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ETHANOL INDUCED NEUROTOXICITY THROUGH DYSREGULATION OF AMPK IN A FETAL ALCOHOL SYNDROME MODEL

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THESIS

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

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Lexington, Kentucky

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ABSTRACT OF THESIS

ETHANOL INDUCED NEUROTOXICITY THROUGH DYSREGULATION OF AMPK IN A FETAL ALCOHOL SYNDROME

Ethanol consumption during pregnancy is rising in the U.S., including the rate of binge drinking. It is reported around 1 in 27 women engage in binge drinking activities while pregnant. The third trimester is a sensitive period of neuronal growth in which ethanol induced neurotoxicity can cause many harmful effects including Fetal Alcohol Spectrum Disorders. It has been shown that ethanol decreases the activity of AMPK through increasing lipid peroxidation, both of which are correlated to neurotoxicity. AICAR is a synthetic analog of AMP which significantly increases AMPK activity and may have beneficial effects in an organotypic hippocampal model of third trimester binge drinking. The purpose of this study is to evaluate if pharmacologically increasing the activity of AMPK could reduce the degree of ethanol induced neurotoxicity to provide a potential therapeutic target for Fetal Alcohol Spectrum Disorders.

KEYWORDS: Alcohol, AMPK, Neurotoxicity, Hippocampal Slice Culture, AICAR

Jessica Elise Gebhardt

11/18/2020

Date

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DEDICATION

To all the educators, family, and friends that have inspired, encouraged, and helped me pursue my dream to become a Neuroscientist.

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CHAPTER 1. INTRODUCTION

1.1 Prevalence and Impact of Drinking during Pregnancy

Drinking during pregnancy can cause stillbirths, miscarriages, birth defects, and Fetal Alcohol Spectrum Disorders (FASDs) (Denny et al., 2019). FASDs is an overarching term that includes: Fetal Alcohol Syndrome (FAS), Partial FAS, and Alcohol-Related Neurodevelopmental disorder (ARND) (Brown, Connor, & Adler, 2012). FASDs covers a range of symptoms that include anatomical abnormalities, behavioral problems, and impaired intellectual development (Denny et al., 2019). Many of these consequences are due to permanent brain damage and will affect an individual for their lifetime (Brown, Connor, & Adler, 2012). The leading global environmental factor that causes intellectual disability is ethanol exposure during pregnancy. This is true for both developing and developed countries. (Welch et al., 2016). Many individuals with FASDs go on to have problems throughout their educational and employment experiences and have a higher chance of being incarcerated (Brown, Connor, & Adler, 2012). In the United States, it is estimated that 1.1%-5.0% of children in the first grade have FASDs (Denny et al., 2019). The lifetime cost of one case of FASDs is estimated to be approximately $2 million (Luo, 2014).

Using the Behavioral Risk Factor Surveillance System the CDC collected data of self-reported drinking behavior in 6,814 pregnant women between the ages of 18 and 44. The most current data set from 2015 to 2017 shows that 11.5% reported drinking at least one alcoholic drink and 3.9% reported binge drinking (4 or more drinks in one sitting) in the past month. Of those that engaged in binge drinking the average frequency was 4.5 episodes per month, and the average intensity was 6 drinks in a sitting. Both the percentage

of women that engaged in drinking and binge drinking behaviors had increased from the 2011 to 2013 estimate, which was 10.2% and 3.1% respectively. Data was not given on trimester; however, as gestation week increases the occurrence of drinking tends to decrease. As this data was self-reported it is assumed the data is a low estimate (Denny et al., 2019).

1.1.1 The Third Trimester as a Critical Period

The third trimester of pregnancy and the following several years after birth in humans is characterized by rapid synaptogenesis and is a period in which ethanol can produce deleterious effects (Saito et al., 2007). Studies have even shown that both low and high doses of ethanol can cause changes in the cerebral vasculature by inducing brain micro-hemorrhages when given during the third trimester. Alteration in the cerebral vasculature have been linked to the pathophysiology of FASDs. Disruption in cerebral vasculature can cause neuronal loss, reactive astrogliosis, and microglial activation (Welch et al., 2016). Microglia have been shown to play a role in brain development, with early activation potentially disrupting neurodevelopmental trajectories and/or triggering neurodevelopmental disorders, brain injuries, and neurodegenerative disease (Saito, Saito, & Das, 2019). Other factors that contribute to the sensitivity of ethanol during this period include: altered neurotransmitters, altered neurotrophic factors, and a less developed antioxidant capacity of the fetus/child (Saito et al., 2007). The last of which is thought to be responsible for the higher levels of proinflammatory cytokines in the fetus compared to the mother during prenatal alcohol exposure (Saito, Saito, & Das, 2019).

1.2 Metabolism of Ethanol

Ethanol readily passes through biological membranes so that its concentration is broadly distributed throughout the body. It can easily enter the brain, as well as pass through the placenta, making it teratogenic (Brown, Connor, & Adler, 2012). Ethanol can be oxidized through alcohol dehydrogenase (ADH), catalase, and P450 (CYP2E1); all of which are present in the brain. These reactions produce acetaldehyde, which can be reactive with other cellular components to produce cytotoxic molecules. Aldehyde dehydrogenase (ALDH) then oxidizes acetaldehyde to acetate. Most of the consumed alcohol is oxidized by ADHI in the liver, though ADH1 is found in many other places including the hippocampus in the brain. ALDH2 is located in the mitochondrial matrix, and in the brain cortex it has a high affinity for acetaldehyde. All of these reactions reduce NAD pools, contributing to disturbances in metabolism (Wilson & Matschinsky, 2020).

Alcohol effects nearly every tissue in the body, and intemperate consumption can dysregulate and damage the brain, heart, liver, pancreas, lungs, bone, skeletal muscles, endocrine, and immune systems (Luo, 2014). In the liver, alcohol leads to the inhibition of gluconeogenesis, reduced fatty acid oxidation, decreased AMP, and increased lactate/pyruvate ratio. In the brain ethanol significantly disrupts brain metabolism and results in decreased glucose uptake, disrupted amino acid metabolism, stimulation of dopaminergic activity, and inhibition of glutamatergic activity. When alcohol is consumed it can account for 12% to 20% of cortical oxidative metabolism (Wilson & Matschinsky, 2020).

1.3 Physiological Role of AMPK

AMPK is a serine/threonine kinase that is a regulator of cellular metabolism as well as cell polarity, autophagy, apoptosis, and cell migration. Its activation can be an adaptive response to energy stress, where it leads to the suppression of energy consuming processes

and enhancement of ATP-producing pathways (Ronnett et al., 2009). As ATP remains nearly constant in the cell, AMP is a sensitive measure of metabolic homeostasis within a narrow range. AMPK is activated by metabolic stress when the AMP:ATP ratio is reduced (Wilson & Matschinsky, 2020). This occurs when AMP allosterically binds to AMPK at the Thr 172 residue. AMPK is composed of an alpha, beta, and gamma subunits with the alpha unit being catalytic and the beta and gamma units being regulatory (Ronnett et al., 2009). AMPK can also be activated independent of energy metabolism by reactive oxygen species (ROS) including H_2O_2, calcium, cellular pH, redox status, creatine/phosphocreatine ratio, and hormones. The three upstream kinases include liver kinase B1 (LKB1), TGFB-activated kinase 1(TAK1), and calcium/calmodulin-dependent protein kinase B (CAMKK2) (Ronnett et al., 2009).

