or NMDA (5um), giving a total of 16 treatment groups (Table 1.). During the 24-hour treatment period, slices were also exposed to the fluorescent marker propidium iodide (PI; 3.74 µM, SigmaAldrich). PI is an indicator of cellular damage because it binds to the DNA of cells whose membranes have been compromised (in this case by EWD-induced toxicity) and produces red fluoresces when exposed to 488nm waves of light. Twenty-two hours later, slices were imaged using fluorescent microscopy. The images were captured using SPOT Advanced version 4.0.9 software for Windows (W. Nuhsbaum Inc., McHenry, IL, USA) using a 5X objective connected to an inverted 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 uptake in the CA1, CA3, and DG cell layers were measured using ImageJ version 1.46 software (National Institute of Health). Raw fluorescent values were obtained by subtracting the background signal from the cell layer fluorescence.

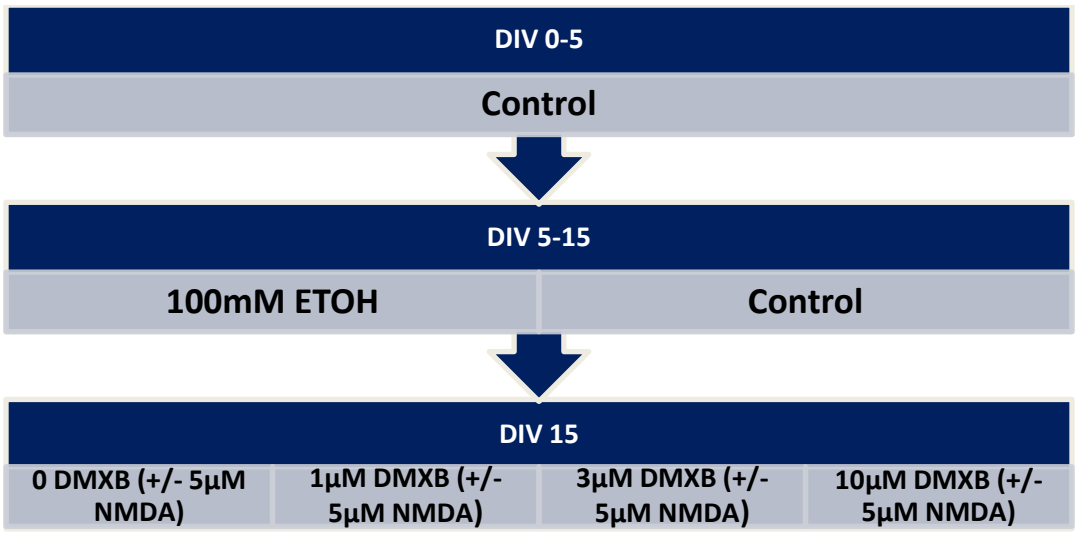

Table 1.

Treatment Groups: 2 control groups, 2 NMDA groups, 6 DMXB groups, 6 NMDA+DMXB groups.

Power Analysis

Before beginning the study, an apriori analysis was run to determine the number of culture preparations needed to achieve 90% power when detecting a medium-sized effect (f= .25) in a study using 16 groups. This required a minimum of 6 culture preparations at roughly 144 slices per prep.

Statistical Analysis

Analyses were conducted using IBM Statistical Package for the Social Sciences (SPSS) version 22 software (IBM Corporation, 2014). First, the three regions of the hippocampus were analyzed together to determine if there were differences in treatment groups between the regions. After this, each hippocampal region was investigated using a 2-way (sex x group) univariate general linear model (GLM) analysis of variance (ANOVA) to explore variance in and among the factors. Each culture replication was also used as a covariate during initial analysis to assess the effect of litter on the individual repetitions of each culture preparation. If significant interactions were detected, further investigation using a post hoc pair-wise comparison with Tukey's

correction for family-wise error was used. For simple graphical representation, mean levels of toxicity for each group were converted to percent control.

Results

To investigate whether there were group differences between the three regions of the hippocampus (CA1, DG, and CA3), an ANOVA was run with region as a repeated measure. The ANOVA was a 2 (sex) x 3 (region) x 16 (treatment) design.

Analysis revealed that there was that there was a main effect of treatment between the three regions, where $F(1, 15)=11.805$,$p<.001$, and sex $F(1,15)=9.753$, $p<.005$, with a non-significant interaction between the two of $F(1,15)=1.420$, $p=.130$. Because of previous research in this lab and others using an OHSC model of EWD supporting variations in outcome between the three regions (specifically the CA1), an a-priori hypothesis warranted the investigation of the individual regions separately.

CA1

In the CA1, using a 2-way ANOVA (treatment x sex), there was a main effect of treatment $F(15,31)=10.851$, $p<.001$ and an interaction between treatment and sex $F(15,31)=1.811$, $p<.05$. Because of this interaction between treatment and sex in the CA1, further analyses in this region were completed separately by sex. For both males and females there was a main effect of treatment $F(15,15)=6.752$, $p<.001$, and $F(15,15)=5.954$, $p<.001$ respectively. Because of this, a Tukey's correction for family-wise error post hoc test was utilized to explore these group differences. Significance was set at $p<.05$.

In slices exposed to ETOH + NMDA that were treated with 1, 3, and 10um of DMXB-A, there was a significant reduction in fluorescence ($p<.005$ for females at all three doses, and $p<.05$ for males at all three doses) compared to ETOH + NMDA alone (fig.1). Female slices treated with ETOH+NMDA alone showed greater fluorescence when compared to controls than did male slices (197% vs 163% fluorescence of control, respectively) (fig.1).

Differences between males and females also persisted with exposure to NMDA. Male slices exposed to NMDA alone expressed greater fluorescence compared to control slices (188% of control, $p<.001$) than did female slices (141% of control, $p<.05$) (fig.2). In males, increasing the dose of DMXB-A reduced PI fluorescence, and at 3 and 10 μm DMXB-A, this difference was significant ($p<.05$ for both doses) (fig.2). However, in females, when treated with 1μm DMXB-A, fluorescence significantly increased compared to NMDA alone ($p<.005$) (fig.2). This increase in fluorescence was reduced to near control levels in females treated with 10 μm DMXB-A.

When treated with ETOH (fig. 3), slices were not significantly different between males and females. Fluorescence in slices treated with ETOH alone was comparable to control slices. However, when treated with 3 and 10 μm DMXB-A, both male and females slices had increased fluorescence compared to controls; this difference was significant for males at 3μm DMXB-A ($p<.05$) and for females at 10μm DMXB-A ($p<.05$).

