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PRECLINICAL DEVELOPMENT OF PHYTOCANNABINOID- AND ENDOCANNABINOID- BASED PHARMACOTHERAPIES FOR THE TREATMENT OF ETHANOL-INDUCED NEURODEGENERATION

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DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Pharmacy at the
University of Kentucky

By
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2013

ABSTRACT OF DISSERTATION

PRECLINICAL DEVELOPMENT OF PHYTOCANNABINOID- AND ENDOCANNABINOID- BASED PHARMACOTHERAPIES FOR THE TREATMENT OF ETHANOL-INDUCED NEURODEGENERATION

Excessive ethanol consumption, characteristic of alcohol use disorders (AUDs), is associated with widespread neurodegeneration and cognitive and behavioral impairments that may contribute to the chronic and relapsing nature of alcoholism. Therefore, identifying novel targets that can afford neuroprotection will undoubtedly aid current treatment strategies for AUDs. The cannabinoids have been shown to provide neuroprotection in a variety of preclinical models of neurodegeneration; however minimal data is available regarding the use of cannabinoid-based pharmacotherapies for treating ethanol-induced neurodegeneration. Therefore, the current dissertation examined the overarching hypothesis: the cannabinoids are a therapeutic strategy to afford neuroprotection in the context of ethanol-induced neurodegeneration. Importantly, this overarching hypothesis was approached with translational considerations in mind. Specifically, the use of many cannabinoids in the clinic is hindered due to multiple unfavorable pharmacokinetic/pharmacodynamic profiles, including high first pass metabolism and untoward psychoactivity. Therefore, the studies herein were designed to circumvent these PK/PD obstacles. The first set of studies examined whether transdermal delivery of the phytocannabinoid, cannabidiol (CBD), could attenuate binge ethanol induced neurodegeneration. Transdermal CBD afforded neuroprotection in the entorhinal cortex and neuroprotection was similar in magnitude as intraperitoneal administration. The second set of studies found that binge ethanol treatment transiently down-regulated the main CNS cannabinoid receptor, CB1R. Interestingly, these changes were not accompanied by alterations in one of the major endogenous ligands, anandamide (AEA), or other related n-acylethanolamides (NAEs). The latter finding is in contrast to other literature reports demonstrating that endocannabinoid content is substantially elevated in response to a CNS insult. Nevertheless, studies were carried out to determine if administration of the AEA and NAE catabolism inhibitor, URB597, could attenuate binge ethanol induced neurodegeneration. URB597 failed to produce neuroprotection in the entorhinal cortex and dentate gyrus of the hippocampus. However, additional studies found that URB597 failed to elevate AEA in the entorhinal cortex, and in general the biological activity of URB597 was impaired by ethanol exposure. Therefore, with further drug discovery/development efforts, it may be feasible to optimize such treatment strategies. In conclusion, the studies within the current

dissertation demonstrated the feasibility of using some cannabinoid-based agents to prevent ethanol-induced neurodegeneration.

KEYWORDS: Alcoholism, endocannabinoids, cannabidiol, ethanol-induced neurodegeneration, binge ethanol exposure

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Student's Signature

07-19-2013

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1. CHAPTER 1

INTRODUCTION

1.1 General introduction

An alcohol is an organic compound with a common hydroxyl functional group bonded to a saturated carbon atom. Among this large class of compounds, one in particular has a long history and significant role in human society. Ethyl alcohol (ethanol), better known as alcohol has been used in nearly every aspect of society including nutrition, medicine, religion and celebration for thousands of years with the earliest evidence of alcohol fermentation dating back to 7000 B.C.E in Neolithic China [1]. Although alcohol ingestion can have marginal beneficial effects on health [2], excessive use can lead to harmful consequences on the individual and to society. Recent reports from the World Health Organization estimate that 4% of all deaths worldwide are attributed to alcohol use, making alcohol consumption the 8th leading risk factor for death [3]. Additionally, alcohol consumption is 3rd among risk factors leading to life years lost globally, only behind childhood malnutrition and unsafe sex. Alcohol consumption is a causal factor in over 60 other diseases and injuries. For example, alcohol consumption is the leading cause of liver cirrhosis, and a major cause of liver cancer, hypertensive heart disease and epilepsy, making alcohol consumption accountable for 4.5% of the global burden of disease and injury [3]. Importantly, in addition to injurious effects to peripheral organ systems, chronic alcohol consumption has deleterious effects on the CNS and 50 - 70% of alcohol-dependent adults show permanent cognitive deficits [4].

Excessive alcohol consumption presents major monetary burdens to the U.S. as the annual economic and direct health costs are 223.5 billion and 24.6 billion, respectively [5]. In the U.S., alcohol use is among the highest globally [6]. For example,

studies estimate that of persons over the age of 12 in the United States, approximately 51% have reported alcohol use within the past month. Maladaptive alcohol consumption in the U.S. is a major concern as it is estimated that at any given time, 8.5% of Americans will meet the Diagnostic and Statistical Manual of Mental Disorders - IV (DSM-IV) criteria for an alcohol use disorder (AUD) [7, 8]. An AUD, commonly referred to as alcoholism, is defined by the DSM-IV as a maladaptive pattern of alcohol consumption that produces clinically relevant distress or impairment and includes inclusion criteria for alcohol abuse and alcohol dependence [9]. AUDs can be characterized by persistent and escalating alcohol consumption, a preoccupation with alcohol use that can develop into uncontrolled alcohol use, tolerance to alcohol's effects and physical dependence [9].

Importantly, many of the individuals that meet the criteria of an AUD can also be classified as binge drinkers and binge drinking is on the rise in the U.S. [10]. Additionally, 76.4% of the total economic burden of excessive drinking in the US can be attributed to binge drinking [5]. Binge drinking is officially classified as 4 standard drinks for women and 5 standard drinks for men within 2 hours, which typically results in blood ethanol concentrations (BECs) around 0.08 mg%. However, alcohol dependent binge drinkers commonly achieve much higher BECs as clinical studies have found that alcoholics admitted to the emergency room are often conscience and alert at BECs exceeding 0.3 mg% [11, 12]. Binge drinking and high BECs associated with this pattern of consumption are particularly detrimental. For example, binge drinking increases the risk for alcohol dependence, co-morbid psychiatric disorders and neuropathology [13-16].

1.2 Ethanol neuropharmacology

Ethanol is a small (molecular weight = 46.07 g/mol) amphipathic chemical without stereoselectivity. Therefore, ethanol's interactions with biological substrates, including

lipids and proteins, are considerably less selective than other drugs of abuse. For example, ethanol has strong interactions with both the hydrocarbon chains and the polar head groups of plasma membrane phospholipids; and can readily interact with proteins by displacing water molecules from hydrophilic pockets [17]. Through these “allosteric” interactions, ethanol can profoundly alter the structure of many proteins, particularly transmembrane receptors, which results in significant functional changes [17]. Importantly, although ethanol is less selective then many other drugs, ethanol does favor interactions with some proteins more than others.

At low millimolar concentrations, which produce intoxication, ethanol interacts with a wide variety of neurotransmitter systems. For example, ethanol directly influences opioid, dopamine, acetylcholine, serotonin, glycine and endocannabinoid (eCB, see section 1.8) neurotransmission, and these interactions are responsible for many ethanol-mediated effects, including intoxication, reinforcement and withdrawal. However, in the context of the current dissertation, the following discussion will focus on ethanol’s effects on glutamate and γ-aminobutyric acid (GABA) neurotransmission, as ethanol-induced neuroadaptations in these neurotransmitter systems are hypothesized to contribute to the neurodegenerative effects of ethanol [18, 19].

L-glutamate is the primary excitatory neurotransmitter in the CNS and is a ligand for both metabotropic and ionotropic receptors. Of these receptors, ethanol has the most profound effects on the N-methyl-D-aspartate (NMDA) receptor subtype. NMDA receptors are heterotetrameric, ionotropic receptors that are permeable to cations, particularly Ca^{2+}, and are critical for many physiological processes, including synaptic transmission and synaptic plasticity. At low millimolar concentrations, ethanol inhibits NMDA-mediated neurotransmission [20, 21], an effect that is greater at NMDA receptors with NR1/NR2A or NR1/NR2B compositions compared the NR1/NR2C or NR1/NR2D subunit compositions [22]. Conversely, chronic exposure has the capability to upregulate

glutamatergic neurotransmission to maintain homeostasis, which contributes to ethanol tolerance. For example, chronic ethanol exposure commonly results in elevated extracellular glutamate concentrations [23, 24], increased surface expression of NMDA receptors [25] and a shifted expression toward the NR1/NR2B subunit composition [26]. Importantly, NMDA receptor up regulation is hypothesized to be a critical mechanism responsible for ethanol withdrawal induced hyperexcitability, resulting in seizure activity and excitotoxic neuronal injury [26-28].

In contrast to the effects of ethanol on NMDA-mediated neurotransmission, ethanol potentiates GABAergic neurotransmission. GABA is the principal inhibitory neurotransmitter in the CNS and consists of both metabotropic and ionotropic receptors. The $GABA_A$ receptor is a Cl^- permeable ion channel that is responsible for hyperpolarizing the postsynaptic membrane and thus increasing the threshold for firing an action potential. Through complex allosteric interactions, ethanol enhances the function of the $GABA_A$ receptor [29, 30], which results in homeostatic changes in the function of GABAergic neurotransmission following chronic ethanol exposure. For example, chronic ethanol exposure decreases mRNA expression of the α1 $GABA_A$ subunit [31] and increases internalization of α1 containing $GABA_A$ receptors [32], effects that are associated with decreased $GABA_A$ function and cell hyperexcitability [33]. Importantly, in conjunction with ethanol-induced neuroadaptations in glutamatergic neurotransmission, reduced GABAergic neurotransmission following chronic ethanol exposure could contribute to ethanol withdrawal induced hyperexcitability and excitotoxic neuronal injury [34-36].

1.3 Pharmacotherapies for alcoholism

To date, the Food and Drug Administration (FDA) has approved four pharmacotherapies for the treatment of AUDs: disulfuram, acamprosate, oral naltrexone

and long-acting injectable naltrexone. Disulfuram (Antabuse®) has been marketed since the 1940s and was the first drug approved for alcohol dependence. Alcohol metabolism primarily occurs though a two-step process where it its first converted to acetaldehyde by alcohol dehydrogenase and then further metabolized to acetate by acetaldehyde dehydrogenase. Disulfuram inhibits acetaldehyde dehydrogenase, which produces aversive physiological effects following alcohol ingestion as a consequence of acetaldehyde accumulation. Reactions to alcohol consumption while being treated with disulfuram include headaches, nausea, vomiting, chest pain and death in severe cases [37, 38]. Therefore, while on this drug, individuals are likely to abstain from drinking. However, the efficacy of disulfuram for the treatment of alcohol dependence is undermined by high rates of noncompliance [38, 39] and its clinical use is declining [40].

Naltrexone (ReVia®), the first central nervous system (CNS) acting drug for alcohol dependence, was approved by the FDA in 1994. Naltrexone is a competitive opioid antagonist with affinity towards the μ- κ- and δ- opioid receptor subtypes and blocks ethanol-induced stimulation of dopamine release in the nucleus accumbens, a major brain region responsible for positive reinforcement [41]. Therefore, it is widely hypothesized that naltrexone prevents heavy drinking by blocking some of the the positive reinforcing properties of ethanol [42, 43]. The efficacy of naltrexone has been examined in 20 clinical trials worldwide to date and these trials have found that naltrexone reduces the likelihood of relapse to heavy drinking and may increase the rate of complete abstinence [38]. However the effect sizes of these outcomes are modest and some reports show a lack of naltrexone efficacy compared to placebo [38, 44]. Noteworthy, genetic variance can predict efficacy of naltrexone treatment. Individuals with a μ-opioid receptor 118G allele rather than 118A, tend to have greater subjective feelings of reward following alcohol consumption [45] and tend to respond better to naltrexone intervention [46-48]. Collectively, these data suggest that naltrexone may be

more beneficial for alcoholics who drink for the positive reinforcing effects of alcohol [49], however this hypothesis remains to be substantiated by published findings. The efficacy of naltrexone is hindered due to patient noncompliance [50], therefore an injectable long-acting form of naltrexone (Vivitrol®) was developed and approved by the FDA in 2006.

Acamprosate (Campral®), was approved by the FDA in 2004 for detoxified and abstinent patients for the maintenance of abstinence. The mechanism of action is not entirely understood, however evidence suggests that acamprosate has interactions with the glutamate system, with potential binding sites at the polyamine site of the NMDA receptor [51, 52] and the mGluR5 receptor [53]. Therefore, it's suggested that acamprosate normalizes hyperglutamatergic activity associated with alcohol withdrawal and abstinence [54]. Multiple clinical trials have been conducted with acamprosate, which, in general, show some efficacy compared to placebo in maintaining abstinence [55]. However, two U.S. placebo-controlled studies failed to show a benefit of acamprosate on percent days abstinent and time to first heavy drinking day [56, 57]. Nevertheless, acamprosate is currently the most prescribed medication for alcohol dependence in the U.S. and is responsible for the dramatic increase use of medications for the treatment of alcohol dependence [40]. Importantly, acamprosate may be more effective in patents who are motivated to abstain from alcohol use [57].

Although these drugs are efficacious for some individuals, in general their effect sizes are small compared to placebo [38]. Additionally, the rate of prescribed medications for patients with an AUD is only 10 -13% [40, 58]. Although multiple factors contribute to the low rate of pharmacological treatment for AUD's, it is certain that low efficacy of current drugs and lack of treatment options plays a critical role [59]. A further understanding of the neurobiology of AUDs is necessary for the development of new and more efficacious pharmacotherapies [59]. Although the current drugs approved for the treatment of AUDs focus on preventing the reinforcing effects of ethanol, it is well known

that excessive ethanol consumption results in structural and functional impairments that influence the initiation and perpetuation of AUDs [60, 61]. Therefore, it is hypothesized that neuroprotective drugs will prevent or even reverse ethanol-induced neurotoxicity, restore cognitive function and aid in the recovery of AUDs. In support of this hypothesis, a recent study found that alcohol-induced deficits in cortical gray volume are a predictor of relapse rates [62].

1.4 Rationale for treating alcohol-induced neurodegeneration: a novel target for the treatment of AUDs

Excessive alcohol use results in numerous structural abnormalities in the CNS, which are theorized to be important pathological mechanisms underlying the neuropsychological and behavioral impairments observed in alcoholics [63]. Operating under this theory, it is hypothesized that alcohol-induced neurodegeneration may contribute to the development and maintenance of an AUD [60, 61]. For example, ethanol-induced damage to the frontal lobe and hippocampus may be associated with poor judgment, perseveration, impulsivity, attention deficits and social withdrawal; behaviors that are associated with AUDs [60, 63, 64]. Therefore, the treatment of AUDs would benefit from a pharmacological approach that could reduce alcohol-induced neurodegeneration and reverse these behavioral deficits that may underlie the chronic nature of alcoholism.

1.4.1 Human evidence of alcohol-induced neurodegeneration

Neuropathological studies, although limited in number, have found structural abnormalities in the alcoholic brain [65, 66]. Many of the neurodegenerative effects of alcohol, have been observed from individuals with comorbid Wernicke-Korsakoff syndrome, including neuronal loss in the thalamus, mammillary bodies, basal forebrain,

raphe and cerebellar vermis [66]. However, evidence also suggests that alcohol-induced neurodegeneration occurs in uncomplicated alcoholics. For example, postmortem studies have found signs of alcohol-induced neurodegeneration including reduced tissue weight [67, 68], increased cerebrospinal fluid filled space [68], white matter loss [67], deficits in dendritic arborization, cell shrinkage and neuronal cell loss [67, 69, 70]. These neurotoxic effects of alcohol appear to be brain region specific as large pyramidal neurons of the superior prefrontal cortex are lost, while neurons are spared in the motor cortex [67, 70]. Additionally, cortical white matter loss is most severe in the prefrontal cortex of the cerebrum [67]. A few reports have also quantified the effects of chronic alcohol use on hippocampal neuron populations in human postmortem brains. An initial report observed significant reductions in neurons in all hippocampal subfields quantified, including Cornu Ammonis (CA) 1 though CA4 and the dentate gyrus [71]. However, a more rigorous study failed to observe neuronal loss in any hippocampal subfield, but was able to attribute hippocampal volume deficits in alcoholic cases to white matter loss [72]. Importantly, these postmortem studies show that alcohol may cause both irreversible (i.e. neuron loss) and reversible structural changes (i.e. dendritic atrophy, cell shrinkage), both of which may be targeted by neuroprotective pharmacotherapies.

Although only a limited number of neuropathological reports examining the neurodegenerative effects of alcohol are available, *in vivo* brain morphometric studies have reported that the alcoholic brain suffers from diffuse reductions in brain volume evidenced by ventricular enlargement and widening of the cortical sulci [63]. Imaging studies have found reduced cortical gray matter and cortical white matter with the most prominent effects in the frontal lobe [73, 74], temporal lobe [75, 76] and the hippocampus [77-80]. Additionally, other subcortical structures including the corpus callosum [81, 82], pons [83], mammillary bodies [84, 85] thalamus [75, 86] and cerebellum [87] are smaller in the alcoholic brain.

1.4.2 Link between alcohol-induced neurodegeneration and neuropsychological deficits associated with AUDs

AUDs are characterized by a well-defined pattern of neuropsychological deficits including impairments in working memory and problem solving, poor judgment, response perseveration, impulsivity, lack of motivation, attention deficits and social withdrawal [63, 88, 89]. In fact, 50-75% of detoxified alcoholics show permanent neuropsychological dysfunction [4]. Although it is thought that the neurodegenerative effects of alcohol underlie the neuropsychological deficits observed in humans, only a few studies have observed direct correlations between discrete brain region volumes and neuropsychological performance dependent on the same region. For example, studies have observed reductions in frontal cortex volume and have found impairments in frontal cortex dependent neuropsychological functions, such as working memory, problem solving and impulsivity [88]; however, in only some instances have correlations between these measures been found [63]. Similarly, reductions in hippocampal volume and deficits in hippocampal-dependent neuropsychological functions, including declarative memory [90], have been observed, however other studies have shown that these measures do not always correlate [77, 91]. The discrepancies between structural and neuropsychological measures are seemingly paradoxical; however emerging data suggests that subtle structural deficits across multiple loci within connected circuitry may be responsible for neuropsychological deficits observed in alcoholism. For example, Sullivan and colleagues show evidence that structural deficits in frontocerebellar circuitry (i.e. pons, thalamus and cerebellum) rather than discrete deficits in the frontal cortex, may underline many executive dysfunctions observed in AUDs, including in working memory and behavioral control [63, 86].

The importance of circuitry dysfunction rather than impairments in discrete brain regions is also emerging between the hippocampus and prefrontal cortex. Evidence

shows that the hippocampus is critically involved in proper functioning of the prefrontal cortex (PFC) [92]. For example, hippocampal glutamatergic efferents terminating in the PFC, are implicated in proper processing of executive function, working memory, contextual information and emotional regulation [92]. Therefore, disruption in the structural integrity of the hippocampus may be an underling substrate for impairments in these functions typically associated with the PFC. Long-lasting deficits in executive function, working memory and emotional regulation are observed following excessive alcohol consumption [88, 93, 94] and it is hypothesized that compromised hippocampal integrity, including neuron loss, white matter atrophy and/or dendritic shrinkage in alcoholics may be important in the expression of these impairments. In support of this hypothesis, a recent study observed a correlation between deficits in executive function and hippocampal volume [75]. Taken together, alcohol-induced neurodegeneration may have a profound impact on multisystem cognition, which may lead to many of the hallmark neuropsychological deficits that are observed in alcoholism and that are theorized to contribute to the chronic relapsing nature of the disease [60, 61].

1.5 Animal models showing ethanol-induced neurodegeneration

The neurodegenerative effects of ethanol were initially described in rodent models of chronic ethanol consumption (CEC) in which mice or rats were fed alcohol in their drinking water from anywhere between 5 to 18 months. CEC was found to produce similar patterns of neurodegeneration as observed in humans, with neuronal loss in dentate gyrus, CA2/3 and CA1 of the hippocampus [95-97] and in layer III of the prelimbic area of the medial prefrontal cortex [98]. Additionally, withdrawal from CEC aggravates cell loss in the hippocampus [99-101], suggesting that both direct neurotoxic effects of ethanol as well as ethanol withdrawal contribute to neurodegeneration. Noteworthy, withdrawal from CEC is associated with dendritic regrowth and

synaptogenesis in the hippocampus and/or cortex, which may represent a mechanism by which recovery of brain volume and neuropsychological function may occur in human alcoholics [95, 98].

In addition to alcohol's neurodegenerative effects on glutamatergic neurons in granular cell layer and pyramidal CA fields of the hippocampus, cholinergic neurons in the hippocampus are also sensitive to the neurotoxic effects CEC [102]. Similarly, studies have shown that GABA-immunoreactive neurons of the hippocampus are lost due to CEC [103, 104], which likely includes dentate basket cells [105] and hilar somatostatin-immunoreactive neurons [106]. Similar to the effects seen for glutamatergic hippocampal granular and pyramidal neurons, alcohol withdrawal exacerbates the loss of both choline acetyltransferase (ChAT)- and GABA- immunoreactive neurons [103, 107], but not somatostatin immunoreactive neurons [106].

Although CEC produces profound effects on brain structure which mimic some features observed in human alcoholic brains, literature suggests that intermittent or binge drinking may be more toxic to the brain then continuous exposure [14]. For example, rats exposed to intermittent intraperitoneal ethanol over the course of one month have hippocampal cell loss, an effect not observed when rats are continuously exposed to ethanol in the drinking water, despite higher total ethanol exposure [108]. Additionally, 2 weeks of intermittent intraperitoneal ethanol exposure in adolescents is associated with DNA fragmentation and elevated caspase-3 activity, indicative of ethanol-induced apoptotic cell death [109].

Other binge-like models also have been utilized to recapitulate the neurotoxic effects of ethanol exposure. Initially developed as a model of alcohol dependence, the Majchrowicz binge model [110], reliably produces patterns of neurodegeneration that are similar to that observed in human [111]. In this model, rats are administered alcohol 3 times daily for 4 days by gastric intubation. Rats are initially administered a priming dose

of 5 g/kg with subsequent doses titrated based on behavioral intoxication scores, such that rats are maintained at highly intoxicating blood ethanol concentrations (BECs) while minimizing mortality. In this model, neurons are lost in the olfactory bulb and throughout the entire cortico-limbic pathway; however neurodegeneration is particularly evident in layer II/III of the entorhinal cortex and the ventral granular cell layer of the dentate gyrus, with occasional degeneration in the CA3 pyramidal layer of the hippocampus [111-113]. Neurodegeneration in this model has traditionally been observed using deOlmos' amino cupric silver stain [111] and/or Fluoro-Jade B (FJB) stain [113, 114], which capture cells currently undergoing degeneration. Corroborating these markers of cell death, a recent study has also shown that total numbers of granule cells in the dentate gyrus are reduced following binge ethanol treatment, an observation likely due not only to cell loss, but also inhibition of ongoing neurogenesis [115]. Additionally, neurodegeneration, visualized by FJB, is detected throughout the corticolimbic pathway following cessation of alcohol for up to one week (figures 1.1 and 1.2) [113], however the role of ethanol withdrawal on neuronal loss in this binge model is still unclear.

A modified Majchrowicz binge model was used for the studies within the current dissertation because it recapitulates many features of human AUDs. For example, studies have shown that binge drinking is the most common form of alcohol intake, is on the rise in the U.S. [10, 116] and is hypothesized to be particularly neurotoxic [14]. Additionally, this model produces BECs between 250 – 450 mg/dL [117] and limbic neurodegeneration [113, 118], which is consistent with human studies [70, 71, 75, 77]. In addition to having face validity, much is known about the cellular and molecular mechanism leading to neurodegeneration and the spatial and temporal profiles of neurodegeneration are well characterized, which aids in experimental design.

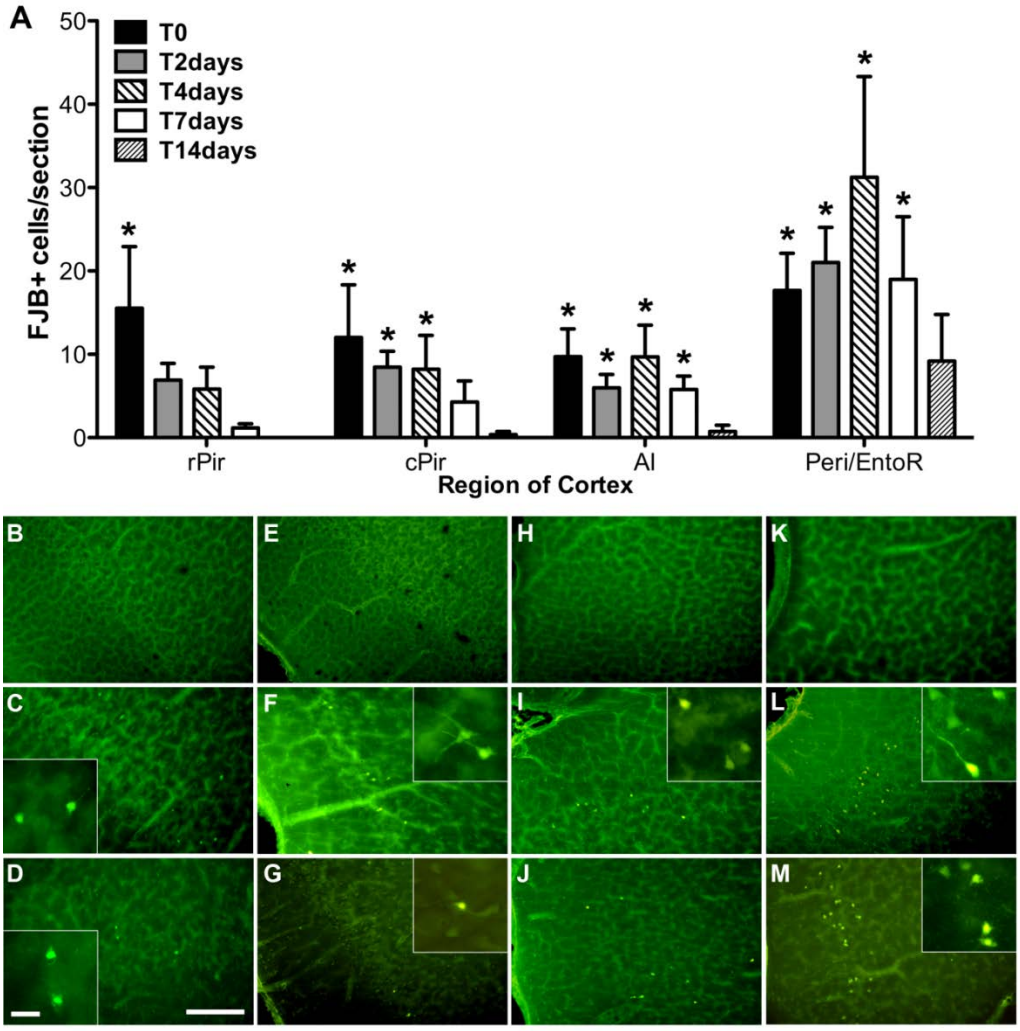

Figure 1.1 Cell death, indicated by FJB, continues for a week into abstinence in corticolimbic regions.

(A) FJB quantification in corticolimbic regions at multiple timepoints following 4-days of binge treatment. (B-M) Representative fluorescent photomicrographs show FJB positive (+) cells in four cortical regions: rostral piriform (rPir; B–D), caudal piriform (cPir; E–G), agranular-insular cortices (AI; H–J), and perirhinal-entorhinal cortices (Per/EntoR; K–M). Control rats (top row B, E, H, K) rarely had detectable FJB+ cells in any brain region. FJB+ cells are shown for T4days (middle row: C, F, I, L) and T7days (bottom row: D, G, J, M). Scale bar in (D) = 100 µm; inset = 10 µm. * $p < 0.05$ compared to controls. (figure from [113])

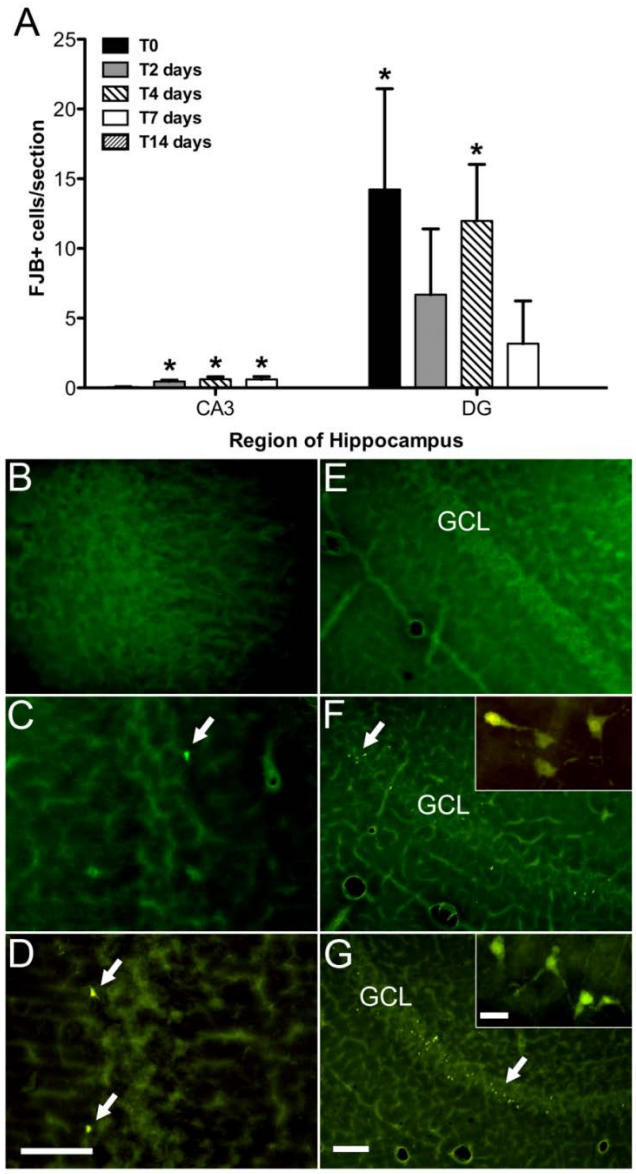

Figure 1.2 Cell death, indicated by FJB, continues for a week into abstinence in the hippocampus.

(A) FJB quantification in CA3 and the dentate gyrus (DG) at multiple timepoints following 4-days of binge treatment. (B-G) Representative fluorescent photomicrographs of FJB staining. In controls, FJB positive (+) cells were rarely observed in the CA3 (B) or DG (E). Conversely, FJB+ cells were observed in CA3 at T4days (C) and T7days (D) and in the DG at T4days (F) and T7days (G). GCL, granule cell layer. Scale bar in (D) = 100 µM, inset = 10 µM. *, $p < 0.05$. (figure from [113])

Importantly, in contrast to other experimental models of ethanol-induced neurodegeneration that require months of treatment [95, 97], substantial neurodegeneration is observed following 4 days of ethanol administration [111, 113] and is evident as early as 1 or 2 days [114, 119]. Thus, the abbreviated nature of this model is amenable to rapid drug screening. In fact, the modified Majchrowicz model has been implemented successfully to evaluate a variety of neuroprotective agents [120-123]. Species, strain and sex are all important considerations for experimental design that may affect outcome measures. Therefore, the studies in the current dissertation used male Sprague-Dawley rats, which is consistent with the majority of previous reports using a modified Majchrowicz binge model to investigate the neurotoxic effects of binge ethanol exposure and to evaluate various neuroprotective agents [114, 118, 120, 124, 125]. This model was also used because much is known about the cellular and molecular mechanism leading to neurodegeneration and the spatial and temporal profiles of neurodegeneration are well characterized. Importantly, in contrast to other experimental models of ethanol-induced neurodegeneration that require months of treatment [95, 97], substantial cell death is observed following 4 days of ethanol administration [111, 113] and is evident as early as 1 or 2 days [114, 119]. Thus, the abbreviated nature of this model is amenable to rapid drug screening. In fact, the modified Majchrowicz model has been implemented successfully to evaluate a variety of neuroprotective agents [120-123].

