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Examination of Hippocampal N-Methyl-D-Aspartate Receptors Following Chronic Intermittent Ethanol Exposure In Vitro

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EXAMINATION OF HIPPOCAMPAL N-METHYL-D-ASPARTATE
RECEPTORS FOLLOWING CHRONIC INTERMITTENT ETHANOL
EXPOSURE IN VITRO

THESIS

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

By

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Lexington, Kentucky

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Lexington, Kentucky

2013

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ABSTRACT OF THESIS

EXAMINATION OF HIPPOCAMPAL N-METHYL-D-ASPARTATE RECEPTORS FOLLOWING CHRONIC INTERMITTENT ETHANOL EXPOSURE IN VITRO

Chronic intermittent ethanol exposure (CIE) is associated with degeneration of hippocampal neurons. The present study used hippocampal cultures to examine the loss of NeuN immunoreactivity, a reliable marker of neuronal density, after 1, 2, or 3 cycles of 5 days ethanol (EtOH) exposure (50 mM), followed by a 24-hour period of ethanol withdrawal (EWD), or continuous EtOH exposure. NeuN immunoreactivity was decreased by 13%, 19%, and 16% in the CA1, CA3, and dentate gyrus after 3 cycles of CIE respectively. Thionine staining confirmed significant cellular losses within each hippocampal subregion. Two cycles of CIE in aged tissue cultures resulted in significant decreases in NeuN immunoreactivity in all hippocampal subregions while continuous ethanol exposure or exposure to one cycle of CIE did not. Exposure to the competitive *N*-Methyl-D-aspartate receptor (NMDAR) antagonist 2-amino-7-phosphonovaleric acid (APV) (30 μ M) during periods of EWD attenuated the loss of NeuN in all hippocampal subregions, while the exposure to APV (40 μ M) prevented the loss of NeuN in the CA1 and the dentate gyrus. These results suggest that the loss of mature neurons after CIE is associated with the overactivation of the NMDAR.

KEYWORDS: hippocampal slice cultures, CIE, NMDA receptor, APV, NeuN

Anna Rebecca Reynolds

January 31, 2013

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Chapter 1: Introduction
Background and Significance

Alcohol Abuse in the United States

The abuse of alcohol is a widespread phenomenon and public health concern in the United States. According to the National Survey on Drug Use and Health, an estimated 131.3 million Americans (12 years of age and older) reported being current alcohol consumers, with 23.1% of all respondents characterized as binge drinkers in 2010 (SAMSHA, 2011). Rates of binge drinking peak in the young adult population (18–25 years of age) with a reported 40.6% of young adults participating in binge drinking episodes in the United States (Substance Abuse and Mental Health Services Administration, 2011). Binge drinking in the United States has been traditionally characterized as consumption of five or more alcoholic beverages for males and four or more for females in an approximate two-hour window. However, the National Institute of Alcohol Abuse and Alcoholism has modified conventional standards by describing binge drinking as any point in which one's level of intoxication exceeds the legal limit for driving (0.08%) (National Institute on Alcohol Abuse and Alcoholism, 2004). It is important to note the legal limit of intoxication for driving in the United States is relatively high when compared to other Western cultures; the Scandinavian kingdoms of Denmark, Norway, and Sweden, for example, have the legal limit of intoxication for driving set at 0.05%, 0.02%, and 0.02% respectively (Popova, Rehm, Patra, & Zatonski, 2007). Given the relative

leniency towards alcohol consumption in regards to legal driving limits, it is no surprise that the American Journal of Preventative Medicine estimated economic expenditures for excessive alcohol consumption in the United States at \$223.5 billion (Bouchery, Harwood, Sacks, Simon, & Brewer, 2011). Costs associated with binge drinking were responsible for \$170.7 billion, or an estimated 75% of the total of these economic expenditures (Bouchery, et al., 2011). Further, costs associated with underage drinking have been estimated at \$62 billion (CDC, 2011). Given the high rates of alcohol consumption (particularly underage drinking) and binge drinking, continued attention and consideration should be aimed at the treatment of alcohol dependence in the United States.

Alcohol Dependence and Brain Morphology

Alcohol dependence is described as a “maladaptive pattern of alcohol use that leads to clinically significant impairment or distress, as manifested by tolerance, withdrawal, or drinking to relieve withdrawals, inability to reduce drinking, drinking for longer than intended, neglect of activities, time spent related to drinking or recovering from drinking, and continued use of alcohol despite alcohol-related psychological or physical problems” (reviewed by Carigulo, 2007, p. S6; American Psychiatric Association [DSM-IV], 2000). Alcohol dependence is associated with marked impairments of brain function (reviewed by Moselhy, Georgiou, & Kahn, 2001), particularly the anterior areas of the brain (i.e., the frontal lobe to medial dorsal nucleus and basal regions) (Tarter, 1975). Necropsy examination analyses indicate that individuals with a history of dependence

demonstrate a significant loss in brain tissue volume (marked by white and grey matter loss of the cerebral hemispheres) as compared to non-alcoholic individuals (Harper, Kril, & Holloway, 1987). It is important to note that white matter volume decreases return to a significant degree with prolonged abstinence from drinking (Pfefferbaum et al., 1995). Analyses conducted via computed tomography (CT) and single-photon emission computed tomography (SPECT), demonstrate significant changes of the central nervous system (CNS) in alcohol-dependent individuals compared to non-dependent controls, including decreases in cerebral blood flow in the frontal lobes (Nicolas et al., 1993), significant reductions of neurons in frontal cortices (Harper, 1987), widening of ventricles (Bergman, Borg, Hindmarsh, Idestrom, & Mutzel, 1980; Carlen, Wilkinson, & Kiraly, 1976; Ron, 1977), degeneration of cortices (Epstein, 1977) and frontal and parietal widening of sulci and fissures (Bergman et al., 1980).

The adolescent brain is particularly sensitive to the effects of alcohol as cortical gray matter of the occipital lobe increases through the early twenties (Geidd et al., 1999). Further, previous research indicates that binge drinking at an early age is associated with decreased volume of grey matter in the frontal brain regions, cerebellum, and brain stem (Pfefferbaum et al., 1992). Notably, the marked cognitive impairments demonstrated in alcoholic patients with a history of repeated detoxifications may also be observed in young adult binge drinkers (Duka, Townshend, Collier, & Stephens, 2002; for a review, see Duka et al., 2004). It is of importance to address adolescent (10-20 years of age) binge

drinking as it may be associated with alcohol dependence in later adulthood (Grant & Dawson, 1997).

The National Institute on Alcohol Abuse and Alcoholism has suggested that duration of alcohol abuse, frequency of abuse, and amount of alcohol consumed may affect the manifestation of cognitive impairments demonstrated in alcohol-dependent individuals (NIAAA, 2000). The low levels of blood flow to the brain observed in alcohol dependence are associated with deficits in short-term memory (Chanraud et al., 2007), working memory (Chanraud et al., 2007), cognitive processes (Sullivan, Rosenbloom, & Pfefferbaum, 2000), executive function (Oscar-Berman, Shagrin, Evert, & Epstein, 1997), recognition of emotional expressions (Kornreich et al., 2002), inhibition of competing responses (Leber, Jenkins, & Parsons, 1981), deficits in anterograde formations of spatial memories (Obernier, White, Swartzwelder, & Crews, 2002), and abnormal response perseveration demonstrated in rodent models (Obernier et al., 2002). Previous studies demonstrate a correlation between Wisconsin Card Sorting Task (WCST) scores and grey matter volumes in the temporal lobe, thalamus, and cerebellum in alcoholic-dependent individuals compared to non-dependent controls (Chanraud et al., 2007). The association between alcohol dependence and cognitive deficits is of particular importance, as it is suggested that alcoholic-dependent individuals may be at risk for development of dementia in later adulthood (Brown, Tapert, Granholm, & Delis, 2000).

While the exact causes of alcohol-associated brain injury are not entirely understood, preclinical research has suggested a role for several different neurotoxic actions of ethanol. For example, previous research has suggested that chronic ethanol exposure produces robust CNS insult and injury, by inhibiting differentiation of progenitor cells into neurons (Santillano et al., 2005), reducing neurogenesis, survival, and dendritic growth (Crews & Nixon, 2009; Wilkins et al. 2006), enhancing apoptosis in maturing neurons (Cheema, West, & Miranda, 2000), enhancing differentiation of reactive oxygen species (Ren et al., 2005), inhibiting function of neurotrophins (Walker et al., 1993), and is associated with reduction in many of the brain regions previously described (Harding, Jalliday, & Kril, 1998).

Additional work suggests a role for ethanol withdrawal in the neurotoxic effects of alcohol dependence. Chronic ethanol exposure increases the sensitivity of the NMDA receptor, modulating hyperexcitability of glutamatergic systems (Lovinger, 1993) producing acute excitotoxicity during withdrawal (Tsai & Coyle, 1998), also characterized as alcohol-induced neurotoxicity (Lewohl, Crane, & Dodd, 1996). It is suggested that alcohol-induced neurotoxicity may develop during periods of heavy drinking (e.g. binge drinking) and withdrawal or periods of lowering of blood ethanol concentrations, which may be associated with anxiety and reinstatement during abstinence (Breese, Overstreet, & Knapp, 2005), and enhanced during periods of withdrawal (Paula-Barbosa, Brandao, Madeira, & Cadete-Leite, 1993; for a review, see Prendergast & Mulholland,

2012). The presumed mechanism of ethanol withdrawal neurotoxicity is classic excitotoxicity. Excitotoxicity is characterized as the overexcitation of neurons associated with the neuronal degeneration observed in hypoxia-ischemia, epilepsy, traumatic brain injury, and neurodegenerative diseases (Choi, 1992). Olney (1986) first coined the term “excitotoxicity” in reference to the overstimulation of excitatory amino acid receptor complexes producing subsequent neuronal death. High concentrations of the excitatory amino acid glutamate can be noxious *in vivo* (Coyle et al., 1981) and *in vitro* (Choi, 1991) with the mechanism of excitotoxicity attributed to the overactivation of *N*-methyl-d-aspartate receptors (NMDARs) (Choi 1992), allowing for an excessive extracellular calcium influx (Choi, 1992), activating phospholipases, endonucleases, and proteases leading the degradation of structural proteins and cell death (Choi, 1995).

Kindling of the Limbic System

Goddard, McIntyre, and Leech (1969) first coined the term “kindling” in reference to repeated, low-intensity electrical stimulations causing epileptic seizures. During initial experiments, electrical stimulations were administered daily at sub-threshold intensities to many regions of the brain with electrographic after-discharges measured with electroencephalograms (EEGs) and behavioral outcomes observed following the stimulations. The authors indicated that initial electrical stimulations did not cause any differential behavioral outcomes or EEGs, whereas after repeated stimulations in areas of the limbic system,

automatisms and convulsions were observed. Subsequently, research has extensively studied the kindling of the amygdala in regards to epilepsy. It is suggested that repeated electrical stimulation to the amygdala produces spontaneous after-discharges in the hippocampus and seizure activity. The dentate gyrus (DG) of the hippocampus shows reorganization after kindling, characterized by the sprouting of axons produced by neurons in the granule cell layer and mossy fiber tract formation in the CA3, which is associated with the lowering the seizure threshold (McNamara, 1988). Other research indicates that following electrical stimulation to the hippocampus, neurons in the granule cell layer die off and there is subsequent neuronal proliferation (Parent et al., 1997).

Multiple Detoxifications as a Stimulus for Kindling

Previous retrospective analyses using hospital records have established a relationship between withdrawal-induced seizures and multiple detoxifications. Gross and colleagues conducted retrospective analyses using the psychiatric hospital records of 567 men admitted for acute alcohol withdrawal; the records revealed that frequent re-admissions were observed in patients who experienced seizures compared to patients who did not (Gross, Rosenblatt, Malenowski, Broman, & Lewis, 1972). Ballenger and Post (1978) also conducted retrospective analyses. They used the records of 200 men who had been admitted to the same hospital for detoxification and who consumed at least seven ounces of alcohol a day for a minimum of six months prior to admissions. They excluded patients with records of brain damage. A positive correlation was found between years of

excessive, daily consumption and severity of withdrawal symptoms regardless of the age of the patient. Brown, Anton, Malcolm, and Ballenger (1988) conducted retrospective analyses using the patient records of 50 alcoholic men with no previous history of seizures, brain injuries, or neurological disorders. Twenty-five of the individuals sampled experienced withdrawal-induced seizures during hospitalization, while 25 of the individuals sampled did not. Analyses demonstrated that out of those patients who experienced a withdrawal-induced seizure, nearly 50% had experienced five or more prior detoxifications, suggesting that previous withdrawal-induced seizures experienced during prior detoxifications are associated with the rate of subsequent withdrawal-induced seizures.

Lechtenberg and Worner (1990) conducted a retrospective observational study using a sample 301 men and women voluntarily admitted to inpatient detoxification programs. Patients with documented neurological impairments were excluded. Analyses indicated that individuals admitted on more than one occasion had a history of previous withdrawal-induced seizures. However, the authors suggested that repeated detoxifications would not increase the likelihood of seizure-related disorders. Lechtenberg and Worner (1991) conducted further analyses on the topic using a sample of 400 men and women who voluntarily admitted themselves to inpatient detoxification programs. Again, patients with documented neurological impairments were excluded. Analyses demonstrated a correlation between a history of multiple detoxifications and seizure incidence

rates in detoxifying patients. The following year, Lechtenberg and Worner (1992) used a sample of 500 men and women who voluntarily admitted themselves to inpatient detoxification programs. Patients with documented neurological impairments were excluded. Data analyses revealed a significant association between multiple alcohol detoxifications and seizure incidence rates, and a significant association between averaged daily alcohol consumption and seizure incidence rates.

Booth and Blow (1993) conducted a database review using a sample of 6,818 compliant male patients undergoing detoxification. The authors suggested that the data analyses revealed further support for the kindling hypothesis, as the extensive review of hospital records demonstrated a significant association between the prevalence of seizures and previous documented detoxifications. Worner (1996) used a sample of 360 men and women who voluntarily admitted themselves to inpatient detoxification programs. Data analyses revealed a significant association between seizure prevalence and multiple detoxifications, as well as a correlation between neurological-related admissions and prior seizure history. Shaw and colleagues (1998) used a sample of 160 male and female patients admitted for detoxification. Data revealed that the degree of alcohol dependence, number of previous detoxifications, and previous manifestations of withdrawal symptoms were predictors of severity of withdrawal. Wojnar, Bizon, and Wasilewski (1999) conducted an extensive study using hospital records from 1,179 male and female patients admitted for detoxification

from 1973 to 1987. Other data analyses also supported alcohol withdrawal as a kindling stimulus, where the manifestation of withdrawal symptoms became progressively worse with each subsequent detoxification (Duka et al., 2002; for a review, see Duka et al., 2004). Collectively, these studies provide support for the hypothesis of alcohol withdrawal as a stimulus for kindling, as documented previous alcohol withdrawal history (i.e., number of prior withdrawals, duration of each drinking and withdrawal episode, spaced intervals between withdrawals, years of chronic abuse) were strongly associated with the severity of latter withdrawal episodes.

Multiple Detoxifications, Cognition, and Brain Morphology

It has been suggested that alcohol-dependent individuals engage in specific and predictable patterns of drinking, where discrete periods of heavy drinking are followed by periods of non-drinking or abstinence (Mello & Mendleson, 1972). It has also been noted that chronic alcohol consumption is marked by neurochemical adaptations leading to inherent physiological symptoms of withdrawal and subsequent cognitive impairments during periods of abstinence and detoxification (Stephens & Duka, 2008). Duka and colleagues suggest that binge drinking and repeated withdrawals may be continuous entities, with underlying similarities often overlooked. Binge drinking may be characterized as the excessive consumption of alcohol during one occasion followed by a period of abstinence, whereas social drinking may be characterized as patterns of weekly drinking where consumption is characteristically maintained

below excessive intoxication thresholds (Duka et al., 2002; for a review, see Duka et al., 2004). In summary, discrete periods of alcohol intoxication followed by periods of abstinence or non-drinking are characteristic of both binge drinking and alcohol dependence.

The direct relationship between repeated detoxifications and changes in brain size has been examined in alcoholic patients using imaging technologies. One study examining alcohol-dependent individuals with a history of at least one prior withdrawal-induced seizure demonstrated significant decreases in temporal white matter compared to healthy controls or those individuals with no history of seizures. (Sullivan et al, 1996). Further, a vast body of literature demonstrates that binge drinkers and alcoholic individuals with a history of multiple detoxifications display greater impairment in particular vigilance tasks associated with impulsivity, spatial working memory (Townshend & Duka, 2005; Weissenborn & Duka, 2003), and immediate and delayed semantic and figural memory (Glenn, Parsons, Sinha, & Stevens, 1988) compared to non-binge drinkers or individuals with no history of previous withdrawals. Additionally, alcohol-dependent individuals with a history of multiple detoxifications may have an impaired conditioned fear response, decreases in long-term potentiation (LTP) (Stephens et al., 2005), enhanced emotional sensitivity to alcohol withdrawal-related stimuli (Duka et al., 2002), and exacerbated fear recognition (Townshend & Duka, 2003). Although some of the cognitive impairments associated with multiple withdrawals (i.e., impulsivity) may recover over time (Loeber et al.,

2009), executive function may be more resistant to recovery (Kuzmin et al., 2011).