In control slices, for both sexes, treatment with 1μm DMXB-A produced levels of fluorescence comparable to controls (fig.3). However, treatment with 3μm of DMXB-A produced a significant increase in PI fluorescence compared to controls (females [$p<.005$], males [$p<.05$])(fig.3). However, only in males did treatment with 10μm DMXB-A produce significantly increased fluorescence when compared to controls ($p<.001$) (fig.4).

CA3 & DG:

In both regions there was a main effect of treatment, $F(15,31)=14.767$, $p<.001$ for the CA3 and $F(15,31)=15.330$, $p<.001$ for the DG. Unlike the CA1, there was no effect of sex and no interaction between treatment and sex. To investigate individual group differences a Tukey's correction for family-wise error post-hoc test was utilized, and significance was set at $p<.05$.

In the CA1, fluorescence in control slices for both the DG and CA3 when treated with 1, 3, and 10 μm DMXB-A was significantly increased when compared to controls ($p<.05$ or greater for all three doses in both regions) (fig.5). This increase in fluorescence

was closer to control levels only in slices treated with 1μm DMXB-A (around 117% fluorescence of control) (fig. 5).

In slices exposed to ETOH, treatment with 3 and 10 μm DMXB-A significantly increased fluorescence when compared to controls in both regions ($p<.05$) (fig. 6). This increase was not present in ETOH slices treated with 1μm DMXB-A for either region. In Slices exposed to NMDA, for both regions, there was a significant increase in fluorescence compared to controls ($p<.05$) (fig. 7). This also occurred in NMDA exposed slices when treated with 1 and 10μm DMXB-A. However, this increase in fluorescence was not found in NMDA slices treated with 3μm DMXB-A (fig. 7). Finally, in slices exposed to both ETOH and NMDA, for both regions, there was no difference in fluorescence when compared to controls (fig. 8). These results persisted with all three doses of DMXB-A in both regions.

Figures:

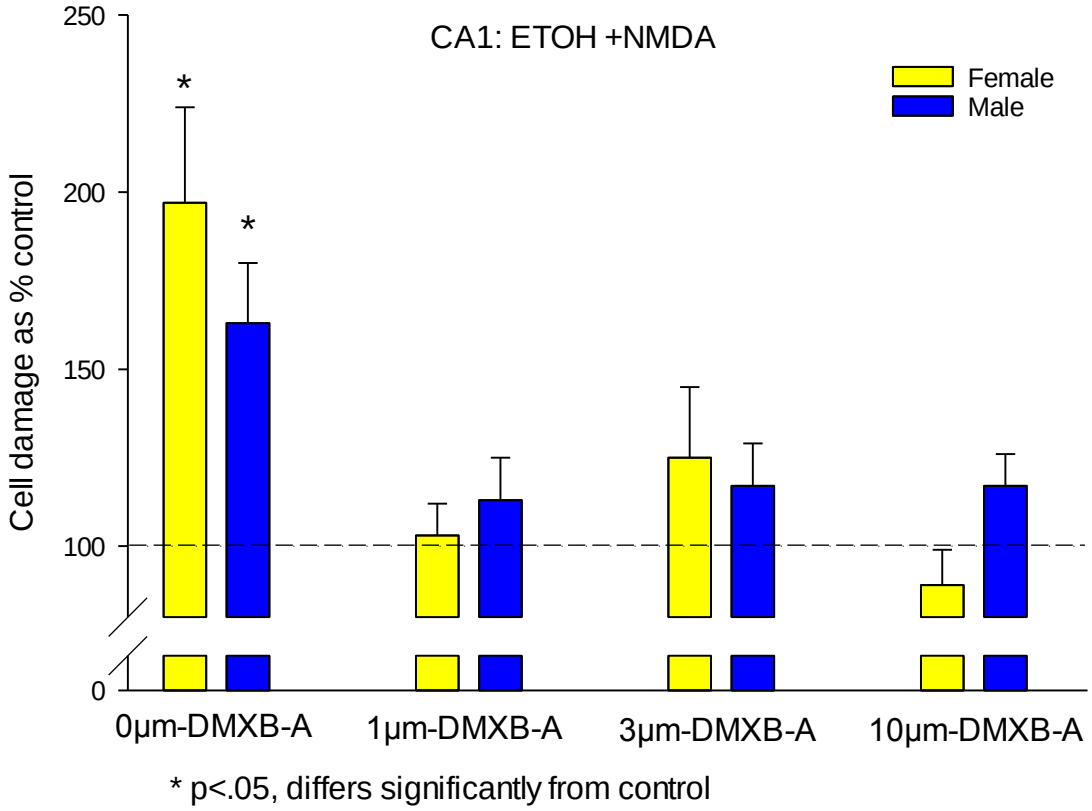

Fig 1. PI uptake expressed as percent control in the CA1 region of the hippocampus, in slices exposed to ETOH+NMDA 24 hours after ethanol withdrawal, treated with four levels of DMXB-A (0, 1, 3, and 10 µm). ETOH/NMDA slices treated with 1, 3, and 10 µm DMXB-A had significantly reduced PI expression compared to ETOH/NMDA slices. *p≤.05, differs significantly from control slices.