1.6 Mechanisms of ethanol-induced cellular damage

Although the specific mechanisms responsible for ethanol-induced neuronal cell death remain elusive, studies have shown that oxidative stress, excitotoxicity, neuroinflammatory signaling and/or tissue edema are critical components [126]. Although each of these components can be characterized by distinct molecular

mechanisms, a complex interaction between them occurs under neurotoxic conditions, creating a self-perpetuating cycle. For example, oxidative stress is a byproduct of excitotoxicity and neuroinflammatory enzyme induction; however, prolonged saturation of endogenous antioxidant mechanisms can result in cellular toxicity, creating a feedback effect on excitotoxicity and neuroinflammatory signaling. Additionally, neuroinflammatory signaling can potentiate excitotoxicity by multiple mechanisms, such as membrane insertion of 2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl) propionate (AMPA) receptors and inhibition of astrocyte-mediated glutamate reuptake [127]. The paragraphs below present further detail on oxidative stress, excitotoxicity, neuroinflammatory signaling and edema and how neurotoxic patterns of ethanol exposure induce these events.

1.6.1 Excitotoxicity:

Excitotoxicity is a common pathological event that occurs in a variety of disease states in the CNS including stroke, epilepsy, trauma and neurodegeneration [128, 129]. In the normal functioning CNS, L-glutamate is responsible for a variety of physiological processes including synaptic transmission, synaptic plasticity, and neuronal maturation during development. Glutamate activates three major ligand-gated cationic channels, including NMDA, AMPA and kainate receptors, which increase neuronal excitability by allowing the influx of Ca^{2+} and Na^{+} and the efflux of K^{+}. However, glutamate can become neurotoxic if normal mechanisms governing its release and/or uptake become dysfunctional. Under excitotoxic conditions, excessive synaptic glutamate concentrations overstimulate ionotropic glutamate receptors, leading to high neurotoxic concentrations of free cytosolic Ca^{2+} [128]. Additionally, excitotoxicity may be governed by plasticity in the expression and function of glutamate receptors. For example, research shows that the NR2B subunit of the NMDA receptor increases current decay time, suggesting that

this subunit increases time for Ca^{2+} entry [130] and enhances susceptibility to excitotoxicity [26]. The molecular mechanisms that mediate Ca^{2+} dependent neurotoxicity are not entirely clear, however it is established that free radical production, activation of Ca^{2+} sensitive proteases, mitochondrial dysfunction and different intracellular cascades, including NF-κB, are involved [128, 129].

Some human reports show that brain volume loss is correlated with withdrawal frequency, suggesting that withdrawal-induced excitotoxicity may be involved in the neurotoxic effects of ethanol [14, 131]. Ethanol-induced neurotoxicity is hypothesized to partially result from neuroadaptive changes in inhibitory and excitatory neurotransmission in the presence of chronic ethanol exposure [18], which results in NMDA receptor-mediated hyperexcitability during withdrawal [27] (also see section 1.2). Numerous studies have observed increases in NMDA receptor density [132], NMDA receptor sensitization [133] and glutamate release during acute withdrawal [24, 27]. This state of hyperglutamatergic activity could result in NMDA receptor mediated aberrant increases in intracellular Ca^{2+}, which causes excitotoxic events as described above, leading to cellular damage and death [19, 28, 134]. However, withdrawal-induced excitotoxicity does not explain all of the neurotoxic effects of ethanol as neurodegeneration is observed prior to ethanol-withdrawal in multiple models [95, 111, 119]. Furthermore, NMDA receptor antagonists have repeatedly failed to prevent ethanol-induced neurodegeneration in a 4-day binge model [118, 121].

1.6.2 Oxidative stress:

The brain is highly susceptible to oxidative stress due to high concentrations of unsaturated fatty acids and transition metals, such as iron and copper, and due to high metabolic demand. The production of ROS and reactive nitrogen species (RNS), such as nitric oxide ($NO^{\cdot}$), peroxynitrite ($ONOO^{-}$), hydroxyl radical ($^{\cdot}OH$), hydrogen peroxide

(H_2O_2) and superoxide (O_2^-), occurs during normal metabolic activity and physiology in the CNS. Thus, the CNS also contains antioxidant systems to prevent excessive free radical production. For example, glutathione scavenges intracellular free radicals; and enzymes such as superoxide dismutase and catalase, convert O_2^- to H_2O_2 and H_2O_2 to CO_2, respectively. However, under neurodegenerative conditions, the balance between free radical production and elimination becomes unbalanced and leads to cellular distress and death, if left unchecked [135]. For example, free radicals attack unsaturated fatty acids which can lead to membrane instability and the formation of malondialdehyde, a reactive hydrocarbon which can form DNA adducts [135]. Additionally, DNA and protein oxidative damage results in protein dysfunction, protein aggregation, protein-DNA adducts and direct DNA damage [135]. Common to most neurodegenerative disorders, free radical production typically results from neurotoxicant metabolism, excitotoxicity, mitochondrial dysfunction and/or neuroinflammation, which is also the case for ethanol-induced oxidative stress.

A primary role of oxidative stress in ethanol-induced neurotoxicity is evident from a variety of experimental studies. Consistent with the hypothesis that ethanol accelerates the formation ROS and RNS, studies have found that alcohol exposure enhances the expression of a variety of free radical producing enzymes, including COX-2, NADPH oxidase (NOX) and iNOS [120, 124, 136]. For example, a recent study found that ethanol administration in mice resulted in increased expression of the NOX subunit $gp91^{phox}$ and subsequent free radical production and cell death, both of which could be attenuated by NOX inhibition [136]. Importantly, increased expression of $gp91^{phox}$ was also observed in postmortem alcoholic brain tissue [136]. Free radical production may also occur by ethanol metabolism and induction of the free radical producing cytochrome P450, CYP2E1 [137, 138]. In addition to the induction of free radical producing enzymes, evidence suggests that ethanol produces ROS by impairing mitochondrial function [137,

139]. For example, binge ethanol exposure reduces mitochondrial bioenergetics, which is associated with protein oxidation and lipid peroxidation [139]. Further support for the role of oxidative stress in ethanol-induced neurodegeneration comes from neuroprotection studies using the modified Majchrowicz model. Administration of a variety of antioxidants, including butylated hydroxytoluene (BHT), α-tocopherol and cannabidiol, attenuate neurodegeneration (Hamelink et al., 2005; Crews et al., 2006a). Interestingly, neuroprotection observed following BHT was associated with decreased NF-κB-DNA binding, COX-2 expression and Iba-1 upregulation, suggesting a role of neuroinflammatory signaling in ethanol-induced oxidative stress and neurodegeneration [120].

1.6.3 Neuroinflammatory signaling:

Neuroinflammation is commonly observed in disorders of the CNS, including neurodegenerative disorders, acutely damaging insults, for example, traumatic brain injury and stroke, and psychiatric diseases, such as depression and addiction [140]. Therefore, it is not surprising that both human and experimental reports also suggest that neuroinflammation is involved in the pathogenesis of alcoholism. Neuroinflammation is broadly defined as a physiological response to tissue damage or infection that involves a variety of cell types, including neurons, glia and infiltrating systemic immune cells, and the induction of a variety of inflammatory mediators including cytokines, eicosanoids and inflammatory enzymes in order to limit tissue damage or eliminate infection [141]. However, inappropriate and/or chronic activation of the neuroinflammatory axis is theorized to contribute to neurotoxicity in the aforementioned CNS diseases [140]. Both pathological and genetic studies underscore the importance of neuroinflammatory signaling in alcoholism [136, 142, 143]. For example, postmortem studies have found that the alcoholic brain shows evidence of neuroinflammation,

including increased expression of MCP-1 (CCL2), NOX ($gp91^{phox}$), toll-like receptors (TLR), high mobility group box 1 (HMGB1, endogenous TLR agonist) and microglia markers Iba-1 and GluT5 [136, 142, 144].

Experimental evidence also suggests that ethanol exposure initiates neuroinflammatory signaling, which may contribute to neurodegeneration. For example, multiple studies have found increased NF-kB translocation and DNA binding following ethanol exposure [120, 136, 145], which is the quintessential proinflammatory transcription factor that is responsible for the induction of multiple inflammatory mediators such as cytokines, chemokines and microglial activation. Interestingly, treatment with BHT reverses NF-κB-DNA binding, COX-2 expression, Iba-1 upregulation and cell death induced by binge ethanol treatment, suggesting that neuroinflammatory signaling is initiated; however whether these effects are responsible for or a result of cell death is unclear [120]. Although these data support a role of neuroinflammatory signaling in ethanol-induced neurodegeneration, a recent report shows that binge ethanol treatment fails to induce markers of classical neuroinflammation, such as TNFα, IL1β, full microglial activation and systemic immune cell infiltration [146]. In light of the current literature, it is likely that the magnitude of ethanol-induced neuroinflammatory signaling and its role in ethanol neurotoxicity is dependent on ethanol exposure protocols and species. For example, in contrast to binge ethanol treatment, 10 days of episodic ethanol exposure in mice causes increased expression of TNFα, IL-6, MCP-1, NOX, TLR3, HMGB1 and Iba-1 immunoreactivity [136, 147, 148]. Similarly, chronic ethanol consumption is associated with TLR4 dependent neuroinflammatory signaling and caspase-3 cleavage [145].

Although the link between ethanol-induced neuroinflammatory signaling and neurodegeneration is yet to be firmly established, current evidence suggests that neuroinflammatory signaling induced by ethanol may contribute oxidative stress-induced

neurotoxicity. For example, NOX inhibition prevents ethanol-induced up regulation of Iba-1 expression, free radical generation, caspase-3 cleavage and cell death [136]. Additionally, inhibition of phospholipase A2 (PLA2) prevents alcohol-induced neurodegeneration in hippocampal entorhinal cortex (HEC) cultures, which is likely due to inhibition of prostaglandin-mediated neuroinflammatory signaling and oxidative stress [149]. Furthermore, inhibition of microglial activation is correlated with neuroprotection following ethanol exposure, suggesting that neuroinflammatory function contributes to some of the neurotoxic effects of ethanol [147].

1.6.4 Edema:

In humans, brain edema has been observed in chronic alcoholics during withdrawal [150] and diuretic treatment results in less severe withdrawal [151]. From these early reports, it has been suggested that brain swelling may be involved in ethanol-induced neuropathology [152]. Over the past decade, evidence has emerged that implicates brain edema as an early event that results in ethanol-induced neuroinflammatory signaling, oxidative stress and neurodegeneration [149, 153-155]. In support of this hypothesis, furosemide, a K^+, Cl^- co-transporter inhibitor, reverses brain water accumulation, ion imbalances and corticolimbic neurodegeneration following episodic alcohol exposure in rats [153]. However, furosemide is a potent free radical trapping antioxidant, which may explain its neuroprotective effects, thus confounding mechanistic interpretation [121]. Further evidence of the edema hypothesis has shown that diuretics, acetazolamide and torasemide, which have negligible antioxidant capacity, also prevent edema and neurodegeneration in either hippocampal-entorhinal cortex (HEC) slice cultures or following *in vivo* episodic alcohol exposure, possibly by varying mechanisms, including inhibition of aquaporin 4 [154]. Proponents of the edema hypothesis suggest that alcohol-induced neurodegeneration may occur though

mechanical disruption of cellular architecture following increased intracranial pressure and/or by induction of neuroinflammatory pathways and oxidative stress [153]. In support of the latter, brain edema is associated with the release of arachidonic acid (AA) from membrane stores, which may in turn lead to oxidative stress, neuroinflammation and induction of a variety of other cytotoxic mechanisms [155]. Interestingly, inhibition of PLA2 prevents alcohol-induced brain damage in HEC slice cultures [149]. Although the edema ↔ neuroinflammation ↔ ROS hypothesis is gaining momentum, additional studies need to establish the exact molecular mechanisms linking these events.

1.7 Cannabinoid physiology and biochemistry

According to archeological evidence, marijuana (*Cannabis sativa*) has been used for thousands of years, particularly for its euphoric and mind altering properties. Before 1990, little was known about the physiological mechanisms of cannabis derivatives except for the identification of Δ^9 – tetrahydrocannabinol (Δ^9 – THC) as the main psychoactive constituent of cannabis. Advances in cannabinoid research took off after the discovery of the first cannabinoid receptor, the cannabinoid 1 receptor (CB1R), in 1990 [156] followed by the discovery of the cannabinoid 2 receptor (CB2R) in 1993 [157].

1.7.1 Cannabinoid receptor expression

The CB1R is commonly called the central cannabinoid receptor because it is predominately expressed in the CNS where it modulates a variety of behavioral and cognitive processes. High expression levels are found in the striatum, hippocampus, substantia nigra and cerebellum, while moderate to low levels are found in other structures including the cerebral cortex, amygdala, hypothalamus and spinal cord [158]. The distribution patterns of CB1Rs throughout the CNS are consistent with the

physiological effects observed following cannabinoid administration including analgesia, learning and memory deficits, addiction, mood regulation and neuroprotection [159]. On the other hand, CB2R expression is abundant in the periphery, particularly on macrophages and in the spleen [157, 159], while central expression remains uncertain and controversial [160]. Recent reports have identified CB2R in the brain on both neuronal and glial processes [161] and are implicated in a wide variety of processes including neuroinflammation and psychiatric disorders [162]. In addition to activation of CB1Rs and CB2Rs, exogenous and endogenous cannabinoid ligands can activate other receptor types including the peroxisome proliferator activated receptors (PPARs), the transient receptor potential cation channel (TRPV1) and the orphan receptors, g-protein receptor 55 (GPR55) and GPR18 [163].

1.7.2 CB1R signal transduction

The cannabinoid receptors are metabotropic G-protein coupled receptors and therefore activate a variety of signal transduction pathways [164]. Both CB1Rs and CB2Rs are sensitive to pertussis toxin and therefore operate primarily though $G_{i/o}$ proteins and reduce levels of cAMP [164]. Additionally, CB1Rs are coupled to a range of ion channels including negative coupling to N, P/Q and L-type voltage-gated calcium channels and positively coupled to GIRK and A-type potassium channels [164]. CB1Rs can activate a variety of kinases including focal adhesion kinase (FAK), mitogen activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) [164].

1.7.3 Endocannabinoid ligands

Cannabinoid receptors are activated by arachidonic acid-derived lipid messengers that are synthesized and released through activity dependent mechanisms, therefore “on demand”. The first ligand discovered, arachidonylethanolamide

(Anandamide, AEA) was isolated in 1992 from porcine brain [165] and was shown to be a partial agonist at CB1Rs and CB2Rs [163, 166]. Additionally, AEA has other non-CB1R and non-CB2R targets, including activation of TRPV1, PPAR isoforms and various other ligand gated ion channels [163]. The other major eCB, 2-arachidonylglycerol (2-AG), was first identified from canine gut, and was found to interact with both CB1R and CB2R as a full agonist [167]. 2-AG has greater efficacy and selectivity at cannabinoid receptors and can be found on the order of nanomoles per of gram tissue (in comparison to picomoles per of gram tissue for AEA), therefore it is considered to be the main cannabinoid neurotransmitter of the brain [159, 163]. In addition to AEA and 2-AG, other bioactive lipids bind to cannabinoid receptors, including dihomo-g-linolenoyl ethanolamide, docosatetraenoyl ethanolamide, 2-arachidonyl glycerol ether, 0-arachidonoylethanolamide and n-arachidonoyldopamine, however their biological significance is less studied [159, 163]. Although not able to bind to cannabinoid receptors, other n-acylethanolamides (NAEs), including palmitoylethanolamide (PEA) and oleoylethanolamide (OEA) have the capacity to interact with the eCBs by competing for cellular reuptake and catabolism by fatty acid amide hydrolase.

1.7.4 Endocannabinoid biochemistry

As mentioned above, both eCBs are synthesized in an activity dependent manner though Ca^{2+} sensitive biosynthetic pathways. The major pathway for AEA synthesis involves a two-step reaction to convert phosphatidylethanoamine into AEA [168]. The first step is the transfer of arachidonic acid from a sn-1-arachidonate-containing phospholipid to phosphatidylethanoamine by N-acyltransferase to produce the intermediate product N-arachidonoyl-phosphatidylethanoamine (NAPE) [169]. This step regulates the activity dependent formation of AEA as N-acyltransferase is Ca^{2+}

sensitive and is thought to be the rate limiting step. The second step requires NAPE – phospholipase D (NAPE-PLD), which performs the hydrolysis of NAPE into phosphatidic acid and AEA [170, 171]. Although this pathway is the primary mechanism of AEA formation and the expression patterns of these enzymes and CB1Rs are relatively consistent [169], other non-NAPE-PLD pathways exist for the formation of AEA and may have biological relevance as NAPE-PLD null mutants have normal AEA content in the brain [172].

AEA is inactivated by cellular reuptake and subsequent hydrolysis by the serine hydrolase, fatty acid amide hydrolase (FAAH). The cellular reuptake of AEA is theorized to be assisted by a facilitated diffusion mechanism, however molecular cloning of a specific transport/carrier protein remains elusive and its existence is highly controversial [173]. In support of a transport system, pharmacological characterization of AEA uptake indicates that this process is saturable and sensitive to small molecular inhibitors [173, 174]. Following reuptake, AEA is hydrolyzed by FAAH into free arachidonic acid and ethanolamine [175-178]. FAAH is expressed widely across the brain, including in the hippocampus and cortex, and is complimentary to CB1R localization [179]. Although FAAH is the major catabolic enzyme for AEA hydrolysis, and pharmacological and genetic deletion of FAAH results in elevated AEA tissue content, other enzymes for AEA catabolism have been suggested, including COX-2, cytochrome P450s and lipoxgenases [168].

Similar to AEA, 2-AG can be synthesized by multiple biosynthetic pathways and acts as an intermediate for further lipid metabolism. However, evidence strongly suggests that the pool of 2-AG responsible for stimulating cannabinoid receptors “on demand” is produced by a two-step pathway requiring phospholipase Cβ (PLCβ) and diacylglycerol lipase α/β (DAGLα/β), which is independent from biosynthetic pathways responsible for basal 2-AG levels [180]. The first step requires the formation of an

intermediate 2-arachidonate-containing diacylglycerol (DAG), mediated by PLCβ hydrolysis of phosphoinositol-bis-phosphate (PIP2). Subsequently, DAGL-mediated hydrolysis of the sn-1 acyl group of 2-arachidonate containing DAG results in the generation of 2-AG. Catabolism of 2-AG primarily occurs though monoacylglycerol lipase (MAGL) to generate arachidonic acid and glycerol. However, similar to AEA, other routes of degradation exist including hydrolysis by FAAH and oxygenation by either 12- and 15-lipoxygenases, cytochrome P450s and COX-2 [180].

1.7.5 Endocannabinoid physiology in the CNS

The eCBs are lipid messengers that do not cause direct synaptic transmission but modulate other neurotransmitter systems through presynaptic inhibition of neurotransmitter release [181-184]. This action has been demonstrated at a variety of synapses including serotonin, acetylcholine, dopamine, glycine, norepinephrine and glutamate; however eCB-mediated inhibition of neurotransmitter release most commonly occurs at GABAergic synapses in multiple brain regions, which include the hippocampus, striatum and neocortex [159]. The initial studies that discovered the mechanism of eCB signaling showed that postsynaptic depolarization [181-183] or activation of mGlu1Rs [184] caused a rapid but transient decrease in either inhibitory postsynaptic currents [181, 183] or excitatory postsynaptic currents [182, 184] that are dependent on eCB signaling. Although it was previously demonstrated that CB1R activation inhibits neurotransmitter release [185, 186], the aforementioned studies confirmed a postsynaptic origin of eCB synthesis/release and presynaptic site of action, thus demonstrating that eCBs act as retrograde messengers. Since these seminal studies, others have shown that various types of eCB-mediated depression of neurotransmitter release exist, which depends on cell type and brain region; and can be manifested as either short-term depression or long-term depression contingent on

stimulation protocols and recruitment of co-modulators [159]. In addition to modulating neurotransmitter release, the eCBs are responsible for affording endogenous neuroprotection (see section 1.9 for detailed discussion).

1.8 Ethanol and endocannabinoids

Over the past 15 years, research has clearly demonstrated that the eCB system is intimately involved with the neurophysiological and behavioral effects of ethanol [187, 188]. For example, genetic and/or pharmacological inactivation of CB1Rs prevents ethanol-induced dopamine transients in the nucleus accumbens and attenuates elevated neuronal firing of ventral tegmental area dopamine neurons following ethanol administration [189-191]. Importantly, ethanol stimulates 2-AG production in the nucleus accumbens, providing direct evidence that eCB signaling is involved in the neuropharmacological effects of ethanol. Behaviorally, CB1R antagonism can decrease ethanol consumption and preference [192-194], an effect that is replicated by genetic deletion of the CB1R [190, 194-196]. Conversely, CB1R agonists can increase ethanol self-administration [197, 198]. Furthermore, genetic or pharmacological inactivation of CB1R influences ethanol-induced hypothermia, ethanol sedation and ethanol withdrawal induced conclusions [194, 195]. In addition to behavioral and neurophysiological studies, evidence shows that ethanol exposure has profound effects on eCB biochemistry which depends on multiple factors, including species, the duration (acute vs. chronic) and pattern (continuous vs. intermittent) of ethanol exposure, ethanol dose, brain region and eCB of interest.

Studies examining the acute effects of ethanol exposure on eCB content have reported complex patters of regulation. For example, ethanol reduces AEA content in the hippocampus, nucleus accumbens, striatum, cerebellum, hypothalamus and amygdala, but not in the prefrontal cortex [199-201]. Conversely, acute ethanol decreases 2-AG

content in the prefrontal cortex, but enhances 2-AG liberation in the nucleus accumbens [200, 201]. Interestingly, acute ethanol administration appears to result in general reductions in OEA and PEA, suggesting that acute ethanol may have a general effect on mechanisms regulating NAE tissue content [199, 200]. However, these effects on NAE content appear to be independent of changes of FAAH and NAPE-PLD activity, even in the presence of ethanol *ex vivo* [199, 202].

On the other hand, chronic ethanol administration has distinct effects on the eCB system compared to observations following acute exposures. For example, 3 or 4 days of ethanol vapor exposure increases AEA content in the cerebral cortex of mice, which is accompanied by reductions in CB1R expression and CB1R agonist induced g-protein activation [203, 204]. However, these changes appear to normalize or even become enhanced following cessation of ethanol exposure [204]. The opposing changes in CB1R expression and AEA suggest that CB1R down regulation results from agonist induced desensitization, however, chronic alcohol could theoretically alter the kinetics of CB1R receptor turnover by either decreasing synthesis or promoting degradation [204]. Following longer periods of ethanol exposure, reductions in CB1R mRNA are observed in selected brain regions including the cingulate cortex, striatum, ventromedial hypothalamic nucleus and hippocampus [205, 206]. In one study, 2-AG elevations coincided with CB1 mRNA down regulation further supporting agonist-induced desensitization; however prolonged withdrawal was associated with elevated AEA, 2-AG and CB1R mRNA [206]. Interestingly, lower CB1R mRNA has been observed in CA1 and CA2 of the hippocampus, but no changes in CA3 and increased expression in the DG [205], suggesting that chronic ethanol exposure may cause reorganization of the eCB system within discrete circuitry. Importantly, not all studies have found changes in CB1R expression following chronic ethanol exposure, a discrepancy that may be accounted for by ethanol dose or pattern of exposure [207, 208]. *In vitro* studies have

found that 50 and 100 mM ethanol (which is equivalent to BECs of 230 and 460 mg/dl) enhances AEA and 2-AG release in hippocampal and cerebellar granular neurons [209-211]. Interestingly, increases in AEA are accompanied by increases in NAPE, suggesting an ethanol effect upstream of AEA biosynthesis. Additionally, another report suggests that ethanol may also inhibit AEA transport and FAAH mediated hydrolysis [210].

Although, the effects of ethanol have been explored in both acute and chronic models, the relationship between the eCB system and ethanol has not been studied in a model with an acutely damaging event such as a 4-day binge model [111]. Neurodegeneration is known to result in the liberation of both AEA and 2-AG (see section 1.8), therefore an interaction between the effects of ethanol and the effects of neurodegeneration on the eCB system are likely to exist. An understanding of this interaction may lead to the development of novel pharmacotherapies for the treatment of alcohol use disorders. For example, the eCBs have emerged as a potent neuroprotective target, thus modulation of this system may afford protection from ethanol-induced neurodegeneration.

1.9 Rationale for targeting endocannabinoids to treat alcohol-induced neurodegeneration

Multiple lines of evidence support the role of eCB signaling in defending the CNS from neurodegeneration and that this system can be modulated to afford additional neuroprotection [212]. This hypothesis has emerged from several studies observing elevated eCB tissue content following acute neuronal injury and during chronic neuroinflammation [213-221]. Furthermore, CB1R null-mutant mice are more susceptible to pathological insults, suggesting that the eCBs are critical for containing and/or attenuating neuronal injury [218, 222]. The neuroprotective properties of the eCB system

have been observed in models of CNS injury including traumatic brain injury and cerebral ischemia, and in models of chronic neuroinflammatory disease including Alzheimer's, disease, multiple sclerosis and Parkinson's disease [219, 223]. The exact mechanisms governing cannabinoid-mediated neuroprotection are not completely elucidated; however it is clear that the effects of cannabinoids are pleiotropic and disease state specific. For example, CB1Rs and CB2Rs have potent effects against excitotoxicity, neuroinflammation, and oxidative stress and are coupled to multiple cell survival pathways that mediate neuroprotection [212].

1.9.1 Excitotoxicity

CB1Rs are localized at presynaptic terminals on the majority of neuronal cell types including GABAergic and glutamatergic neurons [159]. Following stimulation, CB1Rs influence multiple signaling pathways in the presynaptic terminal, including inhibition of cAMP production, inhibition of Ca^{2+} channel conductance and enhancement of inward-rectifying K^{+} channel conductance, resulting in hyperpolarization and suppression neurotransmitter release [159]. During states of hyperexcitability, eCBs are released from the postsynaptic neuron and provide negative feedback inhibition of glutamate release, thus dampening neuronal activity. Importantly, this mechanism has been shown to provide endogenous defense against excitotoxicity. For example, in an elegant study in 2003, genetic deletion of CB1Rs on glutamatergic forebrain neurons increased susceptibility to kainic acid (KA)-induced seizures, hyperexcitability and neuronal cell death. Importantly, the frequency of excitatory post synaptic potentials was greater in mutant mice suggesting a role of CB1Rs in preventing excitotoxicity by suppressing excessive presynaptic glutamate release. Additionally, KA-induced increases in early intermediate genes and brain derived neurotrophic factor (BDNF) were absent in the mutant mice suggesting that multiple CB1R-dependent mechanisms

protect neurons from excitotoxicity. Additional studies examining the molecular mechanisms of CB1R-mediated neuroprotection in models of excitotoxicity have implicated other functions independent of suppression of neurotransmitter release, which include inhibition of nitric oxide production, blockage of TNFα-induced up-regulation of AMPA-type receptors at the post synaptic density, and prevention of Ca^{2+} influx through NMDA receptors through a mechanism involving the release of Ca^{2+} from intracellular stores [224-226]. Regardless of the mechanism, the current literature clearly implicates eCB signaling in preventing excitotoxic neuronal cell death, which makes the eCB system an attractive target for preventing alcohol-withdrawal induced seizures and excitotoxicity. In fact, a recent study demonstrated that CB1R activation was capable of preventing alcohol withdrawal induced excitotoxicity (figure 7.1), presumably by preventing excessive intracellular Ca^{2+} accumulation [227].

1.9.2 Neuroinflammatory function

Neuroinflammation is a complex physiological process that is initiated following exposure to noxious stimuli, including CNS infection and injury. Acute activation of the neuroinflammatory axis is necessary for returning the CNS to homeostasis following a noxious stimulus. However, prolonged activation can result in CNS damage and neurodegeneration [140]. Multiple lines of evidence show that the eCBs are important for modulating neuroinflammatory responses, and that this system can be targeted to attenuate neuroinflammatory functions associated with neurodegenerative disease [228]. Glial cells, including microglia and astrocytes, play an integral role in neuroinflammatory function and these cell types express molecular components of the eCB system. For example CB1R expression is found on microglia [229] and astrocytes [230], while CB2R expression can be stimulated in microglia [231] and is found in primary microglial cultures [229, 232].

In vivo, activation of the eCB system has been shown to attenuate neuroinflammatory function in models of chronic neuroinflammatory disease. For example, administration of synthetic cannabinoid agonists following Theiler's virus infection decreases microglial activation, decreases MHCII expression and decreases CD4+ T cells in the spinal cord [233]. Similarly, the CB1R/CB2R agonist, WIN55,212-2, prevents microglial activation and loss of neuronal markers induced by intracerebroventricular injection of β-amyloid protein [234]. *In vitro* studies suggest that cannabinoids attenuate neuroinflammatory function by preventing microglial activation, inhibiting their migration to injured tissue and preventing the release of proinflammatory cytokines and nitric oxide [215, 229, 234, 235]. Importantly, some *in vitro* studies have found a direct link between the anti-inflammatory effects of cannabinoids and neuroprotection [215, 232]. Interestingly, other *in vitro* studies have shown that neuronal CB1Rs are involved in attenuating neuroinflammatory signaling cascades. For example, activation of neuronal CB1Rs prevents NF-κB and COX-2 dependent neurotoxicity following either excitotoxic or neuroinflammatory stimuli [235]. Although, *in vitro* studies have isolated specific neuronal, astrocyte and microglial dependent actions on neuroinflammatory function, it is likely that these actions act in concert with each other to produce neuroprotection *in vivo* [223]. Whether or not the cannabinoids may prevent neuroinflammatory function associated with ethanol-induced neurotoxicity is yet to be determined. However the aforementioned reports suggest that the eCB system has the capacity to afford such effects.

1.9.3 Oxidative stress

Studies have demonstrated that cannabinoids have neuroprotective effects by attenuating oxidative stress; however the exact mechanisms involved in preventing oxidative stress are debated. The phytocannabinoids, Δ9-THC, cannabinol (CBN) and

cannabidiol (CBD), and some synthetic cannabinoid ligands have phenolic moieties in their chemical structure and therefore are potent antioxidants by scavenging free radicals [236, 237]. In fact, some of the phytocannabinoids have been shown to have greater oxidation potentials than other well-known antioxidants such as butylated hyrdoxytoluene (BHT) and α-tocopherol [121, 236]. Conversely, others have shown that cannabinoids prevent oxidative neuronal injury through CB1R-dependent mechanisms involving protein kinase A (PKA) [238]. Importantly, CBD has been shown to prevent binge alcohol induced neurodegeneration, presumably through its capacity to scavenge free radical production [121].

1.9.4 Summary

The preceding paragraphs outlined the potential mechanisms by which the cannabinoids attenuate neurodegeneration, many of which may be useful for counteracting ethanol-induced neurotoxicity. For example, cannabinoids may prevent ethanol withdrawal induced excitotoxicity primarily through inhibition of excessive glutamate release and/or reducing cell excitability. Additionally cannabinoids may be neuroprotective following binge ethanol exposure by inhibiting neuroinflammatory function and/or by reducing oxidative stress. The studies within this dissertation utilized a 4-day binge model of ethanol exposure that produces neurodegeneration within the cortico-limbic pathway [111, 113]. Importantly, neurodegeneration resulting from ethanol exposure in this model is hypothesized to result from neuroinflammatory signaling cascades and oxidative stress, but not excitotoxicity (section 1.5). Therefore, from current reports on cannabinoid mediated neuroprotection and mechanisms of binge ethanol induced neurodegeneration, it is possible that the cannabinoids may prevent neurotoxicity by preventing neuroinflammatory signaling and oxidative stress, but not excitotoxicity.

1.10 Goals of the dissertation

The overall goal of the current dissertation was to evaluate cannabinoid based agents to prevent ethanol-induced neurodegeneration utilizing a 4-day binge model. Chapter 2 expands on a previous report characterizing the neuroprotective effects of CBD administration during binge ethanol treatment. As CBD has poor oral bioavailability, which limits its clinical use, the studies reported in chapter 2 were aimed at testing CBD transdermal delivery formulations to attenuate binge ethanol induced neurodegeneration. The subsequent chapters were designed to evaluate the effects of binge ethanol exposure on the eCB system and to manipulate eCB signaling to afford neuroprotection. Previous studies have found that brain injury and neurodegeneration engages eCB signaling which is hypothesized to represent an endogenous neuroprotective mechanism. However, this response has not been examined following ethanol-induced neurodegeneration; therefore, chapter 3 presents data validating an analytical method for quantifying eCBs and related lipids, including OEA and PEA, while chapter 4 examined the effects of binge ethanol intoxication and withdrawal on CB1R expression and AEA, OEA and PEA tissue content. Blocking eCB catabolism is emerging as a strategy to afford neuroprotection; therefore, chapter 5 evaluated the neuroprotective effects of FAAH inhibition following binge ethanol-induced neurodegeneration. In summary, these studies expand on current understanding of the neuroprotective effects of cannabinoids and the eCB system. Additionally, this dissertation discusses a vision for future studies to further evaluate the eCB system for the treatment of ethanol-induced neurodegeneration.