When AMP is decreased, the reduction in AMPK causes downregulation in mitophagy, mitochondrial synthesis, glycolysis, fatty acid oxidation, autophagy, and protein synthesis. It also changes gene expression, and causes an upregulation in gluconeogenesis, fatty acid synthesis, and inflammation. Most of the studies involving AMPK focus on its upregulation in conditions such as exercise and hypoxia (Wilson & Matschinsky, 2020). AMPK is a major regulator of lipid metabolism as it prevents the conversion of acetyl Co-A to malonyl-CoA, which is the rate limiting enzyme in fatty acid biosynthesis. Sterol regulatory element-binding protein 1 (SREBP-1), a major regulator of fatty acid metabolism, is regulated by AMPK. (Saito et al., 2007). AMPK has been shown to be inhibited by ethanol in hepatic cells, and possibly contributes to alcoholic fatty liver. (Wilson & Matschinsky, 2020). It has been shown that AICAR (AMPK activator) is beneficial in models of ethanol-induced hepatic steatosis. It decreased SREBP-1c and

reduced fatty acid synthase (FAS), causing decreased triglyceride synthesis (Tomita et al., 2005). AMPK also regulates CAMP-response element-binding (CREB) protein, which is a pro-survival transcription factor (Rehman et al., 2019).

In the brain, AMPK is primarily expressed in neurons, but can be found in astrocytes, oligodendrocytes, and microglia (Saito, Saito, & Das, 2019). It is enriched in the cortex, hippocampus, and hypothalamus. In the hypothalamus, AMPK activity is controlled by nutritional signals such as insulin, leptin, and ghrelin; and it has a significant regulation over feeding behavior (Ronnet et al., 2009). AMPK in the cortex and hippocampus is less influenced by hormonal feeding signals (Saito et al., 2007).

1.4 Ethanol's Interaction with AMPK

In ethanol dependent individuals disruption in protein synthesis and mitochondrial content and morphology is believed to be due to chronic suppression of AMPK (Wilson & Matschinsky, 2020). Oxidation of ethanol and acetate increase the energy state, while decreasing AMPK. Inhibition of AMPK through ethanol oxidation also allows for lipid accumulation to occur. In studies in the hypothalamus, AMPK phosphorylation can decrease by 50% due to alcohol. Suppressed AMPK reduces GLUT1, GLUT3, and GLUT4, which are the main glucose transporters in the brain. In alcohol studies, this reduction can be as much as 71% for GLUT1. In microvessels of the blood brain barrier (BBB), GLUT1 plays an integral role (Wilson & Matschinsky, 2020). AS1600 is phosphorylated by AMPK and promotes the fusion of GLUT4 with the plasma membrane, facilitating glucose uptake (Hill et al., 2016). Ethanol also causes the aspartate-glutamate shuttle to be reduced, which is another way in which glutamate homeostasis is disrupted

(Wilson & Matschinsky, 2020). The functions of AMPK and the developing brain need more clarity; however, it is apparent that AMPK is present and abundant in the developing brain. Knockouts of AMPK β1 can lead to loss of neurons, oligodendrocytes, and astrocyte proliferation (Saito, Saito, & Das, 2019).

1.5 Inflammation

Inflammation is a necessary defense mechanism that protects the body against endogenous and exogenous unwanted agents, such as bacterial, viral, and damaged cells. Microglial have a role in synaptic organization, control of neuronal excitability, and are responsible for the innate immune response in the nervous system. Once activated the homeostatic microglia (M0), can switch between the classical pro-inflammatory (M1) and the alternate anti-inflammatory (M2) phenotypes. The M1 state has been well characterized in cultured cells by treatment with lipopolysaccharide, a type of known pathogenassociated molecular patterns (PAMPS), that mimics the outer membrane of Gramnegative bacteria. This activates Toll-like-receptor 4 (TRL4) on microglia and initiates NFkB (Saito, Saito, & Das, 2019).

NF-kB is a nuclear transcription factor that is important in transcription and synthesis of inflammatory factors (Li et al., 2019). Following NF-kB activation there is the release of proinflammatory cytokines, chemokines, and cytotoxic factors that include: TNF-a, IL-1B, TLRs, NO, ROS, TLRs, and cytokine receptors. In contrast, the M2 state can be triggered by the T-helper type 2 cytokines, IL-4, and IL-3. In response, they release IL-10, transforming growth factor (TGF-B), and arginase-1 (Arg1). The M2 state helps to clear debris, produce trophic factors, and aids in repair. It was once thought that neurodegenerative diseases constituted a M1 phenotype; however, due to differences in

phenotypes, dysregulated microglia are now referred to as disease associated microglia (DAM) or microglial neurodegenerative (MGnD) phenotypes. Microglia are often activated by multiple signals and so it is not uncommon that M1, M2, and DAM phenotypes are all present in an affected area. The inflammation process is also dynamic and so changes in different states are possible (Saito, Saito, & Das, 2019).

An important component of microglial activation is the cellular energy status, which is dependent upon mitochondrial function, glucose availability, and the glycolytic rate. M0 microglia maintain energy through oxidative phosphorylation; however, in the activated state microglia switch to glycolysis. Hypoxia and hyperglycemia promote glucose utilization and the M1 state. Caloric restriction and the ketogenic diet can promote the M2 state in specific circumstances. There are several key signaling molecules that relate the energy status to the microglial inflammatory state. NF-kB is a major contributor and drives the pro-inflammatory state. Silent information regulator 1 (SIRT1) regulates energy metabolism, tissue survival, and is anti-inflammatory. It is activated by AMPK and directly inhibits NF-kB. Peroxisome proliferator-activated receptors y (PPARy) is a type II nuclear hormone receptor that is involved in insulin sensitivity, trophic factor production, metabolism of lipids and glucose, and is overall anti-inflammatory (Saito, Saito, & Das, 2019). It is also necessary, along with mitochondrial transcription factor A, in AMPK's ability to enhance mitochondrial biogenesis (Hill et al., 2016). Nuclear factor erythroid-2related factor-2 (Nrf2) is a regulator of antioxidant responses, and is suppressed under oxidative stress (Rehman et al., 2019). Triggering receptor expressed on myeloid cells 2

(TREM2) is anti-inflammatory as well. All of these signaling molecules are linked to AMPK, with all of them either activating or being activated by AMPK. The exception being NF-kB, which AMPK indirectly inhibits (Saito, Saito, & Das, 2019).

1.5.1 Ethanol and Inflammation

Ethanol has been shown to induce neuroinflammation through toll-like receptor 4 (TLR4) activation of microglia (Saito, Saito, & Das, 2019). This inflammation is more severe in younger nervous systems that are experiencing a higher degree of neuronal plasticity and neurogenesis. Excessive consumption of alcohol can cause neurodegeneration, and neuroinflammation is believed to play a causal role in pathophysiology. Ethanol exposure and most other neurodegenerative diseases display microglia-mediated neuroinflammation and proinflammatory cytokine release (Li et al., 2019). When ethanol is given to P7 mice, it caused acute microglial activation and apoptotic neurodegeneration (Saito, Saito, & Das, 2019).

The hippocampus has been shown to be a region that is vulnerable to alcoholinduced structural damage. Studies have shown that all three regions of the hippocampus experience increased TNF-a and IL-1B levels due to ethanol. In fact, it is believed that chronic alcohol exposure keeps microglia in a phagocytic activated proinflammatory state (M1) (Li et al., 2019).

1.5.2 AMPK and inflammation

Phosphorylated AMPK has been shown to exert anti-inflammatory and immunosuppressive effects in many cell types and models, mainly through supporting the polarization from the M1 to the M2 state (Saito, Saito, & Das, 2019). This is seen in both

genetic and pharmacological manipulation of AMPK. In knockout AMPKB1-/- mice there was increased infiltration and activation of macrophages. Metformin, which is an AMPK activator has shown benefits in chronic inflammatory diseases and cancers that is independent of its effects on normalizing blood glucose levels. These effects include reducing the transcription of TNF-a and IL6. Though most of the studies that observed inflammatory responses in cerebral ischemia and cardiac arrest, metformin was given preemptively or concurrently with the insult rather than subsequently. Acute metformin treatment in stroke injury was shown to be detrimental in some rodent models, in comparison to chronic treatment with metformin which was shown to be neuroprotective. Consequently, the timing, duration, and amount of AMPK activation may be paramount in determining the benefits of AMPK. Compounds such as metformin, rosiglitazone, galgeine, berberine, and resveratrol's beneficial effects, such as reduced inflammation, have been shown to be dependent upon the activation of AMPK (Salt & Palmer, 2012) (Saito, Saito, & Das, 2019). This is also true for betulinic acid (BA),a pentacyclic triterpenoid, ENERGI-F704, an AMPK agonist, as well as Balasubramide, and Salvaoic acid C (Saito, Saito, & Das, 2019).

