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EFFECTS OF CORTICOSTERONE AND ETHANOL CO-EXPOSURE ON HIPPOCAMPAL TOXICITY: POTENTIAL ROLE FOR THE NMDA NR2B SUBUNIT

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ABSTRACT OF DISSERTATION

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ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Arts and Science
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By

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Lexington, Kentucky

Director: Dr. Mark Prendergast, Professor of Psychology
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Chronic ethanol (EtOH) exposure produces neuroadaptations within the NMDA receptor system and alterations in HPA axis functioning that contribute to neurodegeneration during ethanol withdrawal (EWD). Chronic EtOH exposure and EWD, as well as corticosteroids, also promote increased synthesis and release of polyamines, which allosterically potentiate NMDA receptor open-channel time at the NR2B subunit. The current studies investigated effects of 10 day EtOH and corticosterone (CORT) co-exposure on toxicity during EWD in rat organotypic hippocampal slice cultures, and alterations in function and/or density of the NR2B subunit of the NMDA receptor that may mediate CORT-potentiation of toxicity during EWD. We hypothesized that toxicity during withdrawal following EtOH and CORT co-exposure would be greatest in the CA1 region due to increased NMDA NR2B receptor abundance and/or function. Cultures were exposed to CORT (0.01–1 μM) during 10 day EtOH exposure (50 mM) and 1 day EWD. Additional EtOH-naïve cultures were exposed to CORT for 11 days. Propidium iodide (PI) was used to measure toxicity in the CA1, CA3, and DG hippocampal regions. In EtOH-naïve cultures, 11 day exposure to CORT (0.01 – 1 μM) produced modest toxicity and in all regions. Exposure to CORT during EtOH exposure/EWD potentiated CORT-toxicity at all concentrations in the CA1 region. Ifenprodil, an NR2B polyamine site antagonist, significantly reduced toxicity from EtOH and CORT (0.1 μM) co-exposure during withdrawal. Immunohistochemistry and Western blot analyses were conducted for measurement of NR2B immunoreactivity in organotypic cultures, and autoradiography studies were conducted for measurement of polyamine-sensitive NR2B subunits with [^{3}H]ifenprodil. Consistent increases in NR2B subunit protein were not detected with use of any methodology. Additional studies exposed cultures to a membrane impermeable form of CORT (BSA-conjugated CORT; 0.1 μM) with or without EtOH exposure and withdrawal. BSA-CORT exposure did not produce toxicity in any hippocampal region, suggesting that CORT toxicity was not mediated by membrane bound substrates. These data suggest that CORT and EtOH co-exposure result in increased function of polyamine-sensitive NR2B subunits, but this toxicity does not appear dependent on the number of hippocampal NMDA NR2B subunits.

KEYWORDS: Ethanol Withdrawal, NMDA Receptor, Corticosterone, Hippocampus, Alcohol Dependence

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INTRODUCTION

Binge Drinking in the United States

The 2007 National Survey on Drug Use and Health (NSDUH) reported that greater than half of U.S. citizens (aged 12 and older) are characterized as current drinkers, with 56.6% of males identified as current drinkers compared to 46.0% of females (Substance Abuse and Mental Health Services Administration, 2007). Though low levels of daily alcohol consumption have been suggested to have various protective health effects (e.g. cardiovascular diseases; Klatsky, 2009), heavy and binge-type drinking is associated with deficits in neurological, cognitive, heart, liver, and psychosocial functioning (reviewed by Cargiulo, 2007). Alarmingly, 6.9% of NSDUH survey respondents met criteria for heavy drinking (defined as "five or more drinks on the same occasion on each of 5 or more days in the past 30 days"), and 23.3% of survey respondents engaged in binge drinking in the last month. Binge drinking was operationally defined as 4+/5+ drinks (for women and men, respectively) by Wechsler et al. (1994), and this definition has been widely used. In 2004, the National Institute on Alcohol Abuse and Alcoholism (NIAAA) re-defined binge drinking to include a time course of drinking and a functional level of impairment: "Binge drinking is a pattern of drinking alcohol that brings blood alcohol concentration (BAC) to 0.08 gram percent or above. For the typical adult, this pattern corresponds to consuming 5 or more drinks (male), or 4 or more drinks (female), in about 2 hours." Though this newer definition

improves upon Wechsler's criteria, more stringent criteria for defining binge drinking have been suggested (White et al., 2006). White et al. (2006) suggested that using binge drinking as a dichotomous variable is inappropriate and should be expanded to consider grouping individuals based on defining a "binge threshold" of 4/5 drinks (for females and males, respectively). Additional groups would then be characterized as exceeding that threshold by drinking 2 (8+/10+ drinks) or 3 (12+/15+ drinks) times as much alcohol as the standard Wechsler definition, or "binge threshold". White et al. (2006) suggest that neither the definition put forth by Wechsler (1994) nor NIAAA (2004) adequately address the levels of binge drinking that occur, as in their sample 27% of men and 10% of women drank amounts 2 or 3 times more than the "binge threshold". Similarly, in a study of college students, McMillen et al. (2009) reported estimated peak BACs ranging from 2-4 times greater than the "binge threshold" adopted by NIAAA, with relatively equal distribution among the number of individuals within subgroups divided by BACs at 80 mg/dl increments. Using the 30-day Timeline Followback (corrected for gender), the estimated peak BAC was 233 mg/dl, with less than 10% of the sample reporting peak estimated BACs below the legal driving limit (80 mg/dl; McMillen et al., 2009).

Chronic Ethanol Exposure and Neurodegeneration in Human Alcoholics

Given the large population of people who misuse alcohol, neural consequences of heavy and/or binge drinking continue to be studied. Though light to moderate drinking history (88.2 - 181.2 g/week) has been reported to have no negative influence on brain volume compared to alcohol abstainers in MRI comparisons (Kubota et al., 2001), long-

term, heavy alcohol consumption is related to macrostructural neural changes (e.g., reduced structure volume) and microstructural neural changes (e.g., cellular dysfunction). Long-term, heavy alcohol intake impairs metabolism of compounds contributing to cellular integrity (Schweinsburg et al., 2001); reduces cerebral blood flow (Nicolas et al., 1993); reduces volume of cerebellar hemispheres and mammillary bodies (Shear et al., 1996); reduces hippocampal volume in alcohol-dependent males (Beresford et al., 2006); contributes to ventricular enlargement; and increases in cerebrospinal fluid (Agartz et al., 2003; Pfefferbaum et al., 1995); and reduces cortical gray and white matter, most notably in prefrontal and parietal cortices (Fein et al., 2002; Pfefferbaum et al., 1998). Reductions in hippocampal and cortical white matter volume are also age-dependent, with older alcoholics demonstrating more severe loss as compared to younger alcoholics (Kubota et al., 2001; Pfefferbaum et al., 1998; Sullivan et al., 1995). These hallmarks of alcoholic brain injury likely contribute to persisting executive function, visuospatial, and motor deficits that are noted in men who have undergone detoxification in the past month (Sullivan et al., 2000). Additionally, reduced cerebral blood flow in alcoholic patients is correlated with impaired performance in tasks requiring frontal lobe integrity (Nicolas et al., 1993). Specifically in female alcoholics, deficits in motoric function have been noted after months of sobriety. Female former-alcoholics demonstrate increased truncal tremor and postural sway that is associated with decreased volume of the anterior cerebellar vermis, as compared to healthy female control subjects (Sullivan et al., 2010). Comorbid diagnoses may also correlate with reduced neural integrity. For example, gray matter loss is worsened in chronic alcoholic patients who present with Korsakoff's syndrome as compared to chronic alcoholic patients that do not demonstrate amnesic behaviors

(Jernigan et al., 1991). Extreme neurocognitive impairment, termed Wernicke-Korsakoff Syndrome, may be noted in alcoholic patients with thiamine deficiency (Martin et al., 2003).

It must be noted that recovery in white and gray matter integrity, volume, ventricle size (and CSF volume) can occur during abstinence following chronic alcohol use. Reductions in ventricular system pathology (reduced ventricle size and reduced CSF volume) have been observed after 5 weeks of abstinence as compared to baseline MRIs taken at initial alcohol cessation (Schroth et al., 1988). Additionally, short term abstinence (12-32 days) is correlated with decreased CSF and volumes of lateral ventricles and cortical sulci, and a trend toward increased cortical gray matter, whereas long-term abstinence is associated with decreased volume of the third ventricles compared to control subjects and alcoholic subjects who had relapsed (Pfefferbaum et al., 1995). After 5 years of sobriety, former alcoholic patients are similar to control subjects on measures of ventricular enlargement, presumably due to aging, whereas patients who continued to drink demonstrated exaggerated losses in cortical gray matter (Pfefferbaum et al., 1998). Fewer white matter lesions have also been detected in brains of 31-175 week abstinent alcoholics as compared to current, heavily drinking alcoholic patients, which is in parallel with recovery in overall frontal white and gray matter in abstinent alcoholics (O'Neill et al., 2001).

The specific consequences of alcohol exposure versus the consequences induced by withdrawal from long-term alcohol exposure are impossible to fully tease apart in

human studies. However, repeated alcohol detoxifications are correlated with greater long-term neurologic and cognitive deficits in humans, even if benzodiazepines were administered during withdrawal to reduce signs and symptoms of withdrawal (Duka et al., 2003; Duka et al., 2004). These data suggest that neuroadaptations that occur as a result of chronic alcohol exposure confer greater susceptibility to neurological insult after repeated intake and cessation, in keeping with characterization of pathological neuronal hyperexcitability during ethanol withdrawal that leads to functional and/or structural brain-related deficits. In summary, alcohol exposure likely produces brain injury as well as neuroadaptations that make the brain more susceptible to injury during exposure and withdrawal.

Chronic Ethanol Exposure and Neurodegeneration in Rodent Models

As compared to studies of the human brain, rodent models are well-suited for characterization of brain damage that occurs following prolonged ethanol exposure with or without withdrawal. Various models and ethanol exposure paradigms have been employed and have produced various results in terms of alcohol related neuropathology. In a four-day binge model of prolonged ethanol intoxication, necrotic cell death is observable by day two, but becomes exceedingly worse by day four, with necrosis evident in various brain regions, including the agranular insular cortex, anterior piriform cortex, perirhinal cortex, lateral entorhinal cortex, and the hippocampal formation. Signs of necrosis in these regions are no longer apparent 36-72 hours after the last ethanol administration, suggesting that long-term ethanol exposure contributes to

neurodegeneration with and without a period of ethanol withdrawal in this short-term binge model (Obernier et al., 2002a; 2002b; Collins et al., 1996).

Mechanisms of alcohol related neurodegeneration include dysregualtion of excitatory amino acid systems and oxidative stress (Bondy, 1992). Alcohol metabolism in the brain by catalase, alcohol dehydrogenase, or cytochrome P450 causes production of free radicals (e.g. superoxide free radicals, hydrogen peroxide, and hydroxyl radicals) (Zakhari, 2006), leading to initiation of apoptotic cascades and cell death (reviewed by Hampton & Orrenius, 1998). Ethanol also contributes to oxidative stress and cellular injury by inducing mitochondrial damage (e.g., permeability pores). Increased activity of free radicals is accompanied by parallel decreases in endogenous antioxidant concentrations (superoxide dismutase and catalase; Eysseric et al., 2000; Heaton et al., 2003). This is important for cellular integrity, as antioxidants significantly reduce ethanol toxicity in cell culture models with cerebellar granule cells (Heaton et al., 2004), and hippocampal neurons (Marino et al., 2004; Sheth, Tajuddin, & Druse, 2009). For instance, co-exposure to ethanol concentrations ranging from 400-2000 mg/dl for 16 hours and the anti-oxidants Vitamin E or beta-carotene significantly and dose-dependently increase the viability of cultured hippocampal neurons (Mitchell et al., 1999)

Acute and Chronic Ethanol Exposure: Neuroadaptations in Glutamatergic Systems

In addition to damage that may occur during alcohol exposure, withdrawal from long-term alcohol consumption/exposure has been characterized as a state of neuronal

hyperexcitability during which time the brain is susceptible to excitotoxic insult, in part because of adaptive changes induced by chronic ethanol exposure (Hoffman et al., 1990; Hunt, 1983; Littleton, 1998; Prendergast et al., 2004). In rodents, ethanol withdrawal has been shown to exacerbate hippocampal injury and cognitive deficits in comparison with rodents that did not experience ethanol withdrawal (Lukoyanov et al., 1999; Paula-Barbosa et al., 1993). Susceptibility to neuronal injury during ethanol withdrawal after long-term ethanol exposure is associated with a variety of cellular adaptations in membrane proteins and ion channels. Adaptations include up-regulation and increased sensitivity of *N*-methyl-D-aspartate (NMDA) type glutamate receptors (Hu & Ticku, 1995; Kalluri et al., 1998; Prendergast et al., 2000) and L-type voltage-sensitive Ca^{2+} channels (Little, 1991; Watson & Little, 1999); down-regulation of γ-aminobutyric acid type-A ($GABA_A$) receptors (Devaud et al., 1997; Mahtre & Ticku, 1994); potentiation of serotonin type-3 (5-HT3) receptors (Lovinger & Zhou, 1998; McBride et al., 2004); inhibition or stimulation of 5'-triphosphate-gated purinergic (P2X) receptors (Davies et al., 2006); and upregulation or increased sensitivity of adenosine A1 receptors (Butler et al., 2008; Butler et al., 2009; Concas et al., 1996; Daly et al., 1994; Jarvis & Becker, 1998). In particular, primarily by pharmacological manipulation in rodents, the glutamatergic system has been shown to be related to alcohol drinking (Besheer et al., 2008), relapse behavior (Spanagel et al., 1996), hyperexcitability during ethanol withdrawal (Veatch & Becker, 2005), excitotoxicity, and long-term treatment for alcohol dependence (acamprosate; Paille et al., 1995; Whitworth et al., 1996). Glutamatergic receptors include both ionotropic receptors (NMDA, AMPA, and Kainate receptors) and metabotropic receptors (mGluR1-8) that are all affected by ethanol.