Ethanol Withdrawal in Rodent Models as an Indicator of Dependence

Examination of ethanol withdrawal as an indicator of dependence in rodent models has allowed for a fundamental understanding of the neurological adaptations associated with the physiological symptoms of withdrawal, with cognitive impairments, and with reinstatement as a model for relapse (Faingold, 2008; Obernier et al., 2002). Rodent models of ethanol dependence offer the unique opportunity to assess both metabolic and physical tolerance to alcohol after short-term ethanol exposure (Self, Smith, Butler, Pauly, & Prendergast, 2009). The heightened neurochemical adaptations and cognitive impairments associated with multiple alcohol detoxifications observed in humans (Duka et al., 2002) may also be demonstrated in rodents models of repeated withdrawals (Stephens & Duka, 2008). The repeated administration of high doses of ethanol has been used to evoke physical dependence in rodents, determined by the duration of exposure and dose of ethanol administered (Majchrowicz, 1975; Sircar & Sircar, 2005; Wallgren, Kosunun, & Ahtee, 1973). With a four-day, binge-like model of ethanol dependence, high doses of ethanol may produce rapid intoxication and subsequent ethanol dependence resulting in hyperexcitability of the CNS during withdrawal. It has been suggested that as these blood ethanol concentrations decline, physiological manifestations of withdrawal may be observed, including, but not limited to hyperactivity, tremors,

spastic rigidity, and sporadic convulsions (Majchrowicz, 1975). Becker (2000) proposed that the physiological manifestations of alcohol withdrawal might be characterized in three ways: overactivation of the autonomic nervous system (e.g., tachycardia, increased blood pressure, sweating and vomiting), CNS hyperexcitability (e.g., anxiety and seizures), and disturbances related to sensation and perception (e.g., hallucinations and delirium). More recently, Self and colleagues (2009) demonstrated that binge-like dosing of ethanol three times per day (3–5 g/kg) administered for four days produces robust behavioral manifestations of ethanol withdrawal, including cognitive impairments and CNS over-excitation. It is important to note the authors' suggestion that the short-term administration of ethanol may produce metabolic tolerance, in addition to physical dependence, as blood ethanol concentrations (BECs) observed 60 minutes after ethanol administration on the second day (~187.69 mg/dl) differed from BECs observed 60 minutes after ethanol administration on the fourth day (~100 mg/dl) (Self et al., 2009).

Furthermore, the long-term rodent administration of ethanol has been used to examine dependence in rodent models. For example, Devaud and colleagues administered ethanol (~6%) to rodents for 14 consecutive days, with tissue examined after three days of ethanol exposure, after nine days of ethanol exposure, and after a 3-day EWD period following 14 days of ethanol exposure. Data analyses revealed that NR2A and NR2B subunit expression in the hippocampus was increased in male rodents three days following the 14-day

administration of ethanol. The authors conclude notable sex differences in the response to chronic ethanol exposure and withdrawal (Devaud and Alele, 2004). Alele and Devaud (2005) further examined the effects of 14-day ethanol administration (~6%) followed by a 3-day EWD period in male and female rodents on glutamic acid decarboxylase, GABA and glutamate transporters, with females demonstrating higher levels of glutamate transporters compared to male rodents. Further, one study administered ethanol to rodents for 13 months, with some rodents exposed to a subsequent six-week ethanol withdrawal period. The authors reported increased cognitive impairments in the rodents exposed to EWD, reflected by significantly longer swimming times in the Morris Water Maze, as compared to rodents who were not exposed to EWD. Further, rodents exposed to EWD demonstrated significant decreases in hippocampal volume of the pyramidal cell layers of the CA1 and CA3 (Lukoyanov, Mederia, & Paula-Barbosa, 1999). In summary, both short-term and long-term ethanol administration are currently implemented to assess the effects of behavioral effects of ethanol withdrawal and neurochemical changes produced by dependence in rodent models, both of which may have predictive validity for the clinical population and development of novel pharmaceutical compounds for the treatment of alcohol dependence.

Chronic Intermittent Ethanol Exposure in Rodent Models

Previous research suggests that rodents (Stephens et al., 2001; Stephens et al., 2005) and humans (Mello & Mendleson, 1972) display predictable

increases in severity of withdrawal symptoms as the result of repeated detoxifications or repeated withdrawal respectively. Stephens and colleagues indicate that several of the cognitive and behavioral deficits associated with multiple detoxifications were predicted following experimental demonstrations using rodent models of chronic intermittent ethanol exposure (CIE) (Stephens et al., 2001; Stephens et al., 2005). Following CIE, impaired metabolic function in limbic and cortical brain regions may be observed in rodents, as demonstrated by decreases in local cerebral glucose consumption (Clemmesen, Ingvar, Hemmingsen, & Bolwig, 1988). It has been suggested that repeated administrations of ethanol and periods of withdrawals may be associated with kindling of the inferior collicular cortex (McCown & Breese, 1990) and increased neuronal discharges in areas of the limbic system associated with hyperexcitability (Adinoff et al., 1994). It has also been suggested that rodents who have been exposed to kindling of the amygdala may produce increased withdrawal manifestations. Likewise, rodents exposed to CIE may show increased advancement of kindling in the amygdala (Carrington, Ellinwood, & Krishnan, 1984; Pinel, 1980; Pinel & Van Oot, 1975). More recently, EEG activity has been used to examine ethanol-dependent rodents exposed to CIE. Increased spike and sharp wave (SSW) discharges in the hippocampus, as an indication of CNS hyperexcitability following consecutive withdrawals, was of particular interest to researchers. Data revealed that rodents exposed to CIE demonstrated increased SSW discharges during periods of withdrawal

(compared to baseline or during periods of ethanol exposure) with the most robust spikes occurring during the final tested withdrawal (Duka et al., 2004).

The hippocampus has long been implicated in learning and memory formation (Lothman, 1994; Walker et al., 1993), and research suggests that the hippocampus may be particularly vulnerable to epileptiform insult (Lothman, 1994; Shin & McNamara, 1994; Sutula et al., 1994). It has been suggested, therefore, that the cognitive deficits observed in alcoholics may be posited from hippocampal kindling produced by multiple detoxifications. The demonstration of spontaneous EEG activity observed during intermittent ethanol exposure may provide further evidence for ethanol withdrawal as a stimulus for kindling of the limbic system, and specifically, the hippocampus (Lothman, 1994; Shin & McNamara, 1994; for a review, see Duka et al., 2004). It has been demonstrated that CIE may increase the rate, intensity, and duration of subsequent seizures (Veatch & Becker, 2002), may produce a decline in LTP function (Stephens et al., 2005), and may enhance sensitization to convulsant stimuli, potentiating spontaneous seizures (Stevens et al., 2001). Collectively, these findings suggest that CIE may produce pronounced CNS injury in rodents, similar to that observed in human alcohol-dependent individuals. This provides support for CIE as a viable rodent model to study the kindling phenomenon in areas of the limbic system.

Ethanol Exposure: Neurotransmitters

Ethanol exposure affects many neurotransmitters and signaling systems in the mammalian brain: glutamate (Samson & Harris, 1992), γ -aminobutyric acid (GABA) (Littleton & Little, 1994), dopamine (Morikawa & Morrisett, 2010), serotonin (Barr et al., 2005), cannabinoid receptors (Blendov, Cravatt, Boehm, Walker, & Harris, 2007), endogenous opioids (Pastor & Aragon, 2006), corticotrophin-releasing factor (CRF) (Witkiewitz, 2008), and CREB proteins (Acquaah-Mensah, Misra, & Boswal, 2006). Ethanol inhibits calcium flow through L-type voltage-operated calcium channels, decreases LTP (Blitzer, Gil, Landau, 1990), facilitates GABAergic transmission (Samson & Harris, 1992), enhances the release of dopamine (Morikawa & Morrisett, 2010) and may promote long-term depression (Hendricson, Miao, Lippmann, & Morrisett, 2002). With chronic ethanol exposure, ethanol increases the number of L-type voltage-sensitive calcium channels and increases NMDAR mediated calcium influx (De Witte, 2004). Additionally, it is suggested that chronic ethanol exposure reduces activity of GABA at the synapse, inhibits post-synaptic GABA neurons, and reduces dopamine release (Morikawa & Morrisett, 2010).

Ethanol and Glutamatergic Systems

Of the many neurotransmitters influenced by ethanol exposure, glutamate systems are known to have a significant role in dependence, withdrawal and ethanol-related neurotoxicity. Glutamate is the primary excitatory neurotransmitter in the CNS and is involved in learning, memory, LTP, and

neuronal plasticity (Douglas & Goddard, 1975). Ionotropic glutamate receptors are located post-synaptically, generating fast signal transduction, and include NMDARs, AMPARs, and kainate receptors. NMDAR's are heteromeric ion channels comprised of an NR1 subunit and a combination of NR2A-D subunits, allowing for an influx of cations (Dingledine, Borges, Bowie, & Traynelis, 1999; for a review, see Paoletti & Neyton, 2007). It has been suggested that NR2 subunits are particularly sensitive to allosteric modulators (e.g., endogenous polyamines), which may increase channel conductance (Shaw, 1979), influence the duration that the channel is open (Ran, Miura, & Puil, 2003), increase channel-receptor binding (Ransom & Stec, 1988), and are associated with excitotoxicity (Anji & Kumari, 2006). Following the release of glutamate, AMPAR's may become activated and, dependent on the subunit composition, allow for an influx of sodium into the cell that subsequently produces depolarization and removes the magnesium blockade from the ion channel of the NMDAR. Following depolarization, glutamate may activate NMDAR's and allow for an influx of calcium and sodium in the cell that subsequently opens L-type voltage-sensitive calcium channels and produces electrical signaling (e.g., action potential). Furthermore, NMDAR's are critical in synaptic development, LTP, and neuronal plasticity (Castellano, Cestari, & Ciamei, 2001) and are targeted for novel pharmacotherapy development (for a review, see Prendergast & Mulholland, 2012).

Acute Ethanol Exposure: Neurochemical Adaptations in Glutamate

Acute exposure to ethanol produces neurochemical adaptations in many of the neurotransmitter systems, including the glutamatergic system. Acute ethanol exposure non-competitively inhibits NMDAR channel function in the hippocampus, cerebellum, cerebral cortex, nucleus accumbens, amygdala, and ventral-tegmental area (Hoffman, Rabe, Moses, & Tabakoff, 1989; Lovinger, White, & Weight, 1989; for a review, see Hoffman, 2003), non-competitively inhibits AMPAR channel function (Moykkyen, Korpi, & Lovinger, 2003). NMDAR's are particularly sensitive to ethanol exposure (Samson & Harris, 1992). For example, the NR1/NR2A and NR1/2B combinations are sensitive to inhibition caused by acute ethanol exposure (Maswood, Wu, Brauneiss, & Weight, 1993; Mirshahi & Woodward, 1995) and may be vulnerable to adaptations produced by ethanol (Maswood et al., 1993; Mirshahi et al., 1995; for a review, see Prendergast & Mulholland, 2012). It has also been suggested that acute ethanol exposure may influence NMDAR composition by internalizing NR2A subunits (Survarna et al., 2005).

Chronic Ethanol Exposure: Neurochemical Adaptations in Glutamate

It has been demonstrated that chronic ethanol exposure increases the sensitivity of the NMDAR (Lovinger, 1993), potentiating glutamatergic transmission and subsequently increasing the production of NMDAR complexes (Carpenter-Hyland, Woodward, & Chandler, 2004; Floyd, Jung, & McCool, 2003). More specifically, chronic ethanol exposure may increase NMDAR function

(Ahern, Lustig, & Greenberg, 1994), production of NMDAR's (Samson & Harris, 1992), production of NMDAR subunit complexes (Floyd et al., 2003), production of metabotropic GluR1 and GluR2 subunits (Harris et al., 2002), clustering of NMDARs at the synapse (Carpenter-Hyland et al., 2004) and increase size of synaptic spines (Carpenter-Hyland et al., 2004). Chronic ethanol exposure is also associated with the relocation of NR2B subunits to the synapse (Carpenter-Hyland et al., 2004). More specifically, it has been shown that chronic ethanol exposure is associated with significant increases in NR2B expression, whereas continuous ethanol exposure has not been shown to be associated with such adaptations (Sheela, Rani, & Ticku, 2006).

The Organotypic Hippocampal Cell Culture Model

The organotypic slice culture technique is seen as a useful model for the mechanistic examination of ethanol (Collins, Zou, & Neafsey, 1998), excitotoxicity (Vornov, Tasker, & Coyle, 1991), neurotoxicity (Newell, Hsu, Papermaster, & Malouf, 1993), oxidative stress (Wilde et al., 1997), Alzheimer's disease (Kim et al., 2003), Parkinson's disease (Madsen et al., 2003), and stroke (Bonde, Noraberg, Zimmer, 2002) in the hippocampus. The organotypic slice culture model allows for the preservation of the neuropil layers (Zimmer & Gahwiler, 1984), for localized cell death (Noraberg, Kristensen, & Zimmer, 1999), and for the quantification of compromised neurons (Noraberg et al., 1999); it also has the advantage of yielding replicable measures of neuronal overactivation (Gahwiler, 1997).

Summary

In summary, acute and chronic ethanol exposure produce opposing neurochemical adaptations (Freund & Anderson, 1996), which facilitates alcohol-mediated excitotoxicity and hyperexcitability of the CNS (Paula-Barbosa et al., 1993; Wilkins et al., 2006) during a withdrawal process that is marked by physiological symptoms (Wallgren et al., 1973; Majchrowicz, 1975) and cognitive impairments (Obernier et al., 2002; Sircar & Sircar, 2005). Further attention and consideration should be aimed at the robust neurological adaptations associated with CIE in the hippocampus by targeting glutamatergic signaling systems, specifically (for a review, see Prendergast & Mulholland, 2012).

Experimental Rationale

The present study examined the loss of neuron-specific antigen (NeuN) immunoreactivity following binge-like concentrations of CIE *in vitro* in order to provide a better understanding of the neurological underpinnings of CIE previously demonstrated *in vivo* (Veatch et al., 2002; Borlikova et al., 2006; Stephens et al., 2001) and excitotoxicity during withdrawal (Paula-Barbosa et al., 1993; Wilkins et al., 2006). The present study targeted the glutamatergic signaling systems, specifically the overactivation of NMDARs following CIE in hippocampal slice cultures. Based on preliminary data collection, it was hypothesized that multiple cycles of CIE would result in excitotoxicity produced by repeated overactivation of NMDARs *in vitro* as demonstrated by decreased NeuN immunoreactivity and thionine staining when compared to the control

tissue or continuous ethanol exposure. Further, it was hypothesized that this effect would be attenuated by NMDAR antagonism.

Chapter 2: Methods

Procedure

Brains from eight-day-old Sprague-Dawley rats (Harlan Laboratories; Indianapolis, IN) were aseptically removed (after Mulholland et al., 2005) and immediately placed in culture dishes containing chilled dissecting media composed of Minimum Essential Medium (MEM; Invitrogen, Carlsbad, CA), 25 mM HEPES (Sigma, St. Louis, MO), and 50 μ M streptomycin/penicillin (Invitrogen). Bilateral hippocampi were then removed and carefully transferred onto plates containing culture media composed of dissecting medium, distilled water, 36 mM glucose (Fisher, Pittsburg, PA), 25% Hanks' Balanced Salt Solution (HBSS; Invitrogen), 25% (v/v) heat-inactivated horse serum (HIHS; Sigma), and 0.05% streptomycin/penicillin (Invitrogen); excess hippocampal tissue was then carefully removed using a stereoscopic microscope. Unilateral hippocampi were then sectioned at 200 μ M using a McIlwain Tissue Chopper (Mickle Laboratory Engineering Co. Ltd., Gomshall, UK) and three to four intact hippocampal slices were plated onto Millicell-CM 0.4 μ M biopore membrane inserts containing 1 mL of pre-incubated culture media. Excess culture medium was extracted off the top of each well; each plate generated 18 to 24 intact hippocampal slices. The tissue was immediately transferred to an incubator at 37°C with a gas composition of 5% CO₂/95% air for five days, allowing the hippocampal slices to adhere to the Teflon membrane. Care of all animals was carried out in agreement with the University of Kentucky's Institutional Animal Care and Use Committee.

Drug Exposure

Tissue cultures were exposed to one, two, or three cycles of CIE or continuous ethanol exposure. One cycle of CIE was characterized as five days ethanol (EtOH) exposure followed immediately by a 24-hour period of ethanol withdrawal (EWD). Two cycles of CIE was characterized as five days EtOH exposure followed immediately by a 24-hour period of EWD that was then followed by a second cycle of five days EtOH exposure and a subsequent 24-hour period of EWD. Three cycles of CIE was characterized as five days of EtOH exposure followed immediately by a 24-hour period of EWD that was then followed by a second cycle of five days EtOH exposure and a subsequent 24-hour period of EWD that was followed by a third cycle of five days EtOH exposure and a subsequent 24-hour period of EWD. Continuous EtOH exposure was characterized as five days of EtOH exposure accompanied by a discrete 24-hour period of EtOH exposure that was then followed by a second cycle of five days EtOH exposure accompanied by a discrete 24-hour period of ethanol exposure that was then followed by a third cycle of five days EtOH exposure accompanied by discrete 24-hour period of EtOH exposure (i.e., 18 consecutive days of EtOH exposure).