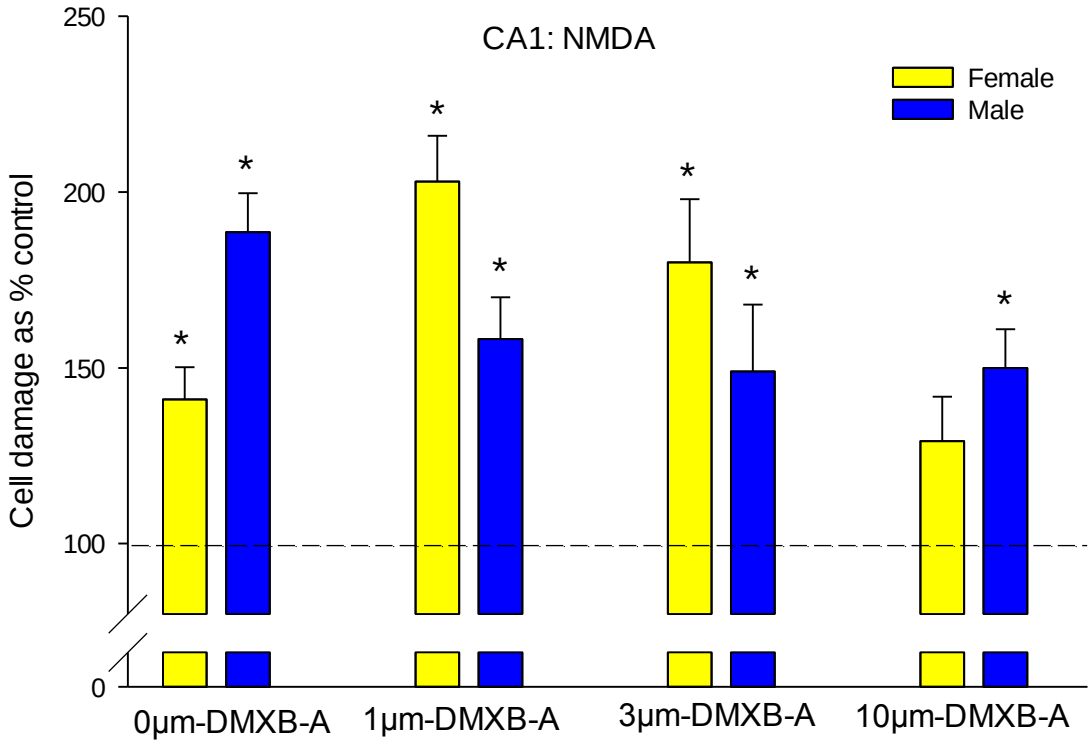

Fig 2. PI uptake expressed as percent control in the CA1, in slices exposed to NMDA, treated with four levels of DMXB-A (0, 1, 3, and 10 µm). Male slices exposed to NMDA alone expressed greater fluorescence compared to control than did female slices (188% vs 141% of control, respectively), but when treated with 1µm DMXB-A, fluorescence in females slices exposed to NMDA was greater than males (203% vs. 158% of control, respectively). *p≤.05, differs significantly from control slices.

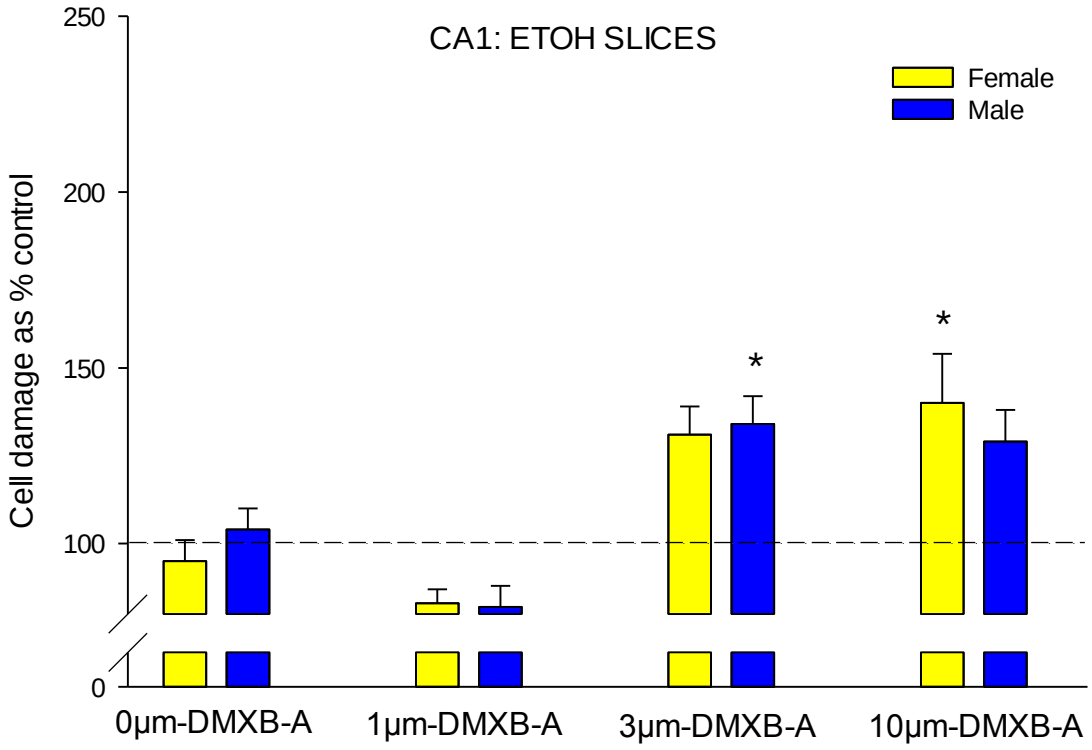

Fig. 3: PI uptake expressed as percent control in the CA1, in slices exposed to ETOH, then treated with four levels of DMXB-A (0, 1, 3, and 10 µm). ETOH exposure alone did not increase fluorescence compared to controls. 3 and 10 µm DMXB-A increased fluorescence in slices treated with ETOH in both sexes. When treated with 1 µm DMXB-A, fluorescence was comparable to control slices. *p≤.05, differs significantly from control slices.