AMPK activation is associated with inhibition of cytokine-stimulated NF-kB activity in cell types such as endothelial, microglial, astrocytes, hepatic stellate, chondrocytes, neutrophils, and macrophages (Salt & Palmer, 2012). The suppression of NF-kB activation is linked to AMPK's ability to inhibit TNF-a, IL-1B, and IL-6 synthesis in macrophages, and to increase IL-10(Salt & Palmer, 2012). It is also important in the tolllike receptor 4 (TLR4) pathway which is causal in microglial activation (Li et al., 2019). Treatment with LPS to induce TLR4 activation causes a reduction in p-AMPK levels, but

can be reversed by AMPK activators (Saito, Saito, & Das, 2019). AMPK has been shown to decrease JNK phosphorylation, and subsequently ER stress. In berberine, LPSstimulated JNK, ERK1/2 and p38 MAPK phosphorylation were inhibited in an AMPK dependent manner in macrophages (Salt & Palmer, 2012). AMPK also reduces cyclooxygenase-2 (COX-2) and inducible nitic oxide synthase (iNOS) (Rehman et al., 2019). These anti-inflammatory processes are associated with a reduction in neurodegeneration (Saito, Saito, & Das, 2019).

1.6 Lipid Peroxidation

Studies have concluded that alcohol consumption enhances astrocyte activation and reactive gliosis, which is characterized by an overexpression of fibrillary acidic protein (GFAP) and the acidic calcium binding protein, S100B. Astrocytes are paramount in the regulation of ionic environments, and their activation is often a first response to physical and chemical insults. At low concentrations GFAP and S100B can be neurotrophic, but elevated levels are neurotoxic (Baydas & Tuzcu, 2005).

During binge ethanol treatments in rats, there is significant brain water elevation, producing brain edema. This is thought to be caused by upregulated expression of astroglial-enriched water channel, aquaporin-4. The swelling triggers phospholipiddependent neuroinflammation and oxidative stress, which the brain is sensitive to, especially in the cortex and hippocampus (Moon et al., 2014) (Rehman et al., 2019). Overactive microglia can release many neurotoxic molecules that include proinflammatory cytokines, metalloproteinanses, nitric oxide (NO), and even more reactive oxygen species (ROS) (Shearn et al., 2014). Lipid peroxidation occurs when oxidative stress causes the degradation of lipids through reactions with free radicals, compromising

membrane integrity (Rehman et al., 2019). Ethanol administration in all age groups has been shown to significantly increase lipid peroxidation (Baydas & Tuzcu, 2005).

Omega-6 and arachidonic acid (AA) are released from the phospholipid membrane in response to ROS. Omega-6 can directly cause the loss of mitochondrial transmembrane potential (MTP), and lead to apoptosis. Normally free levels of AA remain less than 10uM, but they can increase 50-fold in response to an insult when liberated by phospholipase A2 (PLA2), a downstream target of Toll-like receptors. AA is then broken down in eicosanoid products such as prostaglandins and leukotrienes creating an increasing cycle of inflammatory factors. There are three gene products in the PLA2 family: calcium dependent cytosolic PLA2 (cPLA2), calcium independent cytosolic PLA2 (iPLA2), and secretory calcium dependent PLA2 (sPLA2). All three seem to be integral for ethanol's effect on AA. It has been shown that a global inhibitor of PLA2, mepacrin, reduced neurodegeneration in an organotypic hippocampal model. As free lipids with polyunsaturated omega-6 acyl groups result in the production of 4-Hydroxynonenal (4HNE), Mepacrin also blocks ethanol's potentiation of 4-HNE (Moon et al., 2104).

ROS, can disturb the structure and function of proteins, lipids, DNA, and induce cell death (Rehman et al., 2019). Primary markers for measuring increased oxidative stress include 4-HNE and malodialdehyde (MDA), which are produced by peroxidation of lipids in the cellular membrane (Rehman et al., 2019). 4-HNE is an electrophile which reacts with nucleophilic residues such as cysteine, lysine, and histamine (Shearn et al., 2014). Under physiological conditions there is a balance between the production of free radicals and antioxidants (Baydas & Tuzcu, 2005). As such, 4-HNE's damage can be mitigated by cellular controls that include enzyme mediated oxidation, reduction, and glutathione

conjugation (Petersen, 2004). Glutathione is the most important endogenous antioxidant in the central nervous system (Baydas & Tuzcu, 2005). However, if the production of lipid peroxidation is excessive these mechanisms will be overwhelmed and insufficient to adequately handle the stressor (Petersen, 2004). In fact, reduced glutathione levels are often observed after ethanol consumption (Baydas & Tuzcu, 2005).

Studies have shown that melatonin, which is a direct radical scavenger and so an indirect antioxidant, can regulate energy balance through decreasing ROS and increasing p-AMPK in a TBI model. Through increasing AMPK, it also decreased activated JNK and ER stress (Rehman et al., 2019). In an ethanol model, melatonin restored glutathione levels, and stabilized glial activity (Baydas & Tuzcu, 2005). 4-HNE and MDA are negative regulators of AMPK. By decreasing lipid peroxidation levels, this allow for AMPK to regulate energy balance within neuronal tissue; ultimately reducing the degree neurotoxicity and neurodegeneration.

1.6.1 Ethanol and Lipid Peroxidation

Chronic ethanol consumption causes the production of reactive aldehyde species such as 4-HNE, malondialdehyde (MDA), and acrolein (Shearn et al., 2014). In chronic inflammatory hepatic diseases, such as alcoholic liver disease (ALD) and non-alcoholic steatohepatitis (NASH), it has been shown that lipid peroxidation through the production of reactive aldehyde species such as 4-HNE directly inhibits AMPK. This occurs by 4HNE forming Michael addition adducts to the cysteine residues 130, 174, 227, and 304 of AMPKα and cysteine residues 225 on AMPKβ. Of these, 4-HNE's binding to Cys 130, 174, and 304 have been shown to interfere with the active Thr 172 site, substrate access, and inhibition of ROS induced increases. Though most alcohol abuse studies report a

decrease in AMPK, a few have reported an increase in AMPK phosphorylation in hepatic cells. The discrepancy may be due to inconsistencies in the concentration and duration of ethanol, and other contributing variables such as dietary fat. In a few of the studies that reported elevated phosphorylation of AMPKα in response to ethanol, the carbonyl adducts created steric hinderance preventing signal transduction, effectively inactivating the molecule, and creating a misleading statistic (Shearn et al., 2014). It is important to note that those studies do not report a corresponding increase in ACC (Shearn et al., 2014). 4HNE, when given by itself, inhibited the effects of AICAR and H_2O_2 on AMPK, showing that aldehydes can inhibit AMPK under both conditions of oxidative stress and increased AMP(Shearn et al., 2014).