Experiment I: Three Cycles of CIE

The current study examined the hypothesis that multiple cycles of CIE would result in increased excitotoxicity by quantifying the loss of mature neurons

following three cycles of CIE in hippocampal slice cultures, as reflected by decreases in NeuN immunoreactivity and thionine staining. The treatment regimen for the current study is described accordingly. At five days *in vitro*, slices were randomly transferred to new culture plates containing either 50 mM EtOH diluted in 1 mL of culture media or 1 mL of EtOH-naïve media; they were then carefully placed in Tupperware containers inside of Ziploc bags filled with air for five days. At 10 days *in vitro*, slices were transferred to new culture plates containing 1 mL of EtOH-naïve media for a 24-hour EWD period. After the 24-hour EWD period, slices were returned to culture plates containing either 50 mM EtOH diluted in 1 mL of culture media or 1 mL of EtOH-naïve media for five days; they were then carefully placed in Tupperware containers inside of Ziploc bags filled with air for five days. At 16 days *in vitro*, slices were transferred to new culture plates containing 1 mL of EtOH-naïve media for a 24-hour EWD period. After the 24-hour EWD period, slices were returned to culture plates containing either 50 mM EtOH diluted in 1 mL of culture media or 1 mL of EtOH-naïve media and carefully placed in Tupperware containers inside of Ziploc bags filled with air for five days. At 22 days *in vitro*, slices were transferred to new culture plates containing 1 mL of EtOH-naïve media for a 24-hour EWD period. After the 24-hour EWD period, slices were fixed by exposure to 1 mL of 10% formalin solution on the top and bottom of each well for 30 minutes. The slices were then washed twice carefully (1 mL on bottom of the well and 1 mL on top of the well) with phosphate buffered saline (PBS) and stored with 1 mL of PBS on the bottom

of the well overnight at 4°C. The representative timeline for Experiment 1A is presented in Figure 2.1.

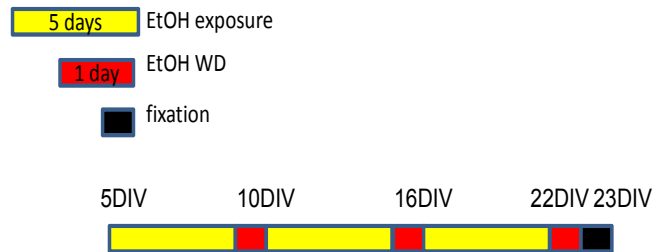


Figure 2.1. Experimental protocol timeline. Cultures were exposed to chronic intermittent ethanol exposure for a total of 3 cycles of 5 days ethanol (EtOH) exposure, followed by one 24 hour period of ethanol withdrawal (EWD). After fixation, cultures were prepared for immunohistochemistry.

Continuous Ethanol Control

The current study further examined the hypothesis that ethanol withdrawal was a required stimulus for neurotoxicity, by exposing tissue cultures to continuous ethanol exposure in the absence of 24-hour EWD periods. The treatment regimen for the current study is described accordingly. At five days *in vitro*, slices were randomly transferred to new culture plates containing 50 mM EtOH dissolved in 1 mL of culture media and carefully placed in Tupperware containers inside of Ziploc bags filled with air for five days. At 10 days *in vitro*, slices were transferred to new culture plates containing 50 mM EtOH dissolved in 1 mL of culture media and carefully placed in Tupperware containers inside of Ziploc bags filled with air for a 24-hour period. After the 24-hour period, slices were transferred to new culture plates containing 50 mM EtOH dissolved in 1 mL of culture media and carefully placed in Tupperware containers inside of Ziploc bags filled with air for five days. At 16 days *in vitro*, slices were transferred to new culture plates containing 50 mM EtOH dissolved in 1 mL of culture media and carefully placed in Tupperware containers inside of Ziploc bags filled with air or a 24-hour period. After this 24-hour period, slices were transferred to new culture plates containing 50 mM EtOH dissolved in 1 mL of culture media and carefully placed in Tupperware containers inside of Ziploc bags filled with air for five days. At 22 days *in vitro*, slices were transferred to new culture plates containing 50 mM EtOH dissolved in 1 mL of culture media and carefully placed in Tupperware containers inside of Ziploc bags filled with air for a 24-hour period. After this 24-hour period, slices were fixed by exposure to 1 mL of 10% formalin solution on the top and bottom of each well for 30 minutes. The slices were then washed

twice carefully (1 mL on bottom of the well and 1 mL on top of the well) with PBS and stored with 1 mL of PBS on the bottom of the well overnight at 4°C. The representative timeline for Experiment 1B is presented in Figure 2.2.

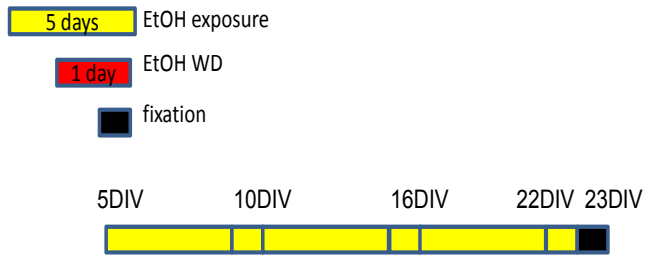


Figure 2.2. Experimental protocol timeline. Cultures were exposed to continuous ethanol exposure for 18 days. After fixation, cultures were prepared for immunohistochemistry.

Experiment II: Two Cycles of CIE

Non-Aged Tissue

The current study examined the hypothesis that multiple cycles of CIE would result in increased excitotoxicity by testing the required number of cycles needed to produce toxicity. Previous research has suggested that young tissue may be less vulnerable compared to aged tissue following excitotoxic insult (Brewer et al., 2007). Therefore, the current treatment regimen examined two cycles of CIE in tissue cultures that were not age-matched to cultures exposed to three cycles of CIE (i.e., the 24-hour EWD periods did not occur on the same day *in vitro*). The treatment regimen for the current study is described accordingly. At five days *in vitro*, slices were randomly transferred to new culture plates containing either 50 mM ethanol diluted in 1 mL of culture media or 1 mL of EtOH-naïve media; they were then carefully placed in Tupperware containers inside of Ziploc bags filled with air for five days. At 10 days *in vitro*, slices were transferred to new culture plates containing 1 mL of EtOH-naïve media for a 24-hour EWD period. After the 24-hour EWD period, slices were returned to culture plates containing either 50 mM ethanol diluted in 1 mL of culture media or 1 mL of EtOH-naïve media; they were then carefully placed in Tupperware containers inside of Ziploc bags filled with air for five days. At 16 days *in vitro*, slices were transferred to new culture plates containing 1 mL of EtOH-naïve media for a 24-hour EWD period. After the 24-hour EWD period, slices were fixed by exposure to 1 mL of 10% formalin solution on the top and bottom of each well for 30 minutes. The slices were then washed twice carefully (1 mL on bottom of the well

and 1 mL on top of the well) with PBS and stored with 1 mL of PBS on the bottom of the well overnight at 4°C. The representative timeline for Experiment 2A is presented in Figure 2.3.

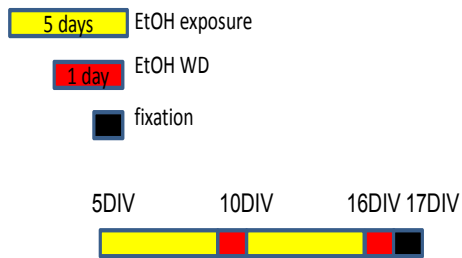


Figure 2.3. Experimental protocol timeline. Cultures were exposed to chronic intermittent ethanol exposure for a total of 2 cycles of 5 days ethanol (EtOH) exposure, followed by one 24 hour period of ethanol withdrawal (EWD). After fixation, cultures were prepared for immunohistochemistry.

Experiment II: Two Cycles of CIE

Aged Tissue

The current study examined the hypothesis that multiple cycles of CIE would result in increased excitotoxicity by testing the required number of cycles needed to produce toxicity. As previous research has demonstrated aged tissue may be more vulnerable to excitotoxic insult (Brewer et al., 2007), hippocampal slice cultures were aged-matched to slices exposed to three cycles of CIE (i.e., the 24-hour EWD periods did occur on the same day *in vitro*). At five days *in vitro*, slices were randomly transferred to new culture plates containing 1 mL of EtOH-naïve media and carefully placed in Tupperware containers inside of Ziploc bags filled with air for five days. At 10 days *in vitro*, slices were transferred to new culture plates containing 1 mL of EtOH-naïve media for a 24-hour period. After this 24-hour period, slices were returned to culture plates containing either 50 mM EtOH diluted in 1 mL of culture media or 1 mL of EtOH-naïve media; they were then carefully placed in Tupperware containers inside of Ziploc bags filled with air for five days. At 16 days *in vitro*, slices were transferred to new culture plates containing 1 mL of EtOH-naïve media for a 24-hour EWD period. After this 24-hour EWD period, slices were returned to culture plates containing either 50 mM EtOH diluted in 1 mL of culture media or 1 mL of EtOH-naïve media; they were then carefully placed in Tupperware containers inside of Ziploc bags filled with air for five days. At 22 days *in vitro*, slices were transferred to new culture plates containing 1 mL of EtOH-naïve media for a 24-hour EWD period. After this 24-hour EWD period, slices were fixed by exposure to 1 mL of 10% formalin solution on the top and bottom of each well for 30 minutes. The slices were then

washed twice carefully (1 mL on bottom of the well and 1 mL on top of the well) with PBS and stored with 1 mL of PBS on the bottom of the well overnight at 4°C. The representative timeline for Experiment 2B is shown in Figure 2.4.

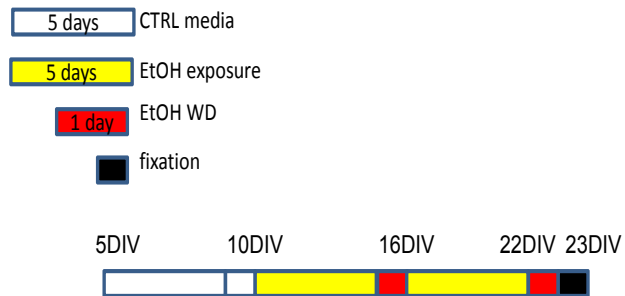


Figure 2.4. Experimental protocol timeline. Cultures were exposed to chronic intermittent ethanol exposure for a total of 2 cycles of 5 days ethanol (EtOH) exposure, followed by one 24 hour period of ethanol withdrawal (EWD) starting on 11 DIV. After fixation, cultures were prepared for immunohistochemistry.

Experiment III: One Cycle of CIE

Non-aged Tissue

The current study further examined the hypothesis that one cycle of CIE would not be sufficient to result in excitotoxicity. Accordingly, the current treatment regimen examined one cycle of CIE in tissue cultures that were not age-matched to cultures exposed to three cycles (i.e., the 24-hour EWD periods did not occur on the same day *in vitro*). At five days *in vitro*, slices were randomly transferred to new culture plates containing either 50 mM EtOH diluted in 1 mL of culture media or 1 mL of EtOH-naïve media; they were then carefully placed in Tupperware containers inside of Ziploc bags filled with air for five days. At 10 days *in vitro*, slices were transferred to new culture plates containing 1 mL of EtOH-naïve media for a 24-hour EWD period. After the 24-hour EWD period, slices were fixed by exposure to 1 mL of 10% formalin solution on the top and bottom of each well for 30 minutes. The slices were then washed twice carefully (1 mL on bottom of the well and 1 mL on top of the well) with PBS and stored with 1 mL of PBS on the bottom of the well overnight at 4°C. The representative timeline for Experiment 3A is presented in Figure 2.5.

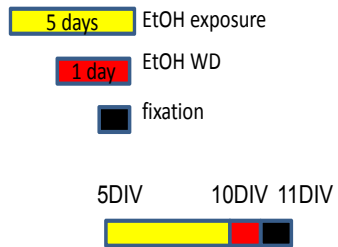


Figure 2.5 Experimental protocol timeline. Cultures were exposed to chronic intermittent ethanol exposure for a total of 1 cycle of 5 days ethanol (EtOH) exposure, followed by one 24 hour period of ethanol withdrawal (EWD). After fixation, cultures were prepared for immunohistochemistry.

Experiment III: One Cycle of CIE

Aged Tissue

The current study further examined the hypothesis that one cycle of CIE would not be sufficient to result in excitotoxicity. Therefore, the current treatment regimen examined one cycle of CIE in aged tissue cultures that were age-matched to cultures exposed to three cycles of CIE (i.e., the 24-hour EWD periods did not occur on the same day *in vitro*). At five days *in vitro*, slices were randomly transferred to new culture plates containing 1 mL of EtOH-naïve media and carefully placed in Tupperware containers inside of Ziploc bags filled with air for five days. At 10 days *in vitro*, slices were transferred to new culture plates containing 1 mL of EtOH-naïve media for a 24-hour period. After this 24-hour period, slices were returned to culture plates containing 1 mL of EtOH-naïve media for five days and carefully placed in Tupperware containers inside of Ziploc bags filled with air for five days. At 16 days *in vitro*, slices were transferred to new culture plates containing 1 mL of EtOH-naïve media for a 24-hour period. After the 24-hour EWD period, slices were returned to culture plates containing either 50 mM EtOH diluted in 1 mL of culture media or 1 mL of EtOH-naïve media; they were then carefully placed in Tupperware containers inside of Ziploc bags filled with air for five days. At 22 days *in vitro*, slices were transferred to new culture plates containing 1 mL of EtOH-naïve media for a 24-hour EWD period. After this 24-hour EWD period, slices were fixed by exposure to 1 mL of 10% formalin solution on the top and bottom of each well for 30 minutes. The slices were then washed twice carefully (1 mL on bottom of the well and 1 mL on top of

the well) with PBS and stored with 1 mL of PBS on the bottom of the well overnight at 4°C. The representative timeline for Experiment 3B is shown in Figure 2.6.

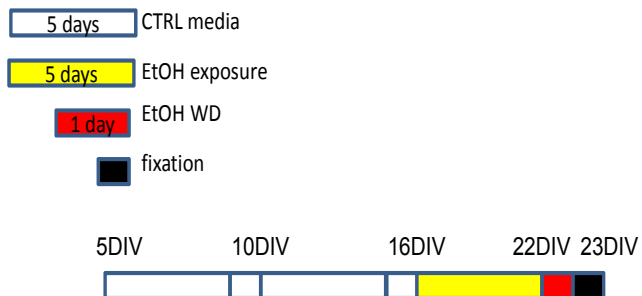


Figure 2.6. Experimental protocol timeline. Cultures were exposed to chronic intermittent ethanol exposure for a total of 1 cycle of 5 days ethanol (EtOH) exposure, followed by one 24 hour period of ethanol withdrawal (EWD) starting on 17 DIV. After fixation, cultures were prepared for immunohistochemistry.

Experiment IV: Three Cycles of CIE + APV

To test the hypothesis that multiple cycles of CIE would result in excitotoxicity produced by repeated overactivation of NMDARs, the NMDAR was specifically targeted. Further, the hypothesis that NMDAR antagonism would attenuate the loss of NeuN produced by multiple cycles of CIE in the current treatment regimen was tested. At five days *in vitro*, slices were randomly transferred to new culture plates containing either 50 mM EtOH diluted in 1 mL of culture media or 1 mL of EtOH-naïve media; they were then carefully placed in Tupperware containers inside of Ziploc bags filled with air for five days. At 10 days *in vitro*, slices were transferred to new culture plates containing 1 mL of EtOH-naïve media or exposed to various concentrations (30, 40, 50 μ M) of (2*R*)-amino-5-phosphonovaleric acid (APV) diluted in 1 mL EtOH-naïve media for a 24-hour EWD period. APV is a competitive NMDAR antagonist with a dissociation constant of approximately 1.3 μ M (Evans et al., 1982).

After this 24-hour period of EWD, slices were returned to culture plates containing either 50 mM EtOH diluted in 1 mL of culture media or 1 mL of EtOH-naïve media; they were then carefully placed in Tupperware containers inside of Ziploc bags filled with air for five days. At 16 days *in vitro*, slices were transferred to new culture plates containing 1 mL of EtOH-naïve media or exposed to various concentrations (30, 40, 50 μ M) of APV diluted in 1 mL EtOH-naïve media for a 24-hour EWD period. After this 24-hour EWD period, slices were returned to culture plates containing 50 mM EtOH diluted in 1 mL of culture media or 1 mL of

EtOH-naïve media; they were then carefully placed in Tupperware containers inside of Ziploc bags filled with air for five days. At 22 days *in vitro*, slices were transferred to new culture plates containing 1 mL of EtOH-naïve media or exposed to various concentrations (30, 40, 50 μ M) of APV diluted in 1 mL EtOH-naïve media for a 24-hour EWD period. After this 24-hour EWD period, slices were fixed by exposure to 1 mL of 10% formalin solution on the top and bottom of each well for 30 minutes. The slices were then washed twice carefully (1 mL on bottom of the well and 1 mL on top of the well) with PBS and stored with 1 mL of PBS on the bottom of the well overnight at 4°C. The representative timeline for Experiment 4 is presented in Figure 2.7.

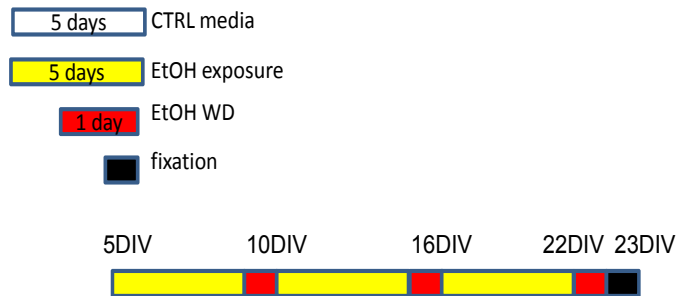


Figure 2.7. Experimental protocol timeline. Cultures were exposed to chronic intermittent ethanol exposure for a total of 3 cycle of 5 days ethanol (EtOH) exposure, followed by exposure to APV during the three 24-hour periods of ethanol withdrawal (EWD). After fixation, cultures were prepared for immunohistochemistry.