1.11 Overall Hypotheses

1.11.1 Hypothesis 1: Transdermal cannabidiol treatment will prevent ethanol-induced neurodegeneration.

Aim1. Determine if transdermal CBD can prevent ethanol-induced neurodegeneration in the entorhinal cortex using an established 4-day binge model.

Aim 2. Determine a target CBD plasma concentration necessary to observe neuroprotection.

Aim 3. Compare the neuroprotective effects of transdermal CBD to the previously established neuroprotective effects of intraperitoneal administration of CBD.

1.11.2 Hypothesis 2: N-acylethanolamide content is amplified in the entorhinal cortex and hippocampus in response to ethanol-induced neurodegeneration as an endogenous protective mechanism and potentiating this response will result in neuroprotection.

Aim 1. Determine if binge ethanol exposure decreases CB1R expression in the entorhinal cortex and hippocampus by examining a timecourse of CB1R expression during ethanol intoxication and withdrawal.

Aim 2. Develop and validate a LC-MS method for the simultaneous quantification of AEA, OEA and PEA in rat brain tissue.

Aim 3. Determine if binge ethanol exposure increases NAE content, including AEA, OEA and PEA, in the entorhinal cortex and hippocampus following multiple timepoints associated with the initiation of ethanol-induced neurotoxicity and peak cell death.

Aim 4. Determine if pharmacological inhibition of FAAH can prevent ethanol-induced neurodegeneration in an established 4-day binge model.

Aim 5. Determine the magnitude of NAE accumulation by acute FAAH inhibition following binge ethanol exposure.

2. CHAPTER 2

TRANSDERMAL DELIVERY OF CANNABIDIOL ATTENUATES BINGE ETHANOL INDUCED NEURODEGENERATION IN A RODENT MODEL OF AN ALCOHOL USE DISORDER

2.1 Introduction

Approximately 8.5% of the U.S. population currently meets the diagnostic criteria for an AUD [8]. Although four pharmacotherapy based interventions are approved in the U.S. for the treatment of AUDs, these drugs have limited efficacy in the patient population [59]. Additionally, these medications primarily target the motivational properties of alcohol, while the neurodegenerative effects of alcohol that impair behavioral control and decision making are not managed by these specific treatments. Therefore, identification of novel targets and development of new therapeutic agents is critical to improve pharmacotherapy based treatment strategies for AUDs.

Neuroprotective agents are hypothesized to have high therapeutic utility for the treatment of AUDs [60]. Excessive alcohol intake, characteristic of AUDs, results in neurodegeneration and cognitive and behavioral impairment, effects which are hypothesized to influence the transition to addiction [60, 61, 63]. Imaging studies have identified gross anatomical abnormalities throughout the brains of human alcoholics including widespread disruption of white matter tracts, atrophied cortical gray matter and increased cerebrospinal fluid filled space [239-241]. These effects have been confirmed in postmortem studies showing significant cortical neuronal loss [67, 70], which is consistent with studies demonstrating long term or permanent deficits in function [88]. Some brain structures appear to be more susceptible to the neurodegenerative effects of alcohol, including frontal cortical regions [67, 73, 136], the temporal lobe [77] and hippocampus [77]. The aforementioned brain regions are involved in problem solving,

attention, information processing, learning and memory and behavioral control, therefore it is not surprising that these functions are impaired in AUDs [88]. Importantly, a recent study described an association between reductions in cortical gray matter and risk for relapse [62]. Therefore, elucidating the mechanism(s) underlying alcohol-induced neurodegeneration and developing neuroprotective pharmacotherapies could improve prevention and treatment strategies for AUDs.

Studies have suggested that chronic alcohol exposure is associated with induction of neuroinflammatory mediators and/or oxidative stress, which leads to neurodegeneration [126, 136]. Consistent with this hypothesis, a variety of antioxidants, including α-tocopherol, BHT and CBD have been effective in reducing binge alcohol-induced neurodegeneration [120, 121]. Neuroprotection mediated by antioxidant treatment is associated with inhibition of NF-κB-DNA binding, reductions of COX-2 expression and microglial activation [120], all of which support the hypothesis that neuroinflammatory signaling and/or oxidative stress contribute to alcohol-induced neurodegeneration [126]. These studies have demonstrated clearly that antioxidants protect against alcohol-induced neurodegeneration, therefore further development of these agents for clinical use is warranted.

CBD is a main constituent of *cannabis sativa*. Unlike the more commonly recognized constituent, Δ^9-THC, CBD does not exhibit psychotropic effects as it is not an agonist at CB1Rs [242]. In fact, CBD is very well tolerated in humans [243]. CBD has a plethora of actions, including anticonvulsive, anxiolytic, anti-relapse and neuroprotective properties [236, 244, 245], which make it an ideal candidate for treating multiple aspects of AUDs. CBD was initially shown to be neuroprotective in an *in vitro* model of excitotoxicity by scavenging reactive oxygen species [236]. Indeed, comparison of CBD with well-known antioxidants including BHT and α-tocopherol, showed that CBD has a higher antioxidant capacity [236]. Extending these findings, another study demonstrated

that administration of CBD was neuroprotective in the modified Majchrowicz binge model of alcohol-induced neurodegeneration, again presumably through its antioxidant activity [121].

Although CBD is efficacious in preclinical models and is safe for human use [243], its clinical use has been minimal because of poor oral bioavailability and low aqueous solubility. Estimated oral bioavailability of CBD is roughly 6% [246, 247]; therefore, it is difficult and expensive to achieve suitable plasma levels for clinical efficacy. These drug delivery obstacles may be circumvented by alternative delivery routes, such as transdermal delivery [248]. Additionally, transdermal delivery is advantageous because it promotes patient compliance as it is non-invasive and pain free compared to injectable formulations, which is especially important in the alcohol dependent population [249]. Therefore, the current study investigated the hypothesis that CBD transdermal systems can attenuate alcohol-induced neurodegeneration using a well-established model of an AUD, the modified Majchrowicz binge model.

2.2 Materials and Methods

2.2.1 Housing and Animals

Adult male Sprague Dawley rats weighing approximately 330 grams on arrival (n = 142, Charles River, Raleigh, NC) were used in these studies. All treatment protocols followed the *Guide for the Care and Use of Laboratory Animals* (NRC, 1996) and were approved by the University of Kentucky Institutional Animal Care and Use Committee. Rats were singlely housed in Plexiglas cages in an AAALAC approved University of Kentucky vivarium on a 12 h light/dark cycle with access to rat chow and water *ad libitum* unless noted. During acclimation, rats were handled daily for at least three days to familiarize rats to experimenters.

2.2.2 Ethanol Treatment

Rats were exposed to ethanol following the modified Majchrowicz binge model [110] as reported previously [117]. This model maintains intoxicating BECs typical of AUDs [12], with minimal mortality and a well-defined pattern of neurodegeneration [111, 113]. Rat chow was removed from home cages and rats were administered either ethanol (25% w/v) in nutritionally complete Vanilla Ensure Plus® (Abbott Laboratories, Columbus OH) or an isocaloric diet consisting of dextrose, water and Vanilla Ensure Plus® every 8 hr for 4 days by intragastric gavage. Ethanol rats initially received a 5 g/kg priming dose, with subsequent doses based off the following intoxication scale: 0, normal (5 g/kg); 1, slightly ataxic and hypoactive (4 g/kg); 2, ataxic with elevated abdomen and intact righting reflex (3 g/kg); 3, delayed righting reflex and lack of abdominal elevation (2 g/kg); 4, lack of righting reflex with intact eye blink reflex (1 g/kg); 5, unresponsive including loss of eye blink reflex (0 g/kg). BECs were measured in plasma from tail vein blood collected 90 minutes after the 7th dose of ethanol (day 3). Approximately 150 µL of blood was collected into microcentrifuge tubes containing heparin (5µL; AAP pharmaceuticals, Schaumberg, IL), centrifuged at 1800 x g for 5 min, and stored at -20°C. BECs were determined in triplicate using a AM1 alcohol analyzer (Analox, Lunenberg, MA) calibrated to a 300 mg/dL external standard.

2.2.3 Cannabidiol Regimen

CBD was synthesized by AllTranz Inc. and formulated for either intraperitoneal (IP) injection or transdermal gel application. CBD (6 mg/mL) and vehicle solutions for IP injections were prepared daily prior to the morning dose. IP solutions were comprised of 76% sterile saline, 21% cremophor and 3% absolute ethanol. The 1%, 2.5%, 5% (w/w) CBD gels and vehicle gels were prepared and loaded into syringes for gel application. The active and vehicle gels prepared by AllTranz Inc. were composed of ethanol,

propylene glycol, sterile water, Transcutol®, preservatives and a crosslinked polyacrylate polymer adjusted to the appropriate pH with triethanolamine to provide suitable rheological properties and pH dependent CBD stability. The optimized formulation described in experiment 2 utilized only a 2.5% (w/w) CBD gel that contained decreased levels of ethanol and an increase in water content. Rats receiving gels had hair removed on their dorsal side using clippers prior to binge treatment and 24 hours before the first gel application. Rats received CBD or vehicle starting after the third dose of ethanol by either daily gel application (11:00 am) or IP injection (20 mg/kg) twice daily (11:00 am & 11:00 pm; see Figure 2.1). This IP dose was chosen based off a previous study demonstrating CBD mediated neuroprotection using a similar binge model (Hamelink *et al.*, 2005). Gels (750 µL) were applied to a 35 cm^2 area and rubbed into the skin for 30 sec with a finger covered by a nitrile glove.

2.2.4 Cannabidiol Quantification

To determine plasma CBD concentrations, tail blood was collected on day 3 and trunk blood was collected during euthanasia. Approximately 250 µL of blood was collected and placed into silanized microcentrifuge tubes containing heparin, centrifuged at 10,000 x g for 3 min and plasma was stored at -70°C until quantification by LC-MS. CBD was extracted according to previously described methods (Paudel *et al.*, 2010). Briefly, CBD was extracted from 50 µL of plasma using 500 µL of acetonitrile (ACN):ethyl acetate (1:1, v/v). Samples were vortexed for 1 min, centrifuged for 20 min at 10,000 x g and supernatants were placed into siliconized test-tubes and evaporated under nitrogen at 37°C. Samples were reconstituted with 100 µL of ACN, vortexed for 1 min and sonicated for 5 min before transfer to HPLC vials with silanized low volume HPLC inserts and placed in a Waters Alliance® 2695 HPLC system.

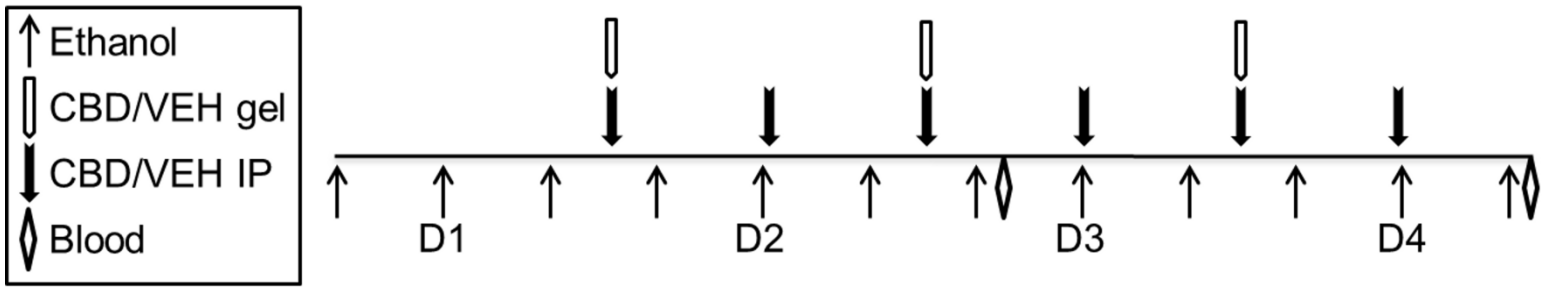

Figure 2.1 Treatment regimen for cannabidiol neuroprotection studies.

Rats were administered ethanol according to a 4-day binge paradigm. In addition to receiving ethanol, rats were co-administered CBD by IP injection twice daily (filled arrows) or by a topical gel formulation daily (open arrows). Plasma samples were collected on day 3 and during euthanasia from tail vein blood or trunk blood, respectively, for determination of BECs and/or plasma CBD concentrations.

CBD was resolved using a Waters Symmetry® C_{18} reversed phase column (5 μm, 2.1 × 150 mm; Milford, MA) fitted with a Sentry Symmetry® C_{18} (3.5 μm, 2.1 x 10 mm) guard column and a mobile phase consisting of ammonium acetate (2mM):ACN (30:70 or 35:65 v/v) at a flow rate of 0.25 mL/min. Electrospray ionization in negative mode was performed for CBD detection (*m/z* 313, retention time 7.7 or 9.8 min) with either a Waters Micromass ZQ™ 2000 mass spectrometer or a Waters Micromass Quattro Micro™ API system (Milford, MA).

2.2.5 Fluoro-Jade B staining and quantification

Following binge treatment, rats were euthanized by an overdose of sodium pentobarbital (Nembutal®, MWI Veterinary Supply, Nampa, ID or Fatal Plus®, Vortech Pharmaceuticals, Dearborn, MI) then perfused transcardially using 0.1 M phosphate buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde (PFA). Brains were extracted, post fixed in 4% PFA at 4°C overnight and stored in PBS at 4°C until sectioning. Brains were cut in a 1:12 series on the coronal plane at 40 μm using a vibrating microtome (Leica Microsystems, Wetzlar, Germany) and stored in cyroprotectant at -20°C. Fluoro-Jade B (FJB) was chosen over amino-cupric silver staining to assess neurodegeneration because it is more cost effective, less time consuming and more consistent [250] and therefore preferable for drug discovery/development studies. Additionally, similar magnitudes of effect are observed following either FJB or silver staining [123], suggesting that FJB is an appropriate alternative to silver stain. FJB staining was performed according to the manufacturer's instructions (Millipore, Billerica, MA) as previously described [114, 115]. A 1:12 tissue series for each animal was washed (3 x 5 min in TBS) then mounted on Superfrost Plus® slides (Fisher Scientific, Pittsburgh, PA) and allowed to air dry overnight. Sections were then rehydrated (5 min, 1% sodium hydroxide in 80% ethanol; 2 min, 70% ethanol; 2

min, ddH_2O), incubated in 0.06% potassium permanganate for 10 min while gently shaking, rinsed in ddH_2O for 2 min and stained with 0.001% (w/v) FJB in 0.1% (v/v) acetic acid for 20 minutes while gently shaking in the dark. Sections were further rinsed (3 x 1 min) with ddH_2O in the dark, dried on a covered slide warmer and cover-slipped in Cytoseal® (Richard Allen Scientific, Kalamazoo, MI). FJB positive (+) cells were quantified at 200X or 400X magnification using an Olympus BX-51 microscope equipped for epifluorescence with a 488λ cube for blue excitation. The entorhinal cortex was defined using a rat brain atlas (Paxinos and Watson, compact 6th edition, 2009) and FJB+ cells were counted by a blinded experimenter in the entorhinal cortex from -3.60 mm through -6.12 mm from bregma and averaged as the number of FJB+ cells/section. Although neurodegeneration can be detected throughout the cortico-limbic pathway, only the entorhinal cortex was quantified as a screen for CBD effects because this brain region has the most reproducible injury severity. Stereology was not used because the entorhinal cortex does not have readily identifiable boundaries necessary for implementing stereological procedures and tissue thickness is difficult to accurately measure with the low background characteristic of FJB staining. Strict criteria were used to identify FJB+ cells: cells were included if they were in cortical layers II or III, displayed a pyramidal cell body characteristic of neurons, and/or had observable proximal dendrites. FJB+ cells were rarely observed in control rats (< 1 cell/section) regardless of CBD treatment and were not significantly different, therefore were collapsed into a single control group for each study.

2.2.6 Statistical analysis

Statistics were performed using Prism (Graphpad version 4.03, La Jolla, CA, USA). Average intoxication behavior was analyzed by Kruskal-Wallis test for non-parametric data followed by Dunn's post-hoc test when appropriate. Average daily dose,

CBD plasma concentrations, and BECs were analyzed by ANOVA followed by Bonferroni post-hoc tests when appropriate. FJB data was analyzed using ANOVA followed by planned post-hoc t-tests. Significant variability in FJB+ cell counts was expected based on previous experience with the binge model; therefore, experiments were designed *a priori* with the intention of collapsing ethanol and ethanol + vehicle rats in order to reduce the number of animals used while maintaining power. Additionally, the experiments were designed *a priori* to collapse control groups as FJB is rarely observed (< 1 cell/section) in these rats. Values are presented as mean ± standard error of the mean and analyses were considered significant at $p < 0.05$.

2.3 Results

2.3.1 Experiment 1: Optimization of CBD transdermal delivery for neuroprotection

Experiment 1 tested the neuroprotective effects of 1.0% (n = 5), 2.5% (n = 4) and 5.0% (n = 6) CBD gels. First, in order to rule out potentially confounding effects of CBD or vehicle treatment on neuroprotection measures; intoxication behavior, ethanol dose and BECs were compared across treatment groups. Rats treated with ethanol only (n = 9) and ethanol plus vehicle gel (n = 6) were indistinguishable across all measured variables, therefore these groups were collapsed. Regardless of treatment, all rats displayed similar intoxication behavior across the four days of binge treatment (Figure 2.2A). The grand mean intoxication behavior was 2.5 ± 0.1 out of 5, which is indicative of rats being intoxicated to the level where they displayed a delayed righting reflex and ataxia. Analysis of mean intoxication behavior (Figure 2.2A inset) revealed a main effect of treatment [$H_{(3)} = 8.258$; $p < 0.05$] and post-hoc tests indicated a significant difference between the ethanol/ethanol + vehicle and ethanol + 1.0% CBD groups ($p < 0.05$).

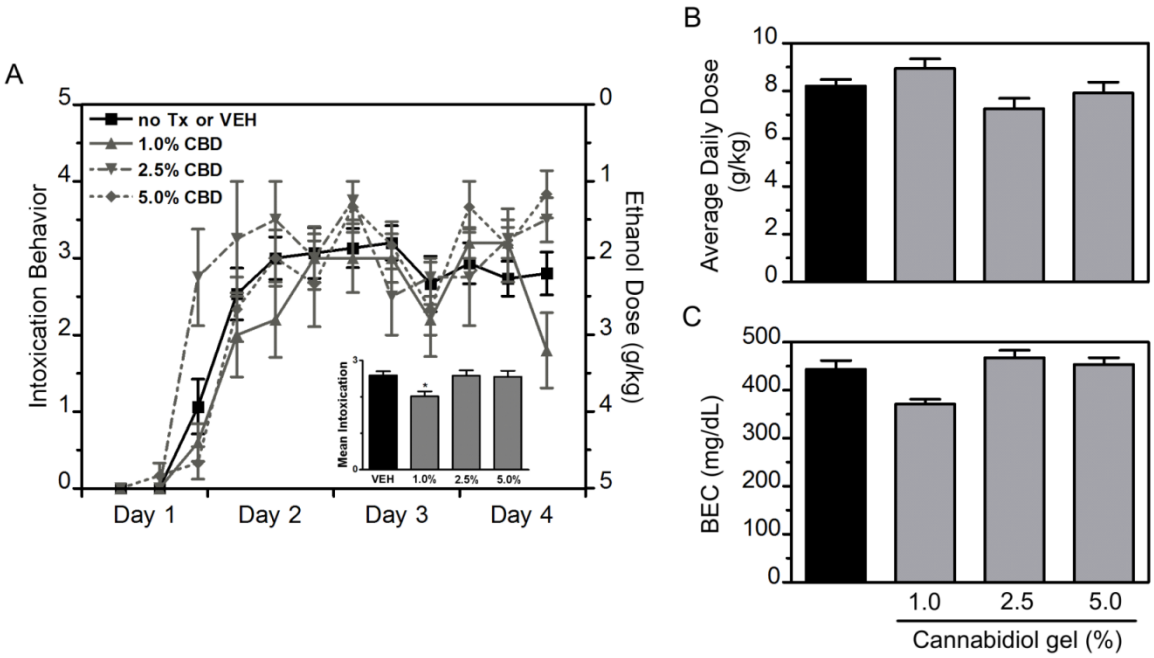

Figure 2.2 Binge data for transdermal CBD optimization experiment.

Rats were treated according to the 4-day binge paradigm and administered nothing, vehicle, 1%, 2.5% or 5.0% CBD gel formulations. Ethanol only and ethanol + vehicle groups were statistically similar and therefore collapsed (black bars). (A) Behavioral intoxication scores were similar across groups regardless of treatment (left axis), therefore received similar doses of ethanol (right axis). (B,C) The average daily doses and BECs also did not differ among treatment groups.

Although a significant difference in intoxication was observed between these two groups, the effect was not large enough to result in different amounts of ethanol administered. The grand mean ethanol dose for rats in this experiment was 8.2 ± 0.2 g/kg/day, which was not different among groups (Figure 2.2B). Accordingly, the grand mean peak BEC was 436.9 ± 11.1 mg/dL. Although one-way ANOVA revealed a significant effect of treatment [$F_{(3,29)} = 3.085$; $p = 0.045$], post-hoc analysis failed to reveal a significant difference between groups (Figure 2C). These data indicate that transdermal vehicle or transdermal CBD did not alter the intoxicating effects or pharmacokinetics of ethanol. Additionally, these binge data are similar to previous reports using the modified Majchrowicz binge model (Morris *et al.*, 2010).

Substantial FJB+ staining was observed in the entorhinal cortex following 4-days of binge ethanol treatment (Figure 2.3). These cells were typically found in cortical layers II and III adjacent to the rhinal fissure and extending ventrally. FJB+ cells were rarely observed in control rats and control groups did not differ significantly, therefore, all controls were collapsed (n = 22). Ethanol only and ethanol + vehicle gel rats displayed statistically similar FJB+ cell counts, therefore these groups were collapsed prior to analysis. One-way ANOVA revealed a main effect of treatment [$F_{(4,47)} = 13.71$, $p < 0.0001$]. Post-hoc tests indicated that rats treated with 1.0% or 2.5% CBD gels had similar FJB+ cell counts as ethanol/ethanol+veh gel rats. However, rats treated with 5.0% CBD gels had a 48.8% reduction in the number of FJB+ cells, which trended to statistical significance ($p = 0.069$).

CBD plasma concentrations were analyzed at the beginning of day 3 and at euthanasia (Figure 2.1). Control rats treated with 2.5% CBD gel were not included in this experiment therefore a two-way ANOVA was not performed. However, a one-way ANOVA of ethanol groups revealed a main effect of CBD gel percentage (Figure 2.4A; [$F_{(2,12)} = 4.492$; $p < 0.05$]).

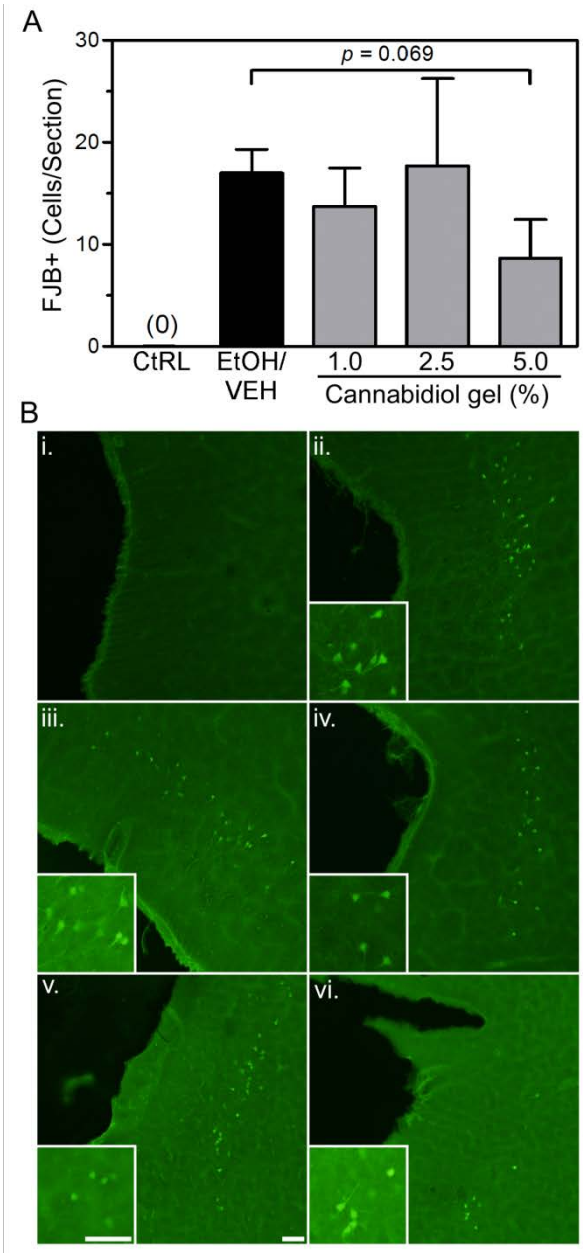

Figure 2.3 Fluoro-Jade B staining in the entorhinal cortex following 4-day binge treatment.

(A) Quantification of FJB. Control rats typically had < 1 FJB+ cell/section therefore were collapsed across treatment groups. Additionally, ethanol and ethanol + vehicle treated rats were indistinguishable, therefore collapsed (black bar). (B) Representative images for each treatment group. i. control; ii. ethanol; iii. ethanol + vehicle; iv. ethanol + 1.0% CBD; v. ethanol + 2.5% CBD; vi. ethanol + 5.0% CBD. Scale bars = 50 μm.

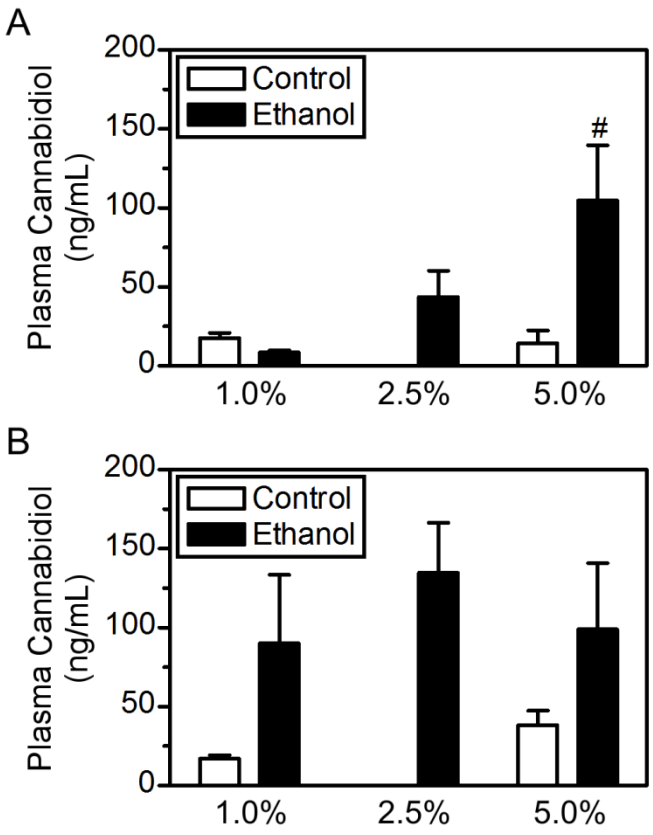

Figure 2.4 Cannabidiol plasma levels following application of transdermal gels containing 1.0%, 2.5% or 5.0% CBD.

A) CBD plasma levels quantified from tail vein blood collected 3 days into binge treatment. B) CBD plasma levels quantified from trunk blood collected at euthanasia. #, $p < 0.05$ compared to ethanol + 1.0% CBD.

Post-hoc analysis showed that 5.0% CBD gels resulted in significantly higher CBD plasma concentrations compared to the 1.0% CBD gel group ($p < 0.05$). However, at euthanasia, CBD plasma concentrations were similar between ethanol groups (Figure 2.4B; [$F(2,13) = 0.29$; $p > 0.05$]).

2.3.2 Experiment 2: Neuroprotective effects of transdermal and IP CBD delivery

Ethanol intoxication measures in this experiment were similar to experiment 1 and the intoxicating effects of ethanol were similar between ethanol only (n = 13), vehicle IP (n = 12), CBD IP (n = 15), vehicle gel (n = 7) and CBD gel (n = 9) groups across the 4-days of binge treatment (Figure 2.5A). The grand mean intoxication score was 2.2 ± 0.1 out of 5 (Figure 2.5A insert); thus rats in this experiment were intoxicated to the level of delayed righting reflexes and ataxia. Additionally, each treatment group in this study received similar doses of ethanol, which on average were 8.4 ± 0.2 g/kg/day (Figure 2.5B). The grand mean peak BEC for this experiment was 380.4 ± 7.8 mg/dL, which did not differ between groups (Figure 2.5C), confirming that the drug treatments had no effect on the intoxicating effects or dosing of ethanol.

Four days of binge ethanol exposure resulted in neurodegeneration as indicated by the presence of FJB+ cells along the entorhinal cortex. The severity of ethanol-induced damage in the entorhinal cortex was similar between experiment 1 (Figure 2.3) and experiment 2 (Figure 2.6). Similar to Experiment 1, controls (n = 34) were statistically similar and therefore collapsed across drug treatment. In contrast to the analysis conducted in experiment 1, ethanol only and ethanol + vehicle groups were not collapsed because the vehicles in this study were delivered by different routes of administration. One-way ANOVA revealed a main effect of treatment [Fig. 2.6; $F_{(5,84)} = 10.63$; $p < 0.0001$].

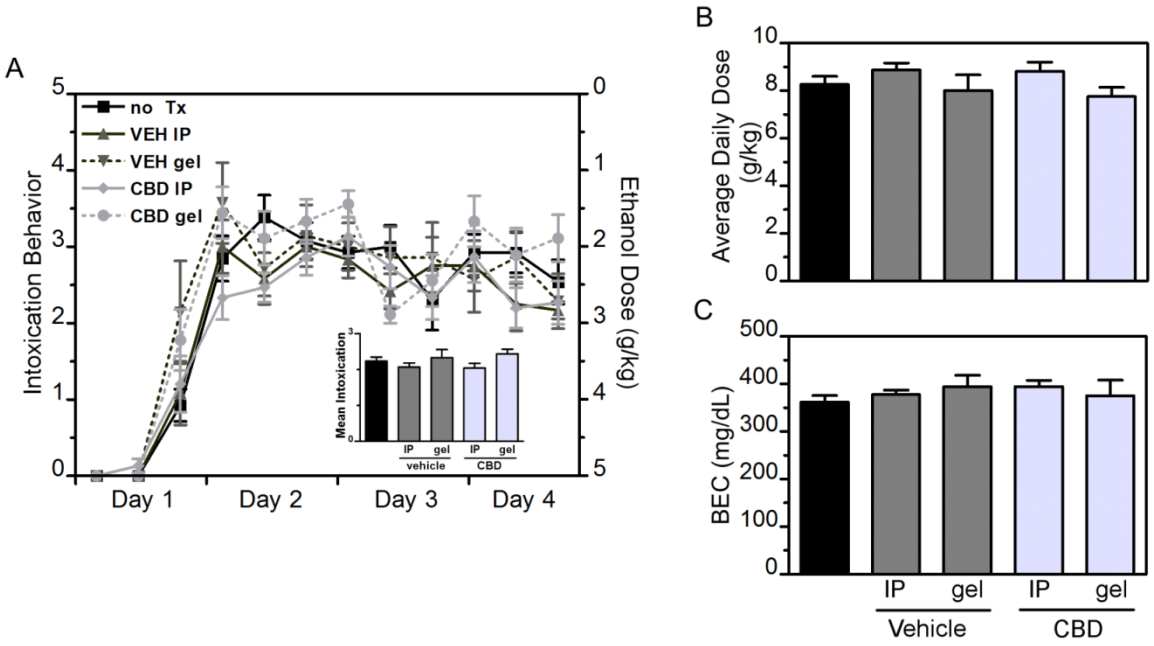

Figure 2.5 Ethanol binge data for rats treated with CBD or vehicle by an optimized transdermal gel or IP injection.

(A) Behavioral intoxication scores (left axis) and ethanol doses (right axis) were similar across groups at all timepoints. (B,C) Average daily ethanol doses and BECs did not differ between treatment groups.