1.7 Ethanol Induced Neurotoxicity

Neurodegeneration is a detrimental outcome of many neurological diseases and injuries that can result in reduced physiological function, reduced mental capacity, or even death. In all of the diseases and injuries that lead to neurodegeneration there are consistent causal factors such as dysregulation of inflammation, oxidative stress, lipid peroxidation, and energy imbalance (Moon et al., 2014) (Rehman et al., 2019). Glial cells appear to be the most vulnerable to neurotoxic insults; however, glial damage can cause glial-neuronal dysfunction and lead to neuronal degeneration. Glial cells are also the most responsive to neuroprotective treatment, which creates a sensitive period in which damage is more readily reversed (Baydas & Tuzcu, 2005).

Studies have shown that immature neurons as well as neurons of increased aged are significantly more intolerant of ethanol compared to a mature nervous system (Baydas & Tuzcu, 2005). In immature neurons this is seen in the degree of neuroapoptosis and is

believed to be the result of an under-developed stress mitigation system, particularly as it involves the unfolded protein response and autophagy. The developing nervous system also has an elevated baseline of pro-apoptotic protein contributing to its vulnerability (Luo, 2014). In FASDs, ethanol is associated with a large reduction of neurons in the cerebral cortex, hippocampus, cerebellum, and olfactory bulb (Naseer et al., 2014). The intensity of exposure is also a factor in the degree cell death, with binge exposure being particular detrimental (Moon et al., 2014).

Ethanol can cause excitotoxicity through overactivation of NMDA receptors from ethanol withdrawal (Naseer et al., 2014). It can also cause damage to mitochondria, which produces intracellular ROS. ROS can regulate autophagy, apoptosis, and activation of NFKB. Ethanol has been shown to induce ER stress, contributing to disruptions in calcium homeostasis. Evidence has also shown that an increase in intracellular calcium can inhibit autophagic flux, allowing more defective proteins to remain in the cell (Luo, 2014). Other markers of neurotoxicity that ethanol induces include cytochrome c, cleaved caspase-3, and PARP-1(Naseer et al., 2014).

Naseer et al., showed that osmotin, a homolog of adiponectin was protective against ethanol induced neurotoxicity in a neonatal rat hippocampus, increasing cell viability by 80%-90%. Though adiponectin is a hormone with many mechanisms of actions, protection was shown to be through activation of AMPK by phosphorylation at Thr^{210}. Metformin was also shown to reduce oxidative imbalance in the brain. Other markers such as normalized intracellular calcium levels, improved mitochondrial function, and reduced activation of caspase-3 were seen as well. When osmotin was given subsequently with ethanol there was a significant reduction in propidium iodide staining (marker of cell

death); however, when osmotin was given post ethanol treatment the reduction in neuroapoptosis was decreased.

Translational studies show that ethanol neurotoxicity in rodents can model human fetal alcohol syndrome through markers such as apoptotic neurodegeneration. In mice, studies have shown that the significant rate of neurodegeneration in response to ethanol exposure abates after 14 days (Saito et al., 2007). In a comparative study between 7- and 19-day old mice, exposure to ethanol in 7-day old mice showed apoptotic neurodegeneration as well as changes in lipid composition. Ethanol was shown to reduce Thr 172 phospohorylation of AMPK (decreasing its activity), along with acetyl-CoA carboxylase which is a substrate of AMPK and a lipogenic enzyme. The levels of triglyceride, cholesterol ester, ceramide, and N-acylphophatidylethanolamine were significantly increased as well. In comparison, the 19-day old mice displayed little neurodegeneration and there were smaller changes in AMPK phosphorylation. Even without ethanol, the basal activity level of AMPK is decreased in 19-day old mice compared to 7-day old mice. It was concluded that ethanol neurodegeneration is more significant and severe in specific developmental periods and is dependent upon lipid composition and AMPK activity (Saito et al., 2007).

1.7.1 AMPK Reduction of Neurotoxicity

AMPK has been shown to be beneficial, providing neuroprotection, in experiments involving ischemic, diabetic, amyotrophic lateral sclerosis (ALS), Alzheimer's Disease, some Parkinson models, and brain injury models. However, in the Parkinson's (MPTP) model, metformin has given conflicting results with some studies showing an exacerbation

dopaminergic decline and others showing a neuroprotective effect (Saito, Saito, & Das, 2019). In AD models, AMPK was shown to decrease amyloid-β plaque, reduce inflammation, and prevent neuronal loss. AMPK is believed to have neuroprotective effects by reducing inflammation, restoring energy balances, enhancing autophagy, and by activating antioxidants. The effects of increasing AMPK are more robust when the initial insult caused a decrease in AMPK, such as in ethanol abuse and traumatic brain injury (Saito, Saito, & Das, 2019). Insults that result in increased AMPK levels, such as ischemia, have the potential for an added activator of AMPK to become neurotoxic (Hill et al., 2016).

Both TBI and ethanol have disturbed metabolism after insult in regions such as the cortex and hippocampus, particularly in the CA1 region of the hippocampus (Rehman et al., 2019). This can contribute to impairment in cognitive abilities in both situations. Hill et. al. showed that TBI decreases CaMKKβ levels, reducing p-AMPK, which can be reduced from 14 days to 1 month after insult. The reduction can be reversed by post-injury administration of AICAR or metformin. In behavioral tests spatial localization was enhanced, suggesting improved long-term memory (Hill et al., 2016). Melatonin was also shown to reduce amyloid-β in the cortex and hippocampus of repetitive mild TBI (Rehman et al., 2019). Other studies have reported that AMPK reduces amyloidogenic pathway in the mouse brain (Ou et al., 2018).

AMPK and GSK-3β are negative regulators of each other (Joo et al., 2016). GSK3 has been shown to inhibit AMPK through phosphorylation. Akt has dual action of inhibiting GSK3 and promoting AMPK activation (Beurel Grieco, & Jope, 2015). In a study evaluating the impact of obesity as a risk factor for Alzheimer's Disease, it was found

that AICAR can reduce apoptosis and inhibit ER stress which reduces tau hyperphosphorylation by GSK-3β (Kim, 2011). Hyperphosphorylation is a biomarker for neurodegenerative diseases such as Alzheimer's, Chronic Traumatic Encephalopathy, and has been reported to be causal in the neurodegeneration of the developing brain when exposed to alcohol (Saito et al., 2010). It disrupts microtubule stability, leading to aggregation and tangles. Lithium has been shown to block ethanol-induced caspase-3 activation and tau phosphorylation through inhibiting GSK-3β. Diets that contain either a greater degree of saturated fatty acids or alcohol both increase the levels of free fatty acids, lipid peroxidation, inflammation, and tau hyperphosphorylation. Insults such as ischemia and trauma follow a similar pathway. Interestingly, the protective effects of leptin on tau hyperphosphorylation is mediated by AMPK. AICAR has even been shown to assist in the dephosphorylation of JNK and tau (Kim, 2011).

1.8 Pharmacotherapy for FASDs

Current therapeutic interventions are only partially efficacious for treating FASDs (Welch et al., 2016). Many focus on behavioral therapies and educational and learning strategies. There is a high rate of comorbid mental health disorders, with attention deficit/hyperactivity disorder (ADHD) being the most common. Though treatment with stimulants often cause more intolerable adverse effects in FASDs individuals. Much of the pharmacotherapy treatment is focused on controlling comorbid mental health disorders rather than targeting FASDs, and currently this area has been studied limitedly. Practitioners often rely on "clinical impressions" for deciding the best treatment options for individuals with FASDs. Other comorbid mental health disorders include depression, bipolar, anxiety, epilepsy, attachment disorders, attention and impulse control problems,

and many other psychiatric conditions. Some evidence with antipsychotics has shown efficacy in FASDs populations (Brown, Connor, & Adler, 2012).

1.8.1 AICAR as a Therapeutic Target

5-aminoimidazole-4caboxamide-1-b-D-ribofuranoside (AICAR) is widely used to increase AMPK activity as its phosphorylated state, ZMP, mimics AMP (Salt & Palmer, 2012). AICAR has been shown to cross the BBB and cause AMPK phosphorylation. Both AICAR and metformin have off-target effects, but AICAR analogs are currently being developed to minimize side-effects. Unlike metformin, AICAR does not inhibit complex 1 of the mitochondrial respiratory chain (Hill et al., 2016).