Immunohistochemistry

Following the 24-hour fixation period, the inserts were transferred to a plate containing 1 mL of permeabilization (wash) buffer (200 mL PBS [Invitrogen], 200 μ L Triton X-100 [Sigma], 0.010 mg Bovine Serum [Sigma]) on the top and bottom of each well. The slices were exposed to the wash buffer for 45 minutes, allowing the buffer to effectively penetrate each hippocampal slice. Following exposure to the buffer, slices were washed twice with PBS, as previously described. Inserts were then transferred to a plate containing 1 mL of PBS on the bottom of each well and were exposed to the primary antibody mouse anti-NeuN (1:200; Sigma) on the top of each well (after Butler et al., 2010). Plates were then stored at 4°C for 24-hours. Following this 24-hour storage period, the slices were washed twice with PBS and transferred to a plate containing 1 mL of PBS on bottom. Slices were then exposed to the goat anti-mouse secondary antibody fluorescein isothiocyanate (FITC) (1:200; Sigma) and stored at 4°C for 24-hours. Following this 24-hour storage period, slices were washed twice with PBS and imaged immediately after.

It is important to note that a range of antibody concentrations was examined prior to the experiments, and the concentrations of NeuN (neuron-specific nuclear protein) and FITC antibodies chosen were shown to have the least amount of background signal while maintaining a strong, specific signal within the regions of interest (Butler et al., 2010). Further, NeuN is expressed in nearly all post-mitotic and differentiating neurons and is detected by the

monoclonal anti-NeuN antibody, which stains many cell types in the nervous system, excluding Purkinje cells and germinal cells of the cerebellum (Mullen, Buck, & Smith, 1992). NeuN has been described as a reliable marker for detecting the density of mature neurons (Butler et al., 2010) and may be particularly useful in detecting the differentiation between small neurons and glia (Gittens and Harrison, 2004). Data that is more recent has identified NeuN as Fox-3, a member of the Fox-1 gene family, which is a splicing regulator of pre-mRNA (Kim, Adelstein, & Kawamoto, 2009).

Following immunohistochemistry, hippocampal slices were observed with SPOT software 4.0.2 (advanced version) for Windows (W. Nuhsbahm Inc.; McHenry, IL, USA) through a 5x objective with a Leica DMIRB microscope (W. Nuhsbahm Inc.; McHenry, IL, USA) connected to a computer and captured with a SPOT 7.2 color mosaic camera (W. Nuhsburg). FITC fluorescence was detected with a band-pass filter at 495 nm (520 nm emission). Densitometry using Image J software (National Institutes of Health, Bethesda, MD) was conducted to measure the intensity of FITC fluorescence. A background measurement was recorded for each slice and then subtracted from the measurement of each region of the hippocampus (the granule cell layer of the DG and the pyramidal cell layers of the CA3 and CA1 regions). Furthermore, in the quantitative analyses of mature neurons, raw values of NeuN immunoreactivity were converted to percent control values within each region of the hippocampus to control for differences among replications.

Thionine

Inserts were removed from storage at 4°C and transferred to a plate containing 1 mL of PBS on the bottom of each well; they were then exposed to 1 mL of 0.2% thionine stock on the top of each well for five minutes. Following exposure to the 0.2% thionine stock, the inserts were transferred to a plate containing 1 mL of PBS on the bottom of each well; they were then exposed to 1 mL of 70% EtOH for a two-minute dehydration period. Following the two-minute dehydration period, the inserts were washed twice with PBS for five minutes each and then imaged immediately after. Hippocampal slices stained with thionine were observed with SPOT software 4.0.2 (advanced version) for Windows (W. Nuhsbalm Inc.; McHenry, IL, USA) through a 5x objective with a Leica DMIRB microscope (w. Nuhsbalm Inc.; McHenry, IL, USA) connected to a computer and captured with a SPOT 7.2 color mosaic camera (W. Nuhsburg). Densitometry using Image J software (National Institutes of Health, Bethesda, MD) was conducted to measure the brightfield intensity. In the quantitative analyses of the density of neurons, raw brightfield values were converted to percent control values within each region of the hippocampus to control for differences among replications.

Thionine is a monochromatic, basic dye used to stain nucleic acids (DNA and RNA) and is useful in the examination of the morphological integrity of hippocampal slice cultures. However, thionine is not a particularly specific stain in regards to differentiating neurons from glia. In the current study, slices from three

cycles of CIE, continuous ethanol exposure and control cultures were exposed to thionine staining following immunohistochemistry to confirm results revealed by NeuN immunoreactivity.

Statistical Analyses

Each experiment was conducted a minimum of two times using different rodent litters, with the exception of the NMDAR antagonist studies. Data from each replication were converted into percent control values and then combined. Recent research has indicated that the CA1 region may be more vulnerable to excitotoxic insult than the CA3 or DG (Butler, et al., 2010); however, a two-way analysis of variance test (ANOVA) was conducted (treatment x sex) for each region of the hippocampus: pyramidal cells of the CA1 and CA3, and granule cells of the DG. Statistical significance was determined as $p < 0.05$. Post-hoc analyses were conducted using Fisher's Least Significant Difference (LSD) test to examine pair-wise comparisons of the means.

Chapter 3: Results

Experiment I: Three Cycles of CIE

To examine the hypothesis that multiple cycles of CIE would result in neurotoxicity, studies were conducted to examine the effects of 50 mM EtOH on NeuN immunoreactivity after three cycles of five days of 50 mM EtOH exposure followed by a 24-hour EWD period (22 days *in vitro*). For statistical analysis, a two-way ANOVA was conducted to assess potential sex differences in hippocampal slice cultures exposed to this treatment regimen. Within the CA1 region of the hippocampus, a significant main effect of treatment (Figure 3.1; $F(1,248)= 12.4, p<0.001$) was revealed in that slice cultures exposed to three cycles of CIE demonstrated significant decreases in NeuN immunoreactivity as compared to control values. Neither a significant main effect of sex nor any significant interaction between treatment and sex was observed in the CA1 region. Consistent with the CA1 region, a significant main effect of treatment (Figure 3.1; $F(1,248)= 15.1, p<0.001$) was detected in that slice cultures exposed to three cycles of CIE demonstrated significant decreases in NeuN immunoreactivity in the CA3 region as compared to control values. There was neither a significant main effect of sex nor any significant interaction between treatment and sex observed in the CA3 of the hippocampal slice cultures. Within the DG region of the hippocampus, a significant main effect of treatment (Figure 3.1; $F(1,248)= 14.8, p<0.001$) was observed in that slice cultures exposed to three cycles of CIE demonstrated significant decreases in NeuN

immunoreactivity as compared to control values. Consistent with the CA1 and CA3 regions, neither a significant main effect of sex or nor any significant interaction between treatment and sex was observed in the DG region of the hippocampal slice cultures. In summary, three cycles of CIE consistently resulted in significant decreases of NeuN immunoreactivity in examined regions of the hippocampus (pyramidal cell layers of the CA1 and CA3, and the granule cell layers of the DG) as compared to control values. Representative images of hippocampal slices labeled with NeuN immunoreactivity are shown in Figure 3.2.

Thionine Analyses (Three Cycles of CIE)

Additional studies were conducted to examine the effects of 50 mM EtOH on thionine staining after three cycles of five days of 50 mM EtOH exposure followed by a 24-hour EWD period (22 days *in vitro*). For statistical analysis, a one-way ANOVA (treatment) was conducted as previous analyses examining NeuN immunoreactivity in slice cultures exposed to this treatment regimen revealed neither significant main effects of sex nor significant sex by treatment interactions. Within the CA1 region of the hippocampus, a significant main effect of treatment (Figure 3.1; $F(1,62)= 15.9, p<0.001$) was observed in that slice cultures exposed to three cycles of CIE had significant decreases in thionine staining, revealed by increases in brightfield intensity, as compared to control values. Within the CA3 region of the hippocampus, a significant main effect of treatment (Figure 3.1; $F(1,62)= 11.5, p<0.001$) was detected in that slice cultures exposed to three cycles of CIE had significant decreases in thionine staining,

revealed by increases in brightfield intensity, as compared to control values. Consistent with the CA1 and CA3 regions, a significant main effect of treatment (Figure 3.1; $F(1,62)=16.7$, $p<0.001$) was detected in that slice cultures exposed to three cycles of CIE had significant decreases in thionine staining, revealed by increases in brightfield intensity, as compared to control values. It is important to note that in these experiments, significant increases in brightfield intensity are indicative of significant decreases in thionine staining. In summary, three cycles of CIE consistently resulted in significant decreases in thionine staining in examined regions of the hippocampus (pyramidal cell layers of the CA1 and CA3, and the granule cell layers of the DG) as compared to control slice cultures. Representative images of hippocampal slices labeled with thionine are shown in Figure 3.2.

Figure 3.1. Effects of CIE with three cycles of five days of exposure to 50 mM EtOH and one day of EWD on (A) NeuN immunoreactivity and (B) brightfield intensity observed in the organotypic hippocampal slice cultures. Three cycles of five days of exposure to 50 mM EtOH and one day of EWD resulted in significant decreases of NeuN immunoreactivity and thionine staining compared to control values within the CA1, CA3, and the DG of the hippocampus.

** = $p < 0.001$ vs. control

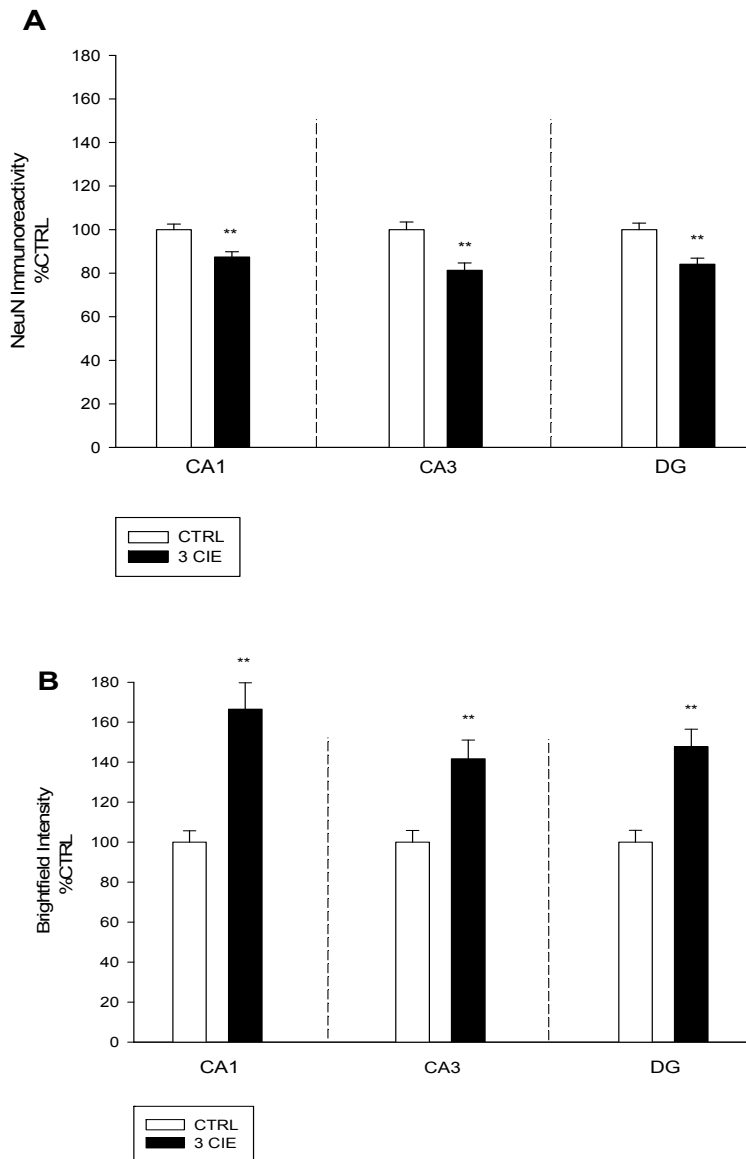
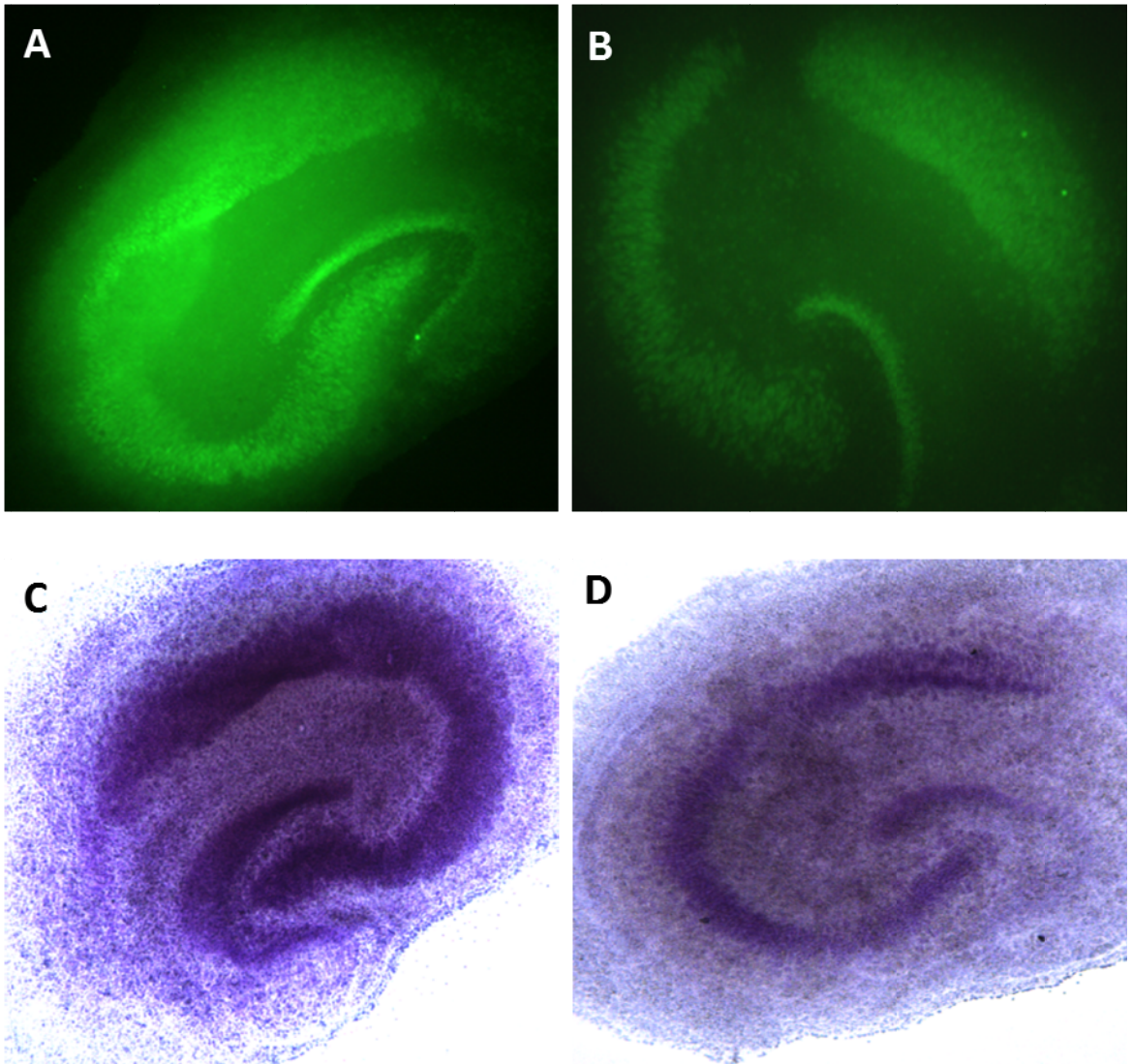


Figure 3.2. Representative images of the effects of CIE with five days of exposure to 50 mM EtOH and one day of EWD on NeuN immunoreactivity and brightfield intensity in organotypic hippocampal slice cultures. (A) three cycles control, NeuN immunoreactivity; (B), three cycles of 50 mM EtOH, NeuN immunoreactivity; (C), three cycles of control, thionine staining; (D), three cycles of 50 mM EtOH, thionine staining



Continuous Ethanol Control

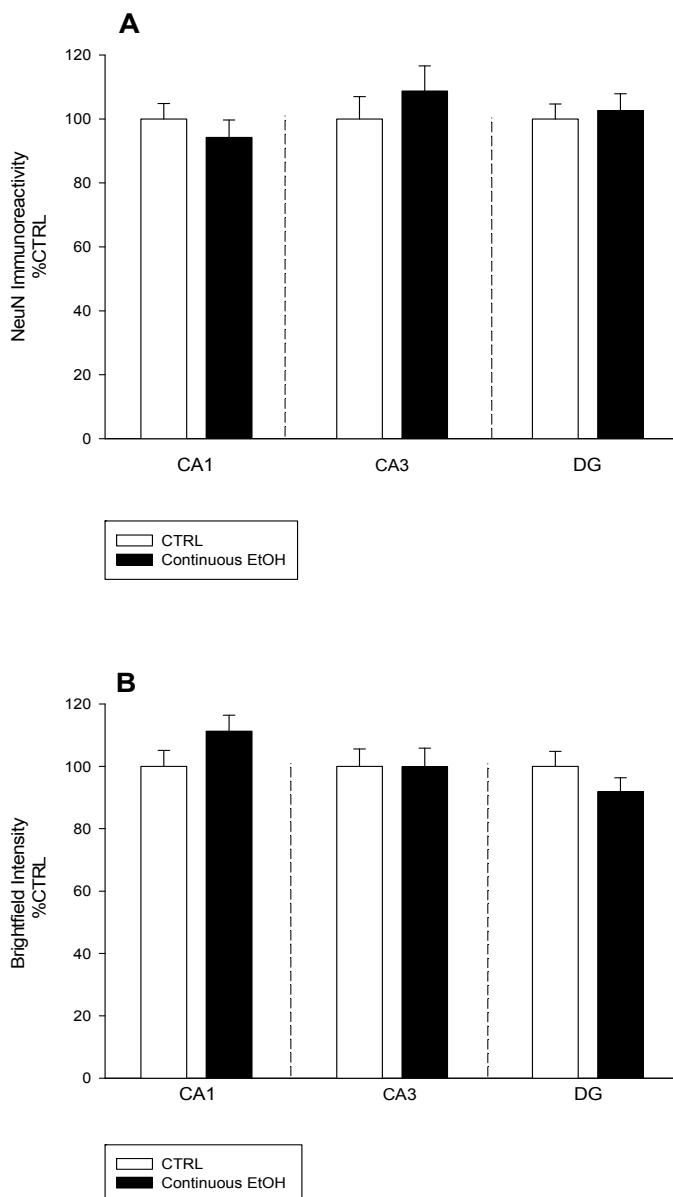
To examine the hypothesis that ethanol withdrawal was a required stimulus for neurotoxicity, tissue cultures to continuous ethanol exposure in the absence of 24-hour EWD periods, studies were conducted to observe the effects of continuous EtOH (50 mM) exposure for 18 consecutive days *in vitro* on NeuN immunoreactivity (Figure 3.3). For statistical analysis, a two-way ANOVA was conducted to assess potential sex differences in hippocampal slice cultures exposed to this treatment regimen. Within the CA1 region of the hippocampus, there were neither significant main effects of treatment or sex nor any significant interaction between treatment and sex observed. Within the CA3 region of the hippocampus, there were neither significant main effects of treatment or sex nor any significant interaction between treatment and sex observed. Within the DG region of the hippocampus, there were neither significant main effects of treatment or sex nor any significant interaction between treatment and sex observed. In summary, continuous ethanol exposure for 18 consecutive days did not result in significant decreases of NeuN immunoreactivity in all examined regions of the hippocampus (pyramidal cell layers of the CA1 and CA3, and the granule cell layers of the DG) as compared to control values.