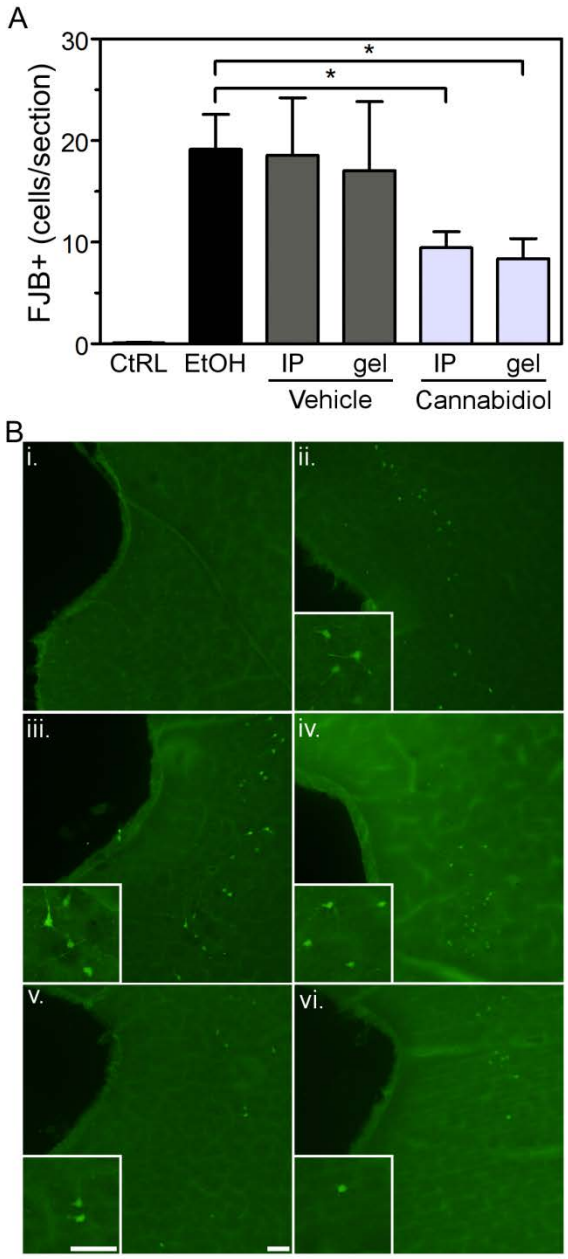

Figure 2.6 FJB staining in the entorhinal cortex following 4-day binge treatment. (A) Quantification of FJB. Control rats typically had < 1 FJB+ cell/section therefore were collapsed across treatment groups. (B) Representative images for each treatment group. i. control; ii. ethanol; iii. ethanol + VEH IP; iv. ethanol + VEH gel; v. ethanol + CBD IP; vi. ethanol + 2.5% CBD gel. *; $p < 0.05$. Scale bars = 50 μm.

Post-hoc analysis indicated that administration of CBD by IP administration significantly reduced FJB+ cells in the entorhinal cortex by 50.6% compared to the ethanol only group ($p < 0.05$). Similarly, transdermal administration of CBD significantly reduced FJB+ cells in the entorhinal cortex by 56.1% compared to the ethanol only group ($p < 0.05$). Although IP and transdermal CBD administration reduced FJB+ cells by 49.0% and 51.0% compared to their respective vehicle controls, this effect did not reach statistical significance ($p > 0.05$).

The mean plasma concentration from the 5% CBD gel group in experiment 1 (Figure 2.4) was used as a target concentration for experiment 2 as this group displayed promising neuroprotective effects. Therefore, a plasma concentration of ~100 ng/mL was targeted following transdermal CBD treatment using a second generation gel formulation from AllTranz Inc. Although the new formulation in experiment 2 only contained 2.5% CBD; day 3 target plasma concentrations of ~100 ng/mL was attainable on average (Figure 2.7A). Two-way ANOVA revealed main effects of diet [$F_{(1,25)} = 7.480$; $p < 0.05$] and time point [$F_{(1,25)} = 14.75$; $p < 0.001$], with a signification interaction [$F_{(1,25)} = 7.398$; $p < 0.05$]. Post-hoc analysis revealed that CBD plasma levels were significantly lower in binge ethanol treated rats at the day 3 time point compared to controls ($p < 0.01$). Additionally, control CBD plasma levels during euthanasia were significantly lower than at day 3 ($p < 0.001$). CBD plasma levels following IP administration (40.0 mg/kg/d) were substantially higher than concentrations achieved following transdermal application (Figure 2.7B) and were indistinguishable between control and ethanol treated rats.

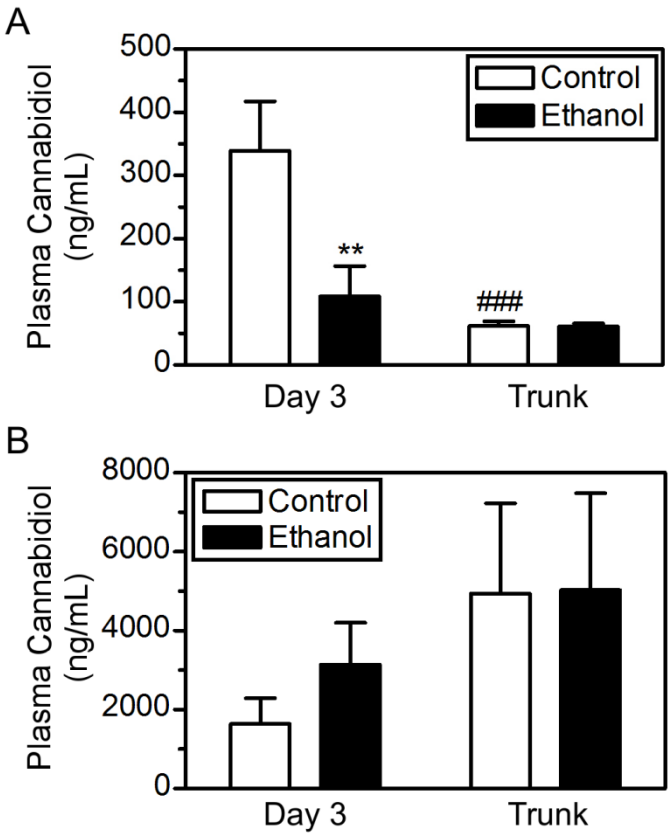

Figure 2.7 Cannabidiol plasma levels following application of an optimized cannabidiol transdermal gel formulation or after IP injection of cannabidiol.

(A) CBD plasma levels on day 3 and at euthanasia quantified following transdermal gel application (B) CBD plasma levels on day 3 and at euthanasia quantified following IP injection. **, p <0.01, ###, p < 0.001 compared to day 3 control.

2.4 Discussion

The current study examined the neuroprotective effects of transdermal CBD systems in an accepted model of an AUD that produces substantial neurodegeneration in the cortico-limbic pathway. The first experiment was a pilot study to determine CBD plasma concentrations necessary to observe neuroprotection following transdermal CBD treatment. The 5% gel formulation in this experiment produced promising neuroprotective effects, a 48.8% decrease ($p = 0.069$), while the 1.0% and 2.5% gels were ineffective (Figure 2.3). The mean day 3 CBD plasma concentration for the 5% CBD gel group was ~ 100 ng/mL and was used as a target concentration because neuroprotection outcomes were promising for this group (Figure 2.4). In experiment 2, an optimized formulation was developed by AllTranz Inc. to efficiently deliver CBD at the target plasma concentration while using less CBD (Figure 2.7). Importantly, the neuroprotective effects of transdermal delivery of CBD were comparable to the magnitude of neuroprotection observed following IP injection (Figure 2.6). Although the degree of neuroprotection appeared to be modest, a 50-60% reduction in FJB+ cells in the entorhinal cortex is similar to previous studies testing neuroprotective agents using the same 4-day binge model [120-123]. Therefore, these results justify additional preclinical development of transdermal CBD for the treatment of alcohol-induced neurodegeneration. Furthermore, preclinical development of neuroprotective agents for the treatment of AUDs is warranted because alcohol-induced brain damage is hypothesized to be critical in promoting impairments in executive self-regulatory behavior, thus contributing to the downward spiral to addiction [60, 61].

Interestingly, this study showed that transdermal and IP delivery of CBD produced similar magnitudes of neuroprotection although IP administration resulted in substantially higher CBD plasma levels. Although a full dose-response experiment was not conducted and these studies were not designed to examine the entire PK profile, the

current data could suggest that the maximum effective concentration (EC_{max}) of CBD to attenuate FJB+ cells was achieved following both routes of administration. However, an earlier study by Hamelink et al. failed to observe neuroprotection in the same binge model following IP administration of CBD at 20.0 mg/kg/d [121], a dose likely to result in plasma concentrations higher than the levels reached following transdermal delivery in the current study. Therefore, it is unlikely that CBD plasma concentrations following transdermal delivery were above the EC_{max}. Alternatively it is possible that neuroprotection observed following transdermal CBD and IP CBD are mediated though different mechanisms. It has been suggested that the neuroprotective effects of CBD observed during binge alcohol induced neurodegeneration are due to its high antioxidant capacity [121, 236], however, CBD has a plethora of pharmacological targets that may afford neuroprotection. For example, CBD is an inhibitor of eCB cellular reuptake and metabolism and an agonist at adenosine A_{2A}, serotonin 5-HT_{1A} and transient receptor potential cation channel VI (TRPV1) receptors, all targets implicated in neuroprotection [251-254]. Interestingly, many of the receptor mediated effects of CBD follow an inverted u-shaped curve, which is also evident for many of the neuroprotective and anti-inflammatory effects of CBD [167, 252, 255-257]. In fact, a study by Mishima et al., found that CBD prevented cerebral infarction via 5-HT_{1A} receptors at 1.0 and 3.0 mg/kg, but not 0.1 or 10 mg/kg [257]. Therefore, it is possible that CBD plasma concentrations achieved following transdermal delivery are conducive to receptor mediated (possibly 5-HT_{1A}) neuroprotection, while higher IP doses, although out of the range for receptor mediated neuroprotection, have effects primarily though antioxidant effects. Alternatively, the neuroprotection observed following transdermal CBD and IP CBD could be related to the different pharmacokinetic profiles expected following each route of administration. It is well known that cannabinoids rapidly distribute to fatty tissue including the brain [258] and although CBD concentrations were not measured in the

brain, it would be interesting to determine how transdermal and IP delivery at these doses differentially affect the brain penetrance of CBD. For example, a recent study found that C_{max} and estimated exposure (AUC) in the brain was higher following oral administration compared to IP, which suggests that different routes of administration and their resulting pharmacokinetic profiles differentially affect CBD accumulation in the brain [259]. Therefore, an alternative interpretation to explain the similar magnitudes of neuroprotection following transdermal and IP administration of CBD could be that transdermal administration at these doses optimizes brain distribution of CBD.

Importantly, we observed a positive relationship between CBD gel percentage and day 3 CBD plasma concentrations in ethanol treated rats, while CBD plasma concentrations were similar across the 1.0%, 2.5% and 5.0% CBD groups during euthanasia (Figure 2.4). Although CBD plasma levels were similar at euthanasia, only 5.0% CBD resulted in promising neuroprotective effects (Figure 2.3). These observations highlight the importance of administering CBD at therapeutic levels early during binge ethanol treatment. CBD treatment was initiated following the third dose of ethanol (Figure 2.1), similar to other studies demonstrating neuroprotection following antioxidant treatment [120, 121]. Neuroprotective agents are likely to be more efficacious when administered at these early timepoints because cellular stress and neurodegeneration can be detected following as few as 1 or 2 days of binge ethanol treatment [112, 119]. For example, unpublished observations show significant impairments in mitochondrial bioenergetics following 2 days of binge treatment [139]. Impairment in mitochondrial function is likely a causal factor contributing to alcohol-induced neurodegeneration as these impairments result in the production of oxidative stress [139]. As CBD is thought to be neuroprotective partially through antioxidant properties, it is possible that CBD attenuates oxidative stress caused by impairments in the mitochondrial electron transport chain. Collectively, these results suggest that

neuroprotective agents, including transdermal CBD, need to be administered at therapeutic levels before ethanol-induced neurotoxic events are irreversible.

Enhanced neuroprotection might be observed by administering CBD as a pretreatment in addition to treatment during binge exposure; however this strategy was not implemented in order to mimic a feasible human application for transdermal CBD. For example, an individual could apply a CBD patch if a relapse event occurred and not prophylactically as a pretreatment study would mimic. However, a prophylactic strategy should not be dismissed and may enhance the value of transdermal CBD for the treatment of a variety of other pathologies associated with AUDs in addition to alcohol-induced neurodegeneration. Alcoholism is a cyclical disease consisting of periods of binge intake, acute physical withdrawal, protracted withdrawal and ultimately relapse, which all may be treated by extended release formulations of CBD [244, 245, 260]. For example, CBD has anticonvulsant effects (acute withdrawal), anxiolytic effects (protracted withdrawal/relapse), reduces drug seeking behavior in rodents (craving/relapse) and has neuroprotective properties (binge intoxication). Therefore, a prophylactic strategy for transdermal CBD treatment could be beneficial if future studies demonstrate efficacy for these other pathologies associated with AUDs. Furthermore, transdermal delivery of other medications, such as naltrexone and acamprosate, could enhance the utility of pharmacotherapy based treatments for alcohol dependence in general. Transdermal delivery is a controllable extended release formulation [248], therefore improves patient compliance because medications can be administered less frequently. Additionally, transdermal products are non-invasive which promotes patient friendly usage, in contrast to injectable formulations. These are important considerations for treating alcohol dependence as compliance has been low for currently approved mediations [249].

Although the results of the current study are promising, there are developmental hurdles that need to be overcome in order to translate these findings into a feasible treatment for AUDs. Noteworthy, plasma concentrations achieved by the first generation gel formulation in experiment 1 were consistently higher in ethanol treated rats (Figure 2.4). Additionally, CBD plasma concentrations in these rats were ~100 ng/mL prior to euthanasia regardless of gel percentage. Although the current data is insufficient to definitively explain these observations, it is possible that the high BECs achieved during binge ethanol treatment may interfere with the pharmacokinetics of transdermal CBD. For example, studies have shown that forced ethanol consumption in rodents, producing BECs greater than 100 mg/dL, can result in moisture loss in the stratum corneum [261]. Dehydration of the stratum corneum could theoretically affect CBD transdermal flux. Furthermore, it is well-known that ethanol interferes with the metabolism of som drugs [262]. For example, acute ethanol exposure commonly inhibits hepatic metabolism, while chronic ethanol exposure enhances drug metabolism and clearance [263]. Although it is currently unknown whether altered metabolism of CBD occurs following binge ethanol treatment, this is an important consideration for future drug development efforts. In experiment 2, which utilized the second generation gel formulation, CBD plasma levels in control rats were significantly lower prior to euthanasia compared to day 3 measurements (Figure 2.7). This observation was expected because of the greater lag time between CBD gel application and plasma CBD quantification during euthanasia (see Figure 2.1). In contrast to the first generation CBD gel formulation, the second generation gel formulation resulted in lower CBD plasma concentrations in ethanol treated rats compared to controls at day 3 (Figure 2.7). Although the reason for this discrepancy is currently unknown, this observation may be related to intrinsic differences in the transdermal flux of CBD between the two formulations. Even in light of these technological issues, neuroprotection was observed following transdermal CBD delivery.

Therefore, future drug development studies are warranted and should be focused on further understanding and optimizing transdermal CBD systems in intoxicated rodents.

3. CHAPTER 3

SIMULTANEOUS QUANTIFICATION OF ANANDAMIDE, OLEOYLETHANOLAMIDE AND PALMITOYLETHANOLAMIDE IN RODENT BRAIN TISSUE USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY-ELECTROSPRAY MASS SPECTROSCOPY

3.1 Introduction

The discovery of the eCB system in the early 1990's intensified research on cannabinoid physiology and pathophysiology. The first receptors identified with affinity for Δ^9-THC and other synthetic cannabinoids were the CB1R [156], followed by the CB2R [157]. Simultaneously, the endogenous ligands were being discovered, with the identification of AEA in the porcine brain first [165] followed by 2-AG [167]. Since these seminal discoveries, researchers quickly became aware of the complexities of the eCB system. It is now known that multiple receptors have the capacity to bind cannabinoid ligands such as TRPV1 [264], GPR55 [265] and PPAR isoforms [266], which suggests significant crosstalk among different signaling systems. Although 2-AG and AEA are the most widely studied, they are not the only endogenous ligands with the capacity to bind cannabinoid receptors [159]. Moreover, other bioactive lipids, particularly the NAEs, acylglycerols, and acylamides have been shown to be important in cannabinoid physiology as they act as entourage compounds by enhancing the activity of AEA and 2-AG [267]. For example, both PEA and OEA have been shown to reduce the degradation of AEA through competition for FAAH [268, 269] and by reduction of FAAH expression, as is the case for PEA [270]. Interestingly, OEA and PEA have the capability to displace both [^{3}H]-CP55,940 and [^{3}H]-WIN55,212-2 [269] from CB1Rs and CB2Rs. It has also been demonstrated that both PEA and OEA can increase the affinity of AEA at TRPV1 receptors [271], an effect which contributes to the crosstalk between eCB and other

signaling systems. As the understanding of eCB physiology and pathophysiology requires an understanding of these lipid classes as a whole, rather than in isolation, it is critical to develop analytical techniques that can simultaneously measure several eCBs as well as entourage compounds from biological tissue under normal and pathological conditions. Furthermore, development of easy, accurate, and reproducible analytical techniques to monitor NAEs is of interest because FAAH inhibitors are currently undergoing preclinical and clinical testing.

A variety of analytical methods have been developed for the measurement of eCBs and other related lipids since their initial discovery [272]. Many of the initial methods used gas chromatography – mass spectroscopy (GC-MS) procedures to measure AEA [273] and/or 2-AG [274]; however, eCBs typically need to be derivatized to increase their volatility, which is complex and time consuming [272]. Therefore, more recent methods for eCB quantification take advantage of liquid chromatography – mass spectroscopy (LC-MS) [275, 276] and more commonly LC-MS-MS [277, 278] techniques to quantify eCBs as additional derivatization procedures are not necessary. Although MS-MS systems have benefits over MS systems for the measurement of eCBs and related compounds, such as increased sensitivity, single quadrupole systems may be the only available option for many laboratories, thus development of methods for MS is valuable and necessary.

eCB extraction from biological matrices and sample preparation widely differs among published analytical methods. Chloroform/methanol liquid extraction is commonly used for GC-MS and sometimes for LC-MS and LC-MS-MS, however these methods are typically followed by sample clean-up using solid phase extraction (SPE) or thin-layer chromatography (TLC) [272]. SPE and TLC procedures are time consuming and expensive, therefore not ideal for analysis of multiple biological samples and/or high throughput drug discovery. Moreover, organic solvents such as toluene [279], ethyl

acetate/hexane [277, 280] or ACN [275, 281] have been used for protein precipitation and lipid extraction; however, many of these reported methods still require further sample clean-up using SPE or TLC.

Another major consideration for accurate quantification of eCBs is the effect of biological matrices on process efficiency, which can greatly affect accurate estimation of eCB content. Reports that include validation in biological matrices have demonstrated that extraction efficiencies for eCBs can deviate greatly and this effect is compound specific [277]. Therefore, the biological matrix should be factored in when developing analytical methods and should be considered for each compound of interest.

In this study, simultaneous measurement of AEA, OEA and PEA is reported in rodent whole brain tissue. This study is the first report that demonstrates the feasibility of quantifying this combination of analytes with a simple protein precipitation procedure followed by single quadrupole LC-MS detection using low milligrams of brain tissue. Notably, this method was validated in whole brain matrix while accounting for the endogenous nature of these eCB species. Additionally, AEA, OEA and PEA were quantified following FAAH inhibition by URB597 which demonstrated that URB597-mediated elevations in NAEs are both brain region and compound specific.

3.2 Materials and Methods

3.2.1 Chemicals

Methanol and ACN were HPLC grade, while all other chemicals used were analytical grade. AEA, OEA and PEA were all purchased from Cayman Chemicals (Ann Arbor, MI, USA) and had a purity of ≥ 98%. Methanol and ACN were purchased from VWR International (Batavia, IL, USA), acetic acid was purchased from Fisher Scientific (Fairlawn, NJ, USA) and ammonium acetate was purchased from Mallinckrodt Chemicals (Phillipsburg, NJ, USA). Water was obtained from a Milli-Q® Advantage A10

purification and filtration system (Millipore, Billerica, MA, USA). Mobile phase was filtered at 0.2 µm using a nylon membrane filter (Supelco, Bellefonte, PA, USA).

3.2.2 Calibration and quality control sample preparation

Stock solutions of AEA, OEA and PEA were prepared in ethanol at 2.0 mg/mL and stored at -20°C. From these stock solutions, 50 µg/mL working stocks and working solutions of appropriate concentration were prepared for each compound in ACN. From the working solutions, calibration and quality control (QC) samples were prepared for each compound by adding 5 µL of analyte working solution of appropriate concentration to 95µL of ACN or tissue homogenate. Calibration curves were constructed for AEA using the following calibration concentrations: 2.5, 5, 15, 25, 50 and 100 ng/mL, while curves for OEA and PEA were constructed using the following concentrations: 5, 10, 25, 50, 100, 250 and 500 ng/mL. QC samples for AEA were prepared at three concentration levels including 7.5, 35 and 75 ng/mL, while QC samples for OEA and PEA were prepared at 15, 30 and 90 ng/mL. The concentration range of calibration and QC samples were chosen based on preliminary studies and literature reports for analytes of interest to encompass baseline endogenous levels of these NAEs as well as elevated NAE levels resulting from treatment with the FAAH inhibitor URB597 [174].

3.2.3 Sample extractions

NAEs were extracted from brain tissue using a protein precipitation protocol modified from Chen et al. 2009 [275]. Brain tissue was weighed and homogenized with equal volumes of ice cold saline in a siliconized microcentrifuge tube by rapid sonication on ice using a Sonic Dismembrator (Fisher Scientific, Fairlawn, NJ, USA). Following sonication, 100 µL of homogenate was transferred to a fresh siliconized microcentrifuge tube followed by the addition of 1 mL of ACN. Samples were then vortexed for

approximately 30 sec and centrifuged at 13,000 g for 20 min at 4°C. ACN extracts were then placed into a 5 mL siliconized test tube and gently evaporated under nitrogen at 37°C. NAEs were extracted with ACN two additional times and extracts were pooled before reconstitution by evaporating repeated extractions in the same test tube. Following evaporation, samples were reconstituted in 100 µL ACN. To ensure maximum extraction of the analytes of interest from the dry residue, samples were vortexed for 30 sec, sonicated in an ice cold water bath for 15 min and vortexed again for 30 sec. Samples were then transferred to siliconized microcentrifuge tubes and centrifuged at 13,000 x g at 4°C to remove precipitates following reconstitution. Finally, the reconstituted samples were transferred to HPLC vials fitted with siliconized low volume inserts and placed in a temperature regulated autosampler (4°C) for analysis. A 20 µL aliquot of sample was injected for LC-MS quantification. To ensure maximal extraction of analytes of interest, the effect of one vs. three extraction cycles on NAE recovery was investigated. To that end, homogenates were created and extracted one or three times according to the procedures described above and the relative MS signal between one and three extractions was compared as percent of one extraction cycle.

3.2.4 LC-MS conditions

HPLC was performed using a Waters Alliance 2695 LC pump (Waters, Milford, MA, USA) equipped with a Waters Alliance 2695 autosampler and thermostatic column compartment which was maintained at 37°C. Separation was achieved using a Waters Symmetry® C18 (2.1 x 150 mm, 5 µm) column coupled with a Waters Symmetry® C18 guard column (2.1 x 10 mm, 3.5 µm). A gradient elution protocol was adapted from Patel et al., 2003 [281] with mobile phase A consisting of 1 mM ammonium acetate with 0.1% acetic acid (v/v) in methanol and mobile phase B consisting of 1 mM ammonium acetate with 0.1% acetic acid (v/v) and 5% methanol in water. Initial conditions were set at 70%

A and 30% B. A was increased linearly to 85% over 25 min and maintained for 1 min. A was then increased linearly to 100% over 1 min and held at 100% for 5 min. Finally, A was returned linearly to 70% over 1 min and held for 10 min for column equilibration. Flow rate was maintained at 0.3 mL/min. The MS detector used was a Micromass® ZQ™ (Waters, Milford, MA, USA) with an electrospray ionization probe (ESI). The MS conditions were set according to Chen et al., 2009 [275]: nitrogen desolvation gas 450 L/hr, nitrogen cone gas 50 L/hr, source temperature 120°C, desolvation temperature 250°C, capillary voltage 3.5 kV, cone voltage 25 kV, extractor voltage 5.0 kV and RF lens voltage 0.5 kV. ESI was set to the positive mode and selective ion monitoring was set to the following protonated ions, *m/z* 348.28 $[M + H]^+$ (AEA), *m/z* 326.6 $[M + H]^+$ (OEA) and *m/z* 300.5 $[M + H]^+$ (PEA) with dwell times of 0.3 sec for each ion.

3.2.5 Validation

The method was validated by examining linearity of standard curves, LLOQ, intra- and interday accuracy, intra- and interday precision, process efficiency (PE) and short-term stability of NAEs in brain extracts at 4°C. AEA, OEA and PEA linearity was evaluated over concentration ranges from 2.5 ng/mL to 100 ng/mL for AEA and 5 ng/mL to 500 ng/mL for OEA and PEA in ACN and brain tissue by performing linear regression analysis. The LLOQ for each analyte was defined as the lowest concentration producing a peak height (signal) 10x greater than the baseline height (noise). This concentration was back calculated from a linear regression analysis of signal: noise vs. analyte concentration at analyte levels ranging from 2.5 ng/mL to 25 ng/mL. Accuracy and precision was determined for each analyte at three different QC levels. Intraday QCs were run in triplicate on two separate occasions and accuracies and precisions were reported for intraday1 and intraday2. Interday QCs (n=8) were run on separate days with each day ranging from 1-3 replications. Accuracy was calculated as the following,

accuracy = (calculated concentration / nominal concentration)*100, while precision (% coefficient of variation, CV) was calculated as, %CV = (standard deviation / mean calculated concentration)*100. Accuracy and precision were considered acceptable when within 15%. PE was calculated for each analyte at each QC level and was defined as the ratio between the relative MS signal of the QC extracted from homogenate and the relative MS signal of the QC in ACN; PE = (QC spiked/QC in ACN)*100. To study short-term stability of AEA, OEA and PEA, extracts were stored in the autosampler at 4°C and injected at T=0 hr and T=18 hr. Relative MS signal between the two time points were compared as percent of T=0 hr.

As endogenous AEA, OEA and PEA may interfere with standardization and validation, great care was taken to ensure uniformity of homogenates. To that end, bulk brain homogenate used in the preparation of calibration and QC samples was produced by sonication on ice followed by vigorous vortexing. With this procedure, consistent background levels of AEA, OEA and PEA were achieved as repeated background measurements (n = 5-7) varied by only 8.1%, 6.7% and 3.3%, respectively. Background signal of each analyte was subtracted prior to calculation of the measured calibrators and QC concentrations. In order to accurately measure background levels for each analyte, multiple background measurements were collected so that each batch of bulk homogenate generally had 5-7 background measurements.

3.2.6 Biological application

Adult male Sprague Dawley rats weighing approximately 330 grams (n = 12; Charles River, Raleigh, NC) were used in this study. All treatment protocols followed the *Guide for the Care and Use of Laboratory Animals* by the National Research Council (1996) and were approved by the University of Kentucky Institutional Animal Care and Use Committee. Rats were singlely housed in Plexiglas cages in a University of

Kentucky vivarium on a 12 hr light/dark cycle with access to rat chow and water *ad libitum* during a 5 day acclimation period. Rats were also handled for at least 3 days. During experimentation, rat chow was removed from cages and rats were fed a nutritionally complete diet consisting of Vanilla Ensure Plus© (Abbott Laboratories, Columbus OH) and dextrose in water. Rats received diet by oral gavage every 8 hr for 24 hr. Rats were treated with the FAAH inhibitor (n=6), URB597 (0.3 mg/kg) by intraperitoneal injection at a concentration of 1.0 mg/mL in dimethylsulfoxide (DMSO) or vehicle (n=6). Two hours after injection, rats were euthanized by rapid decapitation, brains were extracted, brain regions dissected and placed in a microcentrifuge tube and flash frozen using a slurry of dry ice and 70% ethanol. The entire process from decapitation to rapid freezing was kept under 6 minutes to minimize post-mortem accumulation of NAEs [282]. Tissue was stored at -80°C until processing for NAE quantification. Fifty milligrams of tissue was sufficient to achieve quantifiable levels of each NAE in hippocampi and entorhinal corticies. System suitability criteria were met prior to sample batch analysis.

3.2.7 Statistical analysis

Statistics were performed using GraphPad Prism (GraphPad version 4.03, La Jolla, CA, USA). Linear regression was used to assess linearity of calibration curves for AEA, OEA and PEA. Student's t-tests were used to compare differences in AEA, OEA and PEA content between vehicle and URB597 treated rats for each brain region. Statistical significance was accepted at $p < 0.05$. Values are given as mean ± SEM unless otherwise indicated.

3.3 Results and Discussion

3.3.1 Method Development

In this report, NAEs were extracted from whole brain tissue. The simultaneous measurement of AEA, OEA and PEA required optimization of the gradient elution protocol used in Patel et al., 2003 [281] to prevent peak interference from co-eluting analytes. AEA, OEA and PEA eluted at 21.32 min, 25.92 min and 24.24 min, respectively, as shown in Figure 3.1. NAE quantification using LC-MS typically requires long run times [275, 276]. Therefore method optimization towards shorter elution times provides significant advantages when analyzing biological samples such as decreasing the possibility of sample instability in the autosampler and increasing sample throughput. With the current protocol, the run time was decreased by 7 min relative to Chen et al., 2009 [275]. Moreover, during method development, a final step involving washing with 100% methanol for 5 min was incorporated into the gradient protocol to ensure elution of peaks that would otherwise cause interference on subsequent runs. Recovery of eCBs from biological tissues is typically performed using liquid-liquid extraction/precipitation protocols [272]. It is suggested that different solvent conditions can result in better extraction efficiencies for specific eCBs [283], thus preliminary experiments were performed using different solvents and solvent combinations previously shown to be effective for eCB extraction. These solvents included ACN [275, 281, 284], methanol [285] and a 9:1 ethyl acetate/hexane mixture [277, 280]. ACN and methanol extraction produced similar chromatograms and extraction efficiencies while the ethyl acetate/hexane extraction was more time consuming and did not offer any significant benefit over the other two protocols (data not shown). Considering the well documented superiority of ACN over methanol as a protein precipitant [286], ACN was selected for NAE extractions.

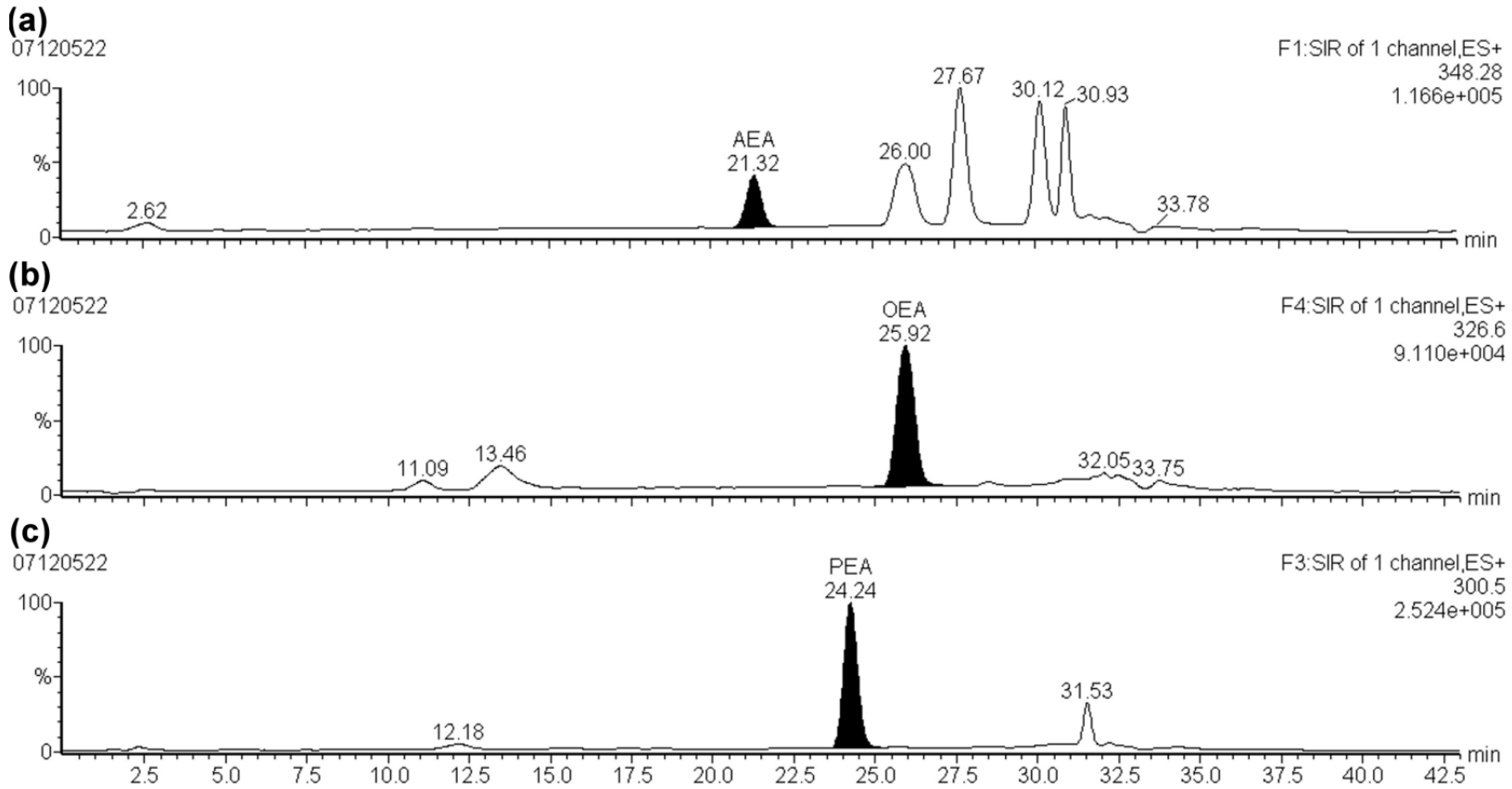

Figure 3.1 Representative LC-MS chromatograms of NAE analytes in whole brain tissue.