Glutamatergic NMDA Receptors and Ethanol

Acute and chronic ethanol exposure can have markedly different effects on glutamatergic receptors. NMDA receptors have been thoroughly studied in models of ethanol exposure and withdrawal, as they are highly permeable to Ca^{2+} and excessive activation of NMDA receptors is related to increased cell death (Choi, 1987). NMDA receptors are heteromeric ion channels that are composed of an obligatory NR1 receptor subunit and some combination of NR2 and/or NR3 subunits (reviewed by Yamakura & Shimoji, 1999). In the hippocampus, NR2A and NR2B subunits are most commonly expressed with the NR1 subunit. The NR2 subunit is important for regulating channel kinetics, with NR2B-containing receptors allowing for greater open channel time and calcium (Ca^{2+}) influx (Chen et al., 1999). Acutely, ethanol inhibits NMDA receptor-mediated synaptic transmission in several neuronal populations, including cerebellar, hippocampal, cortical, and spinal cord neurons (Lovinger, White, & Weight, 1990). Ethanol's acute inhibitory effects are greatest at NR2A and/or NR2B subunit containing NMDA receptors, in comparison with NMDA receptors containing NR2C or NR2D subunits, in both native and recombinant tissues (Allgaier, 2002). Chronic ethanol exposure results in upregulation and increased sensitivity of NMDA receptors and/or NMDA receptor subunits in multiple brain regions of mice and rats as a result of various ethanol exposure regimens. Upregulation of MK-801 binding sites has been noted *in vitro* and *in vivo*, though it should be noted that MK-801 is a noncompetitive NMDA receptor antagonist that binds inside the NMDA receptor channel at the phencyclidine site, but is not entirely specific for the NMDA receptor channel (e.g., MK-801 competitively inhibits

monoamine transporters; Nishimura et al., 1998). Prolonged exposure to ethanol concentrations ranging from 50-100 mM increase both [^{3}H]MK-801 binding sites and NMDA receptor-mediated Ca^{2+} influx in cortical mouse neurons (Hu & Ticku, 1995) and cerebellar rat neurons (Hoffman et al., 1995; Iorio et al., 1992), as well as NR2B subunit gene expression in cortical neurons (Hu, Follessa, & Ticku, 1996). *In vivo* self-administration models that produce ethanol dependence have also noted increased [^{3}H]MK-801 binding sites in the frontal cortex and hippocampus of male rats and mice (Gulya et al., 1991; Rudolph et al., 1997; Snell et al., 1993), and in the parietal cortex, entorhinal cortex, striatum, thalamus, and medulla (Gulya et al., 1991). MK-801 is non-specific for the NMDA receptor channel, however, significant upregulation in number and/or function has been noted for individual NMDA NR subunits. *In vivo*, significant increases in NR1, NR2A, and NR2B subunits in adult male rat cerebral cortex and hippocampus have been noted (three administrations daily for 6 days; Kalluri et al., 1998). *In vitro*, ten day continuous ethanol exposure (100 mM) in organotypic hippocampal slices also results in significant increases in the NR1 and NR2B subunit polypeptide levels and NMDA receptor function, as inferred by potentiated cell death produced by NMDA receptor agonism in ethanol pre-exposed slices. Additionally, ethanol withdrawal toxicity and potentiated Ca^{2+} influx is ameliorated by co-exposure with MK-801 (Harris et al., 2003; Prendergast et al., 2000; Prendergast et al., 2004). Upregulation of these sites appears transient, however, as increased [^{3}H]MK-801 binding and NR subunit density is no longer noted 48 hours following ethanol exposure *in vitro* (Hu & Ticku, 1995; Kalluri et al., 1998), and by 24 hours following ethanol exposure *in vivo* (Gulya et al., 1991).

Increased number and/or function of NMDA receptors contributes to behavioral excitability after chronic ethanol exposure, and NMDA receptor antagonism has been shown to reduce ethanol withdrawal signs in rodents. Valverius et al. (1990) demonstrated that withdrawal seizure prone (WSP) male mice had significantly higher basal MK-801 binding sites than withdrawal seizure resistant (WSR) male mice, and that chronic ethanol exposure increased hippocampal [^{3}H]MK-801 binding sites in both lines, though to a greater extent in WSP versus WSR mice after chronic ethanol exposure. Moreover, acute administration of the competitive NMDA receptor antagonist CGP 39551 before peak ethanol withdrawal reduces ethanol withdrawal tremor and seizure activity in mice (Liljequist, 1991), as does MK-801 administration in rats and mice (Morrisett et al., 1990; Veatch & Becker, 2005). Blockade of the NMDA receptor channel with MK-801 administration in mice also reduces ethanol withdrawal behaviors in a chronic ethanol exposure model that upregulates the number of hippocampal [^{3}H]MK-801 binding sites, whereas NMDA administration potentiates withdrawal behaviors (Grant et al., 1990). However, repeated MK-801 administration to alleviate ethanol withdrawal handling-induced convulsions in mice has been shown to increase susceptibility to seizures when left untreated during ethanol withdrawal (Veatch & Becker, 2005).

Non-NMDA Ionotropic Glutamate Receptors

The ionotropic family of glutamatergic receptors also includes AMPA and kainate receptors. Though AMPA and kainate receptors are preferentially activated by their

respective agonists, these families of receptors were initially very hard to distinguish pharmacologically (Lees, 2000). Thus, initial experimental pharmacologic manipulation of these glutamatergic ion channels was often non-specific, and such effects were referred to as mediated by AMPA/Kainate receptors. Development of selective pharmacological inhibitors of AMPA receptors in the mid-1990s, however, provided a mechanism for studying individual AMPA and kainate receptor-mediated components of non-NMDA glutamatergic receptor activity (Pelletier et al., 1996). AMPA and kainate receptors are ion channels that allow Na^+ influx upon activation (Peruche & Krieglstein, 1993), with some subunit combinations allowing Ca^{2+} influx (Hollmann et al., 1991). AMPA receptors may be homo- or heteromeric, and are comprised of subunits GluR1-4 (reviewed in Hollmann & Heinemann, 1994). Kainate receptors were originally grouped into two subfamilies, distinguished as GluR5-7 or KA1-2 (reviewed in Hollmann & Heinemann, 1994), but have recently been re-classified as GluK1-3 (previously GluR5-7) and GluK4-5 (previously KA1 and KA2; Collingridge et al., 2009). AMPA receptors mediate fast excitatory synaptic transmission, thus contributing to neuroplasticity characterized by increased synaptic strength and insertion of AMPA receptors in the post-synaptic membrane (i.e., long-term potentiation; Nayak et al., 1998). Studies have shown the greatest abundance of AMPA receptors are located in the cytoplasm of dendrites in immature hippocampal neurons, though they are continually inserted rapidly and removed from the plasma membrane (Shi, 2001). Similarly, kainate receptors facilitate long-term potentiation (LTP) at hippocampal mossy fiber terminals independent of NMDA receptor activity (Bortolotto et al., 1999), though they have also been shown to

act as autoreceptors to either facilitate (Lauri et al., 2001) or inhibit (Kidd et al., 2002) synaptic signaling.

Like NMDA receptors, AMPA and/or kainate receptors are also acutely inhibited by ethanol in a non-competitive manner (reviewed in Narahashi et al., 2001). AMPA/kainate receptors expressed in Xenopus oocytes are just as sensitive at NMDA receptors to the acute inhibitory effects of ethanol (Dildy-Mayfield & Harris, 1995). Acute inhibition of AMPA/kainate receptor currents in HEK293 cells and rat cortical neurons by ethanol (10-400 mM) occurs irrespective of subunit composition, though inhibition of transfected receptors was greater than inhibition of receptors in cultured neurons (Lovinger, 1993). Kainate receptors are densely populated in the pyramidal cells of the CA3 region of the hippocampus, where acute ethanol ($\geq$ 20mM) has been noted to inhibit kainate receptor post-synaptic currents in the presence of a specific AMPA receptor antagonist (Weiner et al., 1999). Conversely, chronic intermittent ethanol exposure increases postsynaptic neurotransmission via kainate receptors, and blunts kainate receptor-mediated synaptic plasticity (Lack et al., 2009). Prolonged ethanol exposure and twenty-four hour withdrawal have also been shown to increase kainate receptor mediated currents and protein abundance of GluR6/7 subunits in cultured rat hippocampal neurons (Carta et al., 2002).

Acute ethanol has also been reported to inhibit AMPA currents in rodent hippocampal and cortical neurons (10-500 mM; Costa et al., 2000; Moykkynen et al., 2003). Recent work has shown that ethanol stabilizes (Moykkynen et al., 2003) and

enhances the rate of desensitization of AMPA receptors expressed in HEK293 cells, with co-expression of AMPA receptor regulatory proteins (TARPs) further prolonging receptor desensitization (Moykkynen et al., 2009). Chronic intermittent ethanol exposure increases the amplitude of AMPA-mediated excitatory postsynaptic currents (Lack et al., 2007). Further, prolonged ethanol exposure in rat primary cortical cultures has been noted to increase NMDA and AMPA receptor expression, but not kainate receptor expression (Chandler et al., 1999), suggesting differential effects of ethanol on ionotropic glutamatergic receptors.

Metabotropic Glutamate Receptors

There are eight subtypes of metabotropic glutamate receptors, which are organized into three families based on their signaling pathways, similarities in protein sequence, and activation by agonists (Groups I-III; Pin & Duvoisin, 1995). Acute and chronic ethanol exposure affects mGluR signaling, though differences may be noted dependent on brain region, mGluR subtype, and developmental timepoint. Non-specific activation of metabotropic glutamate receptors (mGluRs) with the agonist (1S,3R)-1-aminocyclopentane-1,3-dicarboxylic acid (ACPD) has been shown to blunt Ca^{2+} influx after chronic ethanol exposure in rat cerebellar purkinje neurons, though ACPD potentiated Ca^{2+} influx after withdrawal from chronic ethanol exposure (Netzeband et al., 2002). Group I mGluRs (mGluR1 and mGluR5) have been most extensively studied for their role in ethanol-related behavior and neuronal signaling. Group I mGluRs are G_q-coupled, leading to activation of phospholipase C (PLC), increased intracellular Ca^{2+}

release, and phosphorylation of proteins via activation of protein kinase C (PKC). These receptors are located primarily post-synaptically (Cartmell & Schoepp, 2000). In Xenopus oocytes, acute ethanol (20-200mM) inhibits glutamate currents via mGluR5, but not mGluR1, which can be prevented by a PKC inhibitor (in Narahashi et al., 2001). Pharmacological antagonism of mGluR5 has also been shown to affect ethanol-seeking behaviors by decreasing ethanol self-administration in male alcohol-preferring rodents (Schroeder, Overstreet, & Hodge, 2005), and decreasing binge-like drinking in male mice infused with an mGluR5 antagonist into the shell of the nucleus accumbens (Cozzoli et al., 2009). Group II mGluRs (mGluR2 and mGluR3) are G_i-coupled receptors and are located both pre- and perisynaptically (Cartmell & Schoepp, 2000). This subfamily of mGluRs has been characterized for their importance in mediating stress- and cue-related increases in ethanol-seeking behavior after extinction (relapse), with activation of mGlu2/3 receptors attenuating the increase in ethanol-seeking behavior observed after stressful stimuli (Zhao et al., 2006). Group III mGluRs (mGluR4, mGluR6-8) are also G_i-coupled receptors. To date, investigation into the role of Group III mGluRs in regard to ethanol-related behaviors is sparse, though data have shown a lack of the stimulatory effect of acute ethanol on locomotor activity in male mice lacking the mGluR4 subtype, with no effects noted between knockout mice and wild-type mice in ethanol preference or withdrawal behaviors (Blednov et al., 2004). These data suggest a role for Group III mGluRs in the acute, motor-activating effects of ethanol.

The Hypothalamic-Pituitary-Adrenal Axis

Ethanol effects on the CNS are vast beyond the glutamatergic system, and an extensive amount of literature has investigated ethanol interactions with the hypothalamic-pituitary-adrenal (HPA) axis. The principal components of the HPA axis include hypothalamic nuclei, the pituitary gland, and the adrenal gland, and the hormones which are synthesized and secreted to regulate the daily circadian cycle and in response to stressors. Circadian fluctuations in steroid secretion include approximately hourly pulses of glucocorticoid secretion in rodents (reviewed in Lightman et al., 2008). The role of the HPA axis in regulation of the physiologic stress response is aimed at restoration of physiologic homeostasis. In the CNS, the paraventricular nucleus (PVN) of the hypothalamus contains neurons that release corticotrophin releasing hormone (CRH) and arginine vasopressin (AVP). These hormones travel from the hypothalamus to the pituitary gland where they synergistically stimulate the release of adrenocorticotropin hormone (ACTH). From the pituitary gland, ACTH travels in the bloodstream to the cortex of the adrenal gland, which is the site of synthesis and release of glucocorticoids (GCs). GCs complete a negative feedback loop by traveling back to the PVN and the pituitary gland to suppress further hormone secretion (reviewed in Nader, Chrousos, & Kino, 2010). Acutely, elevation of GCs helps mobilize resources for energy consumption, and promotes return to physiologic homeostasis (Munck, Guyre, & Holbrook, 1984). Chronically, however, GCs can exert profound negative effects on various peripheral organs and the CNS, resulting in dampened HPA axis responsiveness to stressors (Jacobsen, 2005).

Detrimental effects of HPA axis dysregulation were first studied in the context of aging and led to the Glucocorticoid Cascade Hypothesis (Sapolsky, Krey, & McEwen, 1986). This hypothesis suggests that prolonged high levels of CORT result in downregulation of GRs. GR activation is required for cessation of the HPA response by downregulation of mRNA for CRH, AVP, and ACTH. Thus, downregulation of GRs impairs cessation of the HPA response by leading to CORT accumulation and neurodegeneration (Cullinan et al., 2008). This hypothesis also highlighted CORT glial hypertrophy as a consequence of CORT-related damage (Sapolsky, Krey, & McEwen, 1986). Long-term alcohol exposure negatively impacts HPA axis function and contributes to neurodegeneration in humans and rodents.

Corticosterone Receptors: Glucocorticoid and Mineralocorticoid Receptors

Glucocorticoids are highly lipophilic, thereby easily penetrating the cell membrane to bind to intracellular Type I (mineralocorticoid; MRs) and Type II (glucocorticoid; GRs) receptors. Upon glucocorticoid binding, the complex moves to the nucleus and acts as a transcription factor by binding to hormone responsive elements and altering protein synthesis (reviewed in Nishi, 2010). Corticosterone (CORT) is the primary glucocorticoid in rodents (equivalent to cortisol in humans) and it has nearly a ten-fold greater affinity for MRs than GRs: 0.5 nM and ~2.5-5 nM, respectively (Reul & de Kloet, 1985). MRs are occupied by low levels of circulating CORT, or during troughs of hormonal cycling throughout the circadian rhythm. GR occupation requires increased levels of circulating CORT, as observed during peak HPA axis activity (late evening

hours) and during exposure to a stressor (Reul and de Kloet, 1985). Autoradiographic localization following [^{3}H]CORT injection shows dense labeling in the pyramidal and granule cell layers of the hippocampus of male rats, with modest labeling in other hippocampal cell layers, the entorhinal cortex, lateral septum, and amygdala (Sapolsky, McEwen, & Rainbow, 1983). Using a GR-specific antibody within the subfields of the hippocampus, dense labeling is observed in granule cells of the dentate gyrus (DG) and pyramidal cells of the cornu ammonis (CA)1 region, though GR immunoreactivity is sparse among CA3 pyramidal neurons (van Eekelen et al., 1987; Sarabdjitsingh et al., 2009). MR distribution is predominantly restricted to the hippocampus and the septum, whereas GRs are ubiquitous throughout the brain, and are found in both neurons and glial cells (reviewed in de Kloet et al., 2005; Reul and de Kloet, 1985). As MRs are activated under basal conditions, they have been considered for their role in maintenance of hippocampal excitability, whereas GR activation that occurs with increased CORT concentrations generally suppresses excitatory hippocampal output (de Kloet et al., 1998). Long-term increases in CORT levels, and therefore GR activation, however, results in neurotoxicity and pruning of hippocampal dendrites (Gould et al., 1990; Woolley et al., 1990). Developmentally, GR abundance increases in the rat brain at approximately one week of age, in parallel with increases in CORT levels. In adulthood, the GR system appears to "autoregulate" itself; that is, high circulating levels of CORT lead to reductions in GR density, whereas adrenalectomy (i.e., decreased CORT levels) leads to upregulation of GR density (Meaney, Sapolsky, & McEwen, 1985a; Reul et al., 1987). Specifically in the hippocampus, significant increases in GR density occur around

postnatal day 9 and continue to increase to adult levels (Meaney, Sapolsky, & McEwen, 1985b).