Thionine Analyses (Continuous Ethanol Control)

Tissue cultures to continuous ethanol exposure in the absence of 24-hour EWD periods with analyses conducted to examine the effects of continuous EtOH (50 mM) exposure for 18 consecutive days *in vitro* on thionine staining

(Figure 3.3). For statistical analysis, a one-way ANOVA (treatment) was conducted, as previous analyses examining NeuN immunoreactivity in slice cultures exposed to this treatment regimen revealed no significant main effects of sex or significant sex by treatment interactions. Within the CA1 region of the hippocampus, there was no significant main effect of treatment. Within the CA3 region of the hippocampus, there was no significant main effect of treatment. Within the DG region of the hippocampus, there was no significant main effect of treatment. In summary, continuous ethanol exposure for 18 consecutive days did not result in significant decreases of thionine staining in all examined regions of the hippocampus (pyramidal cell layers of the CA1 and CA3, and the granule cell layers of the DG) as compared to control values.

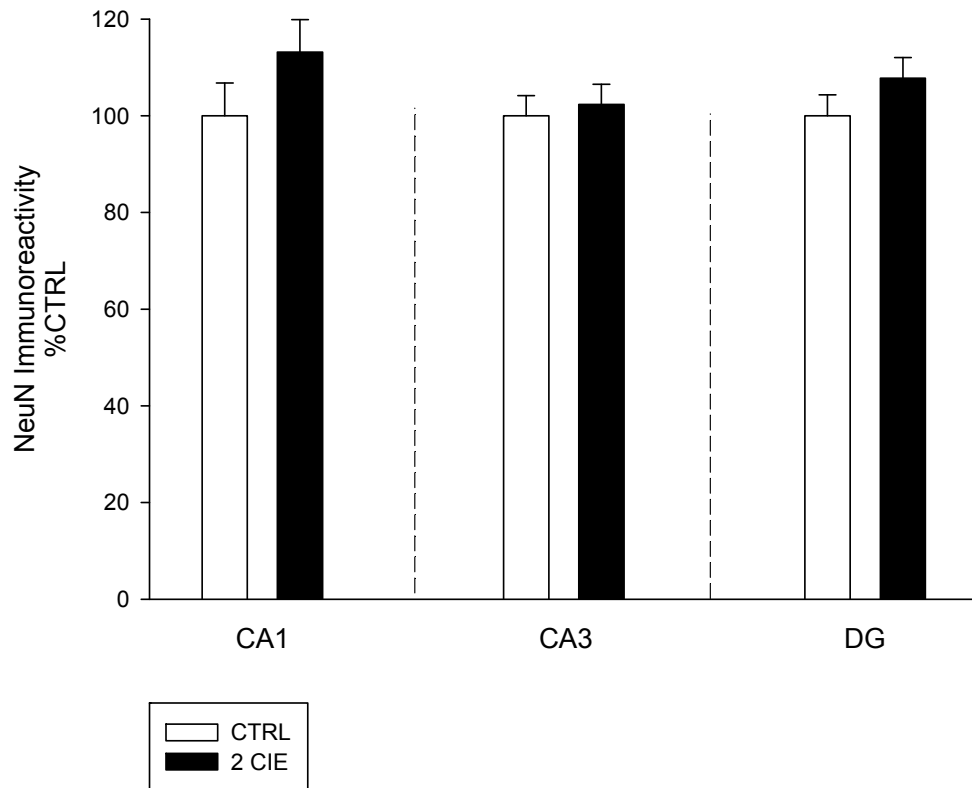
Figure 3.3. Effects of continuous ethanol exposure for 18 consecutive days *in vitro* to 50 mM EtOH on (A) NeuN immunoreactivity and (B) brightfield intensity observed in the organotypic hippocampal slice cultures. Continuous ethanol exposure did not result in significant decreases of NeuN immunoreactivity or thionine staining within the CA1, CA3, or the DG of the hippocampus as compared to control values



Experiment II: Two Cycles of CIE (Non-Aged Tissue)

Studies were conducted to examine the effects of 50 mM EtOH on NeuN immunoreactivity after two cycles of five days of 50 mM EtOH exposure followed by a 24-hour EWD period in non-aged tissue (17 days *in vitro*) (Figure 3.4). Hippocampal slice cultures exposed to two cycles of CIE were not aged-matched to slices exposed to three cycles of CIE (i.e., final 24-EWD period for both conditions did not occur the same day *in vitro*). For statistical analysis, a two-way ANOVA was conducted to assess potential sex differences in hippocampal slice cultures exposed to this treatment regimen. Within the CA1 region of the hippocampus, there were neither significant main effects of treatment or sex nor any significant interaction between treatment and sex observed. Within the CA3 region of the hippocampus, there were neither significant main effects of treatment or sex nor any significant interaction between treatment and sex observed. Within the DG region of the hippocampus, there were neither significant main effects of treatment or sex nor any significant interaction between treatment and sex observed. In summary, two cycles of CIE in non-aged tissue did not result in significant decreases of NeuN immunoreactivity in all examined regions of the hippocampus (pyramidal cell layers of the CA1 and CA3, and the granule cell layers of the DG) as compared to control values.

Figure 3.4. Effects of CIE with two cycles of five days of exposure to 50 mM EtOH and one day of EWD on NeuN immunoreactivity observed in non-aged organotypic hippocampal slice cultures. Two cycles of five days of exposure to 50 mM EtOH and one day of EWD in non-aged tissue did not result in significant decreases of NeuN immunoreactivity within the CA1, CA3, or the DG of the hippocampus as compared to control values.



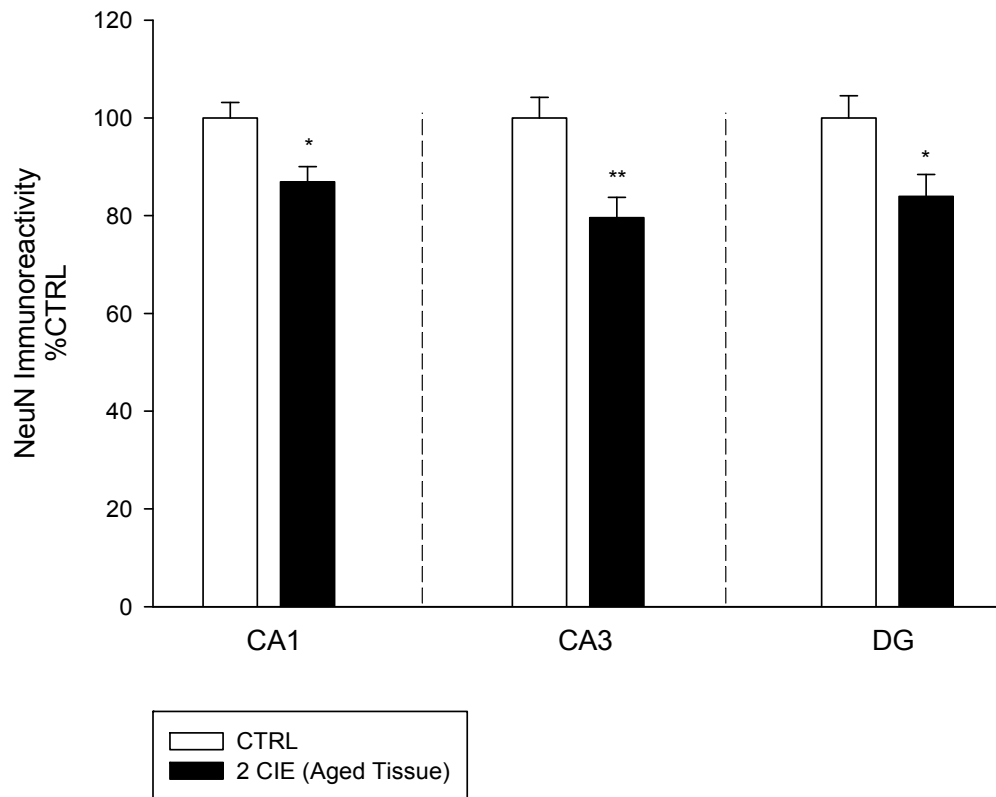
Experiment II: Two Cycles of CIE (Aged Tissue)

Studies were conducted to examine the effects of 50 mM EtOH on NeuN immunoreactivity after two cycles of five days of 50 mM EtOH exposure followed by a 24-hour EWD period in aged tissue (22 days *in vitro*) (Figure 3.5). Hippocampal slice cultures exposed to two cycles of CIE were aged-matched to slices exposed to three cycles of CIE (i.e., final 24-EWD period for both conditions occurred the same day *in vitro*). For statistical analysis, a two-way ANOVA was conducted to assess potential sex differences in slice cultures exposed to this treatment regimen. Within the CA1 region of the hippocampus, a significant main effect of treatment was observed (Figure 3.5; $F(1,114)= 8.7$, $p<0.05$) in that aged tissue cultures exposed to two cycles of CIE demonstrated significant decreases of NeuN immunoreactivity as compared to control values. There was no significant main effect of sex or significant treatment by sex interaction detected in the CA1 region. Consistent with the CA1 region, a significant main effect of treatment was observed (Figure 3.5; $F(1,114)= 11.9$, $p<0.001$) in that aged tissue cultures exposed to two cycles of CIE demonstrated significant decreases of NeuN immunoreactivity in the CA3 region of the hippocampus as compared to control values. Likewise, there was no significant main effect of sex or significant treatment by sex interaction detected in the CA3. Consistent with the CA1 and CA3 regions, a significant main effect of treatment was observed (Figure 3.5; $F(1,114)= 6.4$, $p<0.05$) within the DG region of the hippocampus in that aged tissue cultures exposed to two cycles of CIE demonstrated significant decreases of NeuN immunoreactivity as compared to

control values. There was no significant main effect of sex or significant treatment by sex interaction detected in the DG. In summary, within each region of the hippocampus, two cycles of CIE in aged tissue resulted in significant loss of NeuN immunoreactivity in all examined regions of the hippocampus (pyramidal cell layers of the CA1 and CA3, and the granule cell layer of the DG) as compared to controls.

Figure 3.5. Effects of CIE with two cycles of five days of exposure to 50 mM EtOH and one day of EWD on NeuN immunoreactivity observed in aged organotypic hippocampal slice cultures. Two cycles of five days of exposure to 50 mM EtOH and one day of EWD in aged tissue resulted in significant decreases of NeuN immunoreactivity within the CA1, CA3, and the DG of the hippocampus as compared to control values.

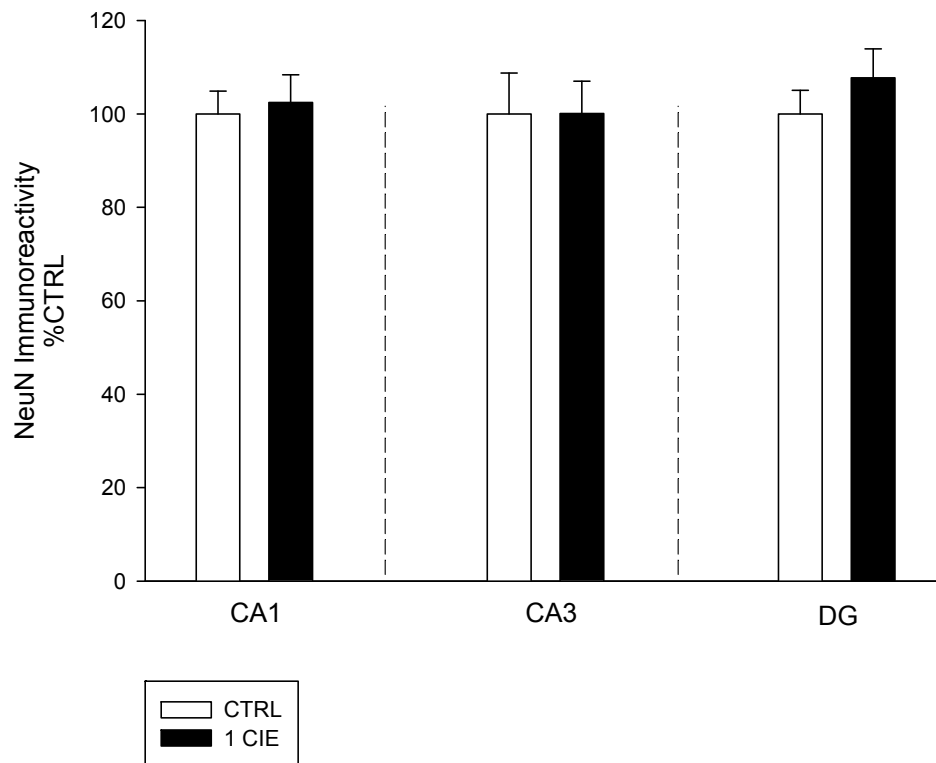
** = $p < 0.001$ vs. control; * = $p < 0.05$ vs. control



Experiment III: One Cycle of CIE (Non-Aged Tissue)

Studies were conducted to examine the effects of 50 mM EtOH on NeuN immunoreactivity after one cycle of five days of 50 mM EtOH exposure followed by a 24-hour EWD period in non-aged tissue (11 days *in vitro*) (Figure 3.6). Non-aged hippocampal slice cultures exposed to two cycles of CIE were not aged-matched to slices exposed to three cycles of CIE (i.e., final 24-EWD period for both conditions did not occur the same day *in vitro*). For statistical analysis, a two-way ANOVA was conducted to assess potential sex differences in hippocampal slice cultures exposed to this treatment regimen. Within the CA1 region of the hippocampus, there were neither significant main effects of treatment or sex nor any significant interaction between treatment and sex observed. Within the CA3 region of the hippocampus, there were neither significant main effects of treatment or sex nor any significant interaction between treatment and sex observed. Within the DG region of the hippocampus, there were neither significant main effects of treatment or sex nor any significant interaction between treatment and sex observed. In summary, one cycle of CIE in non-aged tissue did not result in significant decreases of NeuN immunoreactivity compared to control values in all examined regions of the hippocampus (pyramidal cell layers of the CA1 and CA3, and the granule cell layers of the DG).

Figure 3.6. Effects of CIE with one cycle of five days of exposure to 50 mM EtOH and one day of EWD on NeuN immunoreactivity observed in non-aged organotypic hippocampal slice cultures. One cycle of five days of exposure to 50 mM EtOH and one day EWD in non-aged tissue did not result in significant decreases of NeuN immunoreactivity within the CA1, CA3, or the DG of the hippocampus as compared to control values.



Experiment III: One Cycle of CIE (Aged Tissue)

Studies were conducted to examine the effects of 50 mM EtOH on NeuN immunoreactivity after one cycle of five days of 50 mM EtOH exposure followed by a 24-hour EWD period in aged tissue (22 days *in vitro*) (Figure 3.7). Hippocampal slice cultures exposed to two cycles of CIE were aged-matched to slices exposed to three cycles of CIE (i.e., final 24-EWD period for both conditions occurred the same day *in vitro*). For statistical analysis, a two-way ANOVA was conducted to assess potential sex differences in slice cultures exposed to this treatment regimen. Within the CA1 region of the hippocampus, there were neither significant main effects of treatment or sex nor any significant interaction between treatment and sex observed. Within the CA3 region of the hippocampus, there were neither significant main effects of treatment or sex nor any significant interaction between treatment and sex observed. Within the DG region of the hippocampus, there was no significant main effect of treatment detected. Within the DG, however, a significant main effect of sex was revealed ($F(1,111)= 4.5, p<0.05$) in that female aged tissue demonstrated significant increases in NeuN immunoreactivity as compared to male aged tissue. Furthermore, an unexpected significant treatment by sex interaction (Figure 3.8; $F(1,111)= 4.5, p<0.05$) was detected in that female aged tissue exposed to one cycle of CIE showed significant increases in NeuN immunoreactivity in the DG subregion as compared to male aged tissue exposed to the same treatment regimen. Notably, male tissue showed no significant differences in NeuN immunoreactivity as compared to control values. In summary, one cycle of CIE in

aged tissue did not result in significant decreases of NeuN immunoreactivity in all examined regions of the hippocampus (pyramidal cell layers of the CA1 and CA3, and the granule cell layers of the DG) as compared to control values.