(a) AEA, RT 21.32 min. (b) OEA, RT 25.92 min. (c) PEA, RT 24.24 min.

The effect of single versus multiple extraction cycles was also examined in order to ensure maximal analyte recovery from brain tissue homogenates as shown by others [277]. The effect of three extraction cycles is shown in Table 3.1. Repeated extractions enhanced relative recovery of each NAE while the CV remained at acceptable levels (<15 %). It is also interesting to note that a correlation was observed between the endogenous abundance of each NAE with their recovery following three extractions. The majority of AEA, which has low endogenous levels (low pmol range), was efficiently extracted following 1 extraction cycle, while recovery of PEA, found at higher endogenous levels (high pmol range), was greatly enhanced after 3 extractions (118.4% increase). Thus, this experiment suggests that multiple extractions with 1 mL of ACN are required to overcome a limited capacity of ACN to extract certain NAEs from brain tissue.

3.3.2 Validation

Calibration curves were linear for AEA (R^2 = 0.999) over a concentration range of 2.5 to 100 ng/mL and linear for PEA (R^2 = 0.989) and OEA (R^2 = 0.999) over a concentration range between 5 to 500 ng/mL. The concentration ranges covered anticipated endogenous levels of NAEs and elevated NAE levels in URB597 treated animals. The LLOQs for AEA, OEA and PEA were 1.4, 0.6 and 0.5 ng/mL, respectively. This analytical method is accurate, precise and reproducible for the simultaneous measurement of AEA, OEA and PEA in rodent brain tissue (Table 3.2). The intraday and interday accuracies were acceptable and generally within 15% of the nominal concentration. CVs were also generally well below 15%. These results are acceptable for the developed method; however, the slight discrepancies reported in Table 3.2 are attributed to the endogenous nature of eCBs in biological tissues, which need to be accounted for while validating analytical method.

Table 3.1 Effect of Multiple Extraction Cycles on Analyte Recovery

Analyte	1 Cycle (n = 3)		3 Cycles (n = 3)	
	Mean (% C1)	CV (%)	Mean (% C1)	CV (%)
AEA	100.0	14.1	121.4	11.4
OEA	100.0	8.9	163.7	3.7
PEA	100.0	8.0	218.4	3.3

Table 3.2 Linearity, Intra- and Interday Accuracy and Precision of Analytical Method for NAE Measurement From Brain Matrix

Analyte	R^2	Nominal Concentration (ng/mL)	Intraday 1 (n = 3)			Intraday 2 (n = 3)			Interday (n = 8)		
			Mean	Accuracy (%)	CV (%)	Mean	Accuracy (%)	CV (%)	Mean	Accuracy (%)	CV (%)
AEA	0.999	7.5	8.6	115.0	9.4	7.9	105.8	1.3	8.2	109.5	7.8
		35	31.7	105.7	6.2	34.2	114.0	6.1	33.2	110.8	7.5
		75	68.1	90.8	7.5	72.5	96.7	9.1	68.5	91.4	8.15
OEA	0.989	15	18.2	121.5	18.8	14.8	98.4	5.5	15.9	105.8	17.6
		30	30.9	102.9	5.1	25.1	83.6	2.8	28.4	94.8	13.3
		90	85.8	95.4	5.3	90.6	100.7	4.2	85.9	95.4	6.1
PEA	0.999	15	14.9	99.4	11.6	13.9	93.0	3.6	14.3	95.0	8.6
		30	28.5	95.0	13.2	29.1	97.1	7.2	28.6	95.8	8.7
		90	94.8	105.3	5.4	80.7	89.6	4.9	88.9	98.8	8.9

eCBs are notoriously difficult to quantify accurately because their endogenous nature adds variability during method validation. Methods using biological matrices for validation have to account for endogenous levels of analyte and correct for these basal levels to calculate calibration and QC concentrations. However, this is a difficult task because the basal levels can vary greatly in tissue homogenates. For example, it is well know that eCB species accumulate postmortem and during sample processing [273, 282]. To reduce variability, tissue is rapidly dissected, flash frozen and kept ice cold during processing. Additionally, it is important to prepare a quality homogenate for validation studies. However, this process is not standardized in the literature [277, 278, 280], which may explain different results in validation studies [275, 277]. The method used here for ensuring homogenous background levels of eCBs is different than others, which may have contributed to the good accuracy and precision (Table 3.2). In the current method, bulk homogenates were created each day and great care was taken to ensure uniformity by vigorously vortexing the homogenate while kept ice cold. Additionally, new background measurements were acquired for every batch of homogenate. In Richardson et al., 2007 [277], homogenates were prepared by freezing tissue in liquid nitrogen prior to being ground up using a mortar and pestle and aliquoted for validation experiments. It is possible that these differences in preparation could result in differences in homogenate quality and therefore account for seemingly different accuracies and precisions reported in the literature.

In order to circumvent the obstacles associated with the endogenous nature of eCBs, some reports use surrogate matrices. However, opinions on whether or not the use of artificial matrices is appropriate while developing analytical methods to quantify eCBs vary in the literature. Some reports use alternative matrices commonly consisting of water and bovine serum albumin (BSA) for method validation [278, 284]. On the other hand, other reports use biological matrices and therefore can adjust for extraction

efficiency and potential matrix effects for every analyte of interest [275, 277, 280]. This adjustment is particularly important when not using an internal standard or when only using a representative internal standard for multiple analytes during validation [275]. In the current study, the later approach was used for the reasons mentioned above. Although it appears that significant matrix-analyte interactions were not encountered, it is possible that this could occur when validating and quantifying other eCB species using this method.

Next, the process efficiency was examined for the recovery of AEA, OEA and PEA from whole brain homogenates (Table 3.3). Concentration levels to study process efficiency were selected to reflect both reported levels of endogenous AEA, OEA and PEA [174] and preliminary analysis of analyte content using our developed method. Recovery (PE) values for AEA and OEA ranged from 98.1% to 106.2% and 98.5% to 102.2%, respectively (Table 3.3). These high recovery values indicate that the current extraction method was optimum for recovery of these analytes and that there was no indication of a matrix effect. Moreover, the recovery for PEA ranged from 85.4% to 89.5%. Although a lower recovery was consistently observed for PEA compared to the other two analytes, the calculated %CV was acceptable and the recovery rates were sufficient to achieve accurate and precise quantification of PEA, as indicated in Table 3.2. These high recovery rates for AEA, OEA and PEA are consistent with previous literature using other extraction procedures [277, 278]. However, contrary to other methods, this current method has the advantage of not requiring significant sample clean-up [277, 280, 285]. Due to long run times (43 min), the short-term sample stability was examined in the autosampler at 4°C. The relative MS signals for AEA, OEA and PEA at T=18 hr were 130%, 112.4% and 107% of T0, respectively (Table 3.4). These data suggest that under the specified storage conditions NAE degradation is not occurring.

Table 3.3 Process Efficiency

Analyte	Nominal Concentration (ng/mL)	PE (%, n = 3)	CV (%)
AEA	7.5	106.2	2.4
	35	99.2	2.3
	75	98.1	9.6
OEA	15	102.2	2.2
	30	99.5	16.5
	90	98.5	6.8
PEA	15	89.5	10.4
	30	85.4	17.1
	90	85.8	8.1

Table 3.4 Short-term analyte stability at 4°C

Analyte	0 Hours (n = 3)		18 Hours (n = 3)	
	Mean (% 0h)	CV (%)	Mean (% 0h)	CV (%)
AEA	100.0	13.1	130.5	2.3
OEA	100.0	3.3	112.4	3.5
PEA	100.0	3.7	107.0	4.0

However, in order to reduce inflation of estimated NAE levels, samples were not allowed to remain in the autosampler longer than 12 hr before being analyzed.

3.3.3 Biological application

The present method was used to quantify AEA, OEA and PEA in the rat hippocampi and entorhinal cortices following administration of the FAAH inhibitor URB597 [174]. FAAH is the major enzyme responsible for the degradation of NAEs [176], is expressed throughout the CNS [176, 287] and thus is involved in a variety of physiological and behavioral processes. For example, pharmacological inhibition and/or genetic deletion of FAAH modulates depressive-like behavior [288], reduces inflammatory pain [289], alters drug reward [290] and affords neuroprotection [291]. Therefore, FAAH is under intense investigation for its therapeutic utility in a variety of CNS disorders. In the present study, a significant elevation of all NAEs measured 2 hours following IP administration of URB597 was observed (Table 3.5). In the hippocampus, AEA, OEA and PEA were increased by 57.5%, 475.6% and 986.6%, respectively. On the other hand, an increase in AEA content was not observed in the entorhinal cortex, and much smaller increases of 250.2% and 435.0% for OEA and PEA, respectively. This is the first study to demonstrate brain region and NAE specific alterations following a single dose of URB597. These data are consistent with studies examining NAE content following chronic URB597 administration. After weeks of URB597 administration, Bortolato et al., 2007 [288] observed elevations of AEA in the midbrain, thalamus and striatum, however this effect was absent in the prefrontal cortex and hippocampus [288]. It is not surprising that the effects of FAAH inhibition are brain region specific.

Table 3.5 Effect of URB596 on Endogenous levels of AEA, OEA and PEA

		Vehicle (n = 5-6[#])	URB597 (0.3 mg/kg, n = 6)
AEA (nmol/mg tissue)	Hippocampus	37.9 ± 20.5	59.7 ± 9.4*
	Entorhinal Cortex	43.3 ±12.0	44.0 ± 12.4
OEA (nmol/mg tissue)	Hippocampus	82.9 ± 11.4	477.2 ± 90.2***
	Entorhinal Cortex	171.5 ± 146.6	300.1 ± 56.0***
PEA (nmol/mg tissue)	Hippocampus	155.7 ± 60.1	1691.8 ± 377.6***
	Entorhinal Cortex	85.7 ± 50.0	917.6 ± 195.8***

For example, in the current study, NAE elevation was more pronounced in the hippocampus relative to the entorhinal cortex, a result that is consistent with immunohistochemical data demonstrating that FAAH expression is much more abundant in the hippocampus [179]. Noteworthy, another study examined NAE content in the spinal cord following 4 daily doses of URB597 and found reduced levels of AEA, OEA and PEA relative to single injection [292]. The original characterization of URB597 reported elevations of AEA, OEA and PEA in whole brain tissue [174] following a single dose. However, taken together, the above mentioned studies and the current findings suggest complex and region specific regulation of NAEs which has implications for the pharmacodynamic effects of FAAH inhibition.

3.4 Conclusions

The current study describes a novel method for analyzing eCBs and related compounds from biological samples with acceptable accuracy and precision. This method has been developed to analyze AEA, OEA and PEA from rodent brain tissue and offers multiple advantages over other validated methods for eCB quantification. A simple extraction protocol was used without time consuming and costly sample clean-up, compounds were quantified on a single quadrupole mass spectrometer with satisfactory sensitivity, the method was validated using appropriate biological matrices, which accurately accounts for analyte-matrix interactions, and this report demonstrated the feasibility of measuring these compounds from brain using low milligrams of tissue such as bilateral adult rat hippocampi. Finally, this method was proven effective in detecting elevations of AEA, OEA and PEA in rodent hippocampus and entorhinal cortex following administration of the FAAH inhibitor URB597.

4. CHAPTER 4

CHARACTERIZATION OF THE EFFECTS OF BINGE ETHANOL TREATMENT ON COMPONENTS OF THE ENDOCANNABINOID SYSTEM

4.1 Introduction

Chronic ethanol consumption results in numerous neuroadaptations that are responsible for physical and psychological dependence and ultimately, addiction. Elucidating these neuroadaptive changes may lead to the development of novel targets for the treatment of AUDs. For example, the eCB system is particularly sensitive to ethanol and is critically involved in mediating many of the pharmacological, behavioral and reinforcing effects of ethanol and this neurotransmitter system is prone to significant ethanol-induced neuroadaptations [187, 188]. Therefore, the eCB system represents a promising pharmacotherapeutic target for the treatment of AUDs. Although currently under investigated, the eCBs may be influenced by the neurodegenerative effects of excessive ethanol consumption in a manner different than non-neurotoxic ethanol exposure. This hypothesis is supported by converging evidence demonstrating that excessive ethanol consumption is associated with reductions in brain volume, loss of neuronal populations and compromised structural integrity [63, 66]; and that the eCB system is engaged following experimental brain injury [213, 215, 216, 219]. Therefore, the eCB system may also be a novel target for preventing ethanol-induced neurodegeneration and aid in the recovery from AUDs.

The eCB system consists of two primary g-protein coupled receptors, the CB1R and the CB2R. The CB1R is the principal neuronal receptor, while CB2R expression, although still controversial, has been detected in activated glial cells and to a lower extent in some neuronal populations [158, 159, 161]. The endogenous ligands for these receptors are eicosanoids, including AEA and 2-AG, and are primarily responsible for

presynaptic inhibition of neurotransmitter release, regulation of neuroinflammatory signaling and/or cell homeostasis [159, 163]. In addition to the primary components of the eCBs, significant crosstalk occurs with other signaling systems such as the NAEs. For example, OEA and PEA compete with AEA for cellular reuptake and metabolism [268, 269]. Additionally, AEA is a full agonist at TRPV1, while OEA and PEA potentiate AEA binding to this receptor [264, 271]. Furthermore, the NAEs, including AEA, are ligands for PPAR nuclear receptors [266], which are important for maintaining cellular homeostasis. Together, the eCBs and related lipids represent a complex signaling network with important physiological and neuropsychological functions, including regulating ethanol consumption, drug reinforcement and neuroprotection.

Ethanol exposure produces significant alterations and neuroadaptations in the eCB system [187] (see section 1.8). The literature suggests that these effects are dependent on multiple factors including the duration of ethanol exposure, ethanol concentration, brain region analyzed and eCB of interest. For example, acute ethanol exposure results in reductions in AEA, OEA and PEA in some brain regions but not others [199-201], whereas acute ethanol elevates 2-AG but not AEA in the nucleus accumbens [201, 293]. Conversely, the most consistent effects of chronic ethanol exposure are neuroadaptive elevations in AEA and/or 2-AG and CB1R down-regulation [203-206, 208], however these effects are dependent on exposure protocol and brain region [294, 295].

The eCBs and related NAEs play an important role in the pathogenesis of neurodegenerative disorders (see section 1.9 for detailed discussion). For example, tissue content of 2-AG, AEA and/or other NAEs are elevated in models of traumatic brain injury, stroke and chronic neurodegeneration [213, 215, 216, 219] and it is hypothesized that these elevations represent an endogenous mechanisms by which the CNS counteracts and isolates neuronal damage [296, 297]. Importantly, preclinical

studies have demonstrated that pharmacological enhancement of eCBs and NAEs can afford additional neuroprotection [291], while blocking such events increases susceptibility to neurotoxicity [218]. NAEs and their NAPE precursors do not appear to accumulate in all models of neurodegeneration. For example, Hansen et al., observed significant NAPE accumulation following necrotic cell death, induced by NMDA, but not apoptotic cell death following MK-801 administration [216]. These data suggest that Ca^{2+} -mediated excitotoxicity may be one important trigger for NAPE and subsequent NAE accumulation. Furthermore, production of NAPE intermediates is the rate limiting step of NAE synthesis and requires the activity of the Ca^{2+} sensitive n-acyltransferase, further suggesting an important role of Ca^{2+} for injury-induced NAE formation. Necrotic cell death may also occur through mechanisms independent from NMDA receptors and voltage-gated Ca^{2+} channel dependent excitotoxicity. For example, a form of necrotic cell death termed dark cell degeneration is observed following binge ethanol exposure [114] and is insensitive to NMDA and voltage-gated Ca^{2+} channel antagonists [121, 153]. However, it is currently unclear whether this form necrotic cell death has the capacity to engage eCB and NAE biosynthesis. Importantly, if Ca^{2+} is required for eCB and/or NAE accumulation during neurotoxicity, binge ethanol induced plasma membrane disruption [114] and mitochondrial swelling and functional impairment [114, 139] theoretically could serve as sources for Ca^{2+} stimuli.

Although the studies described in the preceding paragraphs (and in section 1.8) have provided critical insight to the effects of ethanol on the eCB system and in turn on how the eCBs influence ethanol related behaviors, to date, studies examining how the eCB system is altered by ethanol-induced neurodegeneration have not been conducted. This question is particularly important because neurodegenerative insults have independent effects on the eCBs. Therefore concomitant ethanol exposure and neurodegeneration may lead to unique effects on the eCB system. An understanding of

how neurodegenerative patterns of ethanol exposure modulate the eCBs may provide critical insight into new approaches for preventing ethanol-induced neurodegeneration and novel treatment strategies for AUDs. Therefore, the current study examined the effects of binge ethanol on the eCB system using an established model of an AUD, the modified Majchrowicz binge model, which produces substantial neurodegeneration of the cortico-limbic pathway [111, 114]. To that end, CB1R expression and AEA, OEA and PEA bulk tissue content was measured in the cortico-limbic pathway at multiple intoxication and/or withdrawal time points.

4.2 Methods

4.2.1 Animals and housing

Adult male Sprague Dawley rats weighing approximately 330 grams on arrival (n = 77, Charles River, Raleigh, NC) were used in these studies. All treatment protocols followed the *Guide for the Care and Use of Laboratory Animals* (NRC, 1996) and were approved by the University of Kentucky Institutional Animal Care and Use Committee. Rats were singlely housed in Plexiglas cages in an AAALAC approved University of Kentucky vivarium on a 12 h light/dark cycle with access to rat chow and water *ad libitum* unless noted. During acclimation, rats were handled daily for at least three days to familiarize rats to experimenters.

4.2.2 Binge ethanol treatment

Rats were treated with ethanol according to a modified Majchrowicz binge model as described in chapter 2 with the exception that separate groups of rats were administered binge ethanol for 1 (n = 5), 2 (n = 6) or 4 (n=6) days. BECs were measured according to the procedures described in chapter 2 with the exception for 1 and 2 days groups. For these groups, trunk blood was collected at euthanasia, which

was approximately 2 hr after the last dose of ethanol. In addition to rats that were euthanized immediately following 1, 2 or 4 days of binge ethanol treatment, 2 additional groups of rats were euthanized following peak withdrawal (17hr) and 48hr after the cessation of binge ethanol treatment. Withdrawal behavior for these groups was scored on a previously published scale [117, 298] (see Table 4.1) starting 10 h after the last dose of ethanol, which is the time when BECs drop to levels where withdrawal behaviors appear [110]. All withdrawal behaviors (see Table 4.1) were tallied during hourly 30 min blocks over the entire observation period which was 7 h for the 4-Day + T17 (peak withdrawal) group and 16 h for the 4-Day + T48 group. Mean withdrawal was calculated by averaging the highest withdrawal behavior score for each observation block, while the peak withdrawal was defined as the single highest withdrawal behavioral during the entire withdrawal period. Rats receiving control diet (see section 2.2.1; n = 4-6) were included for each timepoint.

4.2.3 CB1 receptor autoradiography

Rats were euthanized by rapid decapitation and whole brains were immediately dissected and flash frozen in isopentane on dry ice. Brains were then stored at -80°C until sectioning. Brains were sectioned at 16 µM in a 1:12 series using a Lecia CM1850 cryostat (Nussloch, Germany) and mounted on Superfrost Plus® slides (Fisher Scientific, Pittsburgh, Pennsylvania). After sectioning, slides were stored overnight at 4°C under desiccation and stored at -80°C. CB1 receptor autoradiography was performed using the nonselective cannabinoid agonist, [^{3}H]-CP55940, similar to previously described (Perkin Elmer, Specific Activity = 173 Ci/mM)[158]. Sections were thawed overnight at 4°C under desiccation and brought to room temperature before binding.

Table 4.1 Withdrawal Scale

Score	Behavior
0	Intoxicated
1.0	Hyperactive
1.4	Tail Tremor
1.6	Tail Spasm
2.0	Caudal Tremor
2.2	Arched back
2.4	Splayed Limbs
2.6	General Tremor
3.0	Head Tremor
3.2	Induced Running
3.4	Wet Dog Shakes
3.6	Chattering Teeth
3.8	Spontaneous Convulsions
4.0	Death

Sections were pre-incubated in Tris-HCl buffer (pH 7.4) containing Tris-HCl (50 mM), NaCl (120 mM), KCl (5 mM), $MgCl_2$ (1 mM), $CaCl_2$ (2.5 mM) and 5% BSA for 30 min. Sections were then placed in fresh Tris-HCl buffer with the addition of 2.5 nM [^{3}H]-CP55940 for 2 hr at 37°C. Following incubation with the radioligand, sections were washed in Tris-HCl buffer (pH 7.4) containing 1% BSA and no binding salts at 4°C for 1 hr. Sections were washed again with fresh Tris-HCl buffer for 3 hr at 4°C . Sections were then washed for 5 min in Tris-HCl buffer at 4°C without BSA or binding salts. Lastly, sections were briefly washed in 4°C dH_2O and then dried under gentle air stream overnight. Following air-drying, all slides were placed in Fisher Biotech autoradiographic cassettes and slides were exposed to Kodak Biomax film for 27 days prior to film development.

Films were imaged using a Power Macintosh based image analysis system (Scion LG-3 frame grabber, Sony XC-77 CCD camera and a Northern Lights desktop illuminator) and quantified using ImageJ (NIH, version 1.59). Initially, CB1R autoradiography was quantified in the entorhinal cortex (Bregma coordinates -5.40 to -6.48) and hippocampus (Bregma coordinates -2.76 to -4.56), the two brain regions most susceptible to binge ethanol induced neurotoxicity. Following initial analysis, the substantia nigra pars reticulate (StN; Bregma coordinates -5.40 to -5.64) was included to determine whether CB1R down-regulation was global or brain region specific. Additionally, subregions of the hippocampus were analyzed to determine which layers were responsible for CB1R down-regulation in the hippocampus. The dorsal dentate gyurs, CA1 striatum oriens and CA3 striatum oriens were measured from bregma coordinates -2.76 to -4.56, while the ventral dentate gyrus was defined as the lateral striatum oriens between bregma coordinates -5.40 to -6.12 and from the most ventral point to the height of the entorhinal fissure.

4.2.4 N-acylethanolamide extraction and quantification

Processing and quantification procedures for AEA, OEA and PEA were performed according to previously described methods (Liput et al., submitted; see chapter 3). Rats were euthanized by rapid decapitation, hippocampi, entorhinal corticies, striatum and cerebellum were dissected and flash frozen and stored at -80°C. Tissue was homogenized, NAEs were extracted than transferred to an HPLC vial containing a siliconized microinsert and stored in an autosampler at 4°C. NAEs were resolved using HPLC and quantified using ESI-MS. NAE concentrations were back calculated using standard curves prepared from tissue standards and quality controls were run periodically. Samples that did not meet quality control standards during processing were excluded from analysis.

4.2.5 Statistical analysis

Statistics were performed using GraphPad Prism (Graphpad version 4.03, La Jolla, CA, USA). Mean intoxication behavior and withdrawal behavior were analyzed by Kruskal-Wallis tests for non-parametric data followed by Dunn's post-hoc test when appropriate. Mean daily ethanol dose and BECs were analyzed by ANOVAs followed by Bonferroni post-hoc tests when appropriate. CB1R autoradiography and NAE quantification were normalized as percent of respective control to correct for differences in film exposures prior to analysis by ANOVAs (treatment x time) followed by Bonferroni post hoc tests when appropriate. Intoxication and withdrawal timepoints and brain regions were analyzed independently. Values are presented as mean ± standard error of the mean and analyses were considered significant at $p < 0.05$.

4.3 Results

4.3.1 Ethanol binge data

Binge intoxication measures are presented in Table 4.2. Mean intoxication, mean daily dose, and BECs were similar between the CB1R (2-day) and NAE (2-day) groups. All groups treated for the entire 4-day binge were compared by Kruskal-Wallis test, which revealed a group difference in intoxication behavior [$H_{(4)} = 9.012$; $p < 0.05$], a group difference in mean daily ethanol dose [$F_{(3,25)} = 3.119$; $p < 0.05$], but no group differences in BECs [$F_{(3,25)} = 2.036$; $p > 0.05$]. Post-hoc analysis revealed a difference in intoxication and mean daily dose for the NAE (4-day) group when compared to the CB1R (4-day + T48) group ($p < 0.05$). Mean withdrawal behavior was similar between the CB1R withdrawal groups; however a significant difference was observed in peak withdrawal behavior ($p < 0.01$). Binge intoxication measures were analyzed across duration of binge treatment. Groups were collapsed by binge duration and rats treated for 1-day, 2-day and 4-day were compared by Kruskal-Wallis test or one-way ANOVA, which, as expected, revealed a group difference in intoxication behavior [$H_{(3)} = 25.61$; $p < 0.0001$], a group difference in mean daily ethanol dose [$F_{(2,42)} = 52.38$; $p < 0.0001$], and a group difference in BECs [$F_{(2,42)} = 6.785$; $p < 0.01$]. Post-hoc analysis of intoxication scores revealed significant differences between 1-day vs. 4-day ($p < 0.001$) and 2-day vs. 4-day ($p < 0.001$). Post-hoc analysis of mean daily dose revealed significant differences between 1-day vs. 2-day ($p < 0.001$), 1-day vs. 4-day ($p < 0.001$) and 2-day vs. 4-day ($p < 0.001$). Post-hoc analysis of BEC revealed a significant difference between 1-day vs. 2-day ($p < 0.01$) and 1-day vs. 4-day ($p < 0.05$).

Table 4.2 Ethanol Binge Data

Group	Intoxication Behavior*	Ethanol Dose (g/kg/d)	BEC (mg/dL)	Withdrawal Behavior** Mean	Withdrawal Behavior** Peak
CB_1 Autoradiography					
1-Day (n = 5)	0.5 ± 0.1	13.4 ± 0.4	304.1 ± 19.0		
2-Day (n = 6)	1.6 ± 0.1	10.3 ± 0.3	403.2 ± 28.0		
4-Day (n = 6)	2.1 ± 0.2	8.8 ± 0.4	404.9 ± 24.4		
4-Day + T17 (n = 7)	2.2 ± 0.1	8.4 ± 0.4	366.1 ± 26.1	1.5 ± 0.4	2.3 ± 0.1
4-Day + T48 (n = 6)	1.8 ± 0.0	9.5 ± 0.2	342.6 ± 24.0	1.1 ± 0.3	3.0 ± 0.1
NAE quantification					
2-Day (n = 6)	1.4 ± 0.2	10.8 ± 0.6	443.8 ± 10.5		
4-Day (n = 7)	2.3 ± 0.1	8.1 ± 0.3	420.5 ± 24.1		

*mean intoxication behavior (0-5 scale), **withdrawal behavior(0-4 scale); see text for details
NAE, n-acylethanolamide; BEC, blood ethanol concentration

4.3.2 Experiment 1: CB1 receptor autoradiography

Experiment 1 was designed *a priori* to examine the effects of binge ethanol exposure on CB1R density in the hippocampus and entorhinal cortex (Figure 4.1) at multiple intoxication time points as these brain regions are susceptible to binge ethanol induced neurodegeneration. CB1R densities were normalized to percent of control for each timepoint in order to correct for inherent differences between batches of autoradiographic film exposures. Two-way ANOVAs (treatment X time) were performed independently for each brain region which revealed a main effect of ethanol for the hippocampus [$F_{(1,44)} = 6.941$; $p < 0.05$] and a main effect of time and a treatment x time interaction for the entorhinal cortex [time: $F_{(4,43)} = 3.134$; $p < 0.05$, interaction: $F_{(4,43)} = 3.132$; $p < 0.05$]. Post hoc analysis revealed a transient down-regulation in CB1Rs that was significant following 2-days of ethanol treatment in the hippocampus and entorhinal cortex ($p < 0.05$; Figure 4.2). Following these initial ANOVAs, additional analyses were performed to determine (1) brain region specificity of CB1R down regulation and (2) sub-layers responsible for CB1R down regulation in the hippocampus. To that end two-way ANOVAs (treatment x time) were performed on the molecular layer of the dorsal dentate gyrus, molecular layer of the ventral dentate gyrus, CA3, CA1 and the substantia nigra pars reticula. This second round of analyses revealed a main effect of ethanol in the molecular layer of the dorsal dentate gyrus [$F_{(1,44)} = 9.622$; $p < 0.01$] and CA1 [$F_{(1,44)} = 4.499$; $p < 0.05$]. Additionally, a main effect of time and a treatment x time interaction was observed in the molecular layer of the dorsal dentate gyrus [time: $F_{(4,44)} = 3.431$; $p < 0.05$; interaction: $F_{(4,44)} = 3.432$; $p < 0.05$]. Post hoc analysis revealed a transient down-regulation in CB1Rs that was significant following 2-days of ethanol treatment in the dorsal dentate gyrus and CA1 ($p < 0.05$; Figure 4.2). No effects were observed in the molecular layer of the ventral dentate gyrus, CA3 and substantia nigra pars reticula.

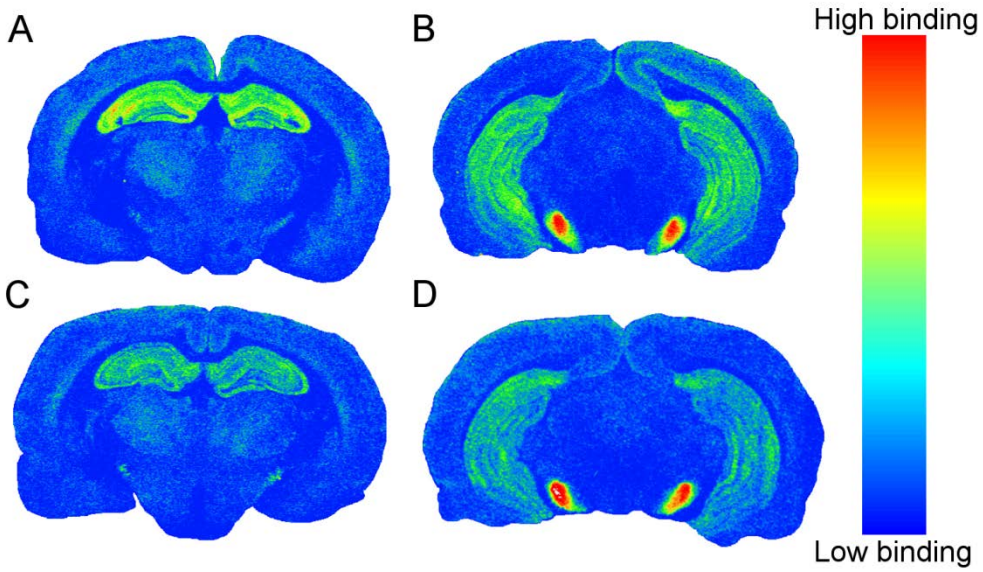

Figure 4.1 Representative CB1R autoradiograms.