AICAR, has been shown to exert anti-inflammatory properties, inhibiting activation of NF-kB, TNF-a, IL1B, IL-6, and iNOS in microglial cells (Saito, Saito, & Das, 2019). It has been proven to attenuate disease progression in rodent models of autoimmune encephalomyelitis, lung injury, and colitis (Salt & Palmer, 2012). In studies focused on AMPK's downregulation in alcohol abuse, it has been shown that AICAR provides protection from alcohol induced fatty liver,and activated AMPK by AICAR is neuroprotective when given before glutamate excitotoxicity and glucose deprivation in cultured hippocampal neurons. However, other studies have shown that overactivation by AMPK can also be detrimental and even pro-apoptotic in hippocampal cells in certain conditions (Wilson & Matschinsky, 2020). Lipid peroxidation (4-HNE) was shown to be decreased by AICAR, which is only partly explained by decreasing adipose tissue. Insulin sensitivity has also been shown to be increased by AICAR in muscle and liver cells in ethanol models (Tomita et al., 2005).

The purpose of this study is to test if the addition of AICAR to an organotypic hippocampal model of binge drinking could compensate for the disturbance created by lipid peroxidation and inflammation through normalizing levels and activity of AMPK, which would theoretically mitigate the degree of neurotoxicity in a Fetal Alcohol Syndrome Model.

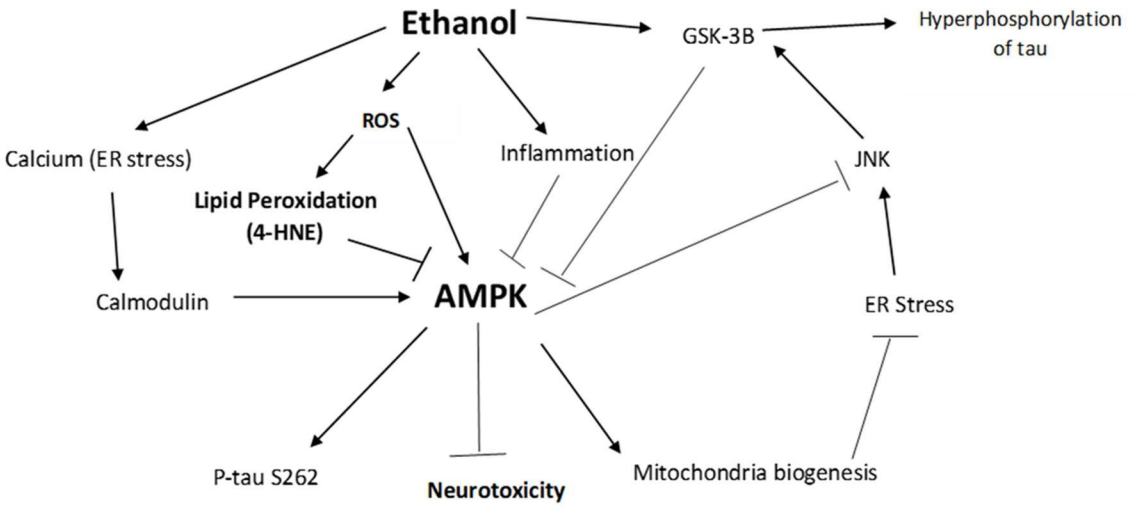

Figure 1.1 Biochemical Pathway Diagram: (Arrows represent upregulation and flipped "T" represents downregulation). This details some of the physiological signaling in response to ethanol consumption. The second pathway to the left in bold is the focus of the thesis and shows how ethanol leads to ROS and lipid peroxidation. This decreases AMPK and leads to a greater degree of neurotoxicity. A point to note, even though ROS causes an increase in AMPK, the decrease from lipid peroxidation is far greater. An increase in AMPK will cause an upregulation in mitochondrial biogenesis, and a downregulation in GSK-3B, JNK (a marker of ER stress), and ultimately prevent hyperphosphorylation of tau.

CHAPTER 2. METHODS

2.1 Hippocampal Slice Culture

Hippocampi were extracted from humanely euthanized eight to ten-day old male and female Sprague Dawley pups (Harlan Laboratories; Indianapolis, IN). Removal of the brains was completed using an aseptic technique. Once the brains were removed, they were immediately placed into cold dissecting media containing Minimum Essential Media (MEM; Invitrogen, Carlsbad, CA), (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), (HEPES; Sigma, St. Louis, MO), streptomycin/penicillin (Invitrogen), and Amphotericin B solution (Sigma). A midsagittal cut was made to the brains to extract the hippocampi. Excess tissue was then cleared away using a scalpel. The hippocampi were chopped in the direction rostral to caudal into 200um think slices using a McIllwain Tissue Chopper (Mickle Laboratory Engineering Co. Ltd., Gomshall, UK). Slices were selected given they met the criteria that all three major regions of the hippocampus were intact (CA1, CA3, and DG). Slices were then placed bipore membrane inserts with four slices per membrane, and care was taken to ensure there was no overlapping between the slices. Excess media was removed to allow for adhesion to the membrane. The membranes were inserted into six well plates that contained approximately 1.5 milliliter of culture medium between the plate and the insert. The six-well plates were incubated for six days prior to treatment to ensure complete adhesion to the membrane surface. This also allowed for damage that occurred during the dissection process to resolve. After treatment(s) the slices were imaged for neurotoxicity using Propidium iodide (PI) stain.

2.2 Treatment

After allowing for the six days of adherence to the Teflon membranes, the slice cultures were randomly assigned to the four treatment groups: Control, Ethanol, AICAR, and Ethanol +AICAR. The media was changed for all four groups at this timepoint. The Control and AICAR groups received control media and placed in a Tupperware container with 50mL of distilled water. This was sealed inside a Ziploc bag, with 5% CO_2 and 95% air. The gases were changed after two days to guarantee the ratio of carbon dioxide to air was consistent, and to account for microleakage within the bag. The Ethanol and Ethanol +AICAR groups were given ethanol media at a concentration of 50 mM, which is equivalent to a blood alcohol level of 0.23%. Similarly, they were placed into a Tupperware container with 50mL of 50mM ethanol media. This was sealed in a Ziplock bag with 5% CO_2 and 95% air, and the air was replaced after two days. After a five-day period of ethanol or control treatment the Control and Ethanol groups were given control media and the AICAR and Ethanol+AICAR groups were given 1mM of AICAR media. Both the control and AICAR media contained 7.48uM of PI. Imaging was completed after 24 hours of treatment. This was repeated for experiment 1 and experiment 2.

2.3 Staining of Cultures with Propidium Iodide

To measure the degree of neurotoxicity we used propidium iodide, which is a fluorescent, polar compound that can interpolate into DNA. As such, it is a biomarker for cell membrane integrity and subsequently cell death. Due to propidium iodide's polarity it can only enter a cell if the bilayer phospholipid membrane is compromised, which occurs during cytotoxic events. After entering, it intercalates between bases with little sequence preference. The binding emits fluorescence (National Center for Biotechnology

Information, 2020). Cultures were imaged using a Leica DMIRB microscope (W. Nuhsbahm Inc.; McHenry, Il, USA). Fluorescence was detected using a mercury lamp under a 5x objective lens. The image was captured through SPOT advanced software for Windows (version 4.0.2), which used a SPOT 7.2 color mosaic camera (W. Nuhsburg). Optical density was analyzed using image J (National Institutes of Health, Bethesda, MD). The CA1, CA3, and DG were all analyzed. For all images, background optical density was subtracted from the area of interest, and the optical density was normalized to a percent of the control to account for variability. The following formula was used where I represents the optical density or intensity of a region, B is the background optical density, and C is the average optical density for the control slices:

$$percent\ of\ control = \frac{I - B}{C}$$

2.4 Statistical Analysis

Statistical analysis of the data provided a quantitative assessment of the effects the four different treatment groups (Control, Ethanol, AICAR, and Ethanol+AICAR) had on the degree of neurotoxicity. A Two Factor ANOVA was performed in SPSS (version 24) comparing treatment and sex with a significance level of $p \leq 0.05$. A Fisher's Least Significant Difference (LSD) post hoc test was used to determine the differences between treatment groups.