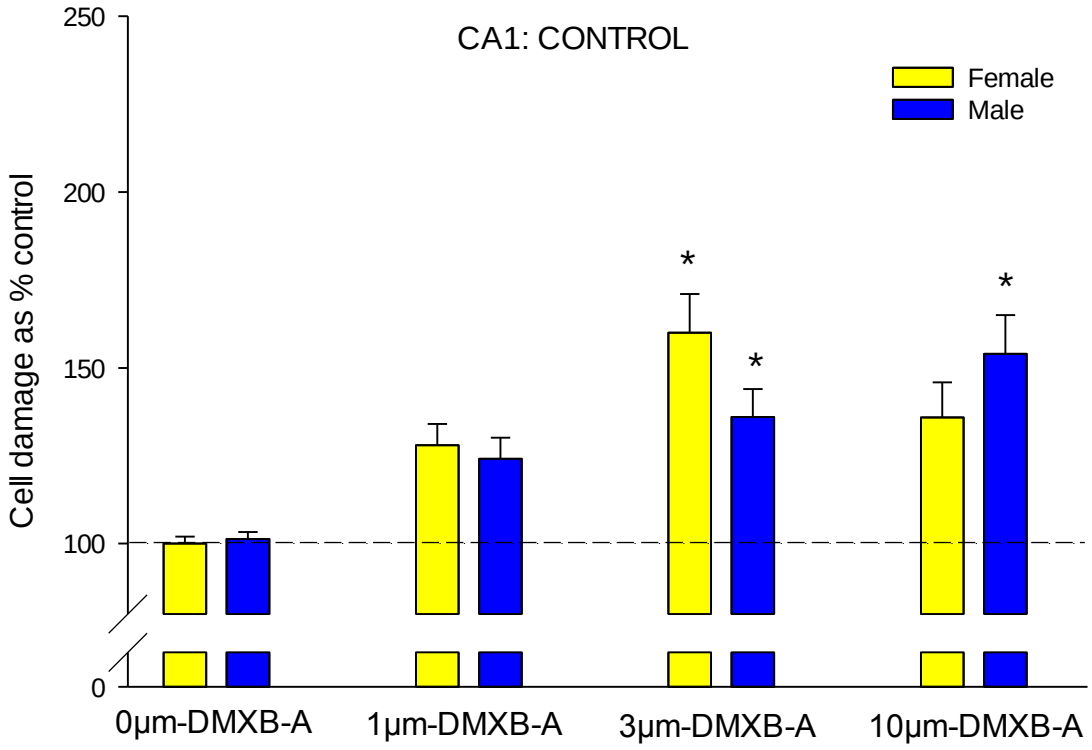

Fig 4. PI uptake expressed as percent control in the CA1, in control slices, treated with four levels of DMXB-A (0, 1, 3, and 10 µm). DMXB-A increased fluorescence with all three levels of DMXB-A in both sexes. Slices treated with 1µm DMXB-A, however, did not differ significantly from control slices. *p≤.05, differs significantly from control slices.

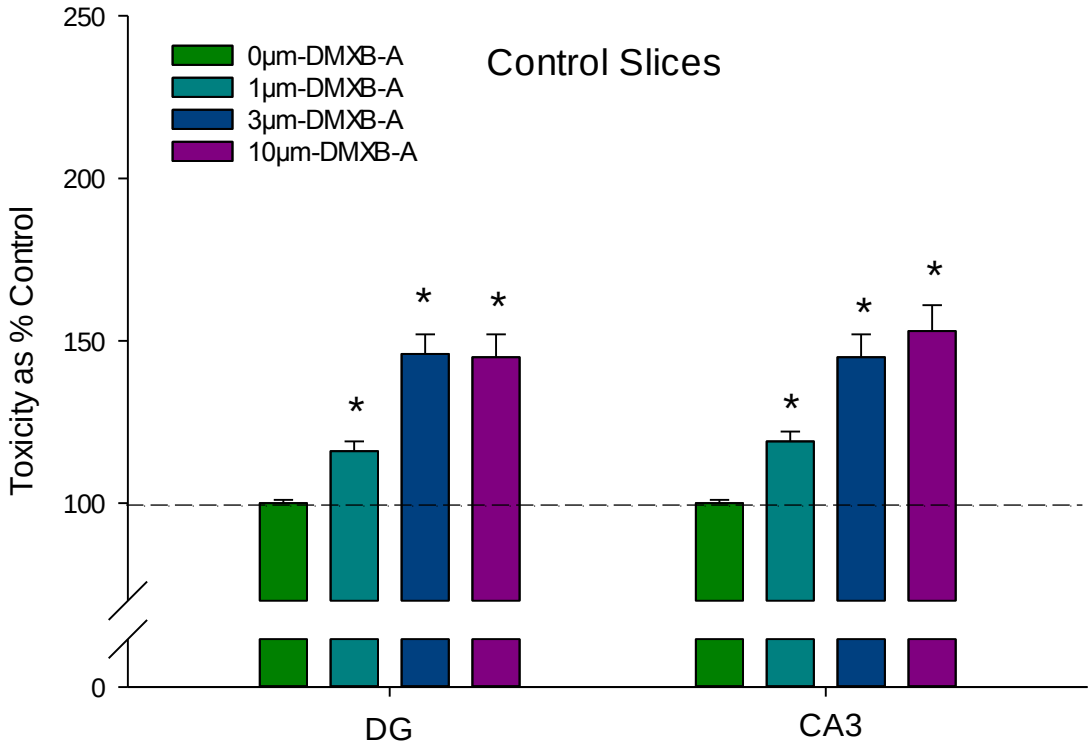

Fig. 5: PI uptake expressed as percent control in the Dentate Gyrus and CA3 in control slices treated with four levels of DMXB-A (0, 1, 3, and 10 µm). For both regions, treatment with DMXB-A significantly increase fluorescence compared to control slices. However, 1µm DMXB-A reduced this fluorescence compared to both 3 and 10 µm DMXB-A. *p≤.05, differs significantly from control slices.