Representative autoradiograms from control (A and B) and ethanol (C and D) rats at the level of the dorsal hippocampus, dorsal dentate gyrus, CA3 and CA1 (A and C) and at the level of the entorhinal cortex, ventral dentate gyrus and substantia nigra (B and D). See methods (section 4.2.3) for details on the bregma coordinates for each region of interest.

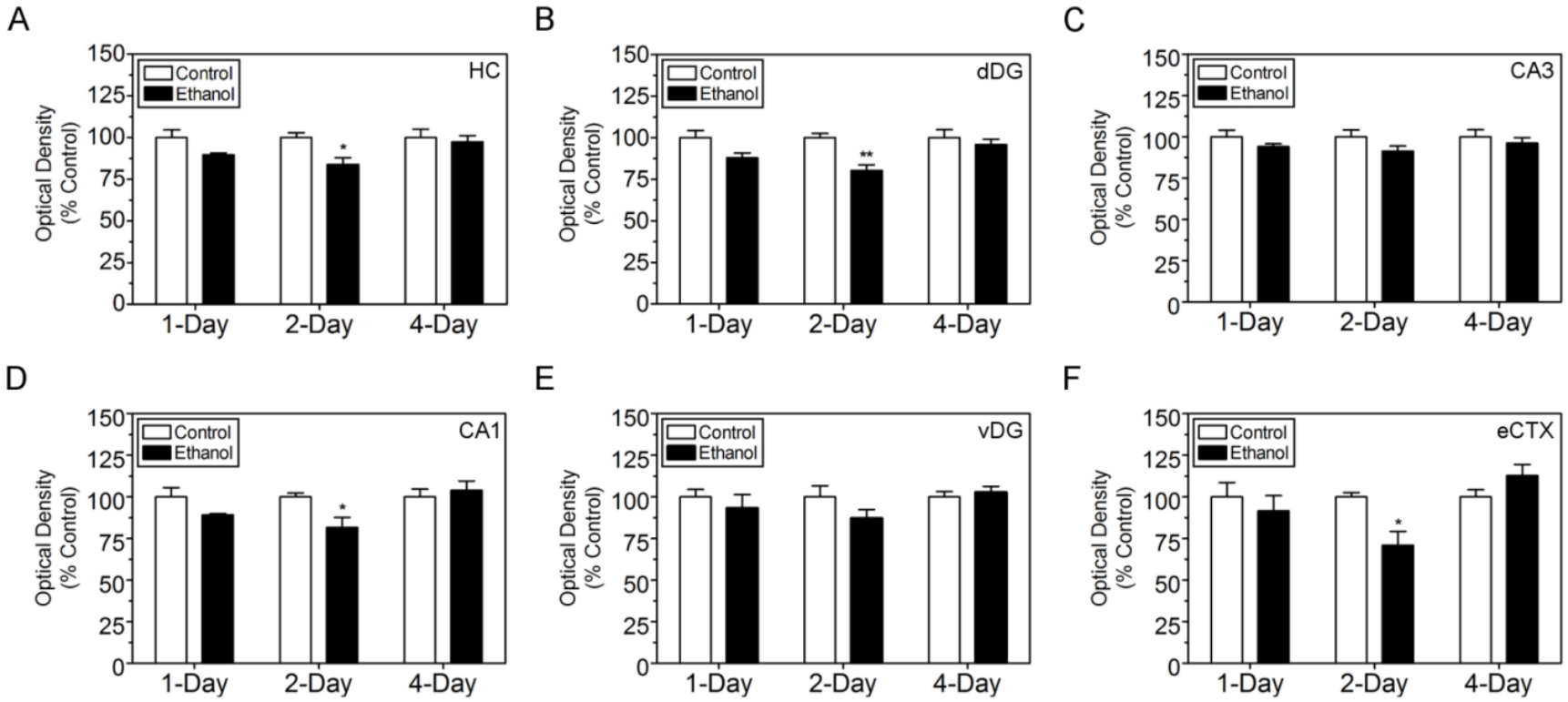

Figure 4.2 CB1R quantification in the hippocampus and entorhinal cortex following 1,2 and 4 days of binge ethanol treatment.

(A) hippocampus, (B) molecular layer dorsal dentate gyrus, (C) stratum oriens of CA3, (D) stratum oriens of CA1, (E) molecular layer of ventral dentate gyrus and (F) layers I-VI of entorhinal cortex. *, $p < 0.05$; **, $p < 0.01$ compared to time matched controls.

Sparate two-way ANOVAs (treatment x time) were performed on withdrawal time points, however no differences in CB1R densities were observed in any brain region at 17 h (peak withdrawal) or 48 h following the last dose of ethanol (Figure 4.3).

4.3.3 Experiment 2: N-acylethanolamide quantification

Effects of binge ethanol treatment on bulk tissue content of AEA, OEA and PEA were quantified in the entorhinal cortex, hippocampus, striatum and cerebellum following 2 and 4 days of binge ethanol treatment (Figure 4.4). Although graphically presented as moles per gram tissue, NAE measurements were converted to percent control prior to analysis to correct for fluctuations in control levels that are believed to be cause by batch processing of 2 and 4 day samples. Additionally, individual brain region samples were excluded from analysis if sample processing did not meet quality control standards. Separate two-way ANOVAs (treatment x time) for AEA, OEA and PEA were performed for each brain region of interest (12 ANOVAs total). In the hippocampus, a main effect of ethanol was observed for AEA [$F_{(1,20)} = 5.559$; $p < 0.05$], however post-hoc analysis failed to reveal a difference at either timepoint. No effects were observed in the entorhinal cortex. There was also a main effect of ethanol treatment on PEA in the cerebellum [$F_{(1,20)} = 5.559$; $p < 0.05$], however post-hoc analysis failed to reveal a difference at either timepoint. In the striatum, main effects of ethanol were observed for AEA [$F_{(1,19)} = 11.73$; $p < 0.01$], OEA [$F_{(1,19)} = 5.386$; $p < 0.05$] and PEA [$F_{(1,19)} = 4.716$; $p < 0.05$] and an interaction was observed for AEA [$F_{(1,20)} = 11.72$; $p < 0.01$]. Post-hoc analysis showed that 2 days of binge ethanol treatment elevated AEA tissue content in the striatum ($p < 0.01$).

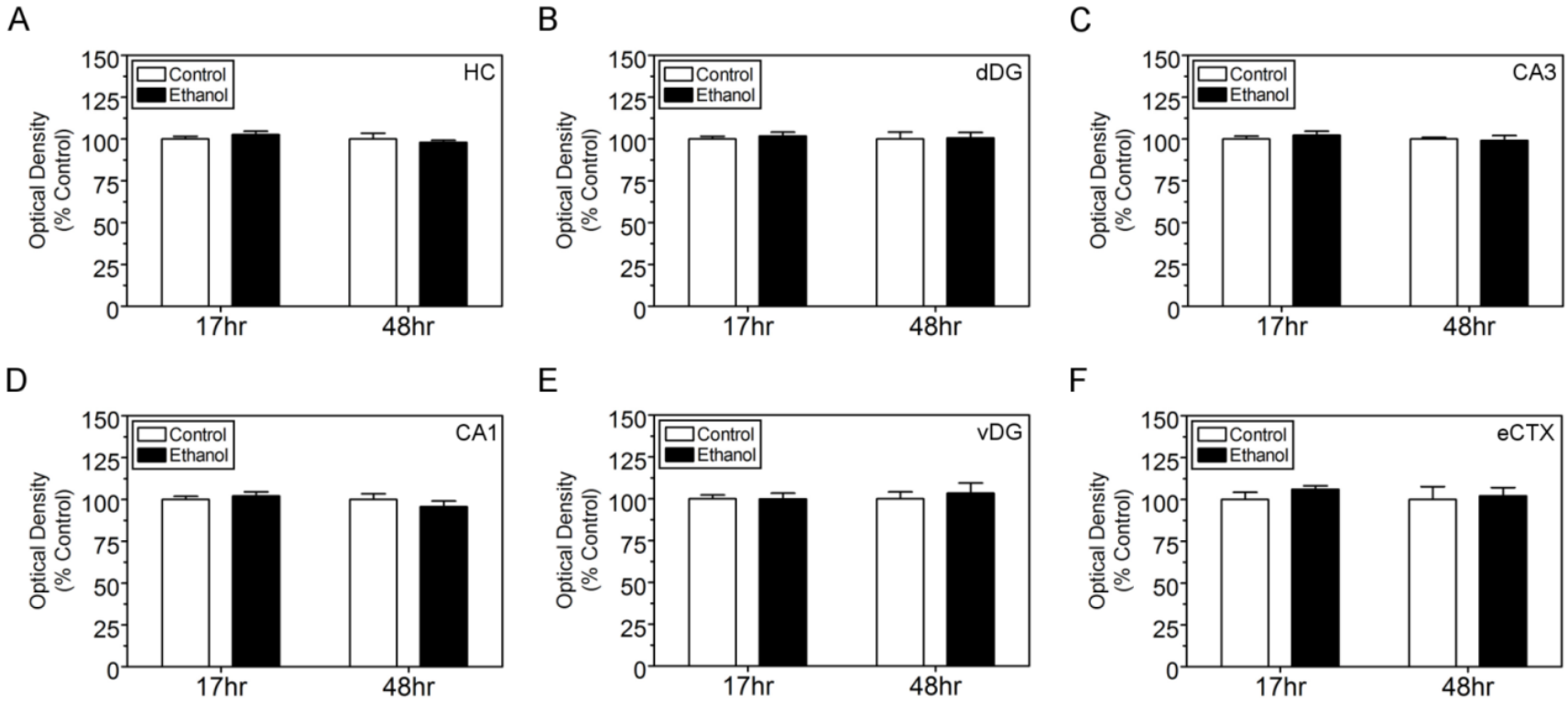

Figure 4.3 CB1R quantification in the hippocampus and entorhinal cortex following either 17 hr or 48 hr withdrawal from binge ethanol treatment.

(A) hippocampus, (B) molecular layer dorsal dentate gyrus, (C) stratum oriens of CA3,(D) stratum oriens of CA1, (E) molecular layer of ventral dentate gyrus and (F) layers I-VI of entorhinal cortex.

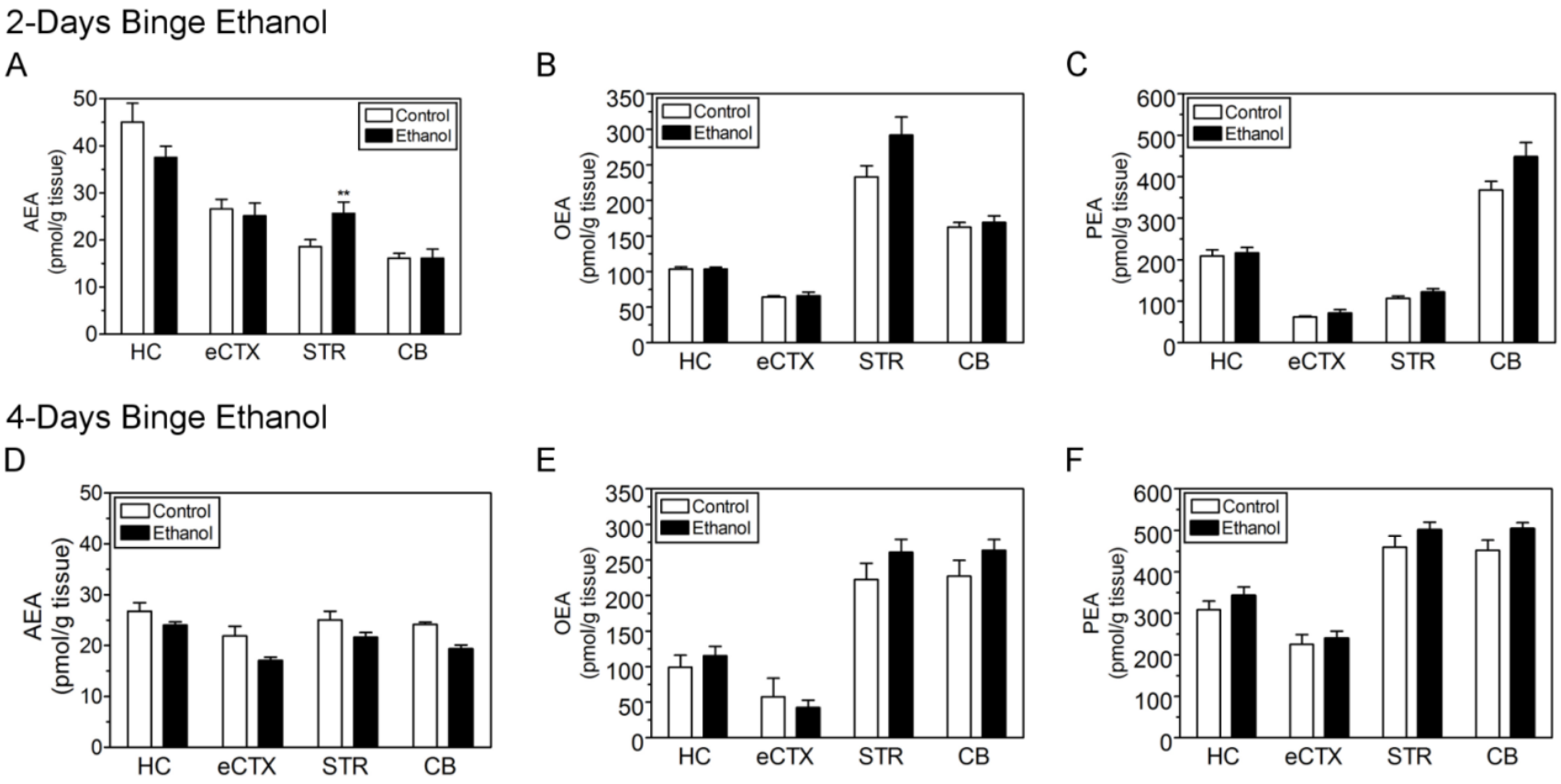

Figure 4.4 N-acylethanolamide quantification following binge ethanol treatment.

(A-C) 2-days of binge ethanol treatment. (D-F) 4-days of binge ethanol treatment. HC, hippocampus; eCTX, entorhinal cortex; STR, striatum; CB, cerebellum. **, $p < 0.05$ compared to respective control.

4.4 Discussion

The current study characterized the effects of binge ethanol exposure on the eCB system by examining CB1R expression and AEA, OEA and PEA tissue content. Significantly decreased binding of the cannabinoid agonist, [^{3}H]-CP55,940, was detected following 2 days of binge ethanol exposure in the entorhinal cortex and hippocampus; however this observation normalized by the 4-day time point. [^{3}H]-CP55,940 binding was also examined at two withdrawal time points, 17 and 48 hours, however no changes in binding were observed at these time points. Furthermore, 2 and/or 4 days of binge ethanol exposure resulted in only minor alterations in NAE tissue content, which was only significant in the striatum at 2 days.

The eCB system is a critical neural substrate mediating many of the neuropharmacological and behavioral effects of ethanol [187, 188]. In humans, variants of the CB1R gene, *CNR1,* predict susceptibility to AUDs [299]. Interestingly, these variants are associated with elevated CB1R binding sites [300], suggesting that enhanced cannabinoid signaling increases susceptibility to AUDs. In addition, CB1R expression is altered by ethanol in humans as decreased expression is observed during early (3-28 days) withdrawal [300], while increased CB1R expression has been reported slightly later into abstinence [301]. Similar to human studies, reports in rodents have observed ethanol-induced down-regulation of the CB1R during intoxication followed by normalization or even elevation of CB1R protein, mRNA and/or g-protein coupling following 1 to 40 days of ethanol withdrawal [204, 206, 208, 302]. Decreased CB1R expression found in the current study is in agreement with these other reports showing decreased CB1R protein or mRNA expression following chronic ethanol exposure [203-205, 208, 302]. However, in the current study, CB1R expression was normalized following 4-days of binge ethanol treatment, before induction of withdrawal. Interestingly, the current results suggest that ethanol mediated changes in CB1Rs are highly dynamic

and cessation of ethanol exposure is not required to see a rebound in CB1R expression as suggested in the aforementioned reports. Importantly, the duration and pattern of ethanol exposure may explain discrepancies between the current study and the literature reports described above. For example, Basavarajappa et al., 1998 and Vinod et al., 2006 observed robust decreases in CB1R expression in the hippocampus and cortex in mice following 3 days of continuous ethanol vapor exposure, a model that results in stable BECs [203, 204]. This is in contrast to the Majchrowicz model used in the current study where BECs rise and fall over the course of repeated gastric intubation [111]. Therefore, continuous exposure to high BECs may result in more pronounced effects on CB1R expression during intoxication and withdrawal. Additionally, other studies observing CB1R down-regulation followed by CB1R enhancement during withdrawal used chronic models in which rats were exposed to intermittent ethanol for up to 160 days, whereas rats in the current study were only administrated ethanol for 4 days [206]. Collectively these data and the current report suggest that pattern and duration of ethanol exposure are important factors that influence CB1R expression.

The mechanisms by which ethanol causes CB1R down-regulation in the current study is unknown, however some suggest that ethanol-induced enhancement of AEA and/or 2-AG results in agonist-induced desensitization by endocytosis [204, 206, 210, 211]. It is unclear whether this mechanism is responsible for CB1R down-regulation in the current study because AEA content was unaltered following 2-days of binge ethanol treatment. Alternatively, enhanced 2-AG signaling may produce CB1R down-regulation; but 2-AG tissue content was not determined in these studies. Conversely, CB1R down-regulation may be a result of diminished NMDA receptor dependent neurotransmission as CB1R mRNA expression is decreased following NMDA receptor inhibition [303] and ethanol directly inhibits NMDA receptors [20, 21]. Additionally, CB1R null mutant mice show diminished basal levels of [^{3}H]MK-801 binding and a loss of chronic ethanol-

induced enhancement of [^{3}H]MK-801 binding [304], further substantiating a functional link between CB1Rs and NMDA receptors. If ethanol mediates CB1R expression by modulating NMDA dependent neurotransmission, then one would expect a rebound in CB1R expression due to ethanol tolerance. Interestingly, CB1R expression was normalized following 4 days of binge treatment; a timepoint where tolerance is readily observed. Thus, the current study further supports a role of NMDA-mediated neurotransmission in mediating the effects of ethanol on CB1R expression. Noteworthy, a previous study examined the effects the Majchrowicz binge model on NMDA receptor expression but did not observe compensatory elevations in NMDA receptors in response to ethanol treatment [305]. However this report does not preclude a role of NMDA-mediated neurotransmission on CB1R expression because compensatory NMDA-mediated neurotransmission through enhanced glutamate release is a possibility and needs further investigation. If an inverse relationship exists between NMDA receptor activation and CB1R expression, it would also predict that ethanol withdrawal could result in elevated CB1Rs. Although elevated CB1Rs have been observed following withdrawal in some studies, no alterations in CB1Rs were observed during peak withdrawal (17 hr) or protracted withdrawal (48 hr) in the current study. Nevertheless, ethanol mediated changes in NMDA receptor activity may influence CB1R expression and the importance this relationship requires future.

Although a transient decrease in CB1Rs was observed in the current study, the physiological significance of this effect is unknown. It was initially hypothesized that loss of CB1R expression could serve as a mechanism that confers susceptibility to ethanol-induced neurotoxicity as CB1Rs are coupled to cell survival pathways [218, 306, 307] and can confer resistance to seizure activity and ethanol withdrawal [195, 218]. However, the transient and modest nature of CB1R down-regulation and the lack of CB1R changes in the ventral dentate gyrus, which is susceptible to ethanol-induced

neurotoxicity [111, 114], does not support this hypothesis. Nevertheless, CB1Rs down-regulation may represent other physiological effects of ethanol. One may speculate that CB1R down-regulation may be a consequence of enhanced eCB signaling, presumably to counteract the neuropharmacological effect of ethanol exposure, as suggested by others [204, 206, 209, 210]. For instance, stimulation of CB1Rs localized on GABAergic interneurons could result in decreased GABAergic neurotransmission to counteract the effects of ethanol. Interesting, activation of CB1Rs has the capacity to block ethanol-induced enhancement of GABAergic neurotransmission in central amygdalar slices [308]. Conversely, other literature reports demonstrate that cannabinoids mediate rather than counteract the pharmacological effects of ethanol. For example, in cultured hippocampal neurons, ethanol inhibits glutamatergic neurotransmission by promoting the formation of 2-AG and AEA, an effect blocked by CB1R antagonism [202]. Regardless CB1R down-regulation may be a consequence of enhanced eCB signaling and represent a maladaptive response to ethanol. It would be interesting to investigate cell-type specific effects of binge ethanol exposure on CB1R expression to gain better insight on the physiological consequence of ethanol-induced CB1R down-regulation. CB1Rs are expressed primarily on cholecystokinin containing GABAergic interneurons within the hippocampus, however, CB1Rs are also expressed on glutamatergic terminals of the perforant pathway [159]. Therefore, CB1R down-regulation observed in the current study, rather than a result of over stimulation, could represent a neuroadaptive effect specific to glutamatergic terminals to counteract ethanol-induced inhibition of excitatory neurotransmission though NMDA receptor-dependent mechanisms discussed in the preceding paragraph. Interestingly, conditional knockout of CB1Rs in glutamatergic forebrain neurons but not GABAergic interneurons increases hippocampal excitation following a kainic acid challenge [218], indirectly supporting the notion that CB1R down-regulation in the presence of ethanol may increase hippocampal excitation. Additionally,

decreased CB1R immunoreactivity in the hippocampus has been observed in pilocarpine treated rats, while CB1R expression in GABA interneurons appeared to be preserved [309], supporting the possibility of cell-type specific regulation of CB1Rs. Clearly, CB1R down-regulation can have multiple physiological consequences. Thus future investigation will need to determine the specificity of ethanol effects on CB1R expression.

The current investigation found brain region specific alterations in CB1R expression, which is in agreement with previous studies [205, 295]. In contrast to CB1R aredown-regulation in the entorhinal cortex and hippocampus, decreased CB1R expression was not observed in the substantia nigra pars reticulate. Additionally, subregions of the hippocampus appeared to be more labile to ethanol-induced CB1R down-regulation, as significant decreases were observed in the molecular layer of the dorsal dentate gyrus and stratum oriens of CA1, but not in stratum oriens of CA3 and the molecular layer of the ventral dentate gyrus. Similar layer specific changes were observed following pilocarpine-induced status epilepticus and were associated with redistribution of CB1R expression within the hippocampus [309]. Collectively, this suggests that CB1R expression is highly sensitive to perturbations in hippocampal function and dynamic alterations in CB1R expression represent neuroadaptations driven by disease states [309].

NAEs and eCBs are known to play an important role in mediating some of the effects of ethanol. For example, genetic and/or pharmacological inactivation of FAAH and concomitant potentiation of AEA, OEA and PEA results in decreased sensitivity to the sedative effects of ethanol, increased ethanol consumption and decreased sensitivity to ethanol withdrawal induced convulsions [310, 311]. Additionally, neurochemical evidence shows that NAEs are influenced by ethanol exposure. For example, acute injection of ethanol or short-term ethanol consumption results in robust reductions in

AEA, PEA and or OEA in multiple brain regions, including the hippocampus [199-201, 227], while chronic ethanol exposure enhances cortical AEA tissue content [204]. NAE tissue content was examined in the current study by quantifying prototypical NAEs, including AEA, OEA and PEA in the hippocampus, entorhinal cortex, cerebellum and striatum; however these species were generally unaffected following binge ethanol exposure. Although discrepancies between the current data and the aforementioned studies are currently unclear, differences in duration and pattern of ethanol exposure and brain regions of interest are likely explanations.

NAEs and/or eCBs are hypothesized to be important for maintaining CNS homeostasis and NAE and/or eCB elevation occurs in response to neuronal damage [213, 216, 219, 312]. The 4-day binge model used in the current study results in necrotic cell death in the cortico-limbic pathway, which is most prominent in the entorhinal cortex and ventral dentate gyrus of the hippocampus [111, 113, 114]. Therefore, it is surprising that NAE elevations were not observed in either the entorhinal cortex or hippocampus, especially following 4-days of binge exposure, a timepoint associated with maximal neuronal damage [112, 113]. It is possible that NAE biosynthesis may not be sensitive to the nature of necrotic cell death following binge ethanol exposure. For example, binge ethanol induced neurodegeneration is independent of NMDA and non-NMDA Ca^{2+} channels [121, 153] and studies suggest that NMDA mediated excitotoxicity and the associated rise in intracellular Ca^{2+} are responsible for NAE accumulation by activation of the Ca^{2+} sensitive N-acyltransferase [216, 296, 313]. Alternatively, the lack of NAE elevation observed in the current study could be due to limitations in the approach used for quantification. Binge ethanol induced neuronal damage occurs in layers II and III of the entorhinal cortex and within the granular cell layer of the ventral dentate gyrus of the hippocampus. However, NAEs were quantified using the entire entorhinal cortex and

hippocampus, therefore potential local elevations in NAE content could be diluted during quantification.

4.5 Conclusions

In summary, binge ethanol exposure resulted in transient down-regulation of CB1R expression in the corticolimbic pathway which is consistent with previous reports. The mechanisms of CB1R down-regulation are currently unknown; however enhanced AEA stimulation of CB1Rs is not a likely factor as AEA elevations were not detected in either the hippocampus or entorhinal cortex. Additionally, engagement of NAE signaling does not appear to occur in response to ethanol-induced tissue damage, which is in contrast to other neurodegenerative stimuli.

5. CHAPTER 5

FATTY ACID AMIDE HYDROLASE INHIBITION DOES NOT PREVENT BINGE ETHANOL INDUCED NEURODEGENERATION OF THE CORTICO-LIMBIC PATHWAY

5.1 Introduction

Current FDA approved pharmacotherapies for the treatment of AUDs have limited clinical utility due to low efficacy and/or compliance [38, 39]. For example, acamprosate is currently the most prescribed medication for alcohol dependence [40], however, only marginal efficacy has been observed in clinical trials. For example, multiple U.S. trials failed to observe a drug effect when compared to placebo on primary outcomes including time to first heavy drinking day or percent days abstinent [38]. The lack of viable treatment options is highlighted by the fact that less than 15% of patients with an AUD are prescribed medication [40, 58]. Multiple factors, such as genetic predisposition, comorbid psychiatric disorders, motivation, neurotoxicity and cognitive dysfunction contribute to AUDs. As AUDs are heterogeneous in nature, it is highly unlikely that a single drug or single target will be effective for all patients [59]. Therefore, current medication development for AUDs is focused on increasing the repertoire of available pharmacotherapies to increase effective treatment options across the spectrum of these disorders. To that end, identification of new biological targets that underlie AUDs, such as ethanol-induced neurodegeneration, should aid in medications development efforts and improve current treatment strategies.

Abnormalities in brain structure, including reductions in cortical gray matter and white matter volume, are commonly observed following long-term ethanol consumption [63, 240]. Ethanol is particularly damaging to the frontal and temporal lobes [70, 73] and the hippocampus [77, 80], which may partially explain deficits in executive function, learning and memory and emotional processing in alcoholics [88, 93]. Compromised

structural integrity and cognitive function is theorized to contribute to the chronic and relapsing nature of alcoholism [60, 62]. Therefore, it is hypothesized that neuroprotective agents will reduce alcohol-induced neurodegeneration, restore cognitive function and improve treatment outcomes for alcoholism.

Over the last two decades, the eCB system has emerged as a potent neuroprotective target in a variety of models of CNS disease, such as Alzheimer's disease, Parkinson's disease, Huntington disease, multiple sclerosis, stroke and traumatic brain injury [223]. The primary mechanisms by which cannabinoid agents afford neuroprotection include attenuation of excitotoxicity, neuroinflammation and oxidative stress [223](see section 1.7). Therefore, the eCB system may be a viable target to prevent alcohol-induced neurodegeneration as these same events are associated with the neurotoxic effects of alcohol [126]. In fact, a recent study has demonstrated that targeting CB1Rs prevents alcohol-induced potentiation of NMDA neurotoxicity *in vitro*, presumably through presynaptic inhibition of excessive glutamate release [314].

Much of the current work on eCBs has utilized CB1R or CB2R agonists to demonstrate neuroprotection, but the use of CB1R and CB2R agonists in a clinical setting may be limited. CB1R agonists are associated with untoward psychotropic effects and abuse liability, which may outweigh the benefits of these agents [315]. Further, although CB2R agonists are not associated with the untoward effects of CB1R agonists and have neuroprotective properties [316], the role of CB2Rs in the CNS is still unclear as the expression and function of CB2Rs appear to be heavily disease state specific. Alternatively, indirect modulation of the cannabinoid system by inhibiting the catabolism of eCBs may prove advantageous for the treatment of neurodegeneration. Inhibition of FAAH recapitulates a distinct subset of CB1R-dependent effects [317], such that the beneficial effects of the eCB system may be exploited, while the untoward effects

avoided. For example, FAAH inhibitors afford neuroprotection in models of kainic acid-induced excitotoxic brain damage and following focal cerebral ischemia [214, 253, 318, 319], while being devoid of classic CB1R adverse responses [320]. Therefore FAAH inhibition may have higher therapeutic value compared to CB1R agonists in many cases. Additionally, FAAH inhibitors are dependent on “on-demand” synthesis of eCBs and, as such, their activity is theorized to be greater in degenerative tissue [291] where endogenous elevations of eCBs are commonly observed [213, 217, 219]. Lastly, FAAH is responsible for the catabolism of all NAEs so inhibition of this enzyme results in elevations of not only AEA but also other neuroprotective NAEs including OEA and PEA [174, 266, 321].

To date, little data exists addressing the neuroprotective effects of the cannabinoid system in models of ethanol-induced neurodegeneration. A single report found that targeting the eCB system is neuroprotective in an *in vitro* model of alcohol withdrawal [314], however withdrawal-induced excitotoxicity is not the sole mediator of ethanol-induced neurodegeneration [126]. In fact, alcohol neurotoxicity is observed in experimental models in the absence of an overt withdrawal syndrome [95, 118, 119]. Further no studies have examined the neuroprotective properties of the eCB system in an *in vivo* model of ethanol-induced neurodegeneration. Therefore, the current study examined the neuroprotective effects of targeting the eCB system by FAAH inhibition using an established model of ethanol-induced neurodegeneration.

5.2 Methods

5.2.1 Animals and housing

Adult male Sprague-Dawley rats weighing approximately 330 grams (n = 81, Charles River, Raleigh, NC) were used in these studies. All treatment protocols followed the *Guide for the Care and Use of Laboratory Animals* (NRC, 1996) and were approved

by the University of Kentucky Institutional Animal Care and Use Committee. Rats were singlely housed in Plexiglas cages in an AAALAC approved University of Kentucky vivarium on a 12 h light/dark cycle with *ad libitum* access to rat chow and water unless otherwise noted. During acclimation, rats were handled daily for at least three days to familiarize rats to experimenters.

5.2.2 Binge ethanol treatment

Rats were treated with ethanol or control diet according to a modified Majchrowicz binge model [110, 115] as described in chapter 2 and outlined in Figure 5.1. BECs were measured 90 min after the 7th (experiment 1) or 3rd (experiment 2) dose of ethanol using plasma collected from tail blood and processed as described in chapter 2 (section 2.2.2).

5.2.3 URB597 Regimen

The FAAH inhibitor, URB597, was dissolved in DMSO at a concentration of 1.0 mg/mL. For experiment 1, nothing (n = 10), URB597 (0.3 mg/kg, i.p.; n = 10)) or vehicle (n = 10) was administered twice daily (11:00 am and 11:00 pm) starting after the third intubation of ethanol or control diet and continuing for the duration of binge treatment (Figure 5.1B). This dosing regimen was chosen based on previous studies demonstrating maximal FAAH inhibition for at least 12 h with 0.3 mg/kg URB597 [174]. For experiment 2, nothing (n = 6), a single dose of URB597 (0.3 mg/kg, i.p.; n = 6) or single injection of vehicle (n = 6) was administered following the third intubation of ethanol or control diet and 2 h prior to euthanasia (Figure 5.1B). This time interval was chosen because URB597 maximally elevates NAEs 2 h following administration [174]. Although preliminary studies found no effect of vehicles on any variables of interest, 4 rats in the ethanol + vehicle group died over the course of the experiment.