Figure 3.7. Effects of CIE with one cycle of five days of exposure to 50 mM EtOH and one day of EWD on NeuN immunoreactivity observed in aged organotypic hippocampal slice cultures. One cycle of five days of exposure to 50 mM EtOH and one day of EWD in aged tissue did not result in significant decreases of NeuN immunoreactivity within the CA1, CA3, or the DG of the hippocampus as compared to control values.

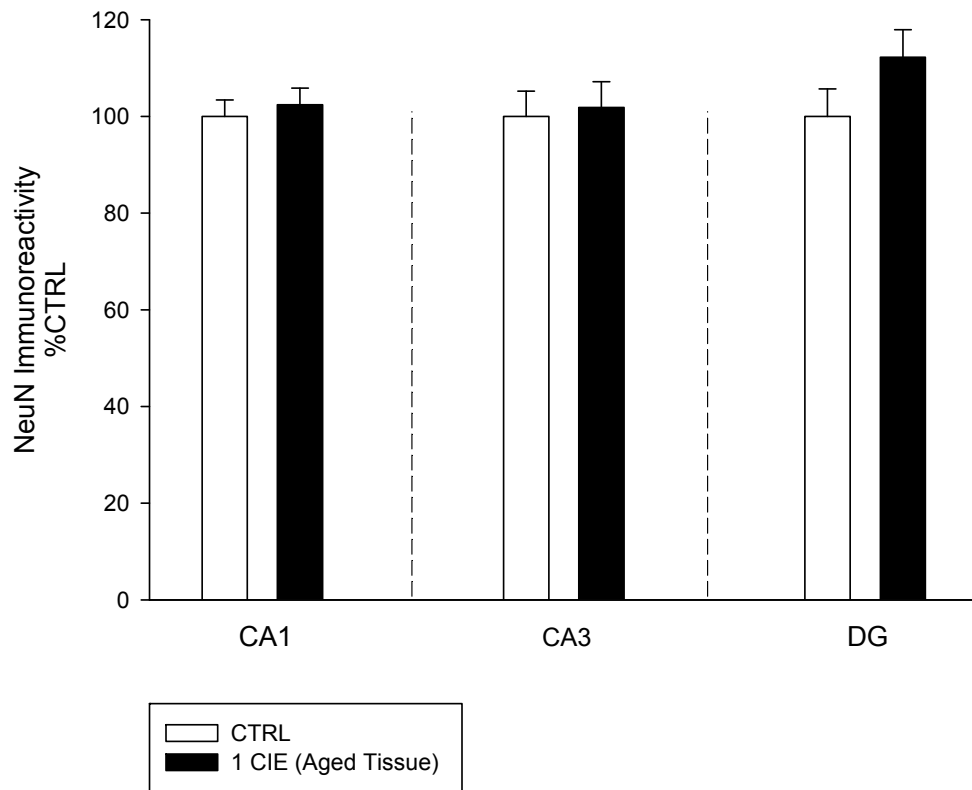
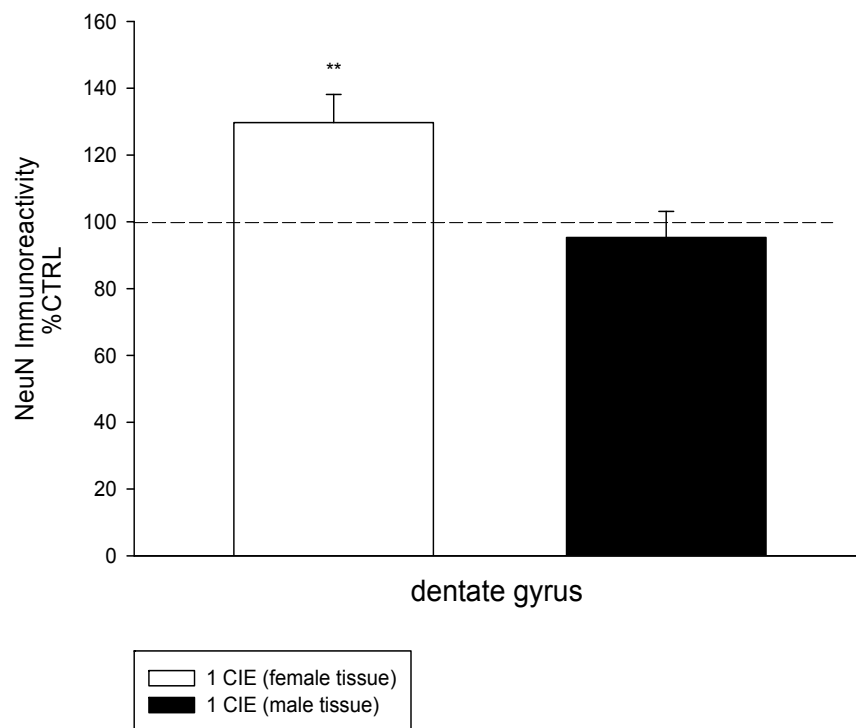


Figure 3.8. Sex by treatment interaction effects of CIE with one cycle of five days of exposure to 50 mM EtOH and one day EWD on NeuN immunoreactivity observed in aged organotypic hippocampal slice cultures. One cycle of five days of exposure to 50 mM EtOH and one day of EWD resulted in increases of NeuN immunoreactivity within the DG of the hippocampus in female tissue as compared to male tissue exposed to the same treatment regimen.

* = $p < 0.05$ vs. male tissue



Experiment IV: Three Cycles of CIE + APV (30 μ M)

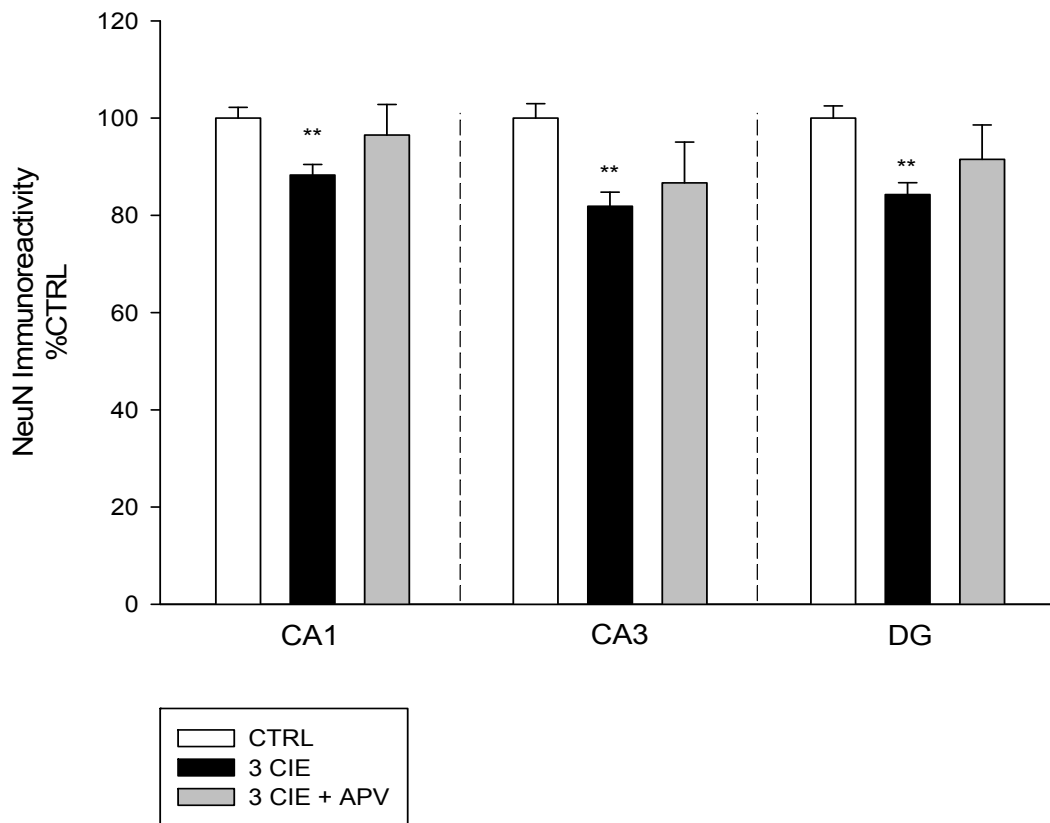
To test the hypothesis that multiple cycles of CIE would result in excitotoxicity produced by repeated overactivation of NMDARs, the NMDAR was specifically targeted to determine if NMDAR antagonism would attenuate the loss of NeuN produced by multiple cycles of CIE. Further, studies were conducted to examine the effects of 50 mM EtOH on NeuN immunoreactivity after three cycles of five days of 50 mM EtOH followed by exposure to competitive NMDAR antagonist APV (30 μ M) during 24-hour EWD periods. For statistical analysis, a two-way ANOVA was conducted to assess potential sex differences in hippocampal slice cultures exposed to this treatment regimen. Within the CA1 region of the hippocampus, a significant effect of treatment was detected (Figure 3.9; $F(3,320)= 5.2, p<0.05$) in that slices exposed to three cycles of CIE demonstrated significant decreases in NeuN immunoreactivity as compared to control values. There was no significant main effect sex or any significant interaction between treatment and sex observed in the CA1.

Within the CA3 subregion, a significant effect of treatment was detected (Figure 3.9; $F(3,320)= 6.5, p<0.001$) in that slices exposed to three cycles of CIE demonstrated significant decreases in NeuN immunoreactivity as compared to control values. There was no significant main effect sex or any significant interaction between treatment and sex observed in the CA3 region.

Within the DG region of the hippocampus, a significant effect of treatment was detected (Figure 3.9; $F(3,320)= 6.8, p<0.001$) in that slices exposed to three cycles of CIE demonstrated significant decreases in NeuN immunoreactivity as compared to control values. There was no significant main effect sex or any significant interaction between treatment and sex observed in the DG region. In summary, exposure to three cycles of CIE resulted in significant decreases of NeuN immunoreactivity in the pyramidal cell layers of the CA1 and CA3, and the granule cell layers of the DG as compared to control values. In summary, exposure to three cycles of CIE and APV (30 μM) during 24-hour EWD periods attenuated the loss of NeuN immunoreactivity the CA1, CA3 and DG.

Figure 3.9. Effects of CIE with three cycles of five days of exposure to 50 mM EtOH and one day of EWD with exposure to APV (30 μ M) NeuN immunoreactivity observed in the organotypic hippocampal slice cultures. Three cycles of five days of exposure to 50 mM EtOH and 1 day EWD resulted in significant decreases of NeuN immunoreactivity within the CA1, CA3, and the DG of the hippocampus as compared to control values.

** = $p < 0.001$ vs. control



Experiment IV: Three Cycles of CIE + APV (40 μ M)

Studies were conducted to examine the effects of 50 mM EtOH on NeuN immunoreactivity after three cycles of five days of 50 mM EtOH exposure followed by exposure to APV (40 μ M) during 24-hour EWD periods to test a higher concentration of APV. For statistical analysis, a two-way ANOVA was conducted to assess potential sex differences in hippocampal slice cultures exposed to this treatment regimen. Within the CA1 region of the hippocampus, a significant effect of treatment was revealed (Figure 3.10; $F(3,318)= 6.7, p<0.001$) in that slices exposed to three cycles of CIE demonstrated significant decreases in NeuN immunoreactivity as compared to control values. Additionally, slice cultures exposed to three cycles of CIE demonstrated significant decreases in NeuN immunoreactivity as compared to slice cultures exposed to three cycles of CIE and APV (40 μ M) during 24-hour EWD periods. There was no significant main effect sex or any significant interaction between treatment and sex observed in the CA1.

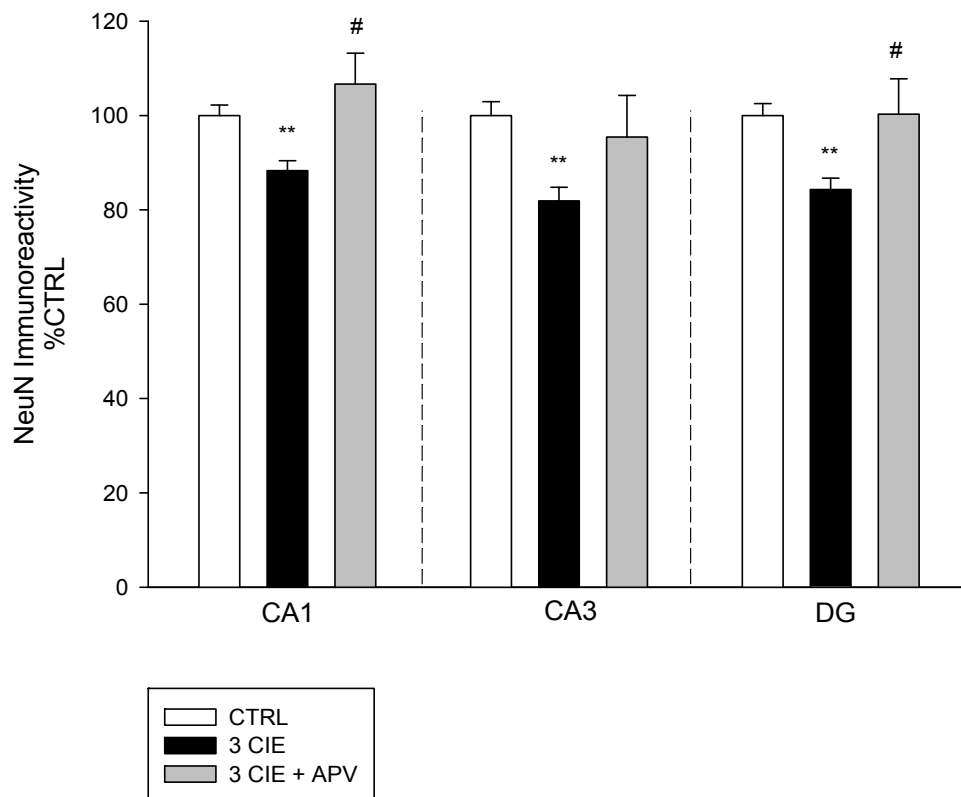
Within the CA3 region of the hippocampus, a significant effect of treatment was revealed (Figure 3.10; $F(3,318)= 7.1, p<0.001$) in that slices exposed to three cycles of CIE demonstrated significant decreases in NeuN immunoreactivity as compared to control values. In contrast to the CA1 region, there were no significant differences observed between slice cultures exposed to three cycles of CIE and slice cultures exposed to three cycles of CIE and APV (40 μ M) during 24-hour EWD periods. There was no significant main effect sex or

any significant interaction between treatment and sex observed in the CA3 region.

Consistent with the CA1 region, a significant effect of treatment was revealed (Figure 3.10; $F(3,318)= 7.9$, $p<0.001$) in that slices exposed to three cycles of CIE demonstrated significant decreases in NeuN immunoreactivity in the DG as compared to control values. Further, slice cultures exposed to three cycles of CIE demonstrated significant decreases in NeuN immunoreactivity as compared to slice cultures exposed to three cycles of CIE and APV (40 μM) during 24-hour EWD periods. There was no significant main effect sex or any significant interaction between treatment and sex observed in the DG. In summary, slice cultures exposed to three cycles of CIE demonstrated significant decreases in NeuN immunoreactivity in the pyramidal cell layers of the CA1 and CA3, and the granule cell layers of the DG as compared to control values. Exposure to three cycles of CIE and APV (40 μM) during 24-hour EWD periods attenuated the CIE-induced losses of NeuN in the pyramidal cell layers of the CA1 and CA3, and the granule cell layers of the DG. Further, exposure to three cycles of CIE and APV (40 μM) during 24-hour EWD periods prevented CIE-induced losses of NeuN in the pyramidal cell layer of the CA1 and granule cell layer of the DG; however, this effect was not demonstrated in the pyramidal cell layer of the CA3.

Figure 3.10. Effects of CIE with three cycles of five days of exposure to 50 mM EtOH and one day of EWD with exposure to APV (40 μ M) NeuN immunoreactivity observed in the organotypic hippocampal slice cultures. Three cycles of five days of exposure to 50 mM EtOH and one day of EWD resulted in significant decreases of NeuN immunoreactivity within the CA1, CA3, and the DG of the hippocampus as compared to control values. Three cycles of five days of exposure to 50 mM EtOH and APV (40 μ M) during EWD periods resulted in significant increases of NeuN immunoreactivity within the CA1 and the DG of the hippocampus as compared to three cycles of CIE.

** = $p < 0.001$ vs. control; # = $p < 0.05$ vs. 3 CIE



Experiment IV: Three Cycles of CIE + APV (50 μ M)

Studies were conducted to examine the effects of 50 mM EtOH on NeuN immunoreactivity after three cycles of five days of 50 mM EtOH exposure followed by exposure to APV (50 μ M) during 24-hour EWD periods to test higher concentrations of APV. For statistical analysis, a two-way ANOVA was conducted to assess potential sex differences in hippocampal slice cultures exposed to this treatment regimen. Within the CA1 region of the hippocampus, a significant main effect of treatment was observed (Figure 3.11; $F(3,341)= 9.7$, $p<0.001$) in that slices exposed to three cycles of CIE demonstrated significant decreases in NeuN immunoreactivity as compared to control values. Slices exposed to three cycles of CIE and APV (50 μ M) during 24-hour EWD periods demonstrated significant decreases in NeuN immunoreactivity as compared to control values. Further, slice cultures exposed to APV alone (50 μ M) demonstrated significant decreases in NeuN immunoreactivity as compared to control values. There was no significant main effect sex or any significant interaction between treatment and sex observed in the CA1.