Corticosterone: Non-Genomic Mechanisms of Action and Injury

Intracellular receptor binding is the classic model of steroid/glucocorticoid actions, but evidence also exists for plasma membrane-bound receptors that bind steroids, and in particular, CORT (reviewed by Moore & Evans, 1999). This mechanism of steroid action was proposed because a genomic mechanism of action could not account for some CORT effects, including: 1. effects that were very rapid; 2. effects that existed even when CORT was made impermeable to the cell membrane by conjugation with bovine serum albumin (BSA); 3. effects that existed when protein synthesis inhibitors were applied; and, 4. specifically for CORT, effects that were not attenuated with GR antagonists (reviewed in Moore & Evans, 1999). Plasma membrane bound receptors that selectively bind CORT and progesterone derivatives have been identified in calf adrenal cortex (K_d = 77 nM, B_{max} = 14nM; Andres et al., 1997). Plasma membrane bound receptors were also identified by [^{3}H]CORT binding in amphibian (K_d=0.51 nM; Orchinik, Murray, & Moore, 1991) and mammalian brain (Orchinik et al., 1997). Functionally, CORT binding to putative plasma membrane bound receptors results in a rapid (less than one minute) increase in excitatory output from rat neurons taken from the reticular formation or locus coereleus (Avanzino et al., 1987a; Avanzino et al., 1987b). Behaviorally, it has also been reported that neither a protein synthesis inhibitor nor GR antagonists attenuated CORT's effect on increasing locomotor activity in rats (Sandi, Venero, & Guaza 1996).

CORT has been shown to interact with the glutamatergic receptor system in mediating rapid membrane receptor effects. In hippocampal neurons, CORT rapidly increases the frequency of AMPA-mediated mEPSCs (Karst et al., 2005). In regard to attenuating neurodegeneration, blockade of excitatory amino acid release by the anti-epileptic drug phenytoin prevents both CORT and stress-induced increases in CA3 pyramidal neuron atrophy (Watanabe et al., 1992a). Stress has also been shown to increase hippocampal glutamate release, which can be reversed by adrenalectomy, and either blockade of steroid formation or NMDA receptor blockade has been shown to protect against atrophy of CA3 pyramidal neurons (reviewed in McEwen & Magarinos, 1997). More recent work has attempted to delineate NMDA receptor subunit-specific signaling pathways that may play a role in CORT-mediated neurodegeneration. Xiao et al. (2010) showed that CORT attenuates neuroprotective signaling by attenuating the neuroprotective NMDA NR2A-ERK1/2 pathway, but CORT does not affect the NMDA NR2B-p38 death pathway in hippocampal neurons. To further demonstrate the CORT effect at the plasma membrane, BSA-conjugated GCs significantly potentiated NMDA receptor-stimulated Ca^{2+} influx that was blocked by a specific NR2A antagonist (Xiao et al., 2010).

HPA Axis Dysregulation

As the HPA axis is essential in physiological homeostasis, chronic HPA axis dysregulation has been associated with many deleterious effects. Allostasis may be defined as the body's adaptation to stressful events, whereas allostatic load refers to

pathophysiology and overuse that result from adaptation to prolonged stressors (McEwen, 1998). HPA axis dysregulation is apparent in several disease states, including major depression (reviewed by Shelton, 2007); post-traumatic stress disorder (reviewed by Yehuda, 2001); anxiety disorders (reviewed by Abelson et al., 2007); schizophrenia (reviewed by Altamura, Boin, & Maes, 1999); and obesity (reviewed by Bose, Olivan, & Laferrere, 2009). Additionally, genetic polymorphisms in GRs and MRs contribute to increased incidence of depression (Derijk & de Kloet, 2008). Major depressive disorder has been associated with increased CORT levels and reduced hippocampal volume in MRI scans (Shah et al., 1998), with reduced hippocampal volume positively correlating with duration of depressive symptoms (Sheline et al., 1996). It has also been shown that inhibitors of CORT synthesis effectively treat psychosis-related depression (Belanoff et al., 2001). This correlation has also been noted in clinical subpopulations with enhanced depressive symptoms, such that multiple sclerosis patients with depressive symptoms have higher CORT levels and reduced volume of the hippocampal CA3 region and DG (Gold et al., 2010). Additionally, MRI studies of patients who have undergone long-term treatment with corticosteroids show decreased hippocampal volume, poorer performance on declarative memory tasks, and more severe depressive symptoms in comparison to control individuals (Brown et al., 2004).

In rodent studies, acute and chronic experimenter-induced stress has been used to investigate structural and hormonal changes at all levels of HPA axis functioning. Blunted HPA axis responsiveness to novel stressors after chronic variable stress exposure has been reported, such that adult male rats had increased CRH mRNA content in the

PVN at 16 hours post-cessation of stressor, though lower ACTH and CORT plasma levels days after stressor cessation (Ostrander et al., 2006). Chronic stress and/or repeated exogenous administration of CORT in adrenalectimized rats reduces the number of receptors present in the cytosol of neurons in the hippocampus and amygdala, without changing the affinity of [^{3}H]dexamethasone, a synthetic glucocorticoid (Sapolsky, Krey, & McEwen, 1984a). This receptor protein adaptation, or reductions in receptor number after chronic stress and/or CORT administration, can be reversed upon treatment cessation (Sapolsky, Krey, & McEwen, 1984a). This downregulation of GRs is of functional importance, as GRs are important for maintaining the negative feedback loop of the HPA axis, and reduced hippocampal GRs impairs termination of the stress response, thus resulting in hypercortisolemia (Sapolsky, Krey, & McEwen, 1984b; Sapolsky, Meaney, & McEwen, 1985). Interestingly, downregulation of GRs appears to reach a trough after long-term stress exposure that fails to decline further when stressors are applied continuously (Sapolsky et al., 1984a).

It is important to note that adrenalectomy or knockout of MR protein can also be detrimental to neuronal integrity. For instance, adrenalectomy in adult male rats significantly decreases the cell body area and dendritic branch points in granule cells of the DG while increasing the number of cells showing chromatin condensation (i.e. pyknosis; and decreasing granule cell number), though deficits are not observed in adrenalectomized rats supplemented with CORT (Gould et al., 1990; Sloviter et al., 1989). These deficits are present as early as 3 days after surgery and worsen over the period of a week, and were likely neurons and not glial cells due to their morphological

appearance. The CA1 and CA3 regions were resistant to significant changes in cell body size and dendritic branching after adrenalectomy, though there was a trend for a decrease in cell body size and dendritic branching in CA3 pyramidal cells (Gould et al., 1990). Although it is unclear why DG granule cells are most sensitive to damage caused by removal of CORT, CORT has been observed to alter the cell cycle and decrease DG hippocampal region neurogenesis (Brummelte & Galea, 2010).

Alcohol and HPA Axis Dysfunction: Human Subjects

In humans, prolonged elevation of glucocorticoids causes damage in the periphery (e.g., muscles, bones), alterations in CNS structures and metabolism (in particular, the hippocampus), and neuronal loss (reviewed by Sapolsky, 2000). Acutely, alcohol activates the HPA axis, as measured by increased release of ACTH, in drinkers that do not meet criteria for dependence (Aguirre et al., 1995). Long-term alcohol consumption also affects HPA axis functioning, both during intoxication/exposure and withdrawal. Excessive alcohol consumption coupled with pathological levels of CORT has the potential to potentiate cellular damage. Secretion of CORT, the primary stress hormone in humans, is increased in male alcoholics that are currently intoxicated and withdrawing. However, CORT levels were greater in male subjects undergoing acute withdrawal (≤ 2 days of abstinence), as compared to subjects that were still highly intoxicated (≥ 100 mg/dl BAC). It should also be noted that salivary CORT levels were similar in male subjects undergoing acute withdrawal (≤ 2 day of abstinence) and subjects that were still intoxicated but with marginal BACs (≤ 100 mg/dl), suggesting that CORT levels rise as

ethanol withdrawal begins (Adinoff et al., 2003). Indeed, it has been reported in a small sample of men that peak ethanol withdrawal correlates with peak CORT levels, which return to control-level after 7 days of abstinence (Adinoff et al., 1991). After periods of prolonged abstinence, however, male alcoholics demonstrate blunted responses to stressors (Adinoff et al., 1998), with reduced responsiveness to pharmacological challenge of HPA axis functioning at each level of the HPA axis (Adinoff et al., 2005; Costa et al., 1996). Importantly for clinical application, male alcoholics that showed a severely dampened CORT response to a psychosocial stressor after 2 weeks of abstinence were much more likely to relapse when interviewed at 6 weeks of abstinence (Junghanns et al., 2003), and attenuation of the blunted response with anti-relapse medication decreased vulnerability to relapse (Kiefer et al., 2006). Though a paucity of data exist examining HPA axis responsiveness in abstinent female alcoholics, conflicting results have been reported in samples of abstinent alcohol-dependent women as to whether they differ from healthy age-matched controls on measures of ACTH reactivity (Adinoff et al., 2010; Brady et al., 2006). And, unlike men, abstinent female alcoholics do not display blunted CORT secretion patterns (Adinoff et al., 2010).

Alcohol, HPA Axis Dysfunction, and Drinking Behavior: Animal Models

Paralleling data gathered from human subjects, alterations in HPA axis functioning have been reported in animal models of acute alcohol exposure and alcohol dependence. Ethanol has been noted to affect CORT plasma levels in male and female adult and peri-adolescent inbred rat strains, as well as in alcohol preferring and alcohol

non-preferring rats. Following an acute injection of ethanol, both alcohol-preferring and alcohol non-preferring rats have increased plasma CORT levels relative to control rats, whether housed in isolation or in groups (Apter & Erikkson, 2006).

Effects of ethanol on plasma CORT varies whether ethanol is given acutely or chronically. In adult male rats, acute alcohol induces increases in CORT and ACTH release, whereas alcohol dependent rodents show blunted CORT release upon alcohol challenge (Richardson et al., 2008). Peri-adolescent (post-natal day (PND) 37-44) male and female rats show similar increases in plasma CORT with acute or binge ethanol exposure. However, plasma CORT levels were significantly greater in rats given an acute dose of ethanol compared to peri-adolescent rats exposed to a binge-ethanol paradigm, suggesting habituation of the CORT response with prolonged, repeated ethanol exposure. Further, it should be noted that the pattern of increased CORT was the same for male and female rats, such that CORT levels were greater under acute ethanol than after binge ethanol. However, the magnitude of the CORT increase was markedly greater in female rats, approximating plasma concentrations of 500 ng/ml compared to values observed in male rats (~100 ng/ml) (Przbycien-Szymanska, Rao, & Pak, 2010). CORT levels are increased in response to acute ethanol administration, but return to baseline in alcohol dependent rats. However, CORT levels are increased again during withdrawal from long-term ethanol adminstration/exposure (Borlikova, Le Merrer, & Stephens, 2006; Janis et al., 1998). Many studies of alterations in CORT levels with acute or prolonged ethanol exposure have depended on measurements of plasma CORT levels. Importantly, plasma CORT levels have been shown to correlate with brain CORT levels during ethanol

consumption, though differences appear during ethanol withdrawal. Plasma CORT levels are initially increased during acute withdrawal but then return to baseline within 24 hours, whereas brain CORT levels have been shown to remain elevated up to 2 months after drinking cessation in mice and rats (Little et al., 2008). Behaviorally, exogenous administration of CORT during withdrawal from an acute dose of ethanol is also able to potentiate withdrawal in withdrawal seizure-prone mice (Roberts et al., 1994).

Exogenous administration of CORT, blockade of CORT synthesis, and/or adrenalectomy has also been shown to affect alcohol drinking behaviors. Male alcohol preferring rats show reduced alcohol drinking after adrenalectomy compared to baseline drinking and sham operated rats, though this decrease in drinking is reversible with chronic CORT administration (Fahlke & Erikkson, 2000). Wistar rats that prefer ethanol to water also demonstrate decreased alcohol drinking when given the CORT synthesis inhibitor metyrapone; an effect that is reversed by concurrent administration of metyrapone and CORT (Fahlke et al., 1994). In additional studies conducted by Fahlke and colleagues (1995), it was shown that male Wistar rats had decreased alcohol intake immediately following adrenalectomy, though alcohol intake returned to baseline levels with or without CORT supplementation. However, if adrenalectomized rats were given prolonged CORT supplementation, alcohol drinking was greater as compared to sham-operated rats. Interestingly, the behavioral effects on drinking in adrenalectomized rats or adrenalectomized rats supplemented with CORT were not altered by subcutaneous injection of a type I or type II GR antagonist, suggesting that CORT-related increases in

drinking behavior were not mediated by activation of intracellular GR receptors (Fahlke et al., 1995).

Alcohol, HPA Axis Dysfunction, and NMDA Receptors

These findings are important, as stress and/or prolonged exposure to CORT results in increased abundance of NMDA receptors, and in particular, NMDA NR2A and NR2B mRNA and subunit protein (Meyer et al., 2004; Weiland et al., 1997) and increased Ca^{2+} influx in hippocampal neurons (Takahashi et al., 2002). CORT has also been shown to potentiate glutamate-induced excitotoxicity in hippocampal neurons *in vitro* (Goodman et al., 1996), and to increase extracellular glutamate levels *in vivo* (Stein-Behrens, Lin, & Sapolsky, 1994). As both long-term ethanol exposure and CORT exposure have been shown to increase glutamatergic neurotransmission and NMDA receptor function and abundance, vulnerability to excitotoxicity during ethanol withdrawal coupled with increased CORT exposure likely promote potentiated neurodegeneration. In fact, it has been shown that CORT administered during ethanol exposure and withdrawal *in vitro* potentiates Ca^{2+} influx and hippocampal cell death, though these effects are reversed by co-exposure to the NMDA receptor channel blocker MK-801 and the GR antagonist RU486 (Mulholland et al., 2005). Additionally, in a model of long-term ethanol exposure that was associated with increases in plasma CORT, decreased neuronal density was noted in the CA3 region of the hippocampus (Hu et al., 2010).

Experimental Rationale

Chronic ethanol exposure leads to numerous structural, behavioral, and functional adaptations that have been studied in both human and animal models. Neuroadaptations that occur in multiple neurotransmitter systems after long term exposure to ethanol likely mediate neurodegeneration and behavioral signs of ethanol withdrawal. Similarly, it has been well-established that chronic ethanol intake alters HPA axis functioning, leading to concentrations of glucocorticoids that promote neurodegeneration. The current studies were designed to investigate effects of CORT exposure during long-term ethanol exposure on toxicity during ethanol withdrawal, and mechanisms that may mediate CORT potentiation of toxicity during ethanol withdrawal in the rodent brain. Effects of CORT and ethanol exposure have been studied extensively in the hippocampus, as the hippocampus is particularly vulnerable to neurodegeneration by both types of insult (Packan & Sapolsky, 1990).