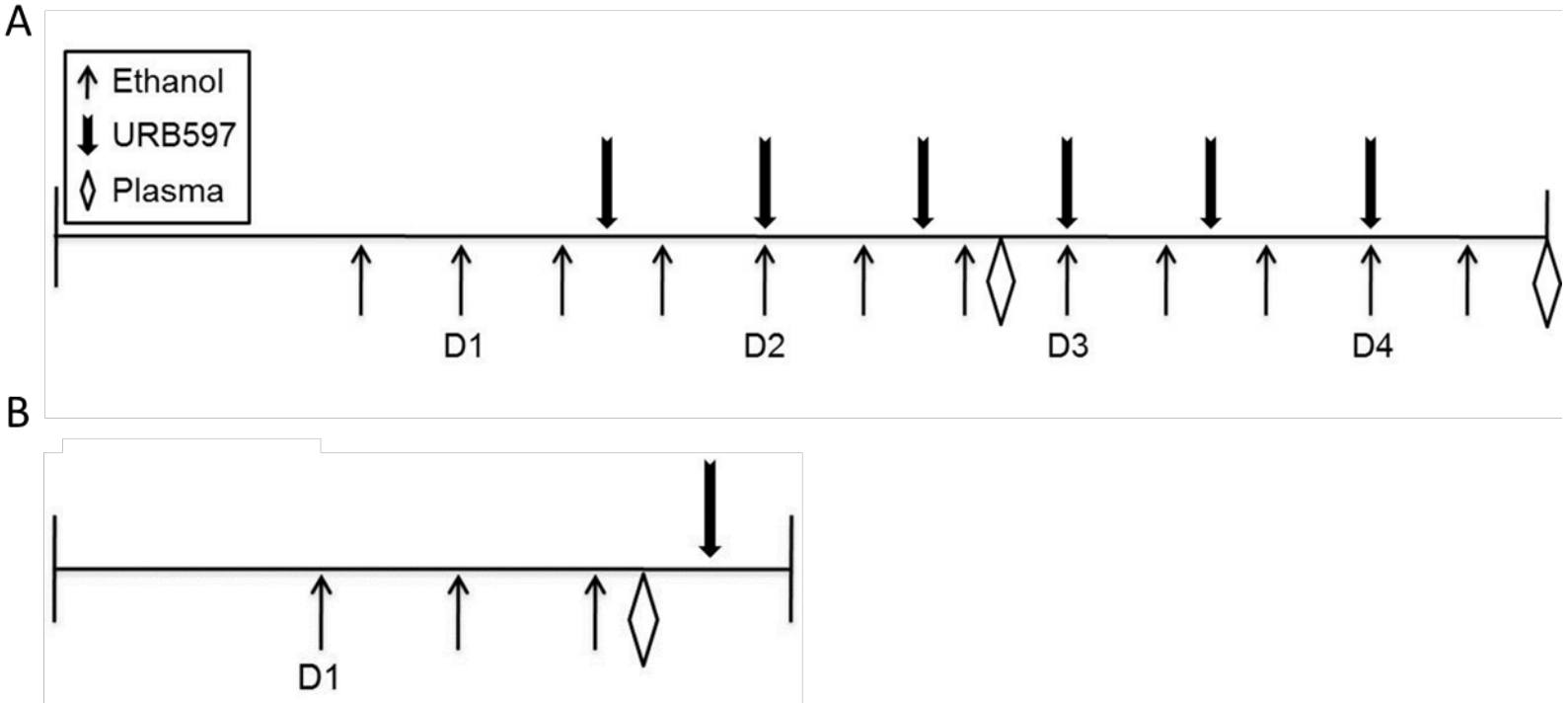

Figure 5.1 Treatment timelines for neuroprotection and "target engagement" experiments.

(A) In experiment 1, rats were administered ethanol 3 times daily for 4 days according to a 4-day binge paradigm and co-administered the FAAH inhibitor, URB597 (0.3 mg/kg), twice daily following the third dose of ethanol. Tail blood was collected 90 minutes after the 7th dose of ethanol and during euthanasia to determine BECs. (B) In experiment 2, rats were administered binge ethanol for one day and then administered a single dose of URB597 (0.3 mg/kg). Two hours following URB597 treatment, n-acylethanolamide content was quantified in the hippocampus and entorhinal cortex.

5.2.4 Fluoro-Jade B staining and quantification

Approximately 2 h after the last dose of ethanol, rats were transcardially perfused and tissue was processed for FJB according to the procedures described in chapter 2 (section 2.2.5) with the exception that a 1:6 tissue series was used. FJB+ cells were quantified by a blinded experimenter in the entorhinal cortex between bregma -5.20 mm and -7.44 mm and in the ventral dentate gyrus between bregma -5.20 mm and -6.84 mm (Paxinos and Watson, compact 6th edition, 2009). Strict criteria were used to identify FJB+ cells: cells were included in ventral dentate gyrus counts if they resided within or adjacent to the granular cell layer, while cells were included in entorhinal counts if they were located in cortical layers II or III. Cells also needed to have a pyramidal cell body characteristic of neurons and/or have observable proximal dendrites. FJB+ cells were rarely observed in control rats (< 1 cell/section) regardless of drug treatment, therefore control rats were collapsed across treatment group.

5.2.5 Endocannabinoid extraction and quantification

AEA, OEA and PEA were quantified in the hippocampus and entorhinal cortex 2 hours following URB597 as described in figure 5.1B. Processing and quantification procedures were performed as detailed in chapter 3 and briefly described in chapter 4 (section 4.2.4). Samples that did not meet quality control standards during processing were excluded from analysis.

5.2.6 Statistical analysis

Statistical analyses were performed using GraphPad Prism (Graphpad version 4.03, La Jolla, CA, USA). Mean intoxication behavior was analyzed by Kruskal-Wallis tests for non-parametric data followed by Dunn's post-hoc tests when appropriate. Mean daily ethanol dose, BECs and NAEs were analyzed by ANOVA followed by Bonferroni

post-hoc tests when appropriate. FJB+ cell counts were analyzed by ANOVA followed by post-hoc planned student t-tests with Welch's corrections for unequal variance. Pearson correlations were performed to assess the relationship between BECs and NAE concentration for rats treated with URB597. All values are given as mean ± standard error of the mean and statistical significance was accepted at $p < 0.05$.

5.3 Results

5.3.1 Experiment 1: Neuroprotection study

Intoxication behavior, ethanol dose and BEC were analyzed to rule out confounding effects of vehicle or URB597 administration on neuroprotection measures (Figure 5.2). Regardless of drug treatment, all ethanol groups displayed similar intoxication behaviors across the 4 days of binge treatment with mean intoxication behaviors being statistically indistinguishable (Figure 5.2A). Additionally, since ethanol doses were calculated from intoxication scores, each treatment group received similar doses of ethanol (Figure 5.2A and 5.2B). Previous reports on the neurodegenerative effects of binge ethanol show that a peak BEC of ~300 mg/dL must be reached to observe cortico-limbic degeneration [111]. In the current study, the grand mean BEC was 424.8 ± 11.7, which is well above the threshold to observe neurodegeneration (Figure 5.2C). Importantly, intoxication, mean daily dose and BECs did not differ between groups, indicating that binge ethanol exposure was similar across all treatment groups.

Consistent with other reports using the modified Majchrowicz binge model to assess neurodegeneration, FJB+ cells were observed in the granular cell layer of the ventral dentate gyrus and layers II and III of the entorhinal cortex [111, 113] (Figures 5.3 and 5.4). These cells displayed typical morphology of neurons in these brain regions including pyramidal cell bodies and/or proximal dendrites.

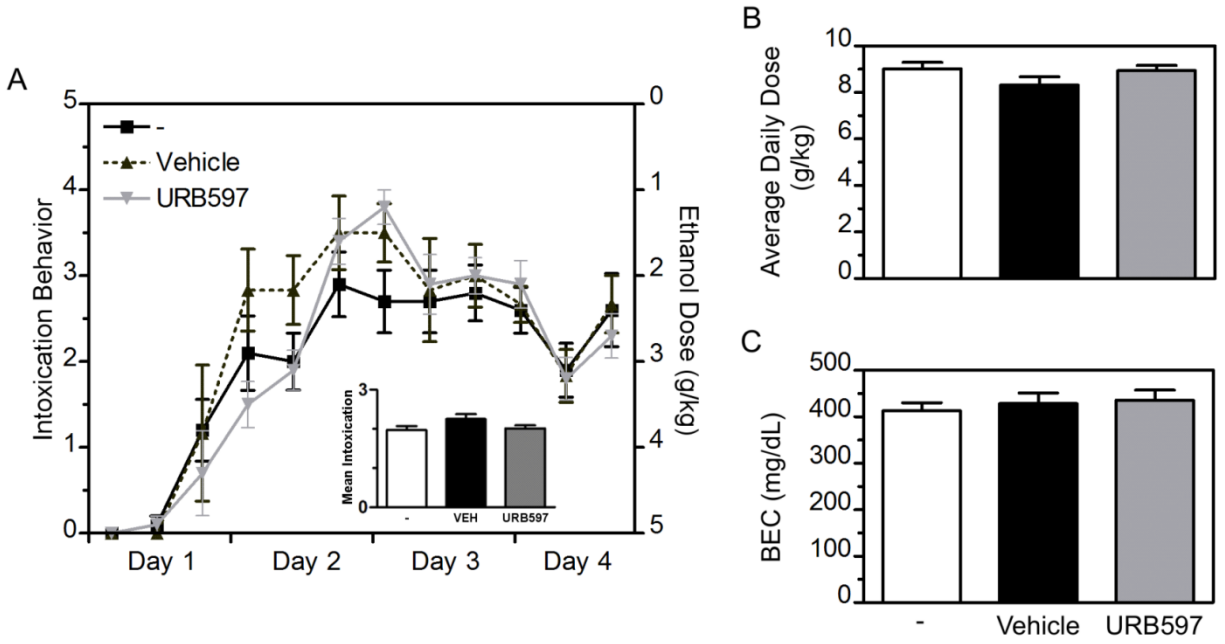

Figure 5.2 Binge intoxication measures for URB597 neuroprotection study.

Rats were treated according to a modified Majchrowicz binge and co-administered nothing (n =10), vehicle (n = 6 or 10, *see methods*) or URB597 (0.3 mg/kg, n = 10)). (A) Intoxication scores (left axis) and ethanol doses (right axis) were similar among all groups during the 4 days of binge treatment. (B) Average daily doses and (C) BECs were not different among groups.

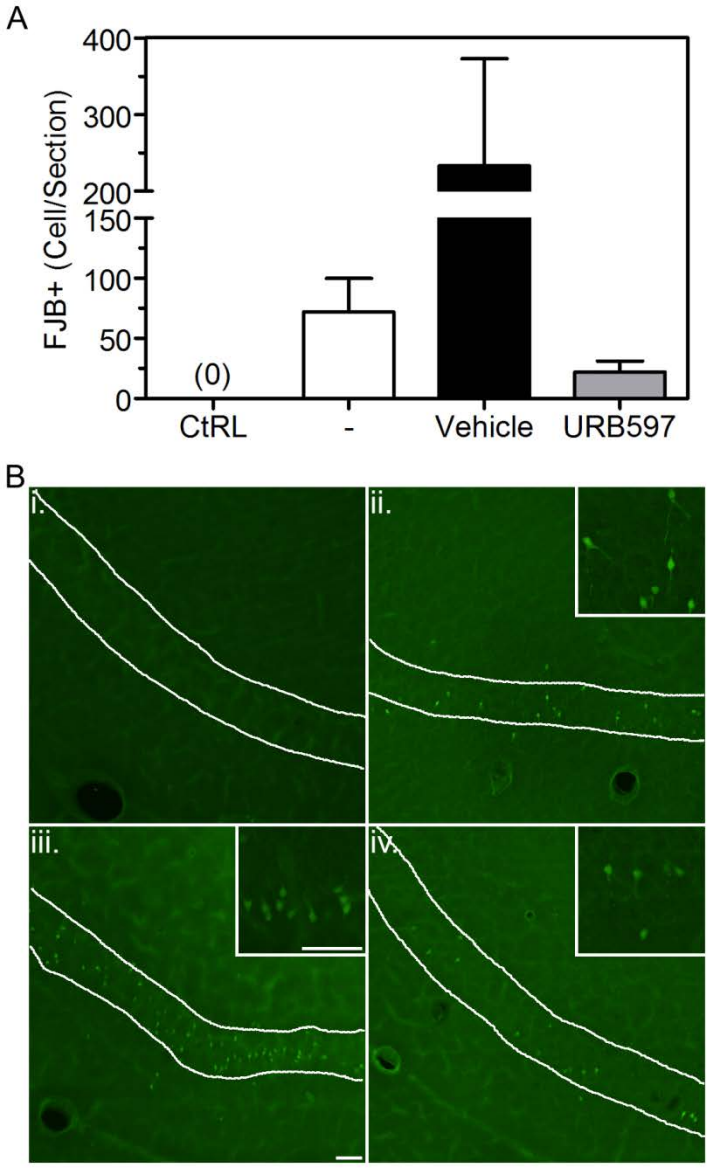

Figure 5.3 FJB staining in the ventral dentate gyrus following 4-day binge treatment.

(A) Quantification of FJB+ cells following binge ethanol treatment. (B) Representative images of FJB staining in the vDG. i. control; ii. ethanol; iii. ethanol + vehicle; iv. ethanol + URB597. Scale bar = 50 µM

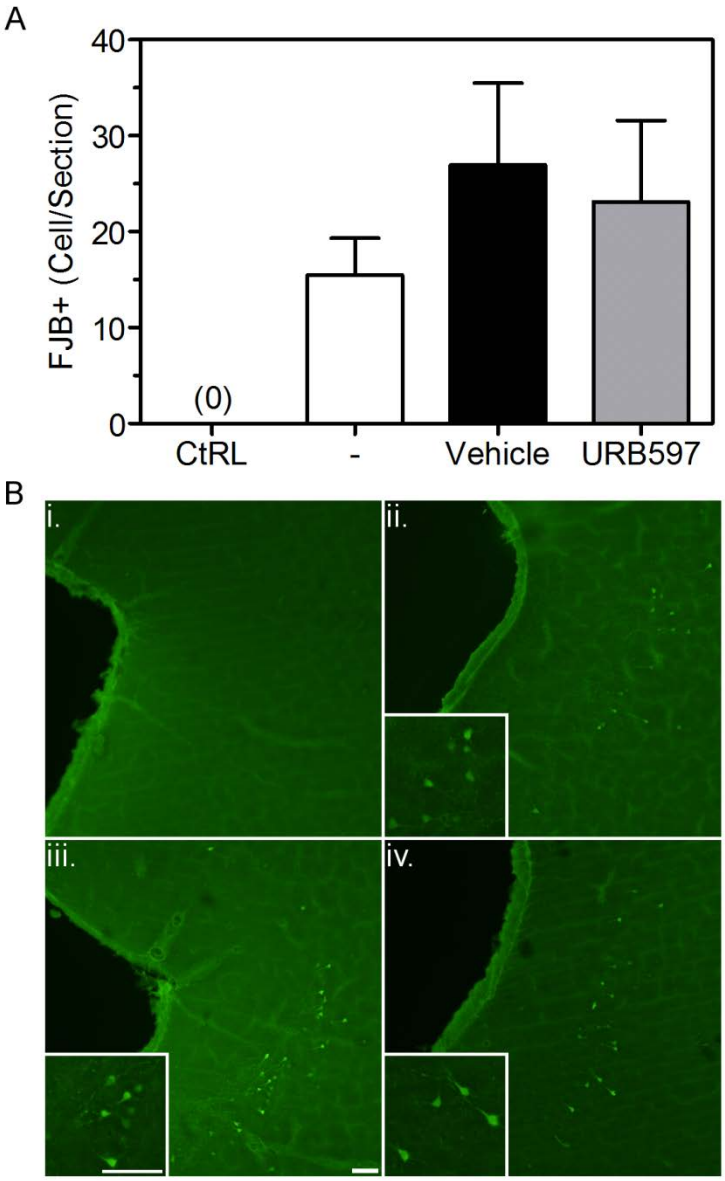

Figure 5.4 FJB staining in the entorhinal cortex following 4-day binge treatment.

(A) Quantification of FJB+ cells following binge ethanol treatment. (B) Representative images of FJB staining in the EC. i, control; ii, ethanol; iii ethanol + vehicle; iv, ethanol + URB597. Scale bar = 50 µM

Additionally, these cells showed evidence of necrosis including shrunken cell bodies, which is consistent with previous characterization of binge ethanol induced cell death [114]. In the ventral dentate gyrus, controls typically had < 1 cell/section, therefore control groups were collapsed before analysis.One-way ANOVA, revealed an effect of treatment ($F_{(3,40)} = 4.634$, $p < 0.01$). Post-hoc analysis showed that binge ethanol treatment resulted in significant increase in FJB+ labeling in the ventral dentate gyrus ($p < 0.001$). Although URB597 administration appeared to reduce FJB+ cell counts (Figure 5.3), this difference failed to reach statistical significance compared to ethanol only ($p = 0.12$) or ethanol + vehicle ($p = 0.19$) groups. Similarly, in the entorhinal cortex, controls also displayed < 1 cell/section and were therefore collapsed across drug treatment before analysis by one-way ANOVA which revealed an effect of treatment ($F_{(3,40)} = 5.937$, $p < 0.01$). Post-hoc analysis showed that binge ethanol resulted in significant FJB+ labeling in the entorhinal cortex ($p < 0.001$), however URB597 treatment did not reduce FJB+ cells compared to the ethanol only or ethanol + vehicle groups ($p > 0.05$, Figure 5.4).

5.3.2 Experiment 2: N-acylethanolamide quantification ("target engagement" study)

Experiment 2 evaluated the effect of URB597 on NAE tissue content in the hippocampus and entorhinal cortex following a single day of binge ethanol exposure. Rats were treated with ethanol or control diet for a single day (3 doses) and were administered an acute dose of URB597 (0.3 mg/kg) 2 hours prior to NAE quantification (Figure 5.1B). Binge ethanol exposure was not different across drug treatment groups as all intoxication measures were statistically similar. The grand mean intoxication behavior was 0.24 ± 0.06, which resulted in a grand mean daily ethanol dose of 14.3 ± 0.2 g/kg/d. BECs were measured 2 h following the 3rd dose of ethanol and the grand mean was 309.2 ± 14.3 mg/dL.

In the hippocampus, two-way ANOVAs revealed significant effects of drug treatment on AEA ($F_{(2,30)} = 10.82$; $p < 0.001$), OEA ($F_{(2,30)} = 36.91$; $p < 0.001$) and PEA ($F_{(2,30)} = 46.74$; $p < 0.001$) tissue content (Figure 5.5A-C). In ethanol naïve rats, post-hoc analyses found that URB597 elevated AEA by 57.6% ($p < 0.05$), OEA by 475.4% ($p < 0.001$) and PEA by 986.6% ($p < 0.001$) in the hippocampus compared to rats treated with vehicle. Although NAE content was much lower in ethanol treated rats following UBR597 administration, post-hoc analyses showed that URB597 elevated AEA by 48.1% ($p < 0.05$), OEA by 188.8% ($p < 0.01$) and PEA by 287.2% ($p < 0.01$) relative to ethanol + vehicle treated rats. Additionally, there was a significant diet x drug interaction for OEA ($F_{(2,30)} = 3.781$; $p < 0.05$) and PEA ($F_{(2,30)} = 6.381$; $p < 0.01$) in the hippocampus. Post-hoc analysis showed that compared to control treated rats, ethanol decreased URB597-mediated elevations of OEA by 36.3% ($p < 0.05$) and PEA by 44.7% ($p < 0.001$) in the hippocampus.

In the entorhinal cortex, two-way ANOVA reveal a significant effect of drug treatment on the levels of OEA ($F_{(2,29)} = 27.40$; $p < 0.001$) and PEA ($F_{(2,29)} = 34.63$; $p < 0.001$), but not on AEA ($F_{(2,29)} = 0.60$; $p > 0.05$; Figure 5.5D-F). In ethanol naïve rats, post-hoc analyses revealed significant effects of URB597 administration compared to rats treated with vehicle for OEA by 250.3% ($p < 0.001$) and PEA by 435.0% ($p < 0.001$). This effect was also evident in ethanol treated rats as URB597-mediated elevations in OEA ($p < 0.001$) and PEA ($p < 0.001$) were 268.6% and 636.7% higher than in ethanol + vehicle treated rats. There was also a main effect of diet on PEA levels in the entorhinal cortex ($F_{(1,29)} = 4.344$; $p < 0.05$). Post-hoc analysis showed that PEA content following URB597 administration in ethanol rats was 36.7% lower than control rats administered URB597 ($p < 0.05$).

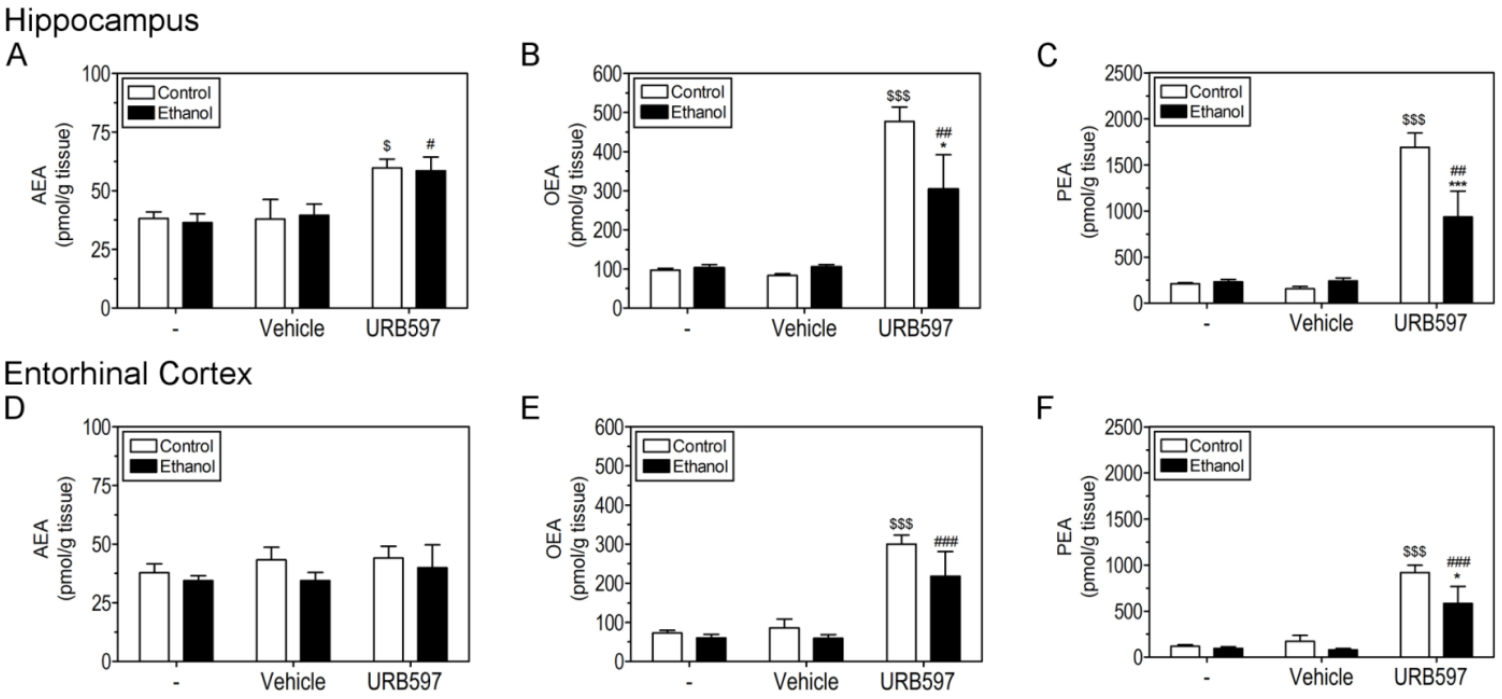

Figure 5.5 N-acylethanolamide quantification in the hippocampus and entorhinal cortex following a single injection of URB597.

URB597 mediated elevations of OEA and PEA in the hippocampus (B,C) and entorhinal cortex (F) were blunted in rats treated with binge ethanol. (D) URB597 mediated elevation of AEA content was absent in the entorhinal cortex. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared to URB597 treated controls. $, $p < 0.05$; $, $p < 0.01$; $$$, $p < 0.001$ compared control or control + vehicle. #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$ compared ethanol or ethanol + vehicle.

Pearson correlations were performed on BECs vs. NAE content to further examine the relationship between ethanol and NAE content following URB597 administration (Figure 5.6A-F). In the hippocampus, OEA ($r^2 = 0.78$; $p < 0.05$) and PEA ($r^2 = 0.74$; $p < 0.05$) were significantly correlated with BECs. In the entorhinal cortex, AEA content was significantly correlated with BECs ($r^2 = 0.75$; $p < 0.05$), while correlations for OEA ($r^2 = 0.62$) and PEA ($r^2 = 0.60$) vs. BECs trended towards significance ($p = 0.07$).

5.4 Discussion

The current study evaluated the neuroprotective effects of FAAH inhibition in a model of ethanol-induced neurodegeneration. However, neuroprotection was not observed in the dentate gyrus of the hippocampus or entorhinal cortex following repeated administration of the FAAH inhibitor, URB597 (Figures 5.3 & 5.4). In light of these results, the effect of binge ethanol treatment on URB597-mediated NAE elevation in the hippocampus and entorhinal cortex was examined (Figure 5.5). In agreement with previous studies [174, 288], an acute dose of URB597 resulted in general elevations of AEA, OEA and PEA, with the exception that AEA elevations were not detected in the entorhinal cortex. NAE elevation was also observed in the hippocampus and entorhinal cortex of binge ethanol treated rats, however this effect was significantly reduced by ethanol. Correlation analysis revealed a negative association between BECs and URB597-mediated elevations in NAE content (Figure 5.6), which further supports that high BECs disrupt the biological activity of URB597. This interaction may be partly responsible for the lack of neuroprotection following FAAH inhibition in this model.

The eCB system is theorized to play a vital role in protecting the CNS from acute brain insults and is important for engaging homeostatic mechanisms for counteracting CNS disease progression.

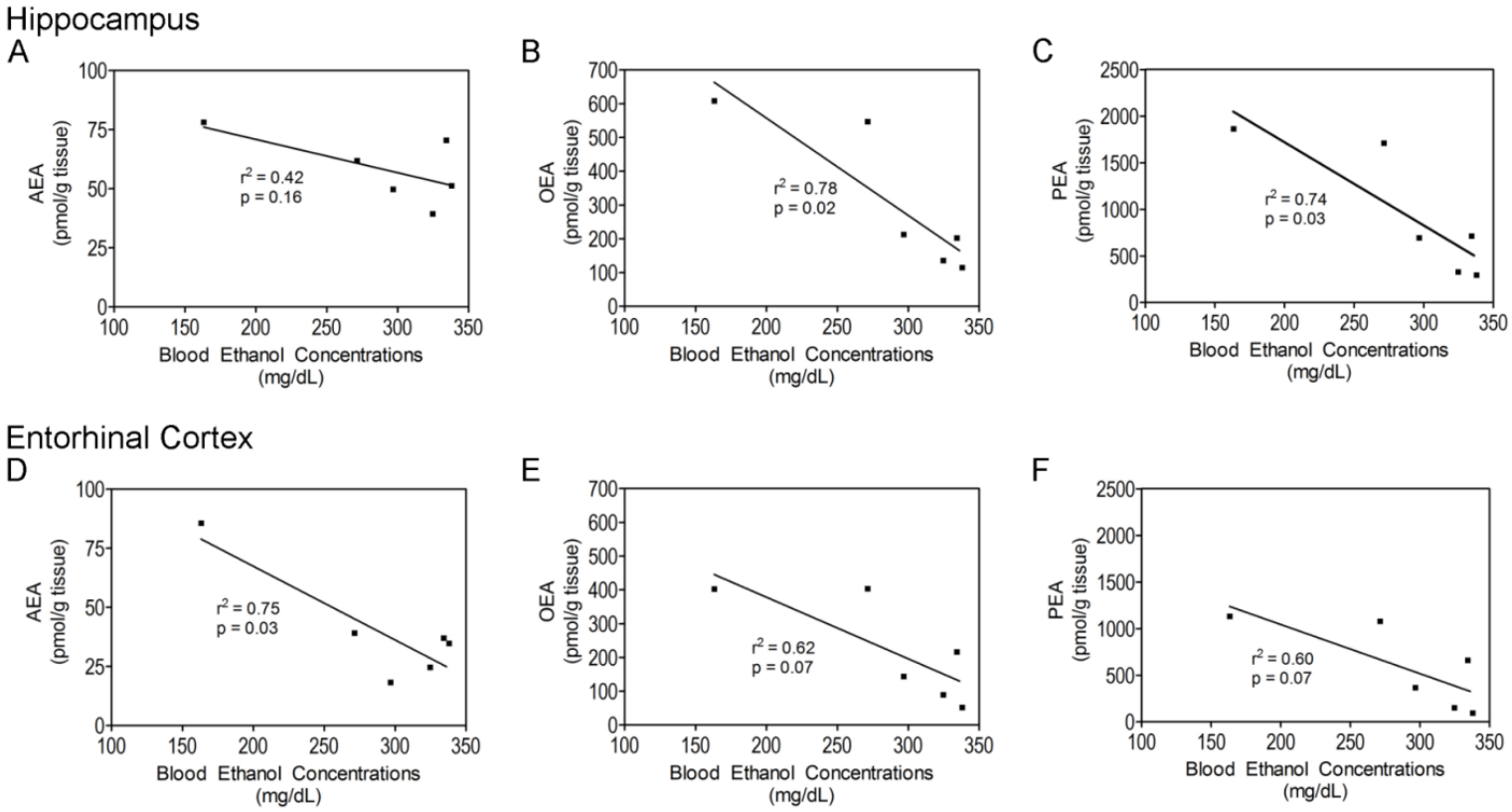

Figure 5.6 Pearson correlations of n-acylethanolamide content and BECs following a single injection of URB597.

AEA, OEA and PEA content in the hippocampus (A-C) and in the entorhinal cortex (D-F). OEA and PEA content was negatively correlated to BECs in the hippocampus (B,C), which was absent for AEA (A). AEA content was negatively correlated to BECs in the entorhinal cortex (A), while OEA and PEA content trended to a negative correlation in the entorhinal cortex (E,F).

For example, studies have shown that AEA and/or 2-AG accumulate following acute tissue damage [213, 214, 216-219] and under chronic neuroinflammatory conditions [215]. Additionally, genetic inactivation of CB1Rs enhances susceptibility to neuropathology [218, 222], while neuroprotection and attenuation of neuroinflammation is observed following CB1R and/or CB2R activation [234, 316, 322].

The exact mechanisms governing cannabinoid-mediated neuroprotection are not completely elucidated; however it is clear that the effects of cannabinoids are pleiotropic and disease state specific. During acute neuronal injury, CB1R activation can decrease neuronal excitability, reduce excessive glutamate neurotransmission and reduce excessive Ca^{2+} influx associated with excitotoxicity [218, 224, 225]. Conversely, during secondary injury progression or chronic neuropathology, the cannabinoids promote neuroprotection through CB1Rs and/or CB2Rs by attenuating pro-inflammatory signaling and modulating microglial activity [215, 234, 235, 316]. Within this general framework, additional reports suggest that eCBs are also responsible for activation of cell survival signaling networks through CB1R- and CB2R- dependent and non-dependent mechanisms [307, 316, 323]. For example, AEA and OEA are ligands for the PPAR nuclear receptors, which accounts for some of the neuroprotective effects of these lipids [266]. Therefore, the eCBs and NAEs are an enticing target for the treatment of ethanol-induced neurodegeneration as excitotoxicity, neuroinflammatory signaling and oxidative stress are mechanisms mediating the neurotoxic effects of ethanol [126, 136]. In fact, CB1R activation prevents ethanol-induced potentiation of NMDA neurotoxicity, possibly through reducing intracellular Ca^{2+} transients [314].

Neuroprotection was not observed in the current study even though FAAH inhibition following a single day of binge treatment was associated with elevations in AEA, OEA and PEA, albeit at a lower magnitude in ethanol-treated rats (Figure 5.5). Although FAAH inhibition induced elevations in NAE content following FAAH inhibition,

this treatment failed to achieve the high tissue concentrations of NAEs typically observed following a neurotoxic insult [214, 217], or concentrations expected following injury-induced NAE production in concert with FAAH inhibition. Compensatory elevations in eCBs and/or NAEs in response to CNS pathologies is thought to be an endogenous mechanism to prevent the spread of neurodegeneration following an insult [296, 297] and it is hypothesized that FAAH inhibitors act by amplifying this endogenous response [291]. Such an endogenous response was examined following binge ethanol induced neurodegeneration (chapter 4), however, elevations in AEA, OEA or PEA were not observed in the hippocampus or entorhinal cortex. Therefore, a lack of endogenous NAE accumulation following binge ethanol induced neurodegeneration may lower the capacity of FAAH inhibitors to produce therapeutic NAE concentrations. Unfortunately, 2-AG tissue content was not measured in chapter 4 as 2-AG has been shown to accumulate following brain damage [219] even in the absence of AEA accumulation [324]. It would be interesting to measure 2-AG in future studies, as this may point to other efficacious treatment strategies to prevent binge ethanol-induced neurodegeneration, such as MAGL inhibitors, duel FAAH/MAGL inhibitors, and/or eCB transport inhibitors [324, 325].