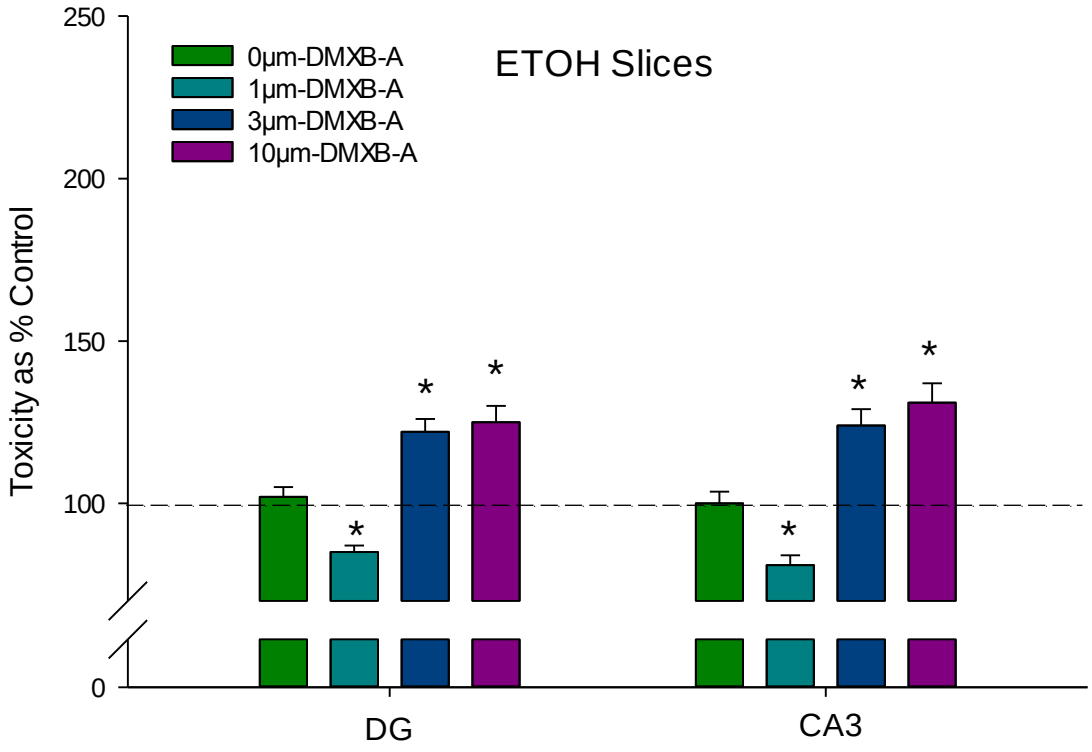

Fig. 6: PI uptake expressed as percent control in the Dentate Gyrus and CA3 in ETOH slices during EWD, treated with four levels of DMXB-A (0, 1, 3, and 10 µm). For both regions, treatment with 3 and 10 µm DMXB-A significantly increase fluorescence compared to control slices. However, 1µm reduced this fluorescence compared both 3 and 10 µm DMXB-A. *p≤.05, differs significantly from control slices.

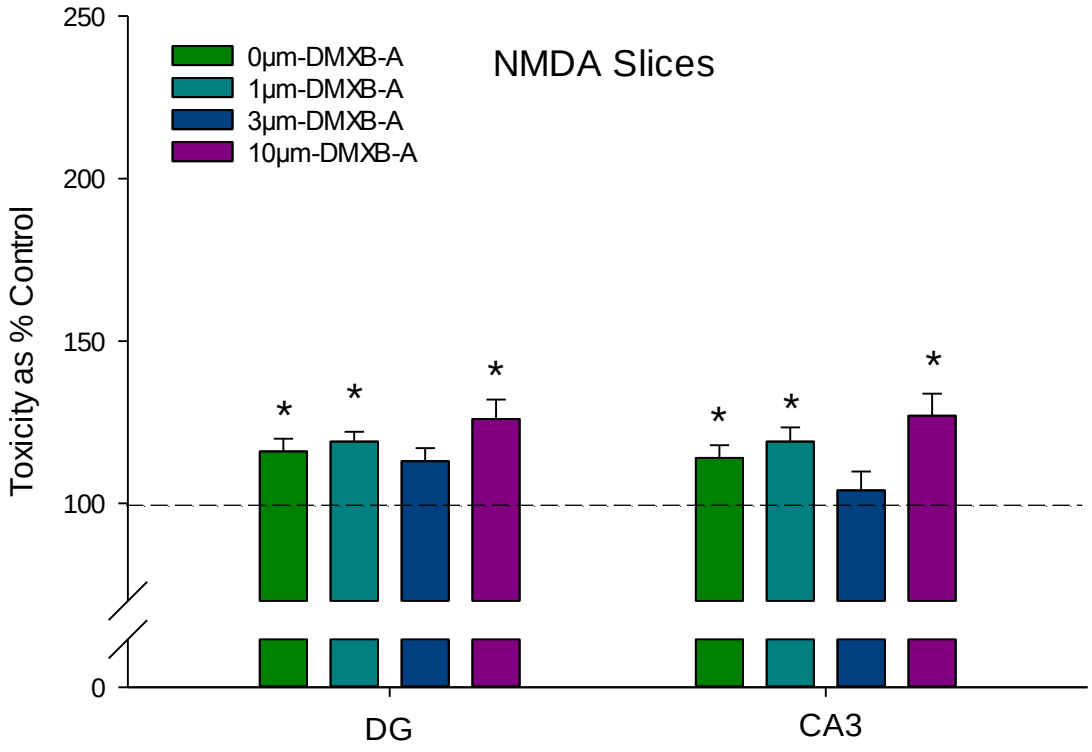

Fig. 7: PI uptake expressed as percent control in the Dentate Gyrus and CA3 in NMDA exposed slices, treated with four levels of DMXB-A (0, 1, 3, and 10 µm). NMDA alone significantly increased fluorescence for both regions when compared to controls. Treatment with 1 and 10µm DMXB-A also significantly increased fluorescence for both regions when compared to controls. Treatment with 3µm DMXB-A in NMDA exposed slices did not significantly differ from controls. *p≤.05, differs significantly from control slices.

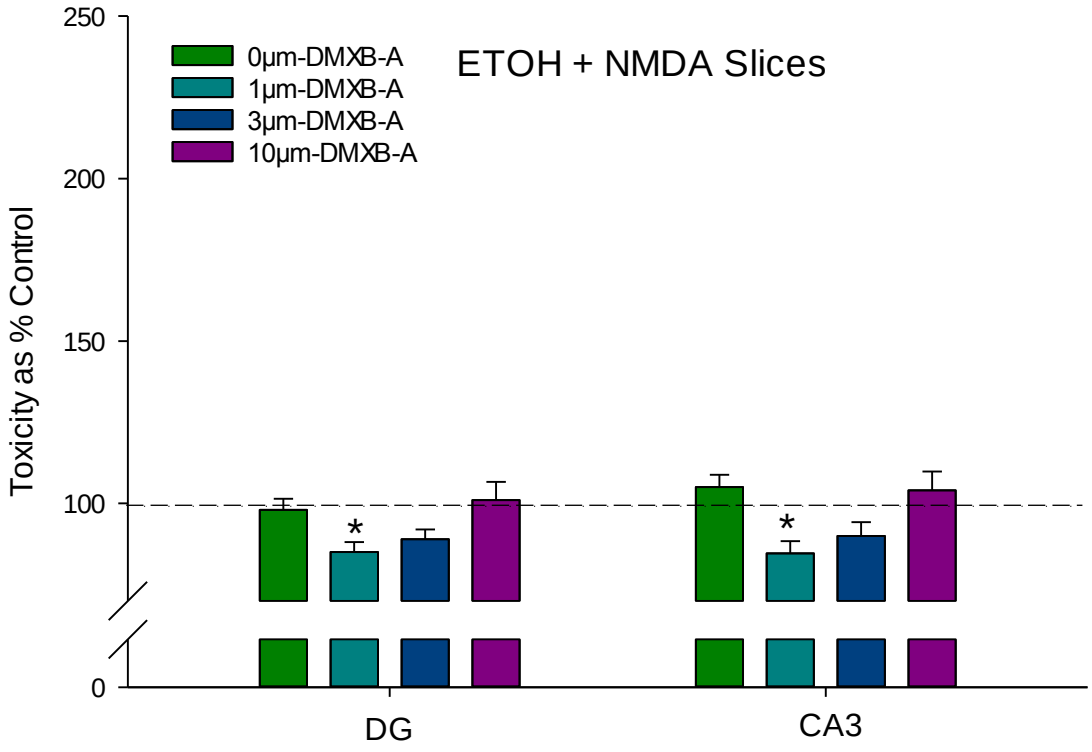

Fig. 8: PI uptake expressed as percent control in the Dentate Gyrus and CA3 in ETOH+NMDA exposed slices during EWD, treated with four levels of DMXB-A (0, 1, 3, and 10 µm). Exposure to ETOH+NMDA alone and ETOH+NMDA slices treated with 3 and 10µm DMXB-A did not significantly differ from control slices. When treated with 1µm DMXB-A, NMDA exposed slices decreased in fluorescence compared to controls. *p≤.05, differs significantly from control slices.

Discussion

DMXB-A's Role in Reducing Toxicity during EWD

In this study, toxicity in PND8 hippocampal slices undergoing various challenges were examined. These challenges included EWD after 10 day chronic binge-like exposure to 100mM ETOH in the presence or absence of an NMDA challenge, NMDA challenge alone, and the effect of various doses of DMXB-A on these groups and in healthy untreated slices. In support of the hypothesis, and in agreement with previous work using DMXB-A, our results suggest that DMXB-A may be neuroprotective against excitotoxicity associated with EWD during an NMDA challenge. This effect was seen in both males and females at all three concentrations of DMXB-A. The fact that DMXB-A reduced this toxicity was not surprising because of previous research supporting the role of the nicotinic system (and in many cases the α-7 nAChR) in protecting against various forms of cellular damage including GLU/NMDA toxicity, EWD related toxicity, β-amyloid toxicity, and hypoxia (Akaike et al., 1994; Dajas-Bailador et al., 2000; Kihara et al., 1997; Hejmadi et al., 2003; Marin et al., 1994; Prendergast et al., 2001 A & B). Previous studies using DMXB-A have also found similar results; where toxicity induced by GLU/NMDA or ETOH/EWD is reduced by treatment with DMXB-A (de Fiebre et al., 2003; Li. et al., 1999 A & B, 2000 & 2002; Shimohama et al., 1997).

After investigating the effect of EWD without NMDA challange, it was found that EWD alone did not produce significant amounts of toxicity. This finding was not surprising and has been supported previously, where 24 hour EWD alone produced significantly less toxicity than slices treated with ETOH given an NMDA challenge (Wilkins et al., 2006). However, the level of ETOH used in this study was actually quite high, averaging about 65mM (roughly equivalent to 299 mg/dl) ETOH over the 10 day exposure period (Prendergast et al., 2004). This minimal toxicity is thought to occur because of insufficient glutamate levels in cultured hippocampi during EWD (Wilkins et al., 2006). In-vivo glutamate levels actually increase during EWD (Dahchour & De Witte, 1999) so, NMDA was used to induce toxicity during EWD in this model because, as described above, most of the toxicity caused by EWD is thought to be mediated by overexcitation of the NMDA type glutamatergic receptor (for review see Tsai et al.,

1998; Kalluri et al., 1998; Grant et al., 1990; Eckardt et al., 2006; or Prendergast et al., [2001a and b]). NMDA induced toxicity in the absence of ETOH was also observed in the current study. Many previous studies (discussed above) use glutamatergic/NMDA insult to produce excitotoxicity without the presence of ETOH/EWD in various models of cellular culture, including OHSC (Akaike et al., 1994; Dajas-Bailador et al., 2000; Prendergast et al., 2001 A & B; Lewis et al., 2012). Yet, in the presence of ETOH, this toxicity caused by the addition of NMDA during EWD is thought to be multiplicative (greater than that of ETOH, or NMDA alone) (Wilkins et al., 2006), and is demonstrated in other studies from this lab (Lewis et al., 2012). However, this effect was only observed in females in our study, where NMDA alone produced a modest increase in toxicity and the addition of NMDA during EWD was significantly more toxic than NMDA alone. Surprisingly, in males, this did not occur and NMDA alone appeared to be more toxic than the addition of NMDA during EWD.

Of the previous studies in this lab using male and female slices in an OHSC model (Barron et al., 2008 and Lewis et al., 2012) looking into NMDA or EWD related toxicity, there were not any reported significant differences between sexes to these insults alone. Yet, modulators of the NMDA receptor (i.e. spermidine) that enhance NMDAR function, have been shown to produce a greater amount of toxicity in females than males in the CA3 region of the hippocampus (Barron et al., 2008). This toxicity was negated by an NMDA antagonist ifenprodil, to significantly below control levels in females. This data was supported by Prendergast and colleagues (2000) who showed similar results in the CA3 region of the hippocampus. Other work using adenosine antagonists during ethanol withdrawal have found that granule cells of the dentate gyrus, and pyramidal cells in the CA3 and CA1 in females showed increased toxicity compared to males (Butler et al., 2008 & 2009). This toxicity from adenosine receptor antagonism was postulated to occur via a NMDAR mediated over activation because co-administration of MK-801 (NMDAR antagonist) reduced this toxicity. These studies indicate that there may be differences between males and females in regard to NMDA and EWD mediated toxicity, but the differences found in these previous studies do not parallel those found in the current study. These results and previous findings support a role of sex in the outcome of

ETOH/EWD and or NMDA toxicity, though it is obvious further work needs to be conducted to further elucidate these differences.

After treating the slices with various levels of DMXB-A, it was found that the drug could be potentially toxic at higher doses, and in the presence of ETOH alone, could be protective against normal culture toxicity. First, the increased PI uptake in both sexes for the CA1, DG, and CA3 in control hippocampal slices when treated with all three concentrations of DMXB-A (1, 3, and 10μm) suggest that DMXB-A may be potentially toxic with the concentrations used in this study. At the lowest concentration of DMXB-A (1μm), the toxicity was reduced to near control levels in the CA1 without reducing the neuroprotective qualities of the drug in slices exposed to both ETOH and NMDA. These results were unexpected because toxicological reports in human clinical trials using DMXB-A have found that the drug is well-tolerated and safe (for review see Kem et al., 2006). However, Li and colleagues (1999) found that acute administration with high levels (30μm) of DMXB-A could cause toxicity. This characteristic could be a result of the drug's potential partial-agonist actions on the α-7 nAChR's (reviewed in Kem, 2006). In fact, in Li's (1999) study, they hypothesized that this toxicity is produced by a rapid desensitization of α-7 nAChR's in response to high levels of DMXB-A, and that lower doses would pro-long receptor activation and cause neuroprotection. However, Treinin and colleagues (1995) found that in a non-desensitizing mutated form of the α-7 receptor there was reduced cellular viability in response to calcium overload. And other studies have shown that using small amounts of selective α-7 antagonists (MLA) can produce some minor neuroprotection (Li. 1999). So there seems to be an ideal amount of receptor activation that promotes neuroprotection and not neurotoxicity. This interaction is unique because this same pattern of toxicity is seen in ETOH slices. Again, ETOH alone did not produce any significant amounts of toxicity, but when ETOH slices were treated with higher levels of DMXB-A (3 or 10 μm) significant levels of toxicity was observed in all three regions of the hippocampus treated with ETOH. Also, like in control slices, the lowest dose (1μm) of DMXB-A in ETOH slices reduced toxicity to below control levels, and actually produced a significant reduction of toxicity below control slices in the CA3 and DG. Though toxicity levels in control slices treated with 1μm DMXB-A were not significantly different from un-treated "healthy" slices, they still displayed significantly

more toxicity than ETOH slices treated with 1μm DMXB-A. This could possibly mean that modulation of the α-7 nAChR in a state of challenge (i.e. EWD) is crucial for its protective abilities, and that activation of this receptor in healthy cells is potentially toxic. Even more interesting though, is that DMXB-A when administered with the combination of ETOH and an NMDA challenge, reduced toxicity to near control levels at all three doses (1, 3, and 10μm); and that treatment with DMXB-A after NMDA challenge alone did not reduce toxicity to control levels at any concentration of the drug. These were a series of surprising findings that had not been previously observed. Because DMXB-A was only neuroprotective against EWD + NMDA induced toxicity, but not NMDA alone, and the fact that both forms of toxicity are thought to work in the same way (e.g. calcium toxicity), it appears that there is a crucial mechanism that exists between the α-7 nAChR, EWD, and NMDA that does not occur otherwise. It may support the idea that DMXB-A is acting as a partial-agonist at the α-7 nAChR, but this relationship seems to be much more complex involving several mechanisms that may or may not directly involve the NMDA receptor in protection against EWD induced toxicity. However, this process remains unclear and further research is needed to determine the NMDA receptors role in this process.

Limitations and Future Directions

In this study we showed that DMXB-A protects against EWD induced toxicity during an NMDA challenge in the hippocampi of PND 8 male and female rats. Future studies could address some potential limitations of this study. It is possible that the underlying mechanism of action of DMXB-A is not solely due to its action on α-7 nAChR activity since DMXB-A could have effect on other ionotropic transmembrane receptors (as discussed above). This question could be addressed by using more highly selective α-7 nAChR antagonists (like MLA & α-BTX) in the presence of DMXB-A. Previous studies (as described above) using this method have found that DMXB-A's neuroprotective effects were negated by the use of selective α-7 nAChR antagonists such as (MLA and α-BTX) (refer to De-Fiebre et al., 2003; Li. et al., 1999, 2000 & 2002; Shimohama et al., 1997), the use of these drugs in our model would provide further evidence of the α-7 nAChR's involvement in reducing toxicity induced by EWD.

However, it is also unclear if direct activation of the α-7 nAChR mediates the actions of other neurotransmitter systems to prevent cellular toxicity. It is thought that EWD and NMDA produce toxicity in a similar manner (Wilkins et al., 2006), but the current findings showed that DMXB-A was only able to reduce toxicity when NMDA and EWD was combined, but not from NMDA challenge alone. It is crucial to investigate the role of the NMDA receptor in this model using DMXB-A. One possible route could be to selectively antagonize the NMDA receptor to see if DMXB-A's toxic and protective actions persist after NMDA has been taken out of the equation. Other neurotransmitters systems found in the hippocampus have been implicated in the α-7 nAChR's protective actions. It has been hypothesized that a reduction in symptoms associated with schizophrenia and the toxicity of β-amyloid is associated with the mediation of GABA-A receptors on hippocampal interneurons by the α-7 nAChR (Benes, 2012; Miwa et al., 2011). Evidence for this modulation comes from research showing that activation of the α-7 nAChR increases GABA-A transmission in cultured hippocampal cells (Arnaiz et al., 2008) and that activation of these receptors can be protective against various forms of cell death and toxicity (Paula-Lima et al., 2005; Rosato-Siri et al., 2006; Miwa et al., 2011). Modulation of these GABA'ergic interneurons in our model could possibly be mediating the protective effect of DMXB-A against EWD toxicity during NMDA challenge. This hypothesis could be tested by co-administration of DMXB-A and selective GABA-A antagonists during EWD during an NMDA challenge. If DMXB-A's protective effects are indeed mediated through activation of GABA'ergic interneurons, then a GABA-A antagonist would negate DMXB-A's protective effects. Other possibilities to investigate the outcomes of DMXB-A treatment is the use of immunohistochemical techniques using compounds like NeuN to investigate neuronal tissue survival. NeuN is a nuclear antigen that stains specifically for mature neurons, and could be used in combination with propidium iodide which stains any cell type that has a compromised membrane (Mullen et al., 1992; Riccardi & Nicoletti 2006). This could help inform us to whether DMXB-A is selectively rescuing neurons and if neuroprotection may also occur for other cell types during EWD (astrocytes, microglia, etc.).

Previous work with both nicotinic agonists, and DMXB-A in both humans and animals have found that these drugs can be protective against behavioral deficits associated with various diseases and forms of toxicity (for review see Kem et al., 2000; Olincy & Stevens 2007). Because of this, future in-vivo work with DMXB-A must be conducted. This work is important for testing the possible therapeutic effects of this drug in our third trimester model of ethanol exposure in rodents because of its current use in clinical trials to treat diseases like schizophrenia and Alzheimer's disease (3 clinical trials in recruiting phase as of 2015; info gathered from clinicaltrials.gov). This increases DMXB-A's translation potential, and makes it a promising candidate for further work in models of fetal ethanol exposure. Completing behavioral work with DMXB-A is necessary to further explore the possibly neuroprotective nature of DMXB-A. Studies using this drug and other nicotinic compounds are critical to increase the understanding the role the nicotinic system plays in protecting against ethanol related deficits and toxicity.

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Curriculum Vitae

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Education

University of Kentucky, Lexington, KY. August 2012-Present
Behavioral Neuroscience and Psychopharmacology, Psychology.
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Publications:

Lutz, J.A., Carter, M.L., **Fields, L.,** Barron, S., & Littleton, J.M. Altered relation between lipopolysaccharide-induced inflammatory response and excitotoxicity in rat organotypic hippocampal slice cultures during ethanol withdrawal. Alcoholism Clinical and Experimental Research. May 2015; 39(5): 827-835

Lutz, J. A., Carter, M. L., **Fields, L**., Barron, S., & Littleton, J.M. The dietary flavonoid rhamnetin inhibits both inflammation and excitotoxicity during ethanol withdrawal in rat organotypic hippocampal slice cultures. Alcoholism Clinical and Experimental Research. September 2015.

Publications in Press:

Carter, M.L., Lewis B., Wellmann, K.A., **Fields, L**., Hawkey, A., Littleton, J.M., & Barron, S. Pre-exposure to ethanol sensitizes the developing brain to a mild hypoxic challenge. *Submitted to Alcoholism Clinical and Experimental Research.*

Presentations and Abstracts:

Fields, L., Cruse, M., Carter, M., Barron, S. DMXB-A's Effects on Spatial Learning and Balance Deficits in a Third Trimester Ethanol Exposure Paradigm. June 2015 (San Antonio, TX).

Fields, L., Carter, M., Hawkey, A., & Barron, S. Neuroprotective effects of DMXB-A against Ethanol Withdrawal in Neonatal Rodent Models. April 2015 (Lexington, KY).

Fields, L., Carter M.L**,** Hawkey, A., Lutz, J.A., Kem, W.E., Barron, S., DMXB-A reduces cell damage following developmental ETOH exposure in a rodent hippocampal slice model. Research Society on Alcoholism. June 2014 (Seattle, WA).

Fields, L., Carter, M.L., Hawkey, A., Kem, W.E., & Barron, S. Dimethoxybenzylidene (DMXB) reduces balance deficits following "3rd trimester" ethanol exposure in female but not male rats. Alcoholism: Clinical and Experimental Research 37: (Suppl 2) 400a.

Carter, M.L., **Fields, L**., Hawkey A., Littleton, J.M., Barron, S. The effects of NMDA receptor modulation following developmental ethanol and hypoxia exposure. Presented at the Research Society on Alcoholism. June 2014 (Seattle, WA).

Carter, M.L., Nall, D., **Fields, L.,** Hawkey, A., & Barron, S. The combination of ETOH and hypoxia produces behavioral deficits in a 3rd trimester rodent model. Presented at the Research Society on Alcoholism, June 2013 (Orlando, FL). Alcoholism: Clinical and Experimental Research 37: (Suppl 2) 126a.

Hawkey, R. Gupta, M. Carter, **L. Fields** & S. Barron. Effects of Developmental Alcohol Exposure in the Absence of Sensory Pre-Exposure on Adolescent Drinking Habits in Rats. Research Society on Alcoholism (2014).

Hawkey, R. Gupta, M. Carter, **L. Fields** & S. Barron. Sex differences in body weight and ethanol consumption in adolescent rats. SEBA (2014).

Hawkey, R. Gupta, M. Carter, **L. Fields** & S. Barron. Exploring the effects of developmental alcohol exposure in the absence of sensory preexposure on adolescent drinking habits in rats. Children at Risk Research Conference. June 2014 (Seattle, WA).

Hawkey, W. Xu, H. Li, M. Carter, **L. Fields**, J. Luo, G. Chen, & S. Barron. Consequences of Prenatal Drinking in the Dark(DID) and Neonatal Ethanol Intubation on Exploration in Juvenile Mice. Research Society on Alcoholism. June 2015 (San Antonio, TX)

A. Hawkey, W. Xu, H. Li, M. Carter, **L. Fields**, J. Luo, G. Chen, & S. Barron. Behavioral Deficits in a Novel "3 trimester" Mouse Model of Developmental Alcohol Exposure. Children at Risk Research Conference. April 2015. (Lexington, KY)

Seminars

Fields, L. "The nAChR Agonist DMXB-A's Effects on a Third Trimester Ethanol Exposure Model: a behavioral approach." Behavioral Neuroscience and Psychopharmacology Departmental Brown Bag, September 2014.

Fields, L., Carter, M., Hawkey, A. "In vitro/In Vivo Methods to investigating Fetal Ethanol Effects in the Barron lab." Behavioral Neuroscience and Psychopharmacology Departmental Brown Bag, December 2014.

Fields, L. “Neuroprotective effects of DMXB-A against ethanol withdrawal in neonatal rodent models.” Behavioral Neuroscience and Psychopharmacology Departmental Brown Bag, March 2014.

Fields, L. “The Baby and the Bottle.” Brain Outreach Program at Bryan Station High School, April 2014.

Funding/Awards:

Spring 2013: 1st place poster at the 3rd annual children at risk research conference.

Teaching Experience:

Fall 2015: Teaching Assistant and Lab Leader for Learning Psy-450 (U. of Kentucky)
Fall 2014: Teaching Assistant and lab leader for Behavioral Neuroscience PSY-456 (U. of Kentucky)
Fall 2014: Guest Lecturer for Behavioral Neuroscience PSY 456 for Dr. Michael Bardo
Fall 2013: Teaching Assistant and lab leader for PSY-100 Introduction to Psychology (U. of Kentucky)

Honors/Activities/Service:

Fall 2015: Created a student Study Aide Coloring Book modeling the sheep, rodent, and human brain for the advanced lecture lab in behavioral neuroscience PSY-456
Fall 2015: Developed course material for a student lab manual for the advanced lecture lab in behavioral neuroscience
Fall 2014 & 2015: Participated in Career Day at Yates Elementary School (information and education about psychology and neuroscience as a career).
Summer 2014 & 2015: Volunteered Audio/Visual Aide for the Research Society on Alcoholism annual meeting

Spring 2014: Brain Day outreach program at Veteran's Park Elementary School

Spring 2014: Abstract Reviewer and Moderator for National Conferences on Undergraduate Research (NCUR)

Spring 2014: Brain outreach program at Bryan Station High School

Fall 2014: Graduate Student Ambassador at Graduate School Night at Transylvania University

Spring 2013: Won first place poster at Fourth Annual Children at Risk Research Conference, U of Kentucky.

Fall 2013- Spring 2014: Behavioral Neuroscience and Psychopharmacology department seminar organizer

Multiple Deans Lists.