Chapter 2

EXPERIMENTAL PROCEDURES

Organotypic Hippocampal Slice Culture Preparation

Eight-day old male and female Sprague-Dawley rat pups (Harlan Laboratories; Indianapolis, IN, USA) were humanely euthanatized for aseptic whole brain removal. Brains were immediately transferred into chilled dissecting medium (4°C) made of Minimum Essential Medium (MEM; Invitrogen Corporation, Carlsbad, CA, USA), 25 mM HEPES (Sigma-Aldrich Co., St. Louis, MO, USA), and 50 µM penicillin/ streptomycin (Invitrogen; adapted from Stoppini et al., 1991). Bilateral hippocampi were removed, cleaned of extra tissue under a dissecting microscope, and placed into chilled culture medium, composed of dissecting medium with the addition of sterile H_20, 36 mM glucose (Fisher Scientific, Florence KY, USA), 25% (v/v) Hanks' balanced salt solution (Invitrogen) 25% heat-inactivated horse serum (HIHS; Sigma-Aldrich Co.), and 0.05% Penicillin/Streptomycin. Hippocampi were sectioned coronally at 200 µm using the McIllwain Tissue Chopper (Mickle Laboratory Engineering Co. Ltd., Gomshall, UK) and placed into fresh culture medium. Slices with intact morphology were selected under a dissecting microscope and placed onto Millicell-CM 0.4 µm biopore membrane inserts that were pre-incubated in 1 ml of culture medium at 37°C in 35 mm 6-well culture plates. Three slices were placed onto each insert, yielding 18 slices per plate for all experiments except for Western blot experiments, for which there were 5-6 slices per insert. Excess medium from the top of the membrane insert was aspirated to allow the

slices exposure to the incubator atmosphere of 5% CO_2/95% air. Slices were allowed five days to attach to the insert membrane before conducting any experiments. Care of animals was carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) and the University of Kentucky's Institutional Animal Care and Use Committee.

11 Day Corticosterone Exposure in Ethanol-Naïve Hippocampal Cultures

Cytotoxicity was measured in a portion of cultures after 11 day exposure to a range of CORT concentrations in ethanol-naïve cultures (after Mulholland et al., 2005). At 5 days *in vitro* (DIV) and 10 DIV cultures were transferred into fresh culture medium containing 0, 0.01, 0.1, or 1 μM CORT (Sigma). CORT was dissolved in dimethyl sulfoxide (DMSO) and diluted to the desired concentration with culture media for a final DMSO concentration of ≤ 0.01% in culture media. For the initial 10 days of CORT exposure, cultures were placed in topless polypropylene containers and surrounded by 50 ml of double-distilled water, in accord with the ethanol exposure regimen described below. At 15 DIV, cultures were placed into fresh culture medium containing their respective CORT concentration with the addition of the nucleic acid marker of dead/dying cells, propidium iodide (PI; 3.74 μM). PI is a polar compound that is only able to enter cells with compromised membranes, after which it binds to DNA and is able to fluoresce when excited with the appropriate wavelength of light (Zimmer et al., 2000). At 16 DIV, after 11 days of continuous CORT exposure, cytotoxicity was measured by fluorescent imaging of PI (described in detail below).

Corticosterone Exposure during Ethanol Exposure and Withdrawal

Additional cultures were continuously exposed to CORT (0.01, 0.1, or 1 μM) during ethanol exposure and withdrawal. We hypothesized that exposure to CORT for the duration of ethanol exposure (10 days) and withdrawal (24 hours) would produce marked toxicity above control values and above any toxicity that may occur as a result of CORT exposure in ethanol-naïve cultures. This hypothesis is in accord with previous studies showing that both long-term ethanol exposure and long-term exposure to CORT result in increased NMDA receptor subunit expression, thereby providing a mechanism for enhanced toxicity upon removal of ethanol. Ten day continuous ethanol exposure was used to model long-term ethanol exposure in hippocampal slices, in accord with the CORT exposure regimen described above. At 5 DIV, slices were randomly transferred to new plates containing either 1 ml of standard culture medium (control) or culture medium with a CORT and a calculated ethanol concentration of 50 mM, or approximately 240 mg/dl. In an attempt to reduce ethanol evaporation, all plates containing ethanol in the medium were surrounded by 50 ml of double-distilled water containing ethanol (at a concentration of 50 mM) in topless polypropylene containers, and plates devoid of ethanol in the medium were surrounded by 50 ml of double-distilled water. Containers were placed into sealable plastic bags and filled to capacity with 5% CO_2/95% air before being placed in the incubator. The same treatment was repeated at 10 DIV. Despite these preventative measures, previous work in our laboratory has shown approximately a 50% decline in ethanol concentration over 5 days when beginning with an ethanol concentration calculated at 50 or 100 mM (Butler et al., 2008; Prendergast et al., 2004).

For studies of the effects of CORT on cell death during ethanol exposure and withdrawal, cultures were removed from media and placed into fresh culture medium without ethanol to begin 24 hour CORT exposure during ethanol withdrawal at 15 DIV. An additional subset of cultures that was exposed to CORT and ethanol for 10 days was co-exposed to CORT and ifenprodil (+)-tartrate salt (ifenprodil; 100 μM) during ethanol withdrawal for 24 hours. Cytotoxicity was measured by densitometry of PI fluorescence. Ifenprodil is a non-competitive polyamine site antagonist. Polyamines act to potentiate NMDA receptor function via their binding site on the NR2B subunit, allowing for greater Ca^{2+} influx. As long-term exposure to CORT can promote polyamine synthesis (Cousin et al., 1982), and long-term ethanol exposure can result in increased NMDA NR subunit expression (Harris et al., 2003), we hypothesized that blockade of NMDA receptor function at the polyamine-sensitive NR2B binding site with ifenprodil would significantly reduce toxicity produced by ethanol and CORT co-exposure during withdrawal. All experiments were replicated 3-5 times. Additional studies were conducted using cultures that were formalin-fixed after 10 day ethanol and/or CORT exposure, but without a 24 hour ethanol withdrawal period for immunohistochemical studies of neuroadaptations following long-term ethanol and CORT co-exposure.

Immunohistochemistry: NMDA NR2B Subunit

Many models of long-term ethanol exposure have shown increases in NMDA receptor subunit proteins, which have been suggested to contribute to ethanol withdrawal-related hyperexcitability. Therefore, we conducted immunohistochemistry with the

hypothesis that 10 day exposure to ethanol would produce significant increases in NMDA NR2B subunit expression. Immunohistochemistry was conducted using male and female oragnotypic hippocampal cultures for measurement of NR2B immunoreactivity in control and ethanol-exposed tissue. At 5 DIV, cultures were exposed to 25, 50, or 100 mM ethanol in culture medium for 5 days, at which time culture medium with ethanol was refreshed, for a total of 10 days of ethanol exposure. Culture medium was changed for control cultures on the same days. The anti-NMDA NR2B subunit antibody used was a polyclonal antibody derived from rabbit and directed against the C-terminal of the rat NR2B receptor (Millipore; product number AB15362).

After 10 days of ethanol exposure, at 15 DIV, cultures were formalin-fixed for immunohistochemistry. For fixation, cultures were placed into plates containing 1 ml of 10% formalin on the bottom and top of the well for 30 minutes. Cultures were then washed twice in 1x phosphate-buffered saline (PBS) and stored with 1 ml of 1x PBS on the bottom of the well at 4°C until immunohistochemistry was conducted. On Day 1 of immunohistochemistry, cultures were incubated in permabilization buffer (PBS buffer with the addition of 0.1% Triton-X and 0.005% bovine serum albumin) for 45 minutes. Primary antibody solution was prepared as a 1:200 dilution of rabbit anti-NMDA NR2B monocolonal antibody in permeabilization buffer. After the initial 45 minute incubation period, cultures were transferred into fresh culture plates and the primary antibody solution was slowly added to the top of the slices, with 1 ml of 1x PBS below the well to reduce diffusion of antibody through the porous insert membrane. Cultures were stored at 4°C for 24 hours. On Day 2 of immunohistochemistry, cultures were removed from

plates containing the primary antibody and washed twice in 1x PBS. Secondary antibody solution contained fluorescein isothiocyanate (FITC)-conjugated secondary antibody (sheep anti-rabbit; 1:500 dilution; Sigma-Aldrich Co.) in permeabilization buffer, and was slowly added to the top of the slices, with 1 ml of 1x PBS below the well. Cultures were stored at 4°C for 24 hours. Fluorescent microscopy following the 24 hour incubation with FITC was used to quantify NMDA NR2B receptor subunit immunoreactivity in the granule cell layer of the dentate gyrus (DG) and the pyramidal cell layers of the *cornu ammonis* 3 (CA3) region and the CA1 region.

Preliminary studies were conducted to measure NR2B immunoreactivity after 10 day exposure to 25 mM, 50 mM or 100 mM ethanol. Additional studies were conducted to measure NR2B subunit immunoreactivity following 10 day exposure to ethanol (50 mM) and CORT (0.1 μM). Similar to the experimental procedures described above for PI measurement after 24 hour withdrawal following 10 day ethanol and CORT co-exposure, cultures were co-exposed to ethanol and CORT for 10 days without ethanol withdrawal. At the end of the 10 day co-exposure period, cultures were fixed and immunohistochemistry for the NMDA NR2B subunit was conducted following the procedure described above.

Fluorescent Microscopy and Statistical Analysis

For PI and immunohistochemistry studies, fluorescent intensity (arbitrary optical units) was measured using densitometry. Images were taken using SPOT Advanced

version 4.0.2 software for Windows (W. Nuhsbaum Inc., McHenry, IL, USA) with a 5× objective on an inverted Leica DMIRB microscope (W. Nuhsbaum Inc.) fitted for fluorescence detection (mercury-arc lamp) and connected to a personal computer via a SPOT 7.2 color mosaic camera (W. Nuhsbaum Inc.). Propidium iodide has a maximum excitation wavelength of 536 nm and was excited using a band-pass filter that excites the wavelengths between 515 and 560 nm. The emission of PI in the visual range is 620 nm. The FITC-conjugated secondary antibody was excited using a band-pass filter at 495 nm (520 nm emission). Fluorescent intensity was analyzed by densitometry using ImageJ software (National Institutes of Health, Bethesda, MD, USA) for the granule cell layer of the DG and the pyramidal cell layers of the CA3 and CA1 regions of the hippocampus. For each labeled hippocampal slice, background fluorescent intensity was subtracted from each region's measurement, and raw fluorescent values were converted to percent control within each region before statistical analysis. Raw values for male and female slices were converted to percent control separately within each hippocampal region (CA1, CA3, and DG). Outliers were removed using Grubb's outlier test (GraphPad). A two-way analysis of variance (ANOVA; treatment × sex) within each hippocampal region was conducted, and when appropriate, Fisher's LSD post-hoc analyses were interpreted. The significance level was set at $P < 0.05$. All experiments were conducted at least 3 times with different rat litters.

[^{3}H]Ifenprodil Autoradiography

Autoradiography was conducted for quantification of ifenprodil binding sites after 10 day exposure to CORT and ethanol in male and female organotypic hippocampal cultures. Ifenprodil is a non-competitive antagonist at the polyamine binding site that is located on the extracellular tail of the NR2B subunit. We hypothesized that ethanol and CORT co-exposure would result in increased ifenprodil binding above that observed in tissue only exposed to ethanol or CORT alone. After 10 days of continuous drug exposure, cultures were removed from culture media and flash-frozen in isopentane on dry ice for storage at -80°C until [^{3}H]ifenprodil binding studies were conducted. For [^{3}H]ifenprodil binding studies, the porous membranes on which the hippocampal slices were cultured were cut and secured onto glass slides. Tissue was then preincubated for 15 minutes in 50 mM Tris-HCl solution (pH 7.4) at room temperature. After 15 minutes, tissue was incubated with the [^{3}H]ifenprodil (20 nM) on ice for 120 minutes in Tris-HCl solution also containing GBR12909 (3 μM; a sigma receptor antagonist to block non-specific binding). Following incubation with the radioligand, tissue was washed with Tris-HCl wash buffer 3 times for 30 seconds, followed by one wash for 10 seconds. After washing, cultures were dried under a fan and placed into a dessicator overnight. Slides were arranged in a light-proof cassette and exposed to Kodak film. Films were developed after approximately 8 weeks of exposure and [^{3}H]ifenprodil binding was quantified using ImageJ software for densitometric analysis of [^{3}H]ifenprodil binding in each hippocampal slice.

Western Blotting

Immunohistochemistry with organotypic slices has the advantage of preserving the cell layers for quantification of immunoreactivity within the CA1, CA3, and DG hippocampal regions. However, analysis is conducted using densitometry, which quantifies overall fluorescence from relatively thick slices (beginning at 200 μm), perhaps rendering this model less sensitive to subtle changes. Therefore, to fully discern if changes in protein density are occurring following long-term ethanol exposure in our model, parallel studies to measure NMDA NR2B receptor subunit hippocampal expression were conducted using Western blots. Western blot analysis of the NMDA NR2B receptor protein was conducted after 10 day exposure to CORT and ethanol. After 10 days of drug exposure, slices were scraped into ddH_2O containing 2% sodium dodecyl sulfate (SDS) and sonicated for 10 seconds at 20% amplitude. A 10 μL aliquot was taken at that time for quantification of protein in each sample using the Pierce BCA Protein Assay Kit (Fisher) and stored separately to avoid protein breakdown with repeated thawing/freezing cycles. All samples were stored at -80°C until the protein assay and immunoblotting procedures were conducted.

Immunoblotting was conducted using protein extracted from male and female organotypic hippocampal slice cultures from at least five different rat litters (n = 6-10 per treatment group). For each sample, an equal amount of protein (20 μg) was loaded per lane. Samples were electrophoresed on 10-lane 7.5% Tris-glycine minigels in 1×Tris/glycine/sodium dodecyl sulfate buffer (Bio-Rad, Hercules, CA, USA) at room

temperature for approximately 45 minutes at 200V using the mini-PROTEAN Tetra system (Bio-Rad). The gels were then removed from their carriages and placed into transfer buffer for 5 minutes before creation of a transfer sandwich. To transfer protein from the gel to nitrocellulose paper, a transfer sandwich was assembled and placed into the mini-PROTEAN Tetra box containing an ice pack and placed on a stir plate for 60 minutes at room temperature (run at 100V). After the transfer step, nitrocellulose papers were washed three times and placed in 1×TBS on a rocker for 15 minutes. After washing, papers were placed into light-proof black boxes containing 5% non-fat dried milk in 1×TBS for 60 minutes to help reduce non-specific antibody binding. Papers were washed again 3 times in 1×TTBS and placed on a rocker for 15 minutes in 1×TTBS. After washing, papers were placed into light-proof boxes containing 1×TTBS with 5% milk solution and anti-NMDA NR2B receptor subunit antibody (1:2000; Millipore) for overnight incubation at 4°C with agitation. This particular NR2B antibody recognizes amino acids 20-271 on the C-terminus. On Day 2, papers were washed 4 times by placement in 1×TTBS on a rocker for 5 minutes, for a total of 20 minutes of washing. After washing, papers were placed into light-proof boxes containing 1×TTBS with 5% milk solution and the fluorescent secondary antibody (IRDye800; Rockland Immunochemicals, Gilbertsville, PA, USA). As before, papers were washed 4 times for a total of 20 minutes before imaging. Papers were imaged using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA). Fluorescent intensity of bands was imaged using ImageJ software, and raw fluorescent were converted to percent control before comparison across treatment groups. This protocol was modified from the laboratories of Dr. James W. Geddes and Dr. Patrick J. Mulholland.

BSA-Conjugated Corticosterone

In line with the traditional view of steroid actions, CORT is known to penetrate the cell membrane and bind intracellular glucocorticoid and mineralcorticoid receptors; travel to the nucleus; and alter gene expression. Some literature, however, has suggested that CORT has effects on neurons that are rapid and occur directly at the cell membrane independently of MR or GR activation and changes in gene transcription. Therefore, additional studies were conducted using BSA-conjugated corticosterone (BSA-CORT), which is a membrane-impermeable form of CORT that is useful for studying effects of CORT that are independent of MR and/or GR activation. Studies were conducted exactly as described in the above methods for measurement of PI uptake and NMDA receptor subunit expression after exposure to BSA-conjugated CORT (0.1 μM) during ethanol exposure and withdrawal. Briefly, male and female cultures were exposed to BSA-CORT (0.1 μM) or co-exposed to BSA-CORT and ethanol (50 mM) for 10 days. After 10 days of continuous drug exposure, cultures began a 24 hour ethanol withdrawal period with exposure to BSA-CORT with the addition of PI to the media for densitometric quantification of dead/dying cells, as described above. A general schematic of the treatment protocol is included (Figure 2.1).

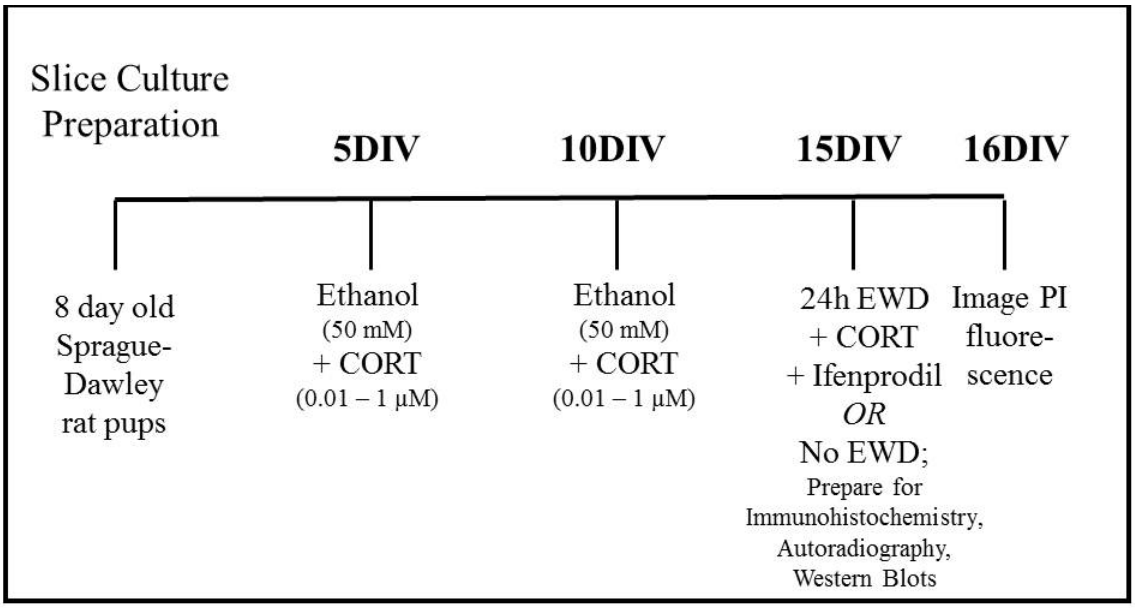

Figure 2.1. Experimental protocol timeline. All cultures were exposed to CORT and/or ethanol for 10 days. Some cultures were allowed a 24 hour ethanol withdrawal (EWD) period with or without ifenprodil, after which PI fluorescence was quantified as a measure of toxicity. Additional cultures were prepared for either immunohistochemistry, autoradiography, or Western blotting after 10 day CORT and/or ethanol exposure without EWD.

Chapter 3

RESULTS

11 Day Corticosterone Exposure in Ethanol-Naïve Hippocampal Cultures

Male and female cultures treated with CORT with or without co-exposure to ethanol withdrawal were compared statistically, but will be discussed separately for clarity and ease of interpretation. Male and female ethanol-naïve organotypic hippocampal cultures were exposed to CORT (0.01 – 1 μM) for 11 days. In both the CA1 and CA3 regions, a two-way ANOVA (treatment × sex) indicated a main effect of treatment ($F(7,565) = 9.674$, $P < 0.001$; $F(7,565) = 17.915$, $P < 0.001$), but no main effect of sex nor significant interaction. Therefore, male and female data were combined and a one-way ANOVA (factor: treatment) was conducted within each region. In the CA1 region, there was a main effect of treatment ($F(7,565) = 10.288$, $P < 0.001$), such that only 0.1 μM CORT increased cell injury and/or death (~10% above control values; Fisher's LSD post-hoc, $P < 0.001$). In the CA3 region, a one-way ANOVA (factor: treatment) indicated a main effect of treatment ($F(7,565) = 17.646$, $P < 0.001$), such that all concentrations of CORT (0.01 – 1 μM) produced significant cell death (~23-33% above control values; Fisher's LSD post-hoc Ps < 0.001). In the DG, a two-way ANOVA (treatment × sex) showed no significant interaction, but a significant main effect of treatment ($F(7, 567) = 16.181$, $P < 0.001$) and a significant main effect of sex ($F(1, 567) = 4.386$, $P < 0.05$). Therefore, a one-way ANOVA (factor: treatment) was conducted separately within the DG for male data and female data, indicating a main effect of

treatment within each sex ($F(7, 283) = 8.008$, $P < 0.001$ for male data; $F(7, 283) = 8.903$, $P < 0.001$ for female data). In male cultures, the main effect of treatment was driven by increased PI uptake in cultures co-exposed to ethanol and CORT, but CORT exposure in ethanol-naïve cultures did not produce toxicity (discussed in further detail below). In female cultures, however, CORT (0.01 μM and 0.1 μM) exposure in ethanol-naïve cultures resulted in significantly greater PI uptake compared to control cultures (Fisher's LSD post-hoc, $Ps < 0.05$). (Figure 3.1). Representative images of PI uptake are presented in Figure 3.2.

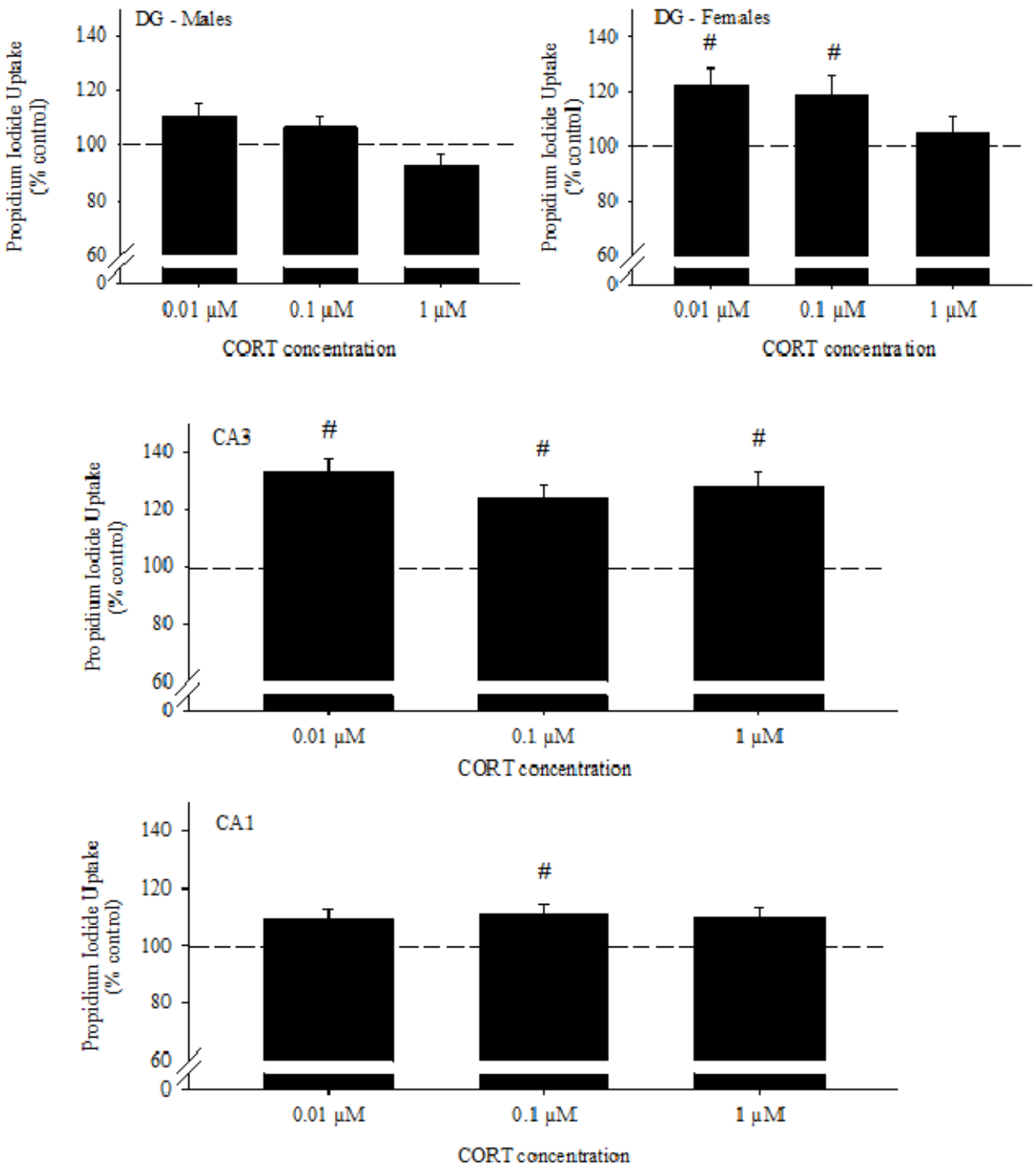

Figure 3.1. Propidium iodide uptake following 11 day exposure to CORT in ethanol-naïve hippocampal cultures. Exposure to some concentrations of CORT (0.01 – 1 µM) produced modest, yet significant, cell death in the primary cell layers of the CA1, CA3, and DG hippocampal regions. #P < 0.05 vs. control.

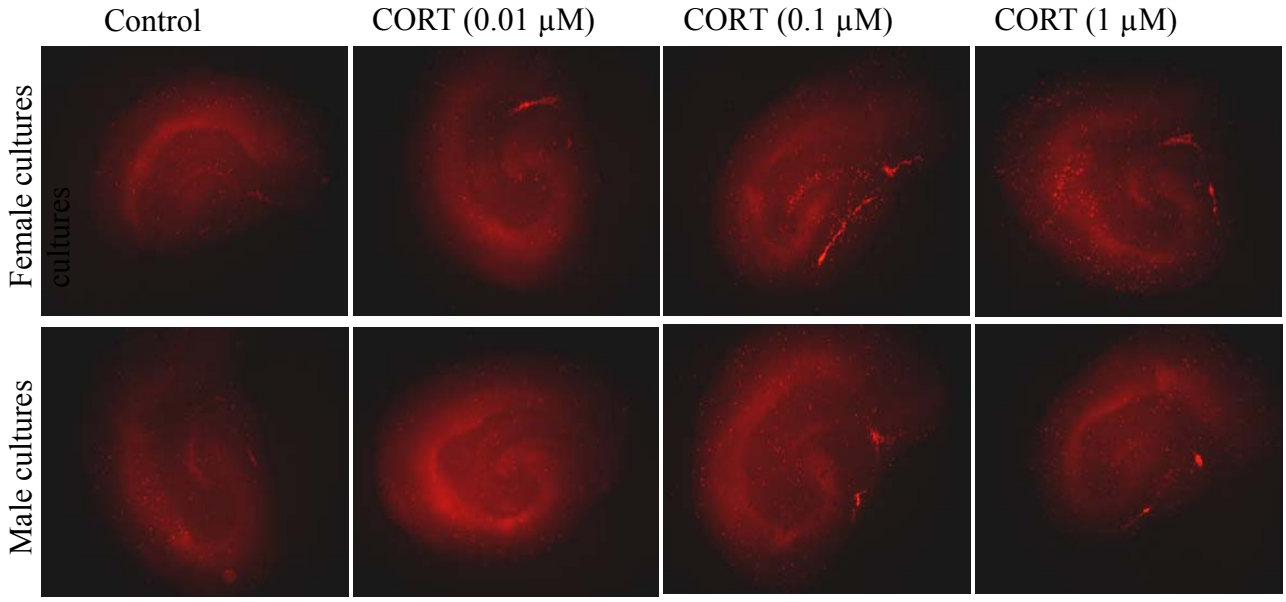

Figure 3.2. Representative images of PI uptake in organotypic hippocampal cultures exposed to CORT (0.01 – 1 μM) for 11 days.

Corticosterone Exposure During Ethanol Exposure and Withdrawal

As discussed above, a two-way (treatment × sex) ANOVA conducted within the CA1 region and within the CA3 region indicated no significant interaction or main effect of sex for either region, and therefore male and female data were combined for statistical analysis. In the CA1 region, the one-way ANOVA indicated a main effect of treatment. Concurrent exposure to ethanol withdrawal and all concentrations of CORT (0.01 – 1 μM) resulted in significantly greater toxicity compared to ethanol withdrawal without CORT and ethanol-naïve CORT-exposed cultures (Fisher's LSD post-hoc, Ps < 0.05). In the CA3 region, the one-way ANOVA indicated a main effect of treatment. Co-exposure to ethanol and each concentration of CORT (0.01 – 1 μM) produced significantly greater toxicity compared to ethanol withdrawal alone (Fisher's LSD post-hoc, Ps < 0.05), though toxicity in CORT and ethanol withdrawal co-exposed cultures was not greater than toxicity produced by CORT exposure in ethanol-naïve cultures (0.01 – 1 μM). In the DG, male and female data were considered separately, as a two-way ANOVA indicated a main effect of sex. For male cultures, exposure to all concentrations of CORT (0.01 – 1 μM) during ethanol withdrawal produced toxicity greater than ethanol withdrawal alone (Fisher's LSD post-hoc, Ps < 0.001). Additionally, exposure to some concentrations of CORT (0.01 μM and 1 μM) during ethanol withdrawal produced significantly greater than toxicity than that observed in ethanol-naïve CORT-exposed cultures (Fisher's LSD post-hoc, Ps < 0.05). Though exposure to 0.1 μM CORT during ethanol withdrawal produced significantly greater toxicity than ethanol withdrawal alone, this toxicity was not greater than that observed in ethanol-naïve CORT (0.1 μM) exposed cultures. In

female cultures, exposure to all concentrations of CORT during ethanol withdrawal produced toxicity significantly greater than ethanol withdrawal (Fisher's LSD post-hoc Ps < 0.001), though the toxicity observed was not different from toxicity produced by CORT exposure in ethanol-naïve cultures. Unexpectedly, in the DG of both male and female cultures, ethanol withdrawal alone resulted in significantly less PI uptake than control cultures (Ps < 0.01). (Figure 3.3). Representative images of PI uptake are presented in Figure 3.4.

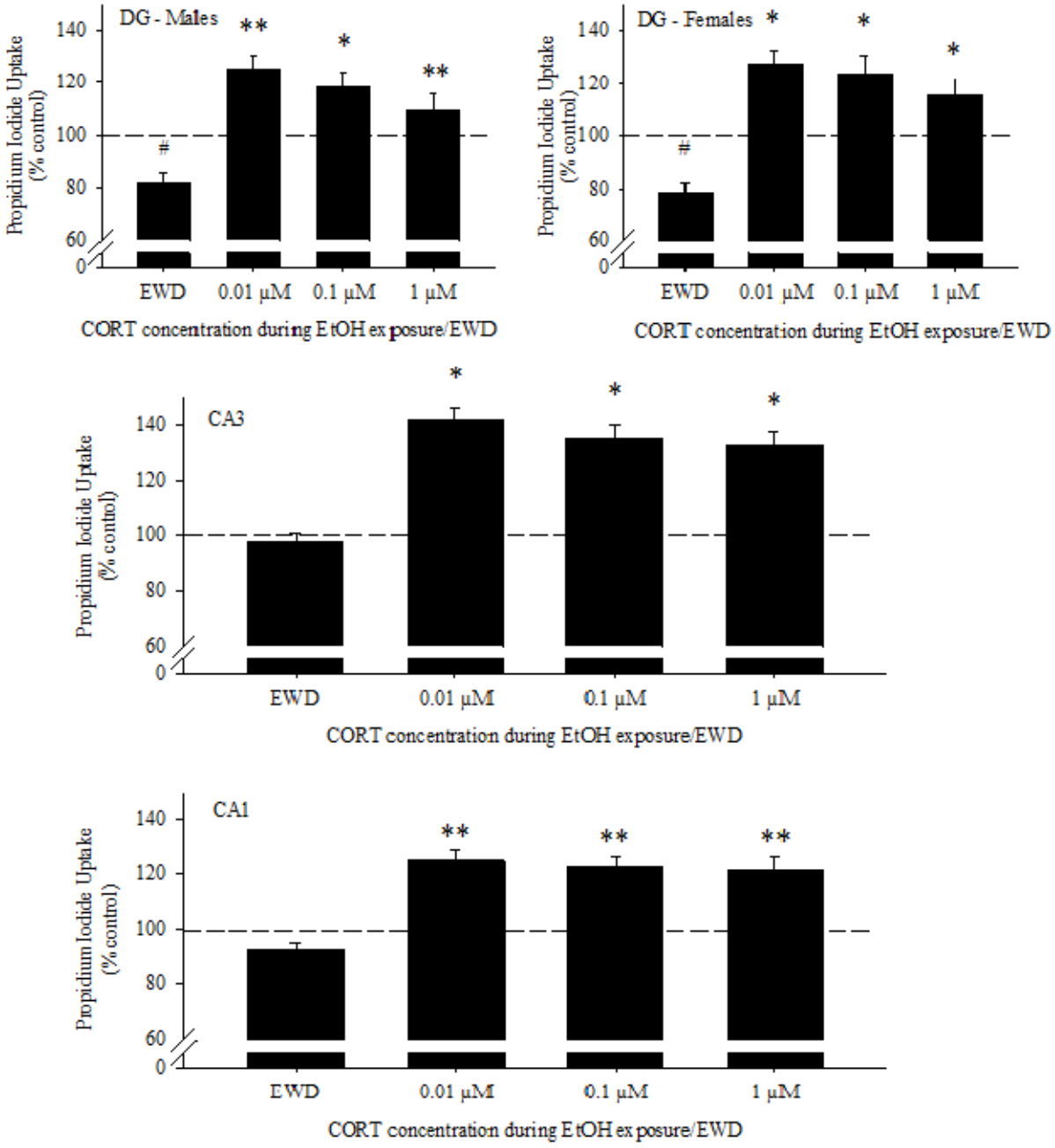

Figure 3.3. Propidium iodide uptake during 24 hour withdrawal following 10 day exposure to CORT (0.01 – 1 µM) and ethanol (50 mM). **P < 0.05 vs. ethanol withdrawal (EWD) and ethanol-naïve cultures exposed to the same concentration of CORT; *P < 0.05 vs. ethanol withdrawal; # P < 0.05 vs. control. Most importantly for the hypothesis governing the current studies, exposure to CORT during ethanol exposure and withdrawal produced significantly greater toxicity at all concentrations in the CA1 region compared to CORT exposure in ethanol-naïve cultures and ethanol withdrawal alone.

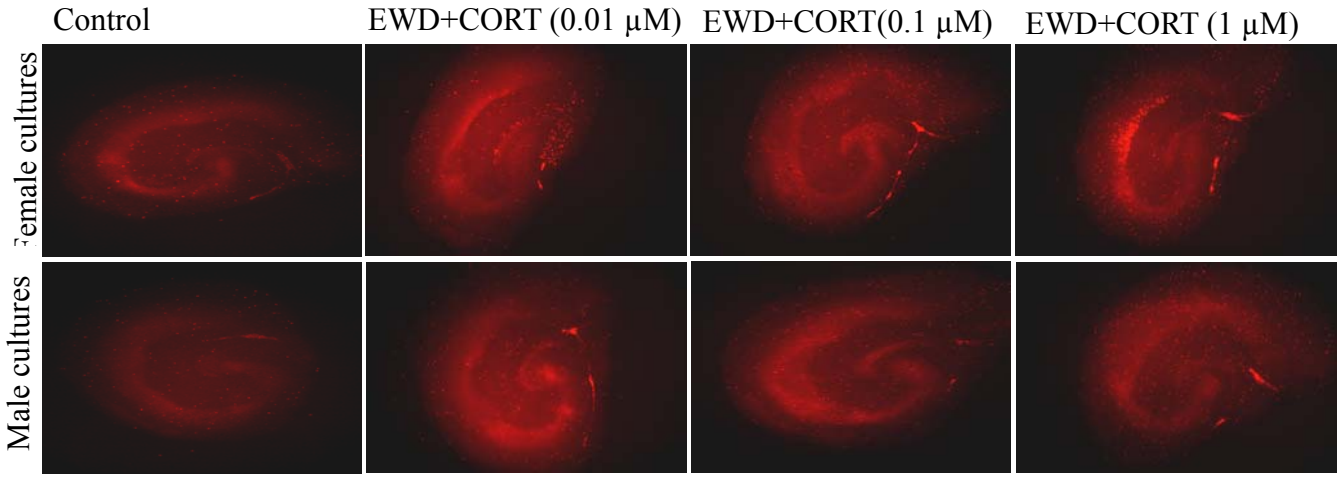

Figure 3.4. Representative images of PI uptake in organotypic hippocampal cultures co-exposed to CORT and ethanol (50 mM) for 10 days followed by 24 hour ethanol withdrawal (EWD).

Ifenprodil and Corticosterone Co-Exposure During Ethanol Withdrawal

A subset of cultures that were exposed to CORT (0.1 μM) for 11 days during ethanol exposure and withdrawal were also exposed to ifenprodil (100 μM) during the 24 hour ethanol withdrawal period (Figure 3.5). As in the previously discussed studies, PI uptake was measured as an indicator of cell damage and/or death after 24 hour ethanol withdrawal. The 0.1 μM concentration of CORT was chosen for these studies because toxicity produced by CORT exposure during ethanol exposure and withdrawal in the previous studies was not concentration dependent, and it is within the range of physiological or stress-relevant CORT levels. A two-way ANOVA (treatment × sex) in the CA1 region indicated a main effect of treatment ($F(5,253) = 4.417$, $P < 0.001$), but no effect of sex. Therefore, male and female treated cultures were combined for a one-way ANOVA analysis (factor: treatment). A one-way ANOVA indicated a main effect of treatment ($F(5,253) = 4.563$, $P < 0.001$). As in previous studies, exposure to CORT (0.1 μM) for 11 days during ethanol withdrawal produced significantly greater toxicity than ethanol withdrawal alone or control (Fisher's LSD post-hoc, $Ps < 0.05$). Most importantly, the addition of ifenprodil to CORT and ethanol co-treated cultures during withdrawal resulted in significantly decreased toxicity compared to cultures co-exposed to CORT during ethanol exposure and withdrawal without ifenprodil (Fisher's LSD post-hoc, $P < 0.001$; Figure 3.5). This supports the hypothesis that the toxicity due to CORT and ethanol exposure during withdrawal was due, in part, to activation of polyamine-sensitive NR2B-containing NMDA receptors. Neither exposure to ifenprodil during

ethanol withdrawal nor ifenprodil exposure in ethanol-naïve cultures resulted in significant toxicity compared to ethanol withdrawal and control, respectively. (Table 1).

In the CA3 region, a two-way ANOVA indicated a main effect of treatment ($F(5,256) = 6.446$, $P < 0.001$) and a main effect of sex ($F(1,256) = 28.904$, $P < 0.001$). As there was a main effect of sex, a one-way ANOVA (factor: treatment) was conducted separately for male cultures and female cultures. Among male cultures, there was a significant effect of treatment ($F(5, 126) = 2.730$, $P < 0.05$). CORT exposure during ethanol withdrawal did not produce toxicity compared to control cultures ($P = 0.828$) or ethanol withdrawal alone ($P = 0.304$). However, PI uptake was significantly reduced in a subset of cultures exposed to ifenprodil during withdrawal following CORT and ethanol co-exposure compared to CORT withdrawal cultures not exposed to CORT (Fisher's LSD post-hoc, $P < 0.05$; Figure 3.5). Twenty-four hour exposure to ifenprodil did not produce toxicity in ethanol-naïve cultures or ethanol withdrawn cultures (Table 1). Among female cultures, there was also a significant effect of treatment ($F(5, 129) = 5.460$, $P < 0.001$). The addition of ifenprodil during withdrawal in CORT and ethanol co-exposed cultures resulted in significantly less toxicity than cultures co-exposed to CORT and ethanol without ifenprodil during withdrawal (Fisher's LSD post-hoc, $P < 0.01$). However, PI uptake produced by CORT and ethanol co-exposure was not different from ethanol withdrawal alone. Neither twenty-four hour exposure to ifenprodil nor ifenprodil exposure during withdrawal resulted in significantly greater toxicity than control or ethanol withdrawal alone, respectively (Table 1).

In the DG, a two-way ANOVA indicated a main effect of treatment ($F(5, 254) = 9.391$, $P < 0.001$), but no effect of sex. Therefore, male and female treated cultures were combined for a one-way ANOVA analysis (factor: treatment). A one-way ANOVA indicated a main effect of treatment ($F(5,254) = 9.656$, $P < 0.001$). Co-exposure to CORT and ethanol did not result in significantly increased PI uptake during withdrawal. However, the addition of ifenprodil during withdrawal in CORT and ethanol co-exposed cultures resulted in significantly less toxicity than cultures co-exposed to CORT and ethanol without ifenprodil during withdrawal (Fisher's LSD post-hoc, $P < 0.05$). Twenty-four hour exposure to ifenprodil in ethanol-naïve cultures and ethanol-withdrawn cultures resulted in significantly increased PI uptake compared to control cultures and ethanol-withdrawn cultures, respectively (Fisher's LSD post-hoc Ps < 0.05; Table 1). It is not clear why ifenprodil increased PI uptake only in the DG region. Representative images of PI uptake are presented in Figure 3.6.

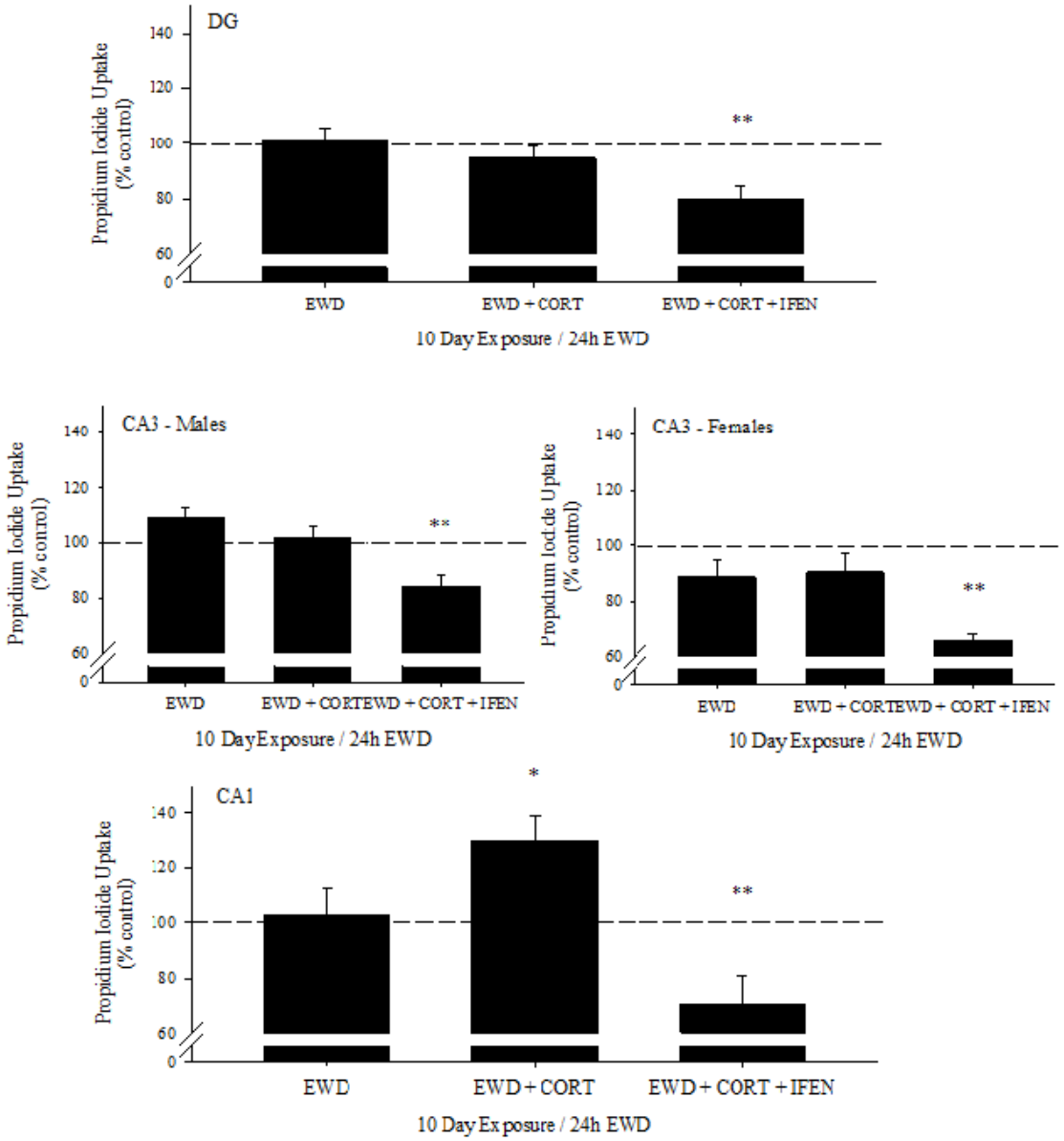

Figure 3.5. Ifenprodil co-exposure during ethanol withdrawal following 10 day ethanol and CORT co-exposure. Within the CA1, CA3, and DG the addition of ifenprodil (IFEN) during ethanol withdrawal (EWD) significantly reduced toxicity produced by CORT and ethanol co-exposure. *P < 0.05 vs. ethanol withdrawal; **P < 0.05 vs. EWD + CORT; #P < 0.05 vs. control.

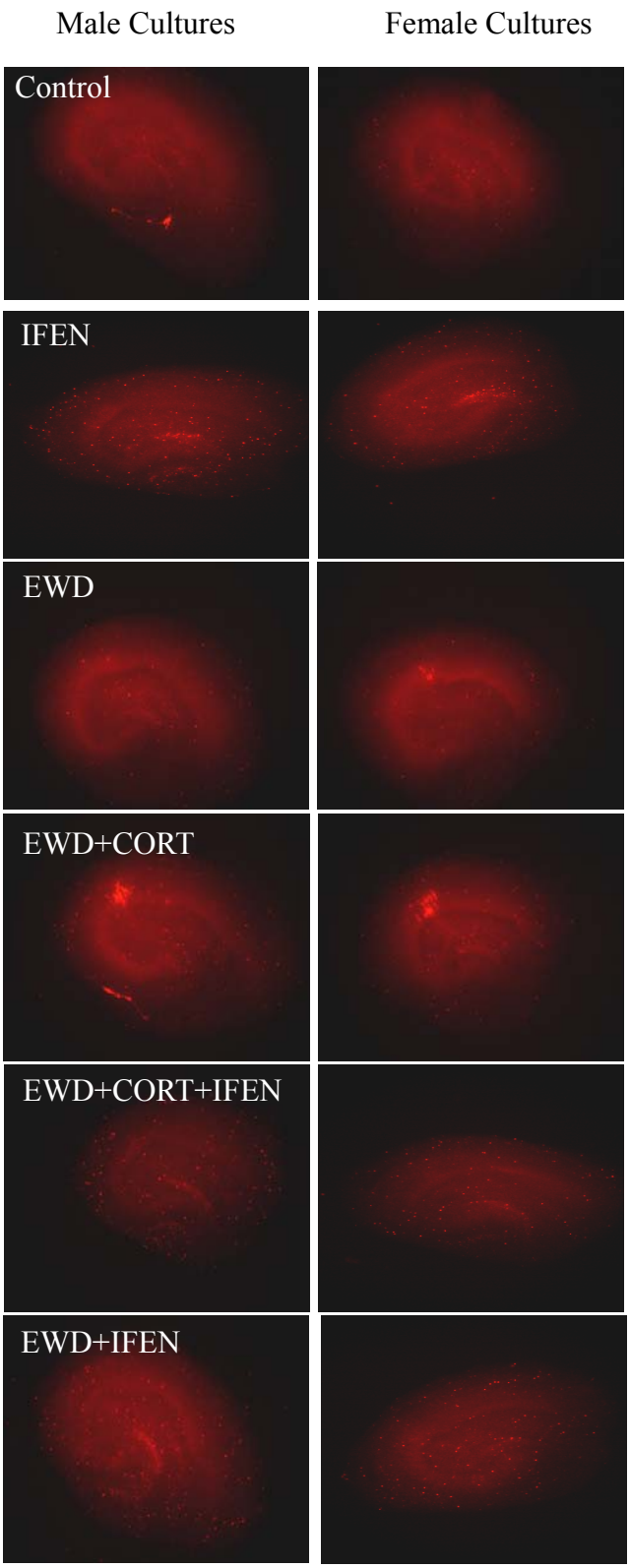

Figure 3.6. Representative images of PI uptake in cultures co-exposed to CORT (0.1 μM) and ethanol for 10 days followed by 24 hour ethanol withdrawal (EWD) and ifenprodil (IFEN) expoure.

Table 3.1. Propidium iodide uptake following 24 hour ifenprodil exposure in ethanol-naïve and ethanol withdrawn hippocampal cultures. Slight toxicity was only observed within the DG. Data are represented as percent control ± SEM. *P < 0.05 vs. ethanol withdrawal; #P<0.05 vs. control.

	24h ifenprodil	**24h ifenprodil during ethanol withdrawal**
DG	120.4 ± 4.8#	115.00 ± 4.6*
CA3 (Males)	110.6 ± 6.9	102.9 ± 6.6
CA3 (Females)	88.7 ± 4.7	77.7 ± 3.5
CA1	103.9 ± 5.7	121.6 ± 11.9

Immunohistochemistry Following 10 Day Ethanol Exposure: NMDA NR2B Receptor Subunit

It was hypothesized that chronic (10 day) ethanol exposure (10 days; 25, 50, or 100 mM) would result in increased abundance of NMDA NR2B receptor subunit protein expression, as previous data have shown increased abundance of the NR2B subunit protein with 10 day exposure to 100 mM ethanol in organotypic hippocampal cultures, though these slices were twice as thick (400 μM) as the slices used in the current studies (Harris et al., 2003). We hypothesized that adaptive changes that may occur in the NMDA receptor subunit expression over the 10 day ethanol exposure period would make the hippocampus more vulnerable to cytotoxic insult upon removal from ethanol and exposure to CORT. Immunoreactivity was measured in the CA1, CA3 and DG hippocampal regions. A two-way ANOVA (treatment × sex) was conducted within each region. As a main effect of sex was not noted in any region, male and female data were combined and a one-way ANOVA (factor: treatment) was conducted within each region. In the CA1 region, there was no effect of treatment ($F(3,222) = 2.258$, $P = 0.083$). In the CA3 and DG regions, however, there was a significant main effect of treatment (CA3: $F(3, 222) = 3.518$, $P < 0.05$; DG: $F(3,222) = 5.27$, $P < 0.01$). In the CA3 region, 10 day treatment with 25 mM ethanol resulted in a significant increase in NR2B immunoreactivity compared to control cultures and cultures exposed to 50 or 100 mM ethanol (Fisher's LSD post-hoc, $P < 0.05$). In the DG region, 10 day treatment with 100 mM ethanol resulted in a significant decrease in NR2B immunoreactivity compared to control cultures and cultures exposed to 25 mM ethanol (Fisher's LSD post-hoc, $P <$

0.01). (Figure 3.7). The decrease and increase in NR2B immunoreactivity within the DG and CA3 region, respectively, are small effects that are hard to interpret. However, most importantly for the hypotheses governing the current studies, no effect of ethanol (25-100 mM) on NR2B immunoreactivity was observed within the CA1 region. Additionally, the concentration of interest in the current studies (50 mM, ~230 mg/dl ethanol) was not associated with changed NR2B immunoreactivity in any region. Representative images of NR2B immunoreactivity are presented in Figure 3.8.

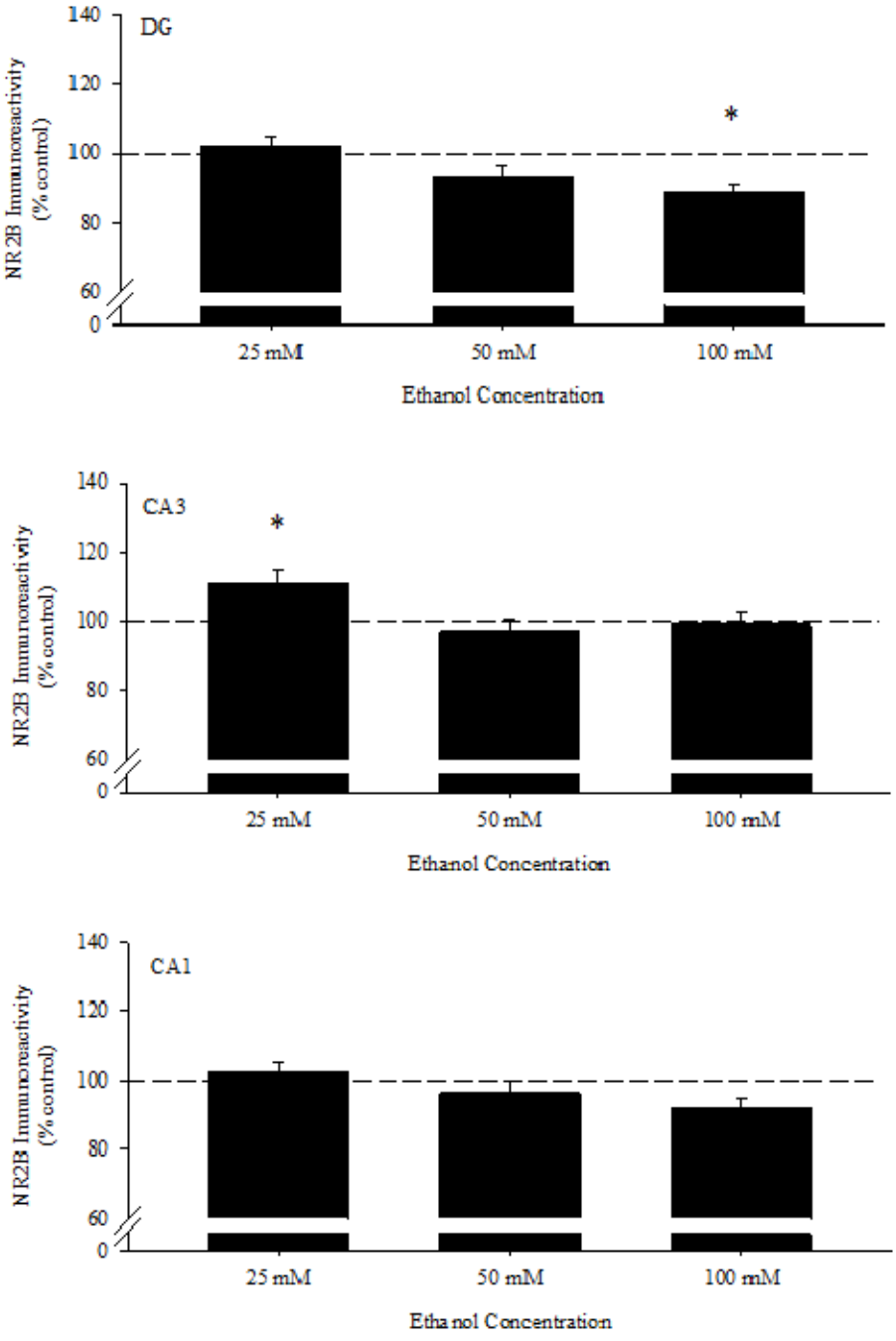

Figure 3.7. NR2B subunit immunoreactivity following 10 day ethanol exposure. *P < 0.05 vs. control.

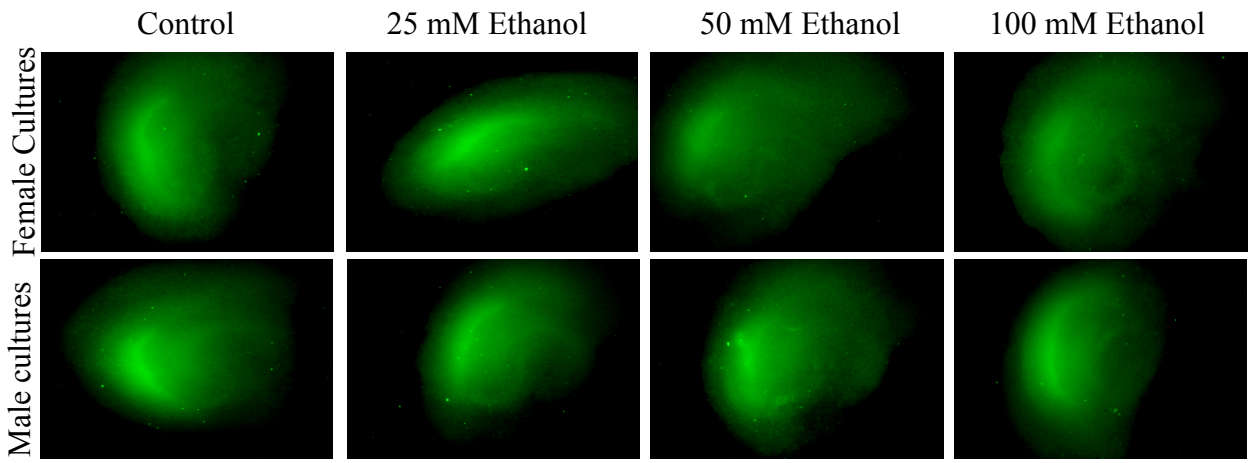

Figure 3.8. Representative images of NMDA receptor NR2B subunit immunoreactitivy in organotypic hippocampal cultures exposed to ethanol (25-100 mM).

NMDA NR2B Subunit Immunoreactivity Following 10 Day Ethanol and Corticosterone Co-Exposure

Immunohistochemistry was conducted to determine if 10 day ethanol and CORT co-exposure resulted in increased NR2B subunit immunoreactivity in organotypic hippocampal cultures. As in the studies described above for measurement of PI uptake, cultures were co-exposed to ethanol (50 mM) and CORT (0.1 μM) for 10 consecutive days before immunohistochemistry was conducted for the NR2B subunit, and immunoreactivity was measured in the primary cell layers of the CA1, CA3, and DG regions. A two-way ANOVA (treatment × sex) was conducted within each region. In the CA1 region there was a main effect of treatment ($F(3,150) = 8.919$, $P < 0.001$) and a main effect of sex ($F(1, 150) = 2.101$, $P < 0.01$). As there was a main effect of sex, male and female data were separated and a one-way ANOVA (factor: treatment) was conducted for male data and for female data. A significant treatment effect was observed in the CA1 region of male cultures ($P < 0.05$). Ethanol and CORT co-exposure resulted in significantly greater NR2B immunoreactivity as compared to ethanol-treated cultures ($P < 0.05$), though the increase in NR2B immunoreactivity in co-treated cultures was not greater than ethanol-naïve cultures exposed to CORT. The increase in NR2B immunoreacitivy following CORT exposure in ethanol-naïve cultures and ethanol and CORT co-exposure resulted in a significant increase above control values (~118% greater than control; $Ps < 0.05$). A significant treatment effect was also observed in the CA1 region of female cultures ($P < 0.001$). Ethanol and CORT co-exposure resulted in significantly greater NR2B immunoreactivity as compared to ethanol exposure alone ($P <$

0.05), though this slight increase was not greater than control values, as ethanol exposure alone resulted in a slight decrease in NR2B immunoreactivity compared to control values (~15%; $P < 0.05$). CORT exposure alone resulted in a slight but significant increase in NR2B immunoreactivity compared to control values (~11%; $P < 0.05$). A two-way ANOVA within the CA3 region indicated a significant main effect of sex ($F(1, 150) = 4.074$), such that across treatments, immunoreactivity was higher in male cultures compared to female cultures (~7% greater). As there was a sex effect, male and female data were separated and a one-way ANOVA (factor: treatment) was conducted for both male data and female data; however, a treatment effect was not detected in either male or female cultures ($F(3, 78) = 1.986$ for male data; $F(3,71) = 1.295$ for female data). Within the DG, a two-way ANOVA indicated a significant effect of treatment ($F(3, 150) = 2.736$, $P < 0.05$). As there was no effect of sex, male and female data were combined and a one-way ANOVA (factor: treatment) was conducted. Although the two-way ANOVA indicated a statistically significant main effect of treatment (but no interaction), the one-way ANOVA did not indicate a statistically significant main effect of treatment after combining male and female data ($F(3, 150 = 2.601$; $P = 0.054$). (Figure 3.9). Representative images of NR2B immunoreactivity are presented in Figure 3.10.

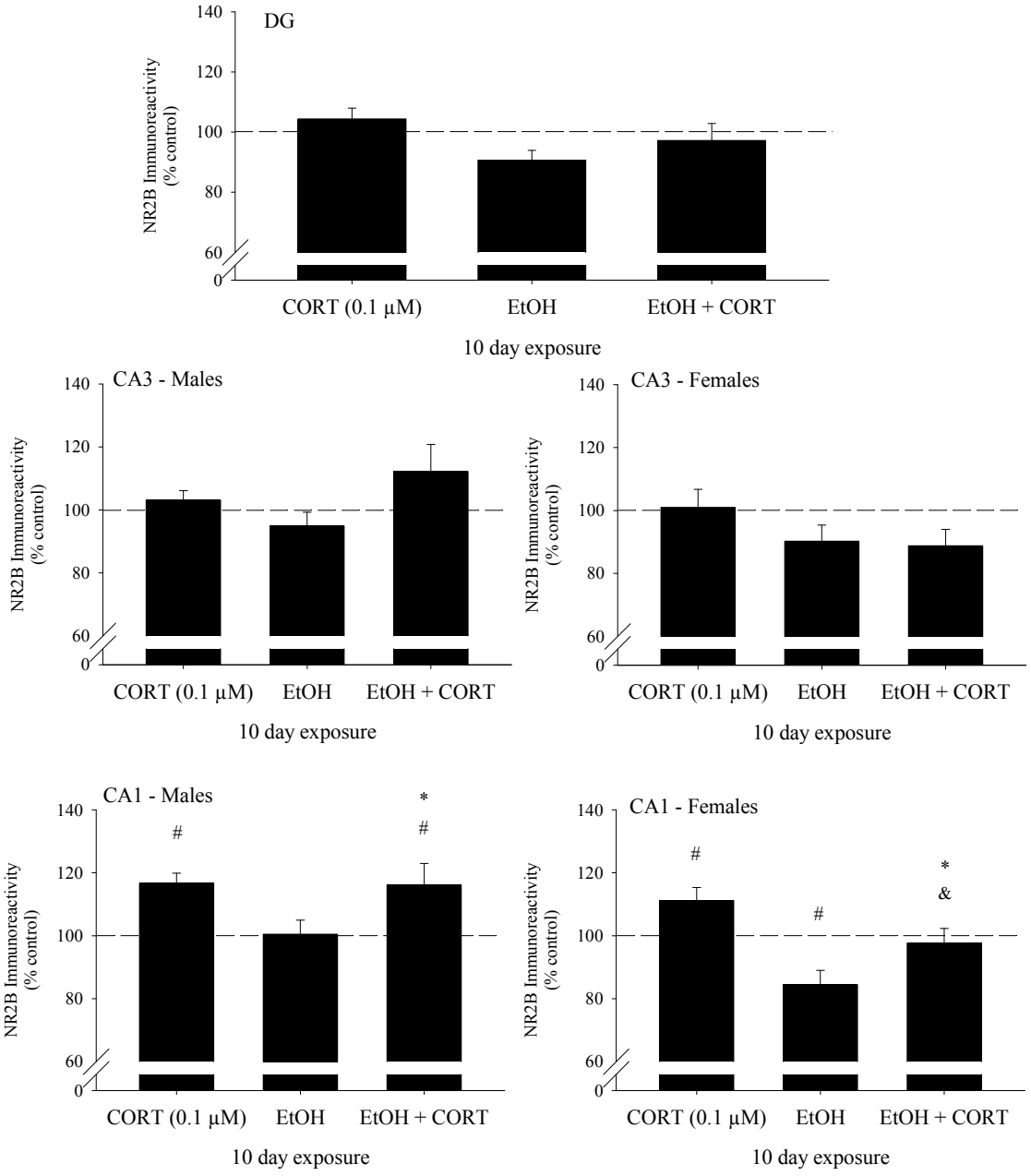

Figure 3.9. NR2B subunit immunoreactivity following 10 day ethanol (50 mM) and CORT (0.1 μM) co-exposure for 10 days. Slight but significant increases in NR2B immunoreactivity were detected in the CA1 region with 10 day exposure to CORT, though this increase was not greater following CORT and ethanol (EtOH) co-exposure. #P < 0.05 vs. control; *P < 0.05 vs. ethanol; & P < 0.05 vs. CORT alone.

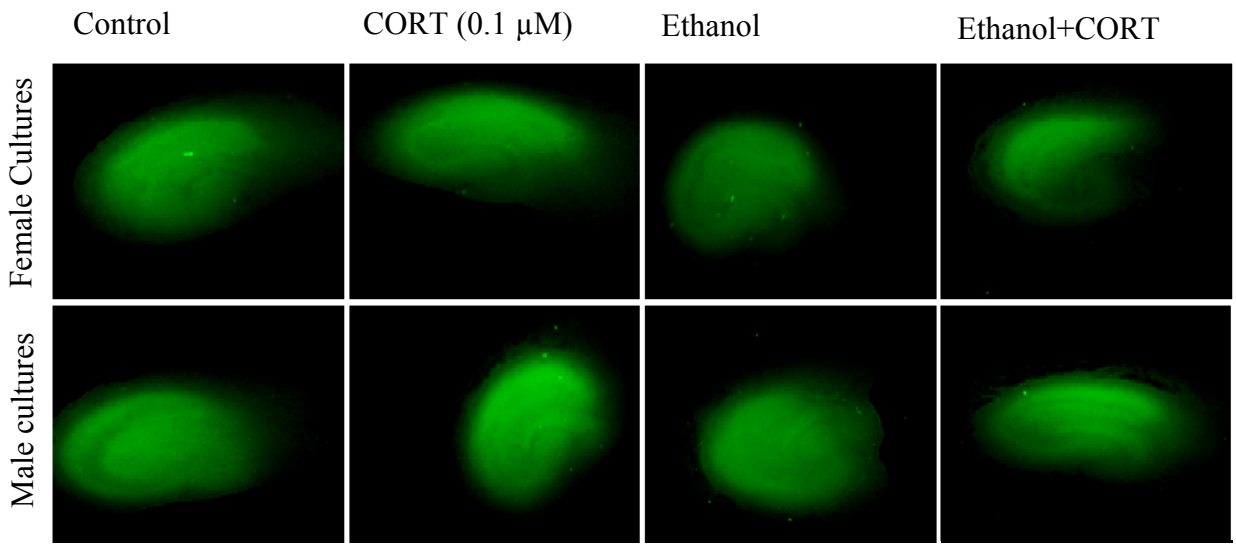

Figure 3.10. Representative images of NR2B immunoreactivity in cultures co-exposed to CORT (0.1 μM) and ethanol (50 mM) for 10 days.

[^{3}H]Ifenprodil Autoradiography

The current studies hypothesized that activation of polyamine-sensitive NMDA NR2B receptors would mediate hippocampal injury following ethanol withdrawal after long-term co-exposure to CORT and ethanol, and this effect would be most marked in the CA1 region. NR2B subunit immunoreactivity after cultures were ethanol and CORT co-exposed for 10 days, however, indicated relatively small effects related to changes in NR2B immunoreactivity in CORT-exposed cultures that were not potentiated by ethanol co-exposure. Therefore, to further address this hypothesis, another subset of male and female hippocampal cultures was exposed to CORT (0.1 μM) and ethanol (50 mM) for 10 days for autoradiographic binding with [^{3}H]ifenprodil. Hippocampal regions were not distinguishable upon film development, and therefore slices were analyzed using densitometric measurement of pixel intensity for the entire slice. A two-way (treatment × sex) ANOVA indicated a main effect of treatment ($F(3, 103) = 27.509$, $P < 0.001$), but no significant interaction nor main effect of sex. Therefore, male and female data were combined and converted to percent control. A one-way ANOVA (factor: treatment) indicated a main effect of treatment ($F(3, 103) = 27.1145$, $P < 0.001$). Cultures exposed to CORT resulted in significantly less [^{3}H]ifenprodil binding compared to all other treatment groups (Fisher's LSD post-hoc, $Ps < 0.001$), showing nearly a 50% reduction in binding below control values. Figure 3.11. Representative images of [^{3}H]ifenprodil-labeled slices are presented in Figure 3.12.

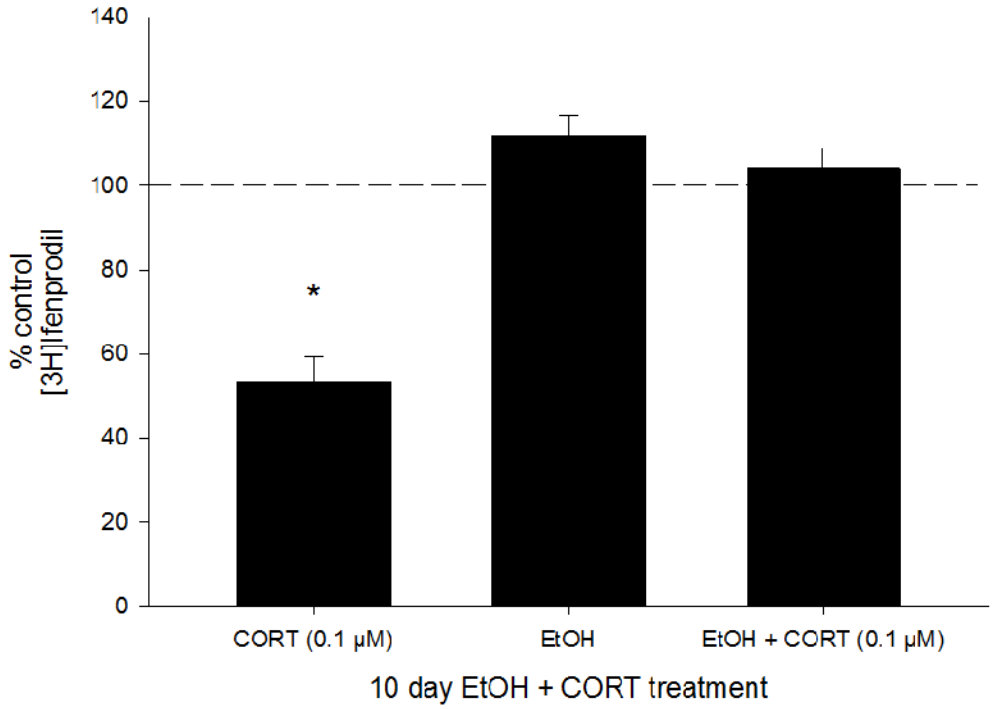

Figure 3.11. [^{3}H]Ifenprodil autoradigraphy following 10 day ethanol (EtOH; 50 mM) and CORT (0.1 µM) co-exposure.

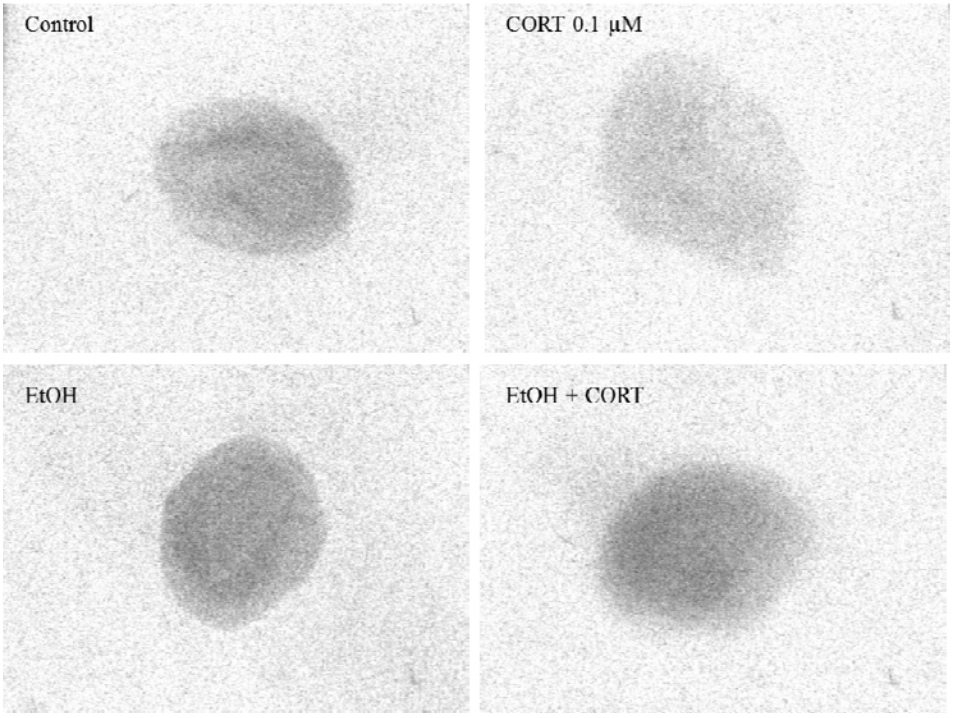

Figure 3.12. Representative autoradigraphy images of organotypic hippocampal cultures exposed to [^{3}H]Ifenprodil following 10 day CORT and ethanol (EtOH) co-exposure. Films were allowed 8 weeks before developing.

Western Blotting: NMDA NR2B Receptor Subunit

In addition to immunohistochemistry and autoradiography studies, Western blots were conducted for quantification of changes in hippocampal NMDA NR2B subunit density following 10 day exposure to ethanol and/or CORT. An additional subset of cultures was also exposed to RU-486. Organotypic hippocampal slices were homogenized for analysis, thus not allowing for measurement of NR2B immunoreactivity in separate hippocampal regions. As a two-way ANOVA (treatment × sex) did not indicate a significant interaction nor main effect of sex, male and female data were combined and a one-way ANOVA was conducted (factor: treatment). No significant main effect of treatment was observed ($F(5,45) = 0.894$, $P = 0.485$). Though not statistically significant, it is notable that changes in NR2B immunoreactivity were in the expected direction in all treatment groups. For instance, we hypothesized that increases in NR2B immunoreactivity may occur in ethanol-exposed cultures, but regardless, CORT and ethanol co-exposure would further increase NR2B immunoreactivity. The current data show 16% greater immunoreactivity in ethanol and CORT co-exposed cultures compared to cultures only exposed to ethanol; an increase that was reduced with the addition of RU-486. (Figure 3.13). Figure 3.14 contains a representative images of a Western blot for NMDA NR2B receptor subunit immunoreactivity.

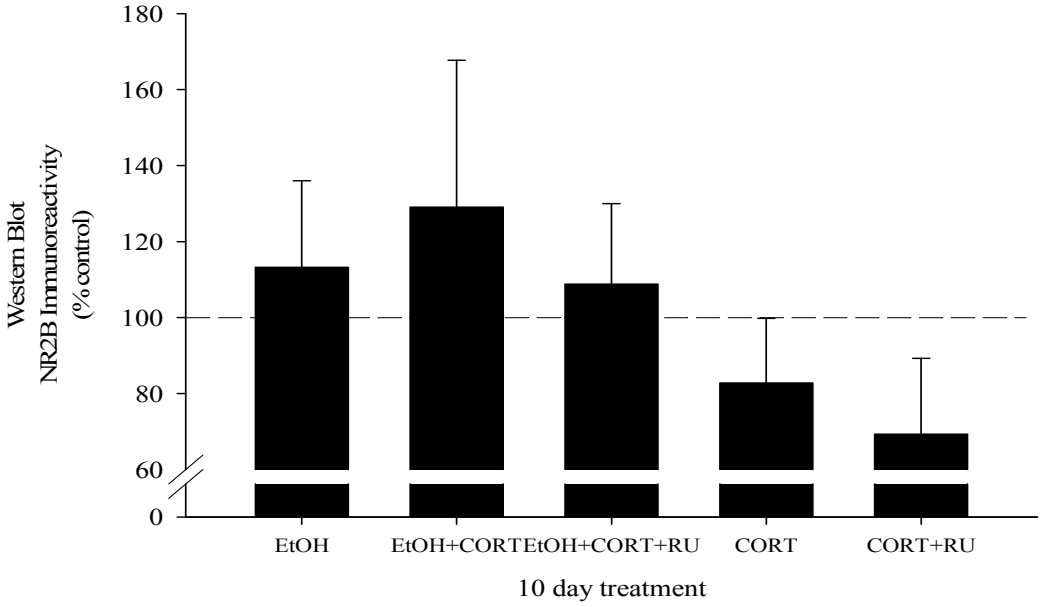

Figure 3.13. Western blot analysis of NR2B subunit immunoreactivity following 10 day drug exposure. No statistically significant changes were observed following 10 day drug treatment.