Within the CA3 region of the hippocampus, there was a significant main effect of treatment demonstrated (Figure 3.12; $F(3,341)= 13.9$, $p<0.001$) in that slices exposed to three cycles of CIE demonstrated significant decreases in NeuN immunoreactivity as compared to control values. Slice cultures exposed to APV alone (50 μ M) demonstrated significant decreases in NeuN immunoreactivity as compared to control values. In contrast to the CA1 region,

slice cultures exposed to three cycles of CIE and APV (50 μ M) during 24-hour EWD periods demonstrated significant decreases in NeuN immunoreactivity as compared to slice cultures exposed to three cycles of CIE. There was no significant main effect sex or any significant interaction between treatment and sex observed in the CA3.

Consistent with the CA3 region, there was a significant effect of treatment demonstrated (Figure 3.13; $F(3,341)= 17.9$, $p<0.001$) in that slices exposed to three cycles of CIE demonstrated significant decreases in NeuN immunoreactivity in the DG as compared to control values. Slice cultures exposed to APV alone (50 μ M) demonstrated significant decreases in NeuN immunoreactivity as compared to control values. Further, slice cultures exposed to three cycles of CIE and APV (50 μ M) during 24-hour EWD periods demonstrated significant decreases in NeuN immunoreactivity as compared to slice cultures exposed to three cycles of CIE. There was no significant main effect sex or any significant interaction between treatment and sex observed in the DG.

In summary, slice cultures exposed to three cycles of CIE demonstrated significant decreases in NeuN immunoreactivity in the pyramidal cell layers of the CA1 and CA3, and the granule cell layers of the DG as compared to control values. Exposure to APV alone (50 μ M) resulted in significant decreases in NeuN immunoreactivity in the CA1, CA3, and DG as compared to control values. In the

CA3 and DG regions, exposure to three cycles of CIE and APV (50 μ M) during 24-hour EWD periods resulted in significant decreases in NeuN immunoreactivity compared to slices exposed to three cycles of CIE.

Figure 3.11. Effects of CIE with three cycles of five days of exposure to 50 mM EtOH and one day of EWD with exposure to APV (50 μ M) NeuN immunoreactivity observed in the organotypic hippocampal slice cultures in the CA1. Three cycles of five days of exposure to 50 mM EtOH and one day of EWD resulted in significant decreases of NeuN immunoreactivity in the CA1 of the hippocampus as compared to control values.

** = $p < 0.001$ vs. control

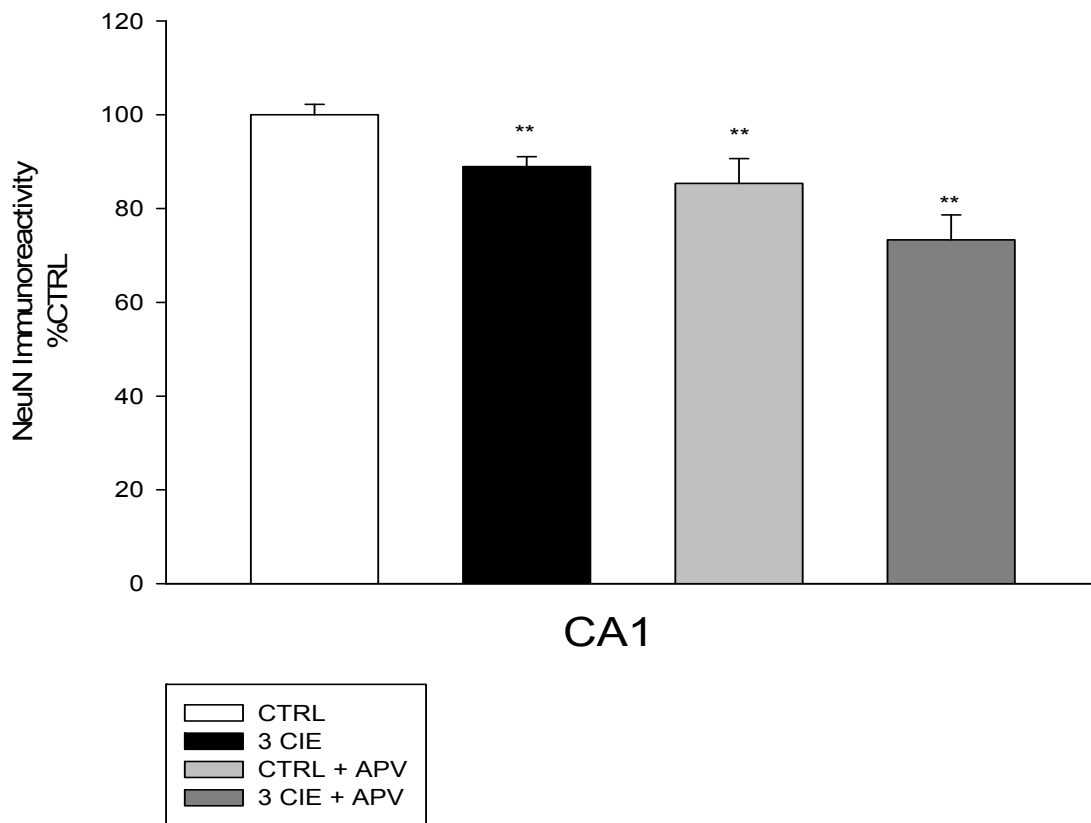


Figure 3.12. Effects of CIE with three cycles of five days of exposure to 50 mM EtOH and one day EWD with exposure to APV (50 μ M) NeuN immunoreactivity observed in the organotypic hippocampal slice cultures in the CA3. Three cycles of five days of exposure to 50 mM EtOH and one day of EWD resulted in significant decreases of NeuN immunoreactivity in the CA3 of the hippocampus as compared to control values.

** = $p < 0.001$ vs. control; * = $p < 0.05$ vs. 3 CIE

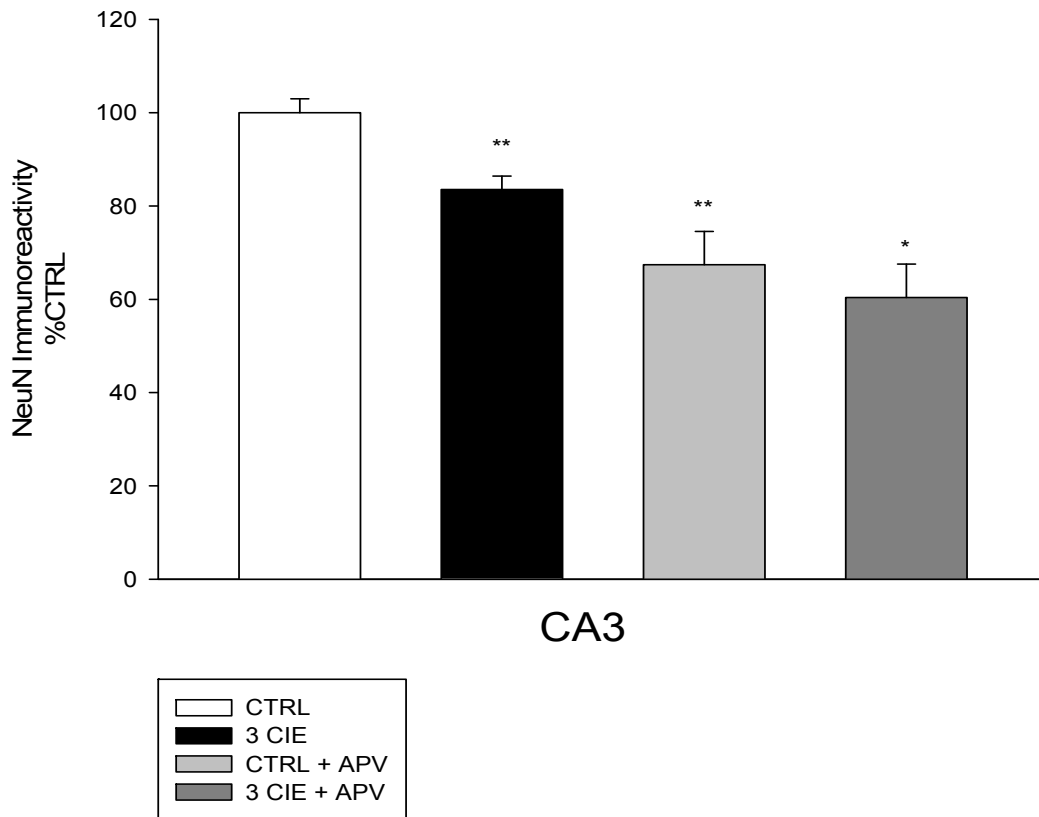
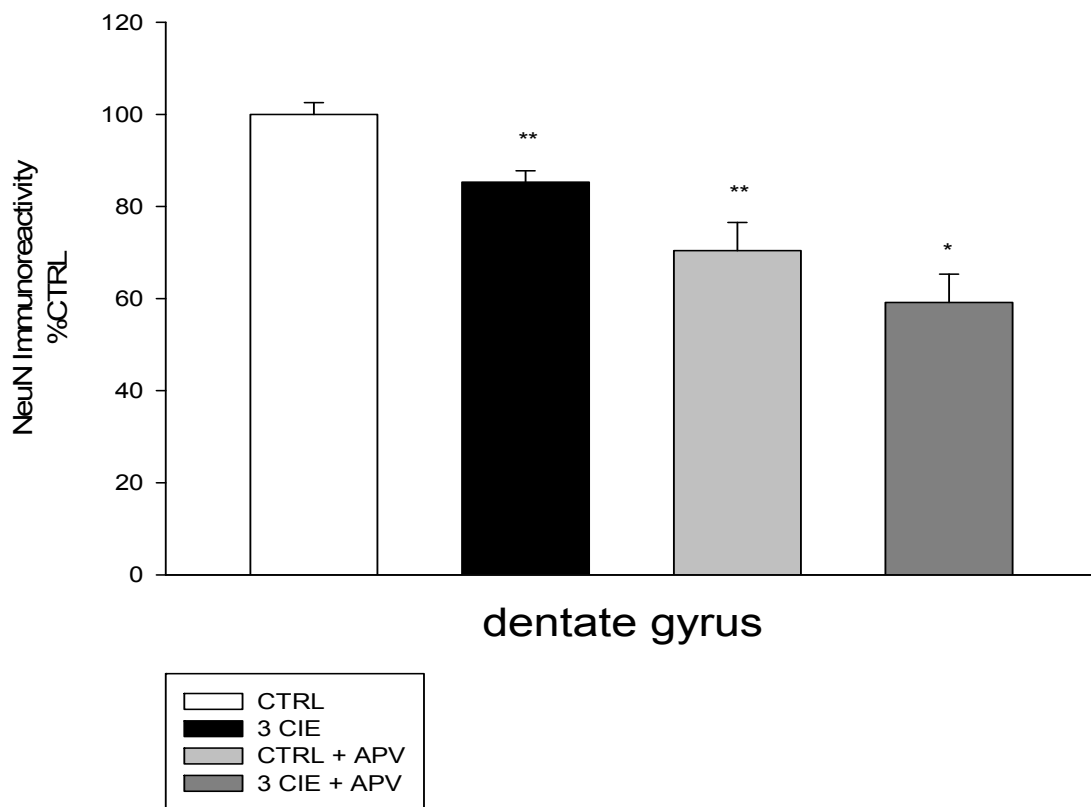


Figure 3.13. Effects of CIE with three cycles of five days of exposure to 50 mM EtOH and one day of EWD with exposure to APV (50 μ M) NeuN immunoreactivity observed in the organotypic hippocampal slice cultures in the DG. Three cycles of five days of exposure to 50 mM EtOH and one day of EWD resulted in significant decreases of NeuN immunoreactivity in the DG of the hippocampus as compared to control values.

** = $p < 0.001$ vs. control; * = $p < 0.05$ vs. 3 CIE



Chapter 4: Discussion

Alcohol-dependent individuals engage in punctuated patterns of consumption: drinking to maintain a specific level of intoxication is discontinued for some amount of time and subsequently resumed following the period of sobriety (Mello & Mendleson, 1972). Binge drinking also follows a pattern of intermittent and high levels of alcohol in the system, which may contribute to enhanced neurodegeneration and cognitive impairments through the “kindling” phenomenon (Hunt, W.A., 1993). “Kindling” was first described in relation to a phenomenon in which repeated low-intensity electrical stimulation to areas of the limbic system produced epileptic seizures. It is important to note that the initial electrical stimulations did not elicit seizure activity; however, repeated stimulations “kindled” differential behavioral outcomes in rodents (Goddard et al., 1969).

Retrospective studies have examined multiple prior detoxifications as stimuli for kindling in the alcohol-dependent population, and it has been found that multiple detoxifications are associated with an increased risk for withdrawal-induced seizures (Brown et al., 1988; Lechtenberg & Worner, 1990, 1991, 1992; Booth & Blow, 1993; Worner, 1996). Rodent models of CIE further demonstrate multiple withdrawals as a stimulus for kindling, as CIE exposure is associated with an increased rate (Becker, H.C., & Hale, R.L., 1993; Veatch & Becker, 2002), intensity, and duration (Becker, H.C., Diaz-Granados, J.L., & Weathersby,

R.T., 1997) of withdrawal-induced seizures (Stephens et al., 2001). Notably, withdrawal-induced seizures are associated with marked brain atrophy. For example, one study demonstrated that patients with a history of at least one prior withdrawal-induced seizure showed significant decreases in white matter volume of the temporal lobe as compared to patients with no history of previous withdrawal-induced seizures or control subjects (Sullivan et al., 1996).

Physiological manifestations of ethanol withdrawal are associated with a hyperexcitability of the CNS that is produced by chronic ethanol exposure. Adaptations in excitatory neurotransmission following chronic ethanol exposure include increased NMDAR sensitivity (Lovinger, 1993), increased number of NMDAR's (Carpenter-Hyland et al., 2004; Floyd et al., 2003), increased expression of NR2B subunits (Sheela et al., 2006), increased aggregation of NMDARs at the synapse (Carpenter-Hyland et al., 2004), and trafficking of NR2B subunits to the synapse (Carpenter-Hyland et al., 2004). The present study tested the hypothesis that multiple cycles of CIE would result in increased excitotoxicity, as compared to a single withdrawal following exposure to a binge-like concentration of ethanol. The present study examined one, two, and three cycles of CIE to binge-like concentrations of (50 mM) for five days followed by a 24-hour EWD period in non-aged and aged tissue cultures. It also targeted NMDAR's by following three cycles of CIE in organotypic slice cultures to determine if overactivation of the NMDAR was associated with the loss of mature neurons produced by multiple cycles of CIE.

Experiment I: Three Cycles of CIE

The current study examined the hypothesis that multiple cycles of CIE would result in increased excitotoxicity by quantifying the loss of mature neurons following three cycles of CIE in hippocampal slice cultures, as reflected by decreases in NeuN immunoreactivity and decreases in thionine staining. Data analyses demonstrated that three cycles of CIE resulted in consistent decreases in the density of neurons in all examined regions as NeuN immunoreactivity was reduced by 13%, 19% and 16% in the CA1, CA3 and DG respectively. As previously discussed, NeuN is expressed in nearly all post-mitotic and differentiating neurons and is detected by the monoclonal anti-NeuN antibody, which stains many cell types in the nervous system, excluding Purkinje cells and germinal cells of the cerebellum (Mullen, Buck, & Smith, 1992). Previous research indicates that decreases in NeuN immunoreactivity are suggestive of decreases in neurons (Wilkins et al., 2006) and mature neuron density (Butler et al., 2010). Likewise, brightfield analyses confirmed decreases in thionine staining in the three hippocampal subregions following three cycles of CIE. The consistent findings between NeuN immunoreactivity and thionine staining in regards to neuronal degeneration are not surprising because thionine staining is typically used to confirm the morphological integrity of hippocampal slice cultures in models of brain injury *in vitro* (Finley, M., Fairman, D., Liu, D., Li, P., Wood, A., & Cho, S., 2004). One study measured the density of neurons in the cingulate cortex using both cresyl violet staining and NeuN immunoreactivity quantitative

analyses. The authors indicate that strong correlations were found between both markers (Gittens and Harrison, 2004).

The current study is consistent with previous studies examining multiple withdrawal episodes as a stimulus for kindling in human studies (Brown et al., 1988; Lechtenberg & Worner, 1990, 1991, 1992; Booth & Blow, 1993; Worner, 1996) and rodent studies (Becker, H.C., & Hale, R.L., 1993; Becker et al., 1997; Stephens et al., 2001; Veatch & Becker, 2002). The present findings are also consistent with previous studies that examined intermittent ethanol exposure *in vivo* and *in vitro* producing neuronal degradation of the hippocampus (Collins, M.A., Zou, J., & Neafsey, E.J., 1998). In a recent study, rodents were administered ethanol (3 g/kg) three times daily for four consecutive days followed by a three-day EWD period. This treatment regimen was repeated four times to mimic binge-like drinking patterns observed in the clinical population. The intermittent administration of ethanol produced neuronal degeneration in the pyramidal cell layers of the CA1 and CA3, and the granule cell layers of the DG of the hippocampus, parietal association cortex, and entorhinal cortex (detected through Flouro-Jade B staining). The authors suggest that the neuronal degeneration observed in the hippocampus, parietal association cortex, and entorhinal cortex were associated with the cognitive deficits in spatial and working memory demonstrated in these experiments (Zhao et al., 2013).