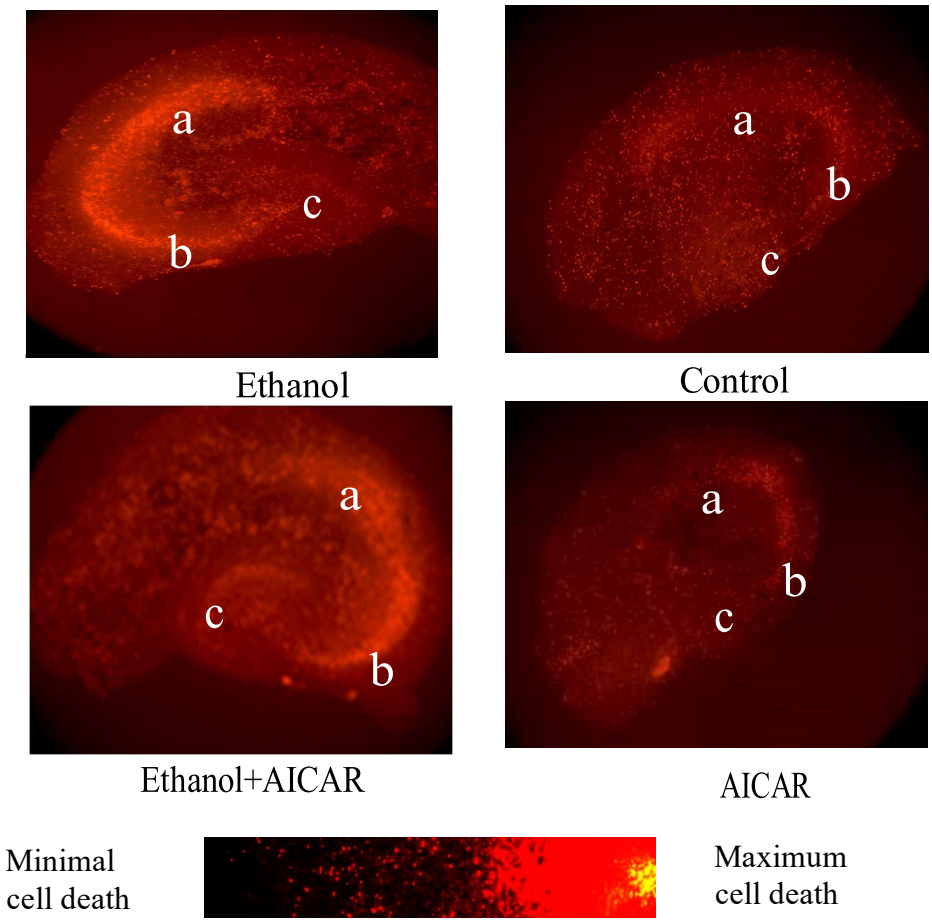

Figure 2.1:Representative Images: All images were stained with propidium iodide and imaged after 24 hours. Clockwise starting at the top left and going around are images representative of the groups: Ethanol, Control, AICAR, and Ethanol+AICAR. All images were labeled with an a, b, and c. Where a represents Cornu Ammonis 1 (CA1), b Cornu Ammonis 3 (CA3), and c Dentate Gyrus (DG). A key is included underneath to show minimal to maximal cell death.

CHAPTER 3. RESULTS

3.1 Experiment 1: Ethanol and AICAR Treatment CA1

There was a significant effect of treatment in the CA1 region at 24 hours after treatment, $F(3, 176)=24.22$, $p<0.001$(Figure 3.1.1). However, no sex effect was observed for the CA1, $p=0.071$. Ethanol ($M=120.02$, $SE= 5.30$) was shown to increase cell death ($p=0.002$) by 20.02% of the control ($M=100.00$, $SE= 4.76$). Both the combined treatment of Ethanol+AICAR ($M=77.68$, $SE=4.044$) and AICAR ($M=71.21$, $SE=3.65$) decreased cell death in comparison to the control by 22.32%($p<0.001$) and 28.79%($p<0.001$) respectively. Both the combined treatment, Ethanol+AICAR and AICAR, decreased cell death in comparison to ethanol($p<0.001$). The effect size was 42.33% for the combined treatment and 48.81% for AICAR treatment.

3.2 Experiment 1: Ethanol and AICAR Treatment CA3

There was a significant effect of treatment in the CA3 at 24 hours after treatment, $F(3,177)= 27.14$, $p<0.001$ (Figure 3.1.2). However, no sex effect was observed for the CA3, $p=0.070$. Ethanol ($M=121.05$, $SE=4.71$) was shown to increase cell death ($p=0.001$) by 21.05% of the control ($M=100.00$, $SE=5.03$). Both the combined treatment of Ethanol+AICAR ($M=78.56$, $SE=4.65$) and AICAR ($M=69.30$, $SE=3.26$) decreased cell death by 21.44% ($p=0.001$) and 30.70%($p<0.001$) respectively. Both the combined treatment, Ethanol+AICAR and AICAR, decreased cell death in comparison to ethanol($p<0.001$). The effect size was 42.49% for the combined treatment and 51.74% for AICAR treatment.

3.3 Experiment 1: Ethanol and AICAR Treatment DG

There was a significant effect of treatment $F(3,177)=28.86$, ($p<0.001$), sex effect $F(1, 177)=13.95$, ($p<0.001$), and sex and treatment interaction in the dentate gyrus, $F(3,177)=3.87$, ($p=0.01$). Ethanol ($M=122.44$, $SE=4.04$) was shown to increase cell death by 22.44% ($p<0.001$) of the control ($M=100.00$, $SE=3.59$) for both males and females. Both the combined treatment of Ethanol+AICAR and AICAR decreased cell death from the control by 23.30% ($p<0.001$) and 23.67% ($p<0.001$) respectively. However, there was a sex difference in both groups. Females ($M=84.36$, $SE=4.90$) and males ($M=69.04$, $SE=4.46$) from the Ethanol +AICAR group and males ($M=61.03$, $SE=3.56$) from the AICAR group were individually found to be significant from the control. Nevertheless, females from the AICAR group ($M=97.74$, $SE=10.08$) failed to be significant from the control ($p= 0.83$). Both the combined treatment, Ethanol+AICAR and AICAR treatment decreased cell death in comparison to Ethanol for both males and females. The effect size was 45.74% ($p<0.001$) for the combined treatment and 46.11% ($p<0.001$) for AICAR treatment. The sex treatment interaction occurred as AICAR was found to be more protective for males in comparison to females for both the Ethanol+AICAR and AICAR treatment groups, but there was no difference in sex in the Control and Ethanol groups.