Limitations in the treatment strategy employed in the current study may have also contributed to the lack of URB597-mediated neuroprotection. Initial characterization of URB597 found that substantial FAAH inhibition occurred for at least 16 hours; however the timecourse of NAE elevation was more constrained as AEA, OEA and PEA content was elevated for only 6 hours [174]. Therefore, since URB597 was administered on a 12-hour schedule, it is unlikely that NAE accumulation was entirely maintained throughout the 3 days of treatment. It is unclear whether more frequent or higher dosing would be beneficial since there is an apparent temporal dissociation between FAAH inhibition and NAE accumulation following URB597 treatment. However, one may speculate that a small fractional recovery in FAAH activity following URB597

administration may be sufficient for maintaining basal NAE levels and therefore a more frequent dosing schedule or higher doses may be required. Additionally, experiment 2 results suggest that FAAH inhibition was compromised by binge ethanol treatment (Figure 5.6). The cause for this impairment is unknown; however it is possible that the PK/PD of URB597 may be altered by ethanol as it is well documented that ethanol can interfere with the PK/PD properties of xenobiotics [262, 263]. Correlations suggested that BECs may be an important factor associated with diminished URB597-mediated NAE elevation. In fact, two rats with BECs approaching 350 mg/dL failed to have detectable elevations in the three NAE's examined following URB597 administration (Figure 5.6). With this in mind, BECs from rats in the neuroprotection study averaged over 400 mg/dL therefore, URB597-mediated NAE elevation may have been severely diminished over the course of binge treatment. Thus, the dosing protocol implemented and/or the reduced efficacy of URB597 in binge ethanol treated rats appears to explain the lack of neuroprotection in the first experiment (Figures 5.3 and 5.4).

The discrepancy between reports that observed neuroprotection following FAAH inhibition and the current study could be related to differences in experimental models. Previous studies reporting on the neuroprotective effects of FAAH inhibition have demonstrated efficacy in models of kainic acid-induced excitotoxicity and focal cerebral ischemia, both of which produce an excitotoxic event [214, 253, 318]. Conversely, the neurotoxic effects of the binge alcohol exposure model utilized in the current study may be independent of excitotoxic mechanisms [118, 121]. Therefore, the current data could suggest that FAAH inhibitors may be particularly efficacious at attenuating excitotoxicity and not for other mechanisms of neurotoxicity. Alternatively, FAAH inhibitors may be more effective as a pretreatment, where NAE levels have time to accumulate (which can take multiple hours with current FAAH inhibitors [174, 318]) prior to the induction of neurotoxicity. In support of this hypothesis, Degn et al., 2007, observed neuroprotection

when URB597 was administered 1.5 h prior to MCAO, but not if URB597 was administered immediately following focal cerebral ischemia [214]. As neurotoxicity is initiated following as little as 1 or 2 days of binge ethanol treatment, it is possible that administration of URB597 was initiated to lake in the current study.

Although FAAH inhibition is associated with ERK phosphorylation presumably though CB1Rs [318], it has yet to be determined whether FAAH inhibitors recapitulate all the neuroprotective mechanisms of CB1R and/or CB2R agonists. For example, cannabinoid agonists can mediate neuroprotection through inhibition of NF-κB-DNA binding [326], reduction of COX-2 and iNOS expression [235, 327] and induction of neurotrophins such as BDNF [218, 307, 316]. However, these effects have not been demonstrated following FAAH inhibition. NF-kB signaling programs are hypothesized to be important inducers of binge ethanol-induced neurodegeneration [126], as neuroprotection from binge ethanol exposure is associated with reductions in NF-kB-DNA binding and attenuation of pro-inflammatory and free radical producing enzymes [120, 136]. Therefore, further understanding of the signaling networks engaged following FAAH inhibition as well as additional mechanisms of ethanol-induced brain damage, would allow for assessment of whether optimization of FAAH inhibition strategies is worth pursuing for future drug discovery/development in the context of ethanol-induced neurodegeneration.

Another important finding in the current study was that URB597-mediated NAE elevation appeared to be brain region and NAE specific (Figure 5.5). For example, enhancement of AEA was observed in the hippocampus but not in the entorhinal cortex. Additionally, URB597 had different relative magnitudes of effect as PEA and OEA were elevated substantially, while AEA was affected marginally. This brain region specific effect is consistent with another study reporting URB597-mediated AEA elevations in the midbrain, thalamus and striatum but not in the prefrontal cortex or hippocampus even

though FAAH activity was substantially inhibited [288]. Similarly, pharmacodynamic tolerance may develop to URB597, as a recent study found that an acute dose of URB597 resulted in increased levels of AEA, OEA and PEA in the spinal cord, though, this effect was lost after repeated dosing [292]. Interestingly, this tolerance was associated with reduced NAPE-PLD protein expression. Therefore, adaptations in NAE biosynthetic pathways could explain the lack of neuroprotection observed in the current study and future studies should examine this possibility.

5.5 Conclusions

There is a critical need for the development of new pharmacotherapies for the treatment of AUDs as current options are less than adequate. Since chronic ethanol consumption is associated with neurodegeneration, neuroprotective drugs should prove to be beneficial treatment options. The current study tested whether enhancing eCB signally by FAAH inhibition could afford neuroprotection in a model of binge ethanol exposure; however these efforts were surprisingly unsuccessful. Nevertheless, this study in combination with other reports point to multiple factors that may be important for targeting the eCB system by interfering with their degradation. With this insight and with future drug discovery efforts, it may be possible to optimize such treatment strategies for preventing ethanol-induced neurodegeneration. Based off the current data, URB597 dose-response relationships for neurochemical response (target engagement) and neuroprotection should be investigated in future studies.

6. CHAPTER 6

GENERAL DISCUSSION AND FUTURE DIRECTIONS

The overall goal of this dissertation was to investigate novel pharmacotherapeutic approaches for the treatment of AUDs, with a focus on preventing ethanol-induced neurodegeneration. The cannabinoids, including phytocannabinoids, synthetic cannabinoids and the eCBs, have emerged as potent neuroprotective agents in a variety of preclinical neurodegenerative models (see section 1.8). However, only a couple of reports to date have investigated cannabinoid based pharmacotherapies for the treatment of ethanol-induced neurodegeneration. For example, the phytocannabinoid, CBD, is a potent antioxidant and has been previously shown to attenuate neurodegeneration following binge ethanol exposure [121]. Additionally, the synthetic CB1R agonist, HU-210, can prevent ethanol withdrawal induced potentiation of NMDA excitotoxicity in primary cortical neurons [314], an effect replicated in organotypic hippocampal slice cultures using the synthetic CB1R agonist, CP-55,940 (figure 7.1).

To date there are three examples of cannabinoids with approval for clinical use [328]. These include nabilone, a Δ^9-THC analogue, used for the treatment of nausea and vomiting caused by chemotherapeutics, dronabinol (Δ^9-THC), used as an appetite stimulant in AIDS patients, and sativex, a Δ^9-THC/CBD co-formulation, used for the treatment of neuropathic pain in multiple sclerosis patients and as an analgesic adjunct therapy for patients with advanced stage cancer. Considering that preclinical research has shown that the eCB system is a potentially useful molecular target for the treatment of numerous CNS and peripherally mediated diseases, it is disappointing that only three cannabinoid based drugs are approved for clinical use. The lack of success for translating cannabinoid based preclinical findings to approved pharmacotherapies is primarily due to unfavorable PK /PD profiles of many cannabinoid derived agents. For

example, CBD has utility for a variety of indications including neurodegenerative disease, addiction, pain, anxiety and epilepsy. However, the clinical use of CBD has failed to come to fruition because of significant first pass metabolism, leading to poor bioavailability [246, 247]. Additionally, the use of cannabinoids acting at central CB1Rs has failed generally due to significant off target effects, primarily neuropsychiatric in nature. For instance, the CB1R antagonist, SR141716 (Rimonabant), underwent substantial clinical development for the treatment of obesity, however trials were suspended due to increased risk of serious neuropsychiatric side effects including anxiety, depression and suicidal ideation [329]. In light of these failures, next generation cannabinoid based pharmacotherapies will need to address these PK /PD obstacles [330, 331].

The studies within the current dissertation were not only designed to investigate the efficacy of cannabinoid based pharmacotherapies for the treatment of ethanol-induced neurodegeneration, but were also designed to circumvent the aforementioned PK/PD obstacles associated with the cannabinoids. In chapter 2, experiments were designed to extend current reports on the neuroprotective effects of CBD following binge ethanol exposure by developing transdermal formulations in order to bypass the first pass effect that has hindered the use of CBD in humans. In collaboration with AllTranz, Inc., multiple topical gel formulations of CBD were developed and evaluated for neuroprotective efficacy using a well-established model of an AUD that produces substantial neurodegeneration in the corticolimbic pathway, the modified Majchrowicz 4-day binge [110, 111]. These experiments were successful in establishing a transdermal formulation that afforded neuroprotection, and importantly transdermal CBD was as efficacious as IP administration of CBD in preventing cell death in the entorhinal cortex (figure 2.6). Although significant challenges were encountered (section 2.4), the results of chapter 2 are promising and warrant further development of transdermal delivery of

CBD for the treatment of ethanol-induced neurodegeneration and for AUDs. Analysis of CBD plasma concentration (figure 2.4) suggested that target plasma levels need to be achieved early during ethanol exposure (discussed in section 2.4), therefore it would be interesting to determine whether pretreatment of transdermal CBD would afford further neuroprotection in future studies. This notion is particularly interesting in the context of one theoretical treatment strategy where an alcohol dependent patient would apply a transdermal formulation prophylactically during recovery, likely as an adjunct therapy. CBD has the potential to be an effective drug for the treatment of AUDs as this phytocannabinoid has pleiotropic effects and has been shown to be effective at preventing convulsions, anxiety, and drug seeking behavior, inhibition of which would be useful in the treatment of AUDs (see section 2.4). Therefore, future preclinical studies should also evaluate the effect of transdermal CBD on these other indications. Although the neuroprotective effects of CBD are attributed to its potent antioxidant capacity, CBD has a variety of other molecular targets. For example, CBD and a major CBD metabolite, (-)-7-OH-CBD, have the capacity to block AEA uptake and AEA hydrolysis by FAAH [251]. Although it is unclear whether these other molecular targets are in any way responsible for CBD mediated neuroprotection following binge ethanol treatment, the potential for CBD to interact with the eCB system lead to the hypothesis that targeting the eCB system by inhibiting AEA metabolism by administering the FAAH inhibitor, URB597, would attenuate binge ethanol induced neurodegeneration (Chapter 5).

Over the past 15 years, it has become increasingly apparent that the eCB system is involved in providing endogenous neuroprotection (see section 1.8). For example, numerous studies have observed 2-AG, AEA and/or NAE accumulation following experimental brain damge and in models of chronic neurodegeneration and importantly, other studies have found that CB1R null-mutant mice are more susceptible to neurotoxicity [218, 222]. Based on these reports, Chapter 4 examined the effect of binge

ethanol induced neurodegeneration on AEA, OEA and PEA content and CB1R expression in the entorhinal cortex and hippocampus, the two brain regions most susceptible ethanol-induced neurodegeneration. Surprisingly, only minor alterations in NAE tissue content were observed following either 2 or 4 days of binge ethanol treatment (figure 4.4) suggesting that ethanol and associated neurotoxicity do not engage the eCB system, at least in the context of this model, which is in contrast to the aforementioned reports. It is possible that binge ethanol treatment may have resulted in more profound effects on 2-AG as seen in other experimental models [219], however the LC-MS method originally developed to measure AEA, OEA and PEA (see Chapter 3) was not effective in accurately quantifying 2-AG levels. Therefore, future studies should determine the relationship between ethanol-induced neurodegeneration and 2-AG tissue content. In regards to CB1R expression, binge ethanol treatment resulted in only a transient down regulation in the corticolimbic pathway, which was most evident following 2-days of binge exposure (figures 4.2 and 4.3). CB1R down regulation following ethanol exposure is consistent with the literature [187], however, unique to the current data, CB1R expression normalized prior to ethanol withdrawal. It was originally hypothesized that binge ethanol-induced down regulation would increase susceptibility to neurodegeneration, which was supported by numerous studies demonstrating that CB1R null-mutant mice are more susceptible to neurotoxicity [218, 222] and dysregulation of CB1Rs can precede neuropathology [332]. Additionally, CB1Rs are coupled to multiple cell survival pathways including PKB/AKT and CREB [212]. However, the modest and transient CB1R down regulation reported in chapter 4 suggests that CB1R down regulation is not a major mechanism of increased susceptibility to neurodegeneration following binge ethanol treatment.

Although elevated NAE tissue content was not observed following binge ethanol treatment, NAEs can be pharmacologically elevated by inhibition of FAAH [174].

Additionally, FAAH inhibition affords neuroprotection in both *in vitro* and *in vivo* models of neuronal injury [291, 318, 319]. Therefore, experiments in chapter 5 tested the hypothesis that FAAH inhibition would attenuate neurodegeneration in the entorhinal cortex and hippocampus following binge ethanol exposure. Although, neuroprotection was not observed following administration of the FAAH inhibitor, URB597 (figures 5.3 and 5.4), preliminary PK/PD evaluation suggested that ethanol impaired the ability of URB597 to elevate NAE content (figures 5.5 and 5.6). Therefore, it is currently unclear whether FAAH is a viable target for preventing neurodegeneration following binge ethanol treatment. Noteworthy, although FAAH inhibition did not significantly attenuate cell death, a trend was observed in the dentate gyrus but not in the entorhinal cortex, which may be explained by brain region specific effects of FAAH inhibition as URB597 mediated elevation of AEA tissue content was only observed in the hippocampus. Interestingly, FAAH expression is substantially higher in the hippocampus compared to the cortex [179], which may explain the brain region specific effects reported in chapter 5. Additionally, studies have found that AEA is a substrate for COX-2 [333], an enzyme that is up regulated following the 4-day binge in the cortex, but not dentate gyrus [124], which may also contribute to lack of AEA elevation and neuroprotection in the entorhinal cortex. Nevertheless, additional studies and optimization of treatment protocols will be necessary to definitively determine the utility of FAAH inhibition as a target to afford neuroprotection following binge ethanol treatment. For example, only a single dose of URB597 was tested for neuroprotective efficacy. Although the dose of URB597 was chosen based off previous reports demonstrating maximum FAAH inhibition following 0.3 mg/kg, this was determined in mice and not in the context of binge ethanol treatment. Therefore, in light of the facts that the rats were used in the current dissertation and that the current results suggest the binge ethanol blunts the

biological activity of URB597, it may be necessary to evaluate higher doses or more frequent dosing intervals to observe neuroprotection.

To date, no studies have been reported that pertain to combination drugs for the treatment of ethanol-induced neurodegeneration. As ethanol neurotoxicity can be initiated by multiple overlapping mechanisms and the fact that all current preclinical reports have not blocked ethanol-induced neurodegeneration, but rather only partially attenuate cell death, it is reasonable to hypothesize that combination treatments may provide more efficacious treatment strategies. In the context of the current dissertation, multiple drug combinations could be examined with reasonable rationale. COX-2 is known to contribute to neurodegeneration and is up regulated by binge ethanol treatment and withdrawal. Additionally, COX-2 is also responsible for metabolizing both AEA and 2-AG as discussed previously. Therefore, the co-inhibition of COX-2 and FAAH may prove to enhance neuroprotection outcomes and should be examined in future studies. A second interesting target for combination drug therapy in context of the current dissertation is MAGL, which is responsible for 2-AG catabolism. 2-AG is a full agonist at both CB1Rs and CB2Rs and has been shown to mediate neuroprotection following a variety of neurotoxic insults by suppressing COX-2 expression and NF-κB activity [219, 235, 334, 335]. Therefore, it is possible that dual inhibition of FAAH and MAGL or eCB transport may be a more efficacious neuroprotective strategy [253, 325].

In conclusion, the studies within the current dissertation examined whether cannabinoid based pharmacotherapies are effective in preventing ethanol-induced neurodegeneration using a well-established model of an AUD. Currently, there are only four approved pharmacotherapies for the treatment of AUDs and these therapies generally have limited efficacy for this heterogeneous disease. Furthermore, these drugs only target the reinforcing effects of ethanol, even though chronic ethanol consumption results in neurodegeneration, cognitive dysfunction and behavioral impairments that

contribute to the development and maintenance of an AUD. Therefore, it is of utmost importance that new pharmacotherapies be developed to increase the repertoire of effective treatments for the treatment of alcoholism. As chronic ethanol intake, characteristic of an AUD, results in neurodegeneration and cognitive deficits that are hypothesized to contribute to the chronic and relapsing nature of these disordes, it likely that neuroprotective agents will have therapeutic utility and future research efforts need to be aimed at translational development these pharmacotherapies.

7. APPENDEX

7.1 Supplementary figures

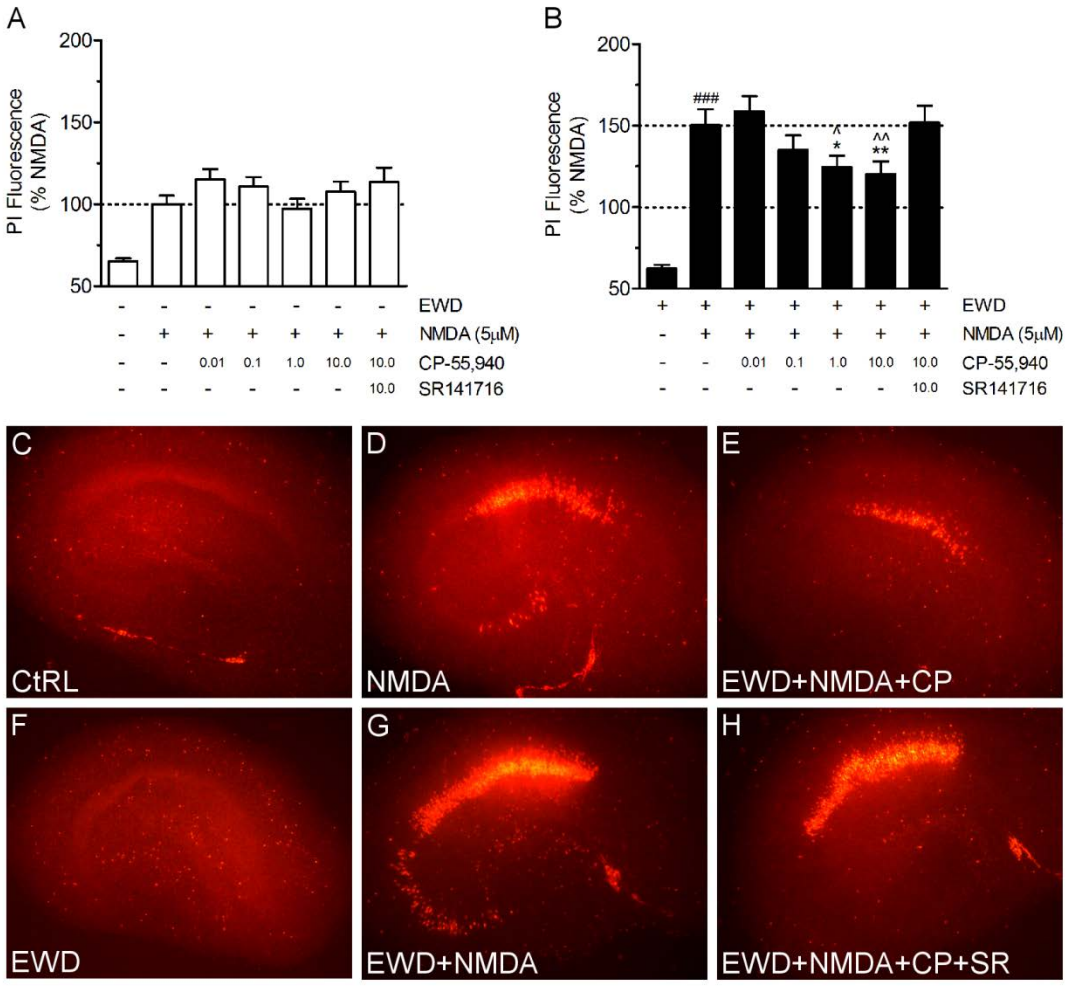

Figure 7.1CB1R activation attenuates ethanol withdrawal induced potentiation of NMDA neurotoxicity.

CP-55,940 dose dependently attenuated ethanol withdrawal induced potentiation of NMDA neurotoxicity in organotypic hippocampal slice cultures (OHCSs), however was ineffective at preventing NMDA neurotoxicity in ethanol naïve OHCSs. Neuroprotection was mediated through CB1R activation as the CB1R selective antagonist, SR141716,

blocked neuroprotection afforded by CP-55,940. (A) Quantification of propidium iodide (PI) uptake in CA1 of ethanol naïve OHSCs. (B) Quantification of PI in CA1 of ethanol withdrawn OHCSs. (C-F) Representative images of PI uptake; EWD, ethanol withdrawal; CP, CP-55,940 (10 μM); SR, SR141716 (10 μM). ###, $p < 0.001$ compared to NMDA; *, $p < 0.05$ and **, $p < 0.01$ compared to EWD + NMDA; ^, $p < 0.05$ and ^^, $p < 0.01$ compared to EWD + NMDA + CP-55,940 (10 μM) + SR141716 (10 μM).

7.2 List of Abbreviations

2-AG, 2-arachidonoylglycerol

AEA, anandamide arachidonoylethanolamide

ACN, acetonitrile

AMPA, 2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl) propionate

ANOVA, analysis of variance

AUD, alcohol use disorder

BDNF, brain derived neurotrophic factor

BEC, blood ethanol concentration

BHT, butylated hydroxytoluene

BSA, bovine serum albumin

CA, Cornu Ammonis

cAMP, cyclic adenosine monophosphate

CCL2, chemokine (c-c motif) ligand 2

CEC, chronic ethanol consumption

ChAT, choline acetyltransferase

CNS, central nervous system

COX-2, cyclooxygenase 2

CV, coefficient of variation

DAG, diacylglycerol

DAGL α/β, diacylglycerol lipase α/β

DMSO, dimethyl sulfoxide

DNA, deoxyribonucleic acid

eCB, endocannabinoid

ESI, electrospray ionization

FAAH, fatty acid amide hydrolase

FAK, focal adhesion kinase

FDA, food and drug administration

FJB, fluoro-jade b

GABA, gamma-amino-butyric acid

GC-MS, gas chromatography mass spectroscopy

GIRK, g-protein coupled inward rectifying potassium channel

GPR55, g-protein receptor 55

HEC, hippocampal entorhinal cortex

HMGB1, high motility group protein B1

Iba1, ionized calcium-binding adapter molecule 1

iNOS, inducible nitric oxide synthase

IL1β, interleukin 1β

IP, intraperitoneal

KA, kainic acid

LC-MS, liquid chromatography mass spectroscopy

MAPK, mitogen activated protein kinase

MCAO, middle cerebral artery occlusion

MCP-1, monocyte chemotactic protein 1

mGLUR5, metabotropic glutamate receptor 5

MS, mass spectroscopy

NAE, n-acylethanolamide

NAPE, n-acyl phosphatidylethanolamide

NAPE-PLD, n-acyl phosphatidylethanolamide – phospholipase D

NMDA, N-methyl-D-aspartate

NOX, NAPDH oxidase

OEA, oleoylethanolamide

PBS, phosphate buffered saline

PD, pharmacodynamics

PE, process efficiency

PEA, palmitoylethanolamide

PFA, paraformaldehyde

PFC, prefrontal cortex

PI, propidium idodide

PI3K, phosphoinositide-3-kinase

PIP2, phosphatidylinositol 4,5 bisphosphate

PK, pharmacokinetic

PLA2, phospholipase A2

PLCβ, phospholipase Cβ

PO, *per os* (by mouth)

PPAR, peroxisome proliferator activated receptor

QC, quality control

ROS, reactive oxygen species

RNS, reactive nitrogen species

SPE, solid phase extraction

TLC, thin-layer chromatography

TLR, toll like receptor

TNFα, tumor necrosis factor α

TRPV1, transient receptor potential cation channel VI

Δ^9 – THC, Δ^9 - tetrahydrocannabinol

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9. VITA

EDUCATION

Degree	Doctor of Philosophy in Pharmaceutical Sciences
2007-Current	College of Pharmacy, University of Kentucky, Lexington, Kentucky Mentor: Kimberly Nixon, Ph.D. Dissertation: *Modulation of endocannabinoids in models of alcohol-induced neurodegeneration: a therapeutic modality?*
Degree Awarded	Bachelors of Science in Biology, College of Liberal Arts and Sciences
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PROFESSIONAL EXPERIENCE

2007-Present	Graduate Student, Department of Pharmaceutical Sciences, College of Pharmacy, University of Kentucky, Lexington, Kentucky Mentor: Kimberly Nixon, Ph.D.
2005-2007	Research Assistant, Biology Department, College of Liberal Arts and Sciences DePaul University, Chicago, Illinois Mentor: Dorothy Kozlowski, Ph.D. Project: *Compensatory neural plasticity following traumatic brain injury*

RESEARCH SUPPORT

2010-2012	Ruth L. Kirschstein National Research Service Award (NRSA) Individual Predoctoral Grant, *Role of anandamide in a rodent model of an alcohol use disorder,* F31AA019853, Awarded by the National Institute on Alcohol Abuse and Alcoholism
2009-2010	Ruth L. Kirschstein National Research Service Award (NRSA) Institutional Training Grant, *Research training in Drug Abuse,* T32DA016176, Awarded by the National Institute on Drug Abuse
2006-2007	DePaul University summer research grant, *Compensatory neural plasticity following traumatic brain injury,* Awarded by the College of Liberal Arts and Sciences

HONORS & AWARDS

2012	Enoch Gordis Research Recognition Awardee, Research Society on Alcoholism
2011-2013	Student merit award, Research Society on Alcoholism
2011	Travel award, International Cannabinoid Research Society
2007	Travel award, National Neurotrauma Symposium

INVITED PRESENTATIONS

2012	Endocannabinoids mediate neurotoxicity and neuroprotection in an *in vitro* model of ethanol-induced neurotoxicity (Research Society on Alcoholism, San Francisco, CA)
2012	Modulation of endocannabinoids in a model of alcohol-induced neurodegeneration (Drug Discovery and Drug Development Symposium, University of Kentucky)
2011	Endocannabinoid modulation attenuates ethanol-induced neurodegeneration during withdrawal (International Cannabinoid Research Society, St. Charles, IL)

PUBLICATIONS

Liput, D.J[†]., Jones, T. A[†]., Maresh, E. L[†]. Donlan, N., Parikh, T. J., Marlowe, D., Kozlowski, D. A. (2012). Use-dependent dendritic regrowth is limited following unilateral controlled cortical impact to the forelimb sensorimotor cortex. J. Neurotrauma, 29(7): 1455-68

Kelso, M.L., **Liput, D.J.**, Eaves, D. W., Nixon, K. (2011). Upregulation of vimentin suggests new areas of neurodegeneration in a rodent model of an alcohol use disorder. Neuroscience, 197:381-93

Nixon, K., Morris, S.A., **Liput, D.J**., Kelso, M.L. (2010). Roles of neural stem cells and adult neurogenesis in adolescent alcohol use disorders. Invited review. Special issue on adolescence, guest editor, D. B. Matthews, Alcohol, 44, 89-98.

Morris, S.A., Kelso, M.L., **Liput, D.J**., Marshall, S.A., Nixon, K. (2010). Similar withdrawal severity in adolescents and adults in a rat model of alcohol dependence. Special issue on adolescence, guest editor, D. B. Matthews. Alcohol, 44, 39-56.

†These authors contributed equally to the manuscript.

SUBMITTED MANUSCRIPTS

Liput, D.J., Tsakalozou, E., Hammell, D.C., Paudel, K., Nixon, K., Stinchcomb, A.L. Simultaneous Quantification of Anandamide, Oleoylethanolamide and Palmitoylethanolamide in Rodent Brain Tissue Using High Performance Liquid Chromatography - Electrospray Mass Spectroscopy. J. Pharmaceutical analysis

Liput, D.J., Hammell, D.C., Stinchcomb, A.L., Nixon, K. (In revision) Transdermal delivery of cannabidiol attenuates alcohol-induced neurodegeneration in a rat model of alcohol abuse. Pharmacol Biochem Behav.

MANUSCRIPTS IN PREPARATION

Liput, D.J., Hopkins, D., Pauly, J., Nixon, K. Binge alcohol exposure causes transient changes in the endocannabinoid system: A target to prevent alcohol-induced neurodegeneration? (expected submission, summer 2013)

Liput, D.J., Nixon, K., Prendergast, M. Endocannabinoid modulation attenuates ethanol withdrawal induced neurodegeneration in hippocampal slice cultures (expected submission, summer 2013)

Geil, C.R., Hayes, D.M., McClain, J.A., **Liput, D.J**., Marshall, S.A., Chen, K.Y. & Nixon, K. Alcohol and adult neurogenesis: promiscuous drug, wanton effects. Review

Liput, D.J., Hopkins, D., Nixon, K., Pauly, J. Binge alcohol exposure induces transient nicotinic receptor plasticity during intoxication. In preparation.

ABSTRACTS

Liput, D.J., Prendergast, M, A., Nixon, K. (2012) Endocannabinoids mediate neurotoxicity and neuroprotection in an *in vitro* model of ethanol-induced neurotoxicity. Alcoholism: Clinical & Experimental Research, 36 (supplement).

Liput, D. J., Prendergast, M. A., Nixon, K. (2011). Endocannabinoid modulation attenuates ethanol-induced neurodegeneration during withdrawal. International Cannabinoid Research Society Abstracts.

Liput, D. J., Pauly, J., Nixon, K. (2011). Binge alcohol exposure causes transient down-regulation of cannabinoid 1 receptors in cortico-limbic regions. Alcoholism: Clinical & Experimental Research, 35 (supplement).

Liput, D. J., Hammell, D.C., Stinchcomb, A.L., Nixon, K. (2010). Transdermal delivery of cannabidiol attenuates alcohol-induced cell death in the piriform cortex in a rat model of an alcohol use disorder. Alcoholism: Clinical & Experimental Research, 34 (supplement).

Liput, D. J., Hammell, D.C., Marshall, S.A., Stinchcomb, A.L., Nixon, K. (2009). Attenuation of alcohol-induced neurodegeneration via transdermal delivery of cannabidiol. Alcoholism: Clinical & Experimental Research, 33 (supplement).

Nixon, K., Pandya, J.D., Butler, T.R., **Liput, D.J**., Morris, S.A., Prendergast, M.A., Sullivan, P.G. (2009). Binge ethanol impairs neuronal mitochondrial bioenergetics - reversal by antioxidants and uncouplers. Alcoholism: Clinical & Experimental Research, 33 (supplement).

Liput, D.J., Hammell, D.C., Deeny, A., Stinchcomb, A.L., Nixon, K. (2009). Transdermal delivery of cannabidiol attenuates alcohol-induced neurodegeneration in a rat model of alcohol abuse. Society for Neuroscience Abstracts.

Liput, D.J., Maresh E., Donlan, N., Marlowe, D., Jones, T., Kozlowski, D. (2007). Bilateral reductions in dendritic processes despite increased use of the ipsilateral forelimb after a controlled cortical impact to the forelimb sensory motor cortex. Journal of Neurotrauma

TEACHING EXPERIENCE

2007-2008	University of Kentucky, College of Pharmacy - teaching assistant for neuropharmacology and geriatric pharmacy

UNIVERSITY SERVICE

2011-2012	Student representative for graduate program committee, College of Pharmacy, University of Kentucky
2011-2012	Chair – American Association of Pharmaceutical Sciences, University of Kentucky student chapter*
2010-2011	Vice Chair – American Association of Pharmaceutical Sciences, University of Kentucky
2010-2011	Graduate program self-review committee, College of Pharmacy, University of Kentucky

* Awarded AAPS student chapter of the year

PROFESSIONAL SOCIETIES

2008 – Present	Research Society on Alcoholism
2008 – Present	Blue Grass Chapter, Society for Neuroscience
2011-2012	International Cannabinoid Research Society
2010-2012	American Association of Pharmaceutical Scientists

2009-2010 Society for Neuroscience