Previous *in vitro* studies demonstrate that exposure to EtOH (100 mM) followed by a period of EWD results in CNS hyperexcitability associated with the NMDAR. For example, Prendergast and colleagues demonstrated that a ten-day exposure to EtOH (100 mM) followed by a 24-hour EWD period produces excitotoxic insult in the pyramidal cell layers of the CA1 subregion of the hippocampus (Prendergast et al., 2000; Prendergast et al., 2004). It has been suggested that the CA1 region of the hippocampus contains the greatest expression of NMDAR complexes and NR1 and NR2B NMDAR subunit complexes as compared to the CA3 and DG. Accordingly, the authors suggest that the pyramidal cells of the CA1 are particularly vulnerable to NMDA-induced excitotoxicity as compared to the CA3 and DG (Butler et al., 2010). However, ten-day exposure to 50 mM ethanol did not produce excitotoxicity, though it did sensitize NMDA receptors to agonist effects of other substances (Self et al., 2004; Self et al. 2005). The current study did not demonstrate regional vulnerability specific to the CA1 in regards to CIE. Significant decreases in NeuN immunoreactivity following three cycles of CIE were consistent within the CA1, CA3, and DG. Although brightfield analyses revealed modest loss of thionine stain in the CA1 region as compared to the CA3 and DG in the current study, this effect was not significant.

Continuous Ethanol Control

The current study further examined the hypothesis that ethanol withdrawal was a required stimulus for neurotoxicity, by exposing tissue cultures to

continuous ethanol exposure in the absence of 24-hour EWD periods. Thus, the current study quantified the loss of mature neurons on NeuN immunoreactivity following continuous EtOH exposure in hippocampal slice cultures. However, exposure to continuous ethanol for 18 consecutive days *in vitro* did not result in the loss of mature neurons, as significant decreases of NeuN immunoreactivity were not observed following continuous ethanol exposure in the three examined regions. Likewise, brightfield analyses revealed no decreases in thionine staining following exposure to continuous ethanol for 18 consecutive days *in vitro* in the three examined regions.

Experiment II: Two Cycles of CIE

The current study further examined the hypothesis that multiple cycles of CIE would result in increased excitotoxicity by testing the required number of cycles needed to produce toxicity. Accordingly, the current study quantified the loss of mature neurons on NeuN immunoreactivity following two cycles of CIE in non-aged and aged hippocampal slice cultures. Previous literature suggests that aged cultures may be particularly vulnerable to excitotoxic insult (Brewer et al., 2007). Thus, aged hippocampal slice cultures were aged-matched to slices exposed to three cycles of CIE in that the final 24-EWD periods for both conditions occurred the same day *in vitro*. Likewise, non-aged slice cultures in the current experiment were not age-matched to slices exposed to three cycles of CIE. The current study demonstrated that two cycles of CIE in non-aged tissue did not result in significant decreases of mature neurons in the pyramidal cell

layers of the CA1 and CA3, and the granule cell layers of the DG as compared to control values.

In contrast, exposure of hippocampal slice cultures to two cycles of CIE in aged tissue resulted in the loss of mature neurons, reflected by significant decreases of NeuN immunoreactivity in the three subregions of the hippocampus. Notably, the loss of mature neurons in each examined subregion in aged tissue following two cycles of CIE is comparable to the loss of mature neurons observed in slice cultures following three cycles of CIE. This effect further validates the hypothesis that multiple withdrawals following binge-like concentrations of ethanol (50 mM) may be required for the loss of mature neurons. These results are consistent with previous rodent studies that suggest that multiple withdrawal episodes are associated with neuronal degradation in the hippocampus (Collins et al., 1998; Zhao et al., 2013).

Influence of Aging on Response to CIE

Aging of the slice cultures *in vitro* demonstrated a significant effect on the outcome of data analyses in regards to slice cultures exposed to two cycles of CIE. Exposure to two cycles of CIE in aged tissue resulted in the significant loss of mature neurons, whereas exposure to two cycles of CIE in non-aged tissue did result in neurodegeneration. Previous research has demonstrated that voltage-dependent calcium influx may increase as a function of age in the CA1 region of the hippocampus (Landfield, P.W. & Pitler, T.A., 1984) and decrease as a

function of age in the granule cell layer of the DG, though dentate granule cells were injured in the present studies (Reynolds, J.N. & Carlen, P.L., 1988). It has been suggested that in the CA1 region of the hippocampus, miniature glutamatergic excitatory post-synaptic currents (mEPSCs) significantly increase from 7 days *in vitro* to 21 days *in vitro* (De Simoni, et al., 2003). Further, aged cell cultures (23–24 days *in vitro*) may demonstrate potentiated damage following excitotoxic insult, reflected by STYOX immunoreactivity, a marker of cell death. Brewer and colleagues (2007) found that exposure to glutamate (30 and 100 μM) produced increased cell death in aged cultures (23–24 days *in vitro*) as compared to young cultures (8–9 days *in vitro*). They also demonstrated that the effective concentration (EC 50) of glutamatergic toxicity was 207 μM for young tissue cultures (8–9 days *in vitro*) as compared to 28 μM for aged tissue cultures (23–24 days *in vitro*). In regards to NMDAR subunit expression and aging of cultures, western blot analyses revealed that the obligatory NR1 subunits and NR2A subunits increased by more than 50% from 7 days *in vitro* to 14 days *in vitro*. Additionally, NR1 and NR2A subunit expression increased by more than double by 21–22 days *in vitro* with no significant changes in NR2B subunit expression observed (Brewer et al., 2007). Given the effects of aging on neurotoxicity in cell cultures, it is plausible to infer that significant loss of mature neurons in the CA1, CA3, and DG following two cycles of CIE in aged tissue may be associated with an increased susceptibility to excitotoxic insult as a function of days *in vitro*.

Experiment III: One Cycle of CIE

The current study quantified the loss of mature neurons on NeuN immunoreactivity following one cycle of CIE in non-aged and aged hippocampal slice cultures. Aged hippocampal slice cultures were aged-matched to slices exposed to three cycles of CIE in that the final 24-EWD periods for both conditions occurred the same day *in vitro*. Accordingly, non-aged slice cultures were not age-matched to slices exposed to three cycles of CIE. The current study demonstrated that one cycle of CIE in non-aged tissue and aged tissue did not result in significant decreases of mature neurons in the pyramidal cell layers of the CA1 and CA3, or the granule cell layers of the DG as compared to control values. In the aged cultures, the current study shows a significant effect of sex and sex by treatment interaction in the DG of the hippocampus following one cycle of CIE. Female aged tissue cultures exposed to one cycle of CIE showed significant increases in NeuN immunoreactivity as compared to male aged tissue cultures; however, male aged tissue exposed to one cycle of CIE did not show significant loss of mature neurons as compared to control values. Further, the current findings in regards to the treatment regimen are consistent with previous *in vitro* studies in that exposure to EtOH (50 mM) for 10 days followed by one 24-hour EWD period did not result in cytotoxicity in the pyramidal cell layers of the CA1 and CA3, or in the granule cells of the DG (Butler et al., 2009; Self et al., 2005). Additionally, studies examining tissue exposed to moderate ethanol concentrations (75 mM) *in vitro* for six consecutive days have demonstrated that moderate ethanol exposure prior to withdrawal may be neuroprotective against

potential cytotoxicity associated with media changes (Thomas, M.P., & Morriset, R.A., 2000).

Experiment IV: Three Cycles of CIE + APV

Concentrations of APV (30, 40 and 50 μM) were chosen based on previous studies demonstrating that the exposure to APV (up to 100 μM) is efficacious in the inhibition of NMDAR's (Godfraind, J.M., & Xu, Y.Z., 2006). Data analyses in the current study demonstrate that exposure to APV (30 and 40 μM) during 24-hour EWD periods following three cycles of CIE attenuated the loss of mature neurons in all examined regions: the pyramidal cell layers of the CA1 and CA3, and granule cell layer of the DG. Further, exposure to APV (40 μM) during 24-hour EWD periods following three cycles of CIE prevented the loss of mature neurons in the CA1 and DG. The current findings, in regards to APV (40 μM), suggest that the overactivation of the NMDAR is involved in excitotoxicity associated with the loss of mature neurons; these findings are consistent with previous studies that have demonstrated that excitotoxic insult is attenuated by NMDAR antagonism *in vitro* (Butler et al., 2010; Mayer et al., 2002; Prendergast et al., 2000; Prendergast et al., 2004; Self et al., 2009) and *in vivo* (Rossetti et al., 1995; Rossetti et al., 1999).

The current study is consistent with previous research indicating that ethanol-induced withdrawal periods may be necessary for the overactivation of NMDARs and neurotoxicity (Prendergast et al., 2004, Rossetti et al., 1995;

Rossetti et al., 1999; Sanna et al., 1993). A study conducted by Sanna and colleagues (1993) demonstrated that rats exposed to binge-like concentrations of ethanol (12–18 g/kg) for six days followed by a 9–24-hour EWD period were more susceptible to the convulsant stimuli Isoniazid (50–250 mg/kg), NMDA (0.5–5 g/5 μ L), and Kainic acid (12 mg/kg) as compared to rats exposed to the same binge-like concentrations of ethanol and tested 1–3 hours after the final ethanol administration (Sanna et al., 1993). In a study examining extracellular glutamate release in ethanol-dependent rodents, glutamate levels during a 12-hour EWD period were observed to be more than 200% higher than glutamate levels observed in control animals. The high levels of glutamate observed during the 12-hour EWD period decreased following the re-administration of ethanol and MK-801, a non-competitive NMDAR antagonist, but the administration of diazepam, a benzodiazepine, did not reduce the withdrawal-induced increases in extracellular glutamate release. The authors conclude that these findings demonstrate that ethanol alone and NMDAR antagonism are efficacious in the treatment of ethanol withdrawal (Rossetti et al., 1995). Rossetti and colleagues also conducted an experiment examining NMDAR overactivation during EWD on extracellular glutamate levels; they found that the administration of NMDA (800 μ M) evoked an increase in extracellular glutamate levels of nearly 600% in the striatum as compared to baseline levels. Consistent with the previous findings, this effect was reduced by MK-801 (1 mg/kg) and ethanol (5 g/kg). However, the administration of NMDA (800 μ M) in ethanol-dependent rodents who were not exposed to the 12-hour EWD period did not evoke increases in extracellular

glutamate levels (Rossetti et al., 1999). Further, Prendergast and colleagues (2004) examined cytotoxicity in hippocampal slice cultures exposed to ethanol (100 mM) for 10 days that was followed by a 24-hour EWD period and exposure to continuous ethanol (100 mM) for 11 days. The results indicated that slices exposed to ethanol for 10 days (100 mM) and followed by a 24-hour EWD period showed significant cytotoxicity in the CA1 region of the hippocampus as reflected by increased propidium iodide (PI) uptake. However, slice cultures exposed to continuous EtOH (100 mM) for 11 days did not result in cytotoxicity. The cytotoxicity demonstrated in the CA1 of the hippocampus was attenuated by exposure to MK-801 (20 μ M) and TTX (500 nM), a selective sodium channel blocker, during the 24-hour EWD period (Prendergast et al., 2004).

Nonetheless, NMDAR antagonism at high concentrations can independently result in excitotoxicity. In the current study, exposure to the high concentration of APV (50 μ M) alone during the 24-hour EWD periods was toxic, as NeuN immunoreactivity was significantly decreased in all three regions of the hippocampus as compared to control values. Additionally, three cycles of CIE followed in the 24-hour EWD periods by APV (50 μ M) in the pyramidal cell layers of the CA3 and the granule cell layers of the DG was toxic as compared to three cycles of CIE alone. The current study examining high concentrations of APV are consistent with previous *in vivo* studies high concentrations of MK-801(2 mg/kg). High doses of MK-801 alone (2 mg/kg) produced marked brain damage in areas of the DG, entorhinal cortices, and olfactory bulbs. High doses of MK-801 (2

mg/kg) in combination with severe ethanol intoxication (~300 mg/dl) were lethal. High doses (2 mg/kg/day) of MK-801 administered with lower levels of intoxication (150 mg/dl) further potentiated the MK-801-induced neurodegeneration (Corso, Mostafa, Collins, & Neafsey, 1998).

Not all studies suggest that the neuronal degeneration associated with intermittent or episodic ethanol exposure is excitotoxic in nature. Collins and colleagues (1998) conducted a study examining the underlying mechanism involved in the neurodegeneration produced by intermittent ethanol exposure *in vivo* and *in vitro*. In the *in vivo* study, a single dose of ethanol (~5 g/kg/day) administered for 5 to 10 days produced swelling of the cerebral cortices and neuronal degeneration in the granule cells of the DG of the hippocampus, entorhinal cortices, and olfactory bulbs. Although ethanol withdrawal periods were not explicitly characterized in this model, the authors note that the daily, single administration of ethanol produced fluctuations in blood ethanol concentrations (BEC's). For example, in the study, BEC's declined to nearly 0 g/kg on the 24-hour time points following each previous ethanol administration. MK-801 (75 µg/kg) and furosemide (20 mg/kg), a diuretic, were administered daily following the administration of ethanol. The administration of MK-801 did not attenuate the neurodegeneration produced by the once daily dose of ethanol (~5 g/kg) in any of the examined brain regions. However, the administration of furosemide twice daily (10 mg/kg) attenuated the swelling observed in the cerebral cortices by more than 75%.

In the *in vitro* study (Collins et al., 1998), slice cultures were exposed for six days to EtOH (~150 mM) for 15 hours per day followed by a nine-hour EWD period. The results revealed that slice cultures exposed to EtOH followed by a nine-hour EWD period for 6 days showed significant increases in media lactate dehydrogenase, reflecting cytotoxic insult. When slice cultures were co-exposed to furosemide (1.1 mM), the release of lactate dehydrogenase was significantly attenuated. The authors concluded the mechanism associated with episodic binge-like EtOH administration may not be excitotoxic but associated with electrolyte accumulation and swelling of the cerebral cortices. Accordingly, swelling of glial membranes is determined as one underlying cause of hyperexcitable epileptiform activity in hippocampal slice cultures leading to the associated generalized tonic-clonic seizures in humans (reviewed by Andrew, R.D., 1991). Given the present findings and the studies conducted by Collins and colleagues (1998), the mechanism associated with neurodegeneration produced by intermittent EtOH *in vitro* may be associated with both excitotoxic and non-excitotoxic mechanisms.

Limitations of Current Study

Two fundamental limitations to this current study may be the use of young tissue (post-natal day eight) for the culturing of hippocampal slices and the artificiality of the organotypic slice culture model as compared to other preclinical models. It has been suggested that hippocampal slice cultures demonstrate preservation of cell layers (Zimmer & Gahwiler, 1984); however, the pyramidal

cell layer may broaden (Zimmer et al., 1984), resulting in decreases in the thickness of the slices. For example, in an experiment conducted by Martens and Wree (2001), hippocampi (post-natal day six) were originally chopped in 400 μM slices. The authors reported that the thickness of the hippocampal slices following 24 days *in vitro* was an estimated 150 μM . It is demonstrated that there are many similarities between *in vitro* slice cultures and age-matched hippocampi *in situ*, including the distribution of glutamatergic MK-801 and AMPA binding sites (Martens & Wree, 2001). Further, hippocampal slice cultures demonstrate many “adult-like features,” such as comparable distributions of astroglia and oligodendrocytes, with comparable synaptic plasticity between the adult hippocampus and hippocampal slice cultures (Baher et al., 1994; Berger and Frotscher, 1994; Muller et al., 1993; for a review, see Baher, 1995). However, hippocampal slice cultures demonstrate the unique additional reorganization and formation of direct pathways between the pyramidal cell layer of the CA1 and the granule cell layer of the DG, attributed to the sprouting of mossy fibers produced by the granule cells. It has been suggested that this artificial pathway between the CA1 and DG subregions demonstrated in hippocampal slice cultures may potentiate epileptiform spikes in the DG (Gutierrez & Heinemann, 1999). This artificial pathway may be implicated in and potentiate the neurodegeneration produced by CIE in hippocampal slice cultures.

Conclusions and Future Directions

The current study supports the hypothesis that multiple cycles of CIE results in excitotoxic insult as two and three cycles of CIE produced a significant loss of mature neurons and neuronal density in the pyramidal cell layers of the CA1 and CA3, and granule cell layer of the DG. The exposure of slice cultures to APV (40 μ M) is efficacious in the attenuation of neuronal degradation following three cycles of CIE in the pyramidal cell layers of the CA1 and CA3 and granule cell layer of the DG. However, an additional replication of hippocampal cultures exposed to three cycles of CIE and APV (40 μ M) will be conducted in order to conclude whether overactivation of the NMDAR is involved in the loss of mature neurons following intermittent ethanol exposure *in vitro*. Given the limitation of the current study in regards to the artificial pathway between the DG and CA1, CIE on the loss of mature neurons should be examined further *in vivo*. The aging of cultures *in vitro* should be a fundamental consideration in interpreting data analyses in future studies aimed at targeting the specific mechanisms associated with the loss of mature neurons following CIE *in vitro*. Additionally, future studies should examine the efficacy of calcium channel blockers in regards to CIE in hippocampal slice cultures. In regards to NMDAR subunit expression, future studies could utilize western blot analyses to examine NMDAR NR1 subunit expression following three cycles of CIE in hippocampal slice cultures. Likewise, western blot analyses should be conducted to examine the NMDAR NR1 and NR2B subunit expression in both aged and non-aged cultures following two

cycles of CIE for a better understanding of the effect of aging on cultures in regards to intermittent ethanol exposure.

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