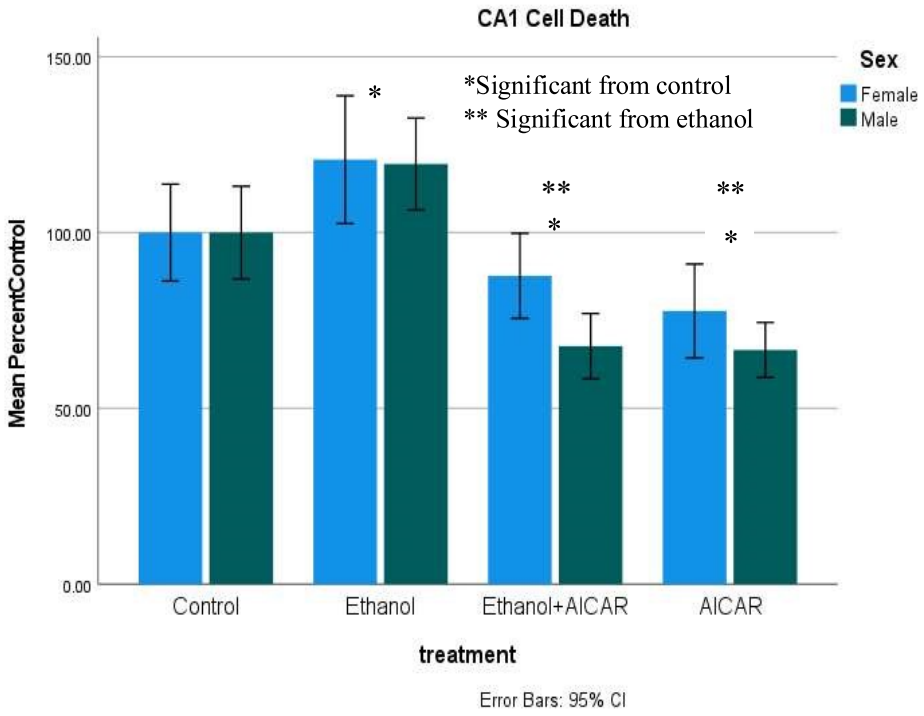

Figure 3.1: The effects of propidium iodide (PI) uptake in the CA1 region of the hippocampus 24 hours after treatment. Ethanol was shown to increase toxicity by 20.02% compared to the control ($p=0.002$). The combination treatment of Ethanol+AICAR was significant from the control ($p<0.001$) and decreased the toxicity by 22.32%. The AICAR treatment group decreased toxicity by 28.79% ($p<0.001$) from the controls. The Ethanol+AICAR group decreased the toxicity compared to ethanol by 42.33% ($p<0.001$), and AICAR had a decreased toxicity compared to ethanol by 48.81% ($p<0.001$).

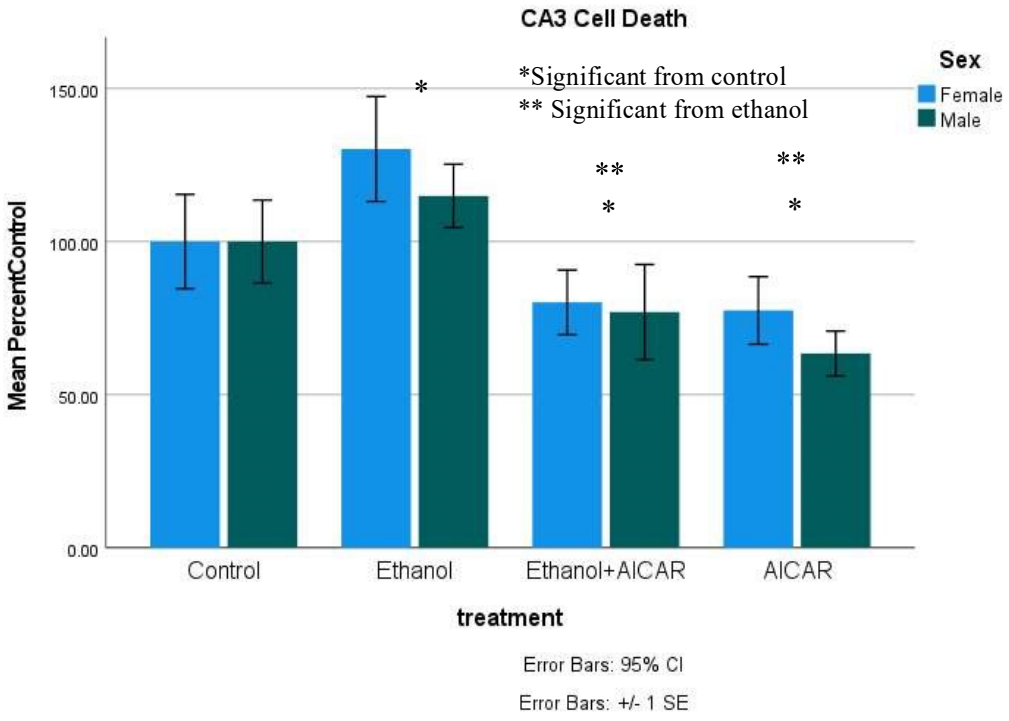

Figure 3.2: The effects of propidium iodide (PI) uptake in the CA3 region of the hippocampus 24 hours after treatment. Ethanol was shown to increase toxicity by 21.05% compared to the control ($p=0.001$). The combination treatment of Ethanol+AICAR was significant from the control ($p=0.001$) and decreased the toxicity by 21.44%. The AICAR treatment group decreased toxicity by 30.70% ($p<0.001$) from the controls. The Ethanol+AICAR group decreased the toxicity compared to ethanol by 42.49% ($p<0.001$), and AICAR had a decreased toxicity compared to ethanol by 51.74% ($p<0.001$).

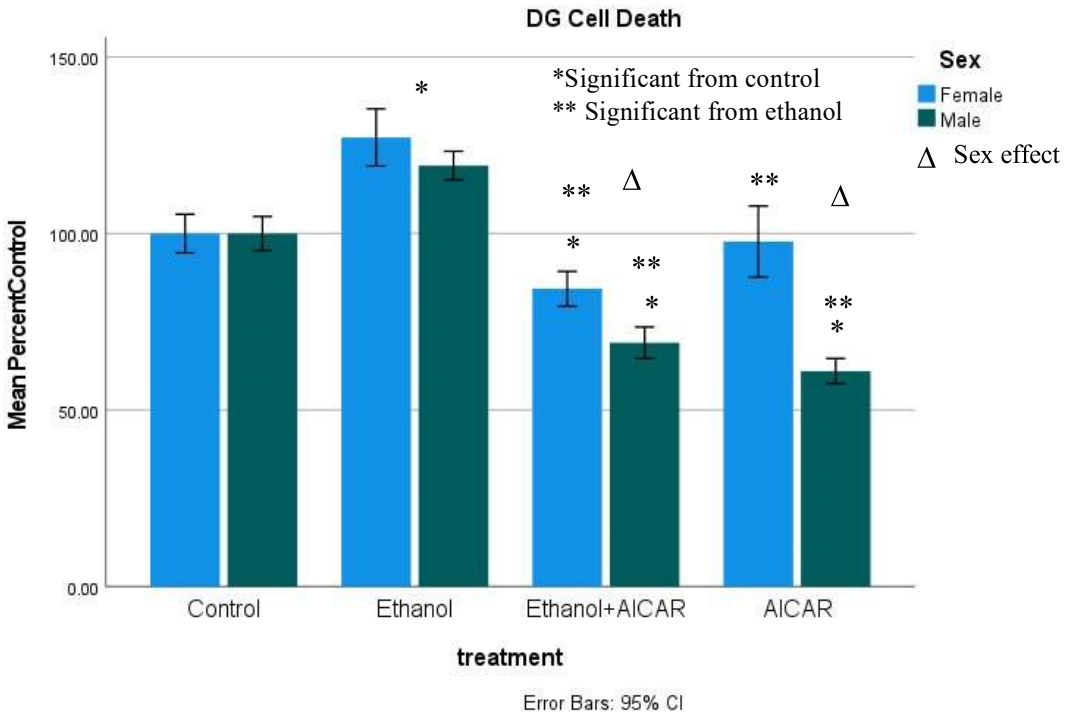

Figure 3.3: The effects of propidium iodide (PI) uptake in the DG region of the hippocampus 24 hours after treatment. Ethanol was shown to increase toxicity by 22.44% compared to the control ($p<0.001$). The combination treatment of Ethanol+AICAR was significant from the control ($p<0.001$) and decreased the toxicity by 23.30%. The AICAR treatment group decreased toxicity by 23.67% ($p<0.001$) from the controls. However, females from the AICAR group failed to be significant from the control ($p= 0.83$). The Ethanol+AICAR group decreased the toxicity compared to ethanol by 45.74% ($p<0.001$), and AICAR had a decreased toxicity compared to ethanol by 46.11% ($p<0.001$). The was a sex effect and a sex treatment interaction.

CHAPTER 4. DISCUSSION

This experiment shows proof of concept that AICAR could have therapeutic potential for treating individuals with Fetal Alcohol Spectrum Disorder. It was shown to be protective in all three regions of the hippocampus (CA1, CA3, and DG) against ethanol induced neurotoxicity in an organotypic hippocampal model of Fetal Alcohol Spectrum Disorders. AICAR was also consistently protective in comparison to the control, excluding the one exception of females in the DG. This protection is most likely attributable to the fact that the hippocampal dissecting process induces trauma that is in itself neurotoxic. By many of the same mechanism in which AICAR is protective against ethanol induced neurotoxicity (reducing inflammation, regulating cellular metabolism, etc.), AICAR may in the same way be protective against trauma from the dissections (Saito, Saito, & Das, 2019).

This experiment also confirmed what is apparent in the scientific literature and in clinical populations, that ethanol is indeed neurotoxic to the developing brain (Wilson & Matschinsky, 2020) (Saito et al., 2007) (Denny et al., 2019). For all regions, ethanol consistently increased the degree of cell death by around 20%. The cell death induced by ethanol is most likely caused by a variety of factors that include ROS, lipid peroxidation products, altered cellular metabolism, and inflammation. It can be observed from this data, that the addition of AICAR, which mechanistically increases AMPK, contributed to neuroprotection as it prevented a significant degree of cell death in comparison to the Ethanol treatment group.

Though there was not a sex effect observed for the CA1 and CA3 regions of the hippocampus, there was a sex effect observed in the DG. Specifically, this was a

sextreatment interaction where males were more responsive to treatment with AICAR in comparison to the females. When age was accounted for this was also only seen in the eight-day old pups. The difference in these findings regionally, are most likely attributable to the distinctions in cells types between the CA1/CA3 regions and the DG region. The CA1 and CA3 are mainly composed of pyramidal cells, and granule cells are mainly found in the DG (Alkadhi, 2019). The sex differences are most likely due to the physiology of endocrine signaling of estradiol in the third trimester. The aromatization hypothesis states that testicularly derived testosterone diffuses into the male brain where it is locally aromatized to estradiol (McCarthy, 2008). Estradiol is then responsible for the masculinization of the brain, with males having levels that are several folds higher than female at this stage (Hilton, Nueza, & McCarthya, 2003). Females are protected from masculinization effects of maternal estrogens by alpha-fetoprotein within neurons. Alpha-fetoprotein is a binding globulin found in late-gestation fetuses and early postnatal pups that has a high affinity for estradiol. The effects of alpha-fetoprotein occur in the prenatal and early postnatal periods only, creating a sensitive period. This could explain the sex differences being present in the eight-day old pups, and absent in the ten-day old pups when age was accounted for (McCarthy, 2008).

Interestingly, there was a study where kainic acid was given to newborn rat pups of both sexes and then the brains were analyzed at postnatal day 7. In both males and females, the CA1, CA2, and CA3 regions had minimal cell death; however, the DG region the females had up to 40% cell loss. Pretreating females with estradiol was shown to prevent this cell death and normalize the levels to that of the male counterparts. Estradiol may have been protective against excitotoxicity in this case due to its

modulating effects on calcium handling or its effect on increasing brain-derived neurotrophic factor (BDNF), though the mechanism was not fully explored. This study showed that the DG region in P7 female rats was prone to an increased neurotoxic response given a stressor (Hilton, Nueza, & McCarthya, 2003). Though no increased neurotoxic effects between males and females in the DG were found in this experiment due to ethanol, females in the DG were found to respond less to treatment with AICAR. Though it should be noted that though there was a sex difference in the DG and that female P8 rats treated with AICAR were not significant from the control, both males and females in the Ethanol+AICAR and AICAR groups were still significant compared to the Ethanol group in the DG. This may suggest that there is an interaction between estradiol and AICAR in the DG for P8 male rat pups, or that for some reason P8 females were unable to respond as effectively to AICAR treatment in the DG. This experiment along with Hilton, Nueza, and McCarthya's findings suggest that males during this time may be more protected against negative stressors, and more responsive to neuroprotective treatment due to the effects of estradiol. Though the mechanism and region selectivity of this finding needs further research for elucidation and confirmation.

Future directions beyond looking into the sex treatment interaction in the DG, will include evaluating the biochemical pathway in which AICAR is protective against ethanol induced neurotoxicity. Targets of these studies will include evaluating the ratio of p-AMPK/AMPK in the four treatment groups: Control, Ethanol, Ethanol+AICAR, and AICAR. Hypothetically, ethanol would decrease the ratio, AICAR would increase the ratio, and Ethanol+AICAR would increase the ratio in comparison to Ethanol based on the mechanism of AICAR and the results of this study. Lipid peroxidation products

should also be evaluated for the treatment groups to observe if lipid peroxidation, induced by ethanol, does indeed cause a decrease in the ratio of p-AMPK/AMPK. Lastly, AICAR should be evaluated to see if increases in the p-AMPK/AMPK ratio could reduce inflammatory markers, possibly being a mechanism in which treatment with AICAR limits the degree of ethanol-induced neurotoxicity. Currently, immunohistochemistry with NeuN is being performed to confirm PI imaging. NeuN, which stains post-mitotic neurons by marking the FOX-3 protein, is a fluorescent marker of living neurons (Duan et al., 2016). From this analysis one will see if cell death from the PI concurs inversely with the amount of neuronal conservation. Alternate explanations if the PI and NeuN do not align, include that glial cell death contributed substantially to the total cell death.

In conclusion, there are many individuals with FASDs that suffer from intellectual disabilities throughout the duration of their lives. The estimated cost of FASDs is $2 million per individual (Luo, 2014). This population is expected to increase in the coming years, as binge drinking among pregnant women is rising (Denny et al., 2019). Currently, there is no treatment or cure specifically for FASDs, and treatment is based on managing symptoms (Welch et al., 2016). This study seeks to provide proof of concept and propose a potential mechanism in which ethanol induces neurotoxicity. To this point, based on the literature and these initial findings, ethanol may induce neurotoxicity by downregulating AMPK, through lipid peroxidation (Shearn et al., 2014). Upregulating AMPK may prevent neurotoxicity by reducing the amount of inflammation (Saito et al., 2010). Though to this effect, this study only supports that AICAR, which's mechanism of action is increasing AMPK, is protective against ethanol induced neurotoxicity in an organotypic hippocampal model of Fetal Alcohol Spectrum Disorders. In this model, AICAR was

shown to be protective when given after ethanol intoxication, which is translational to a real-world scenario in which a therapeutic is most likely given after intoxication. However, further studies should evaluate if the protective effects of AICAR still remain if there is a greater time between alcohol intoxication and treatment intervention, as this would model Fetal Alcohol Spectrum Disorders and possible treatment intervention to a greater degree. Animal studies should also be performed to evaluate if AICAR or a similar pharmacologic intervention could be a possible therapeutic target. Nevertheless, these results are promising in providing proof of concept that AICAR or a similar therapeutic has the potential to become a medicinal target in the treatment of Fetal Alcohol Spectrum Disorders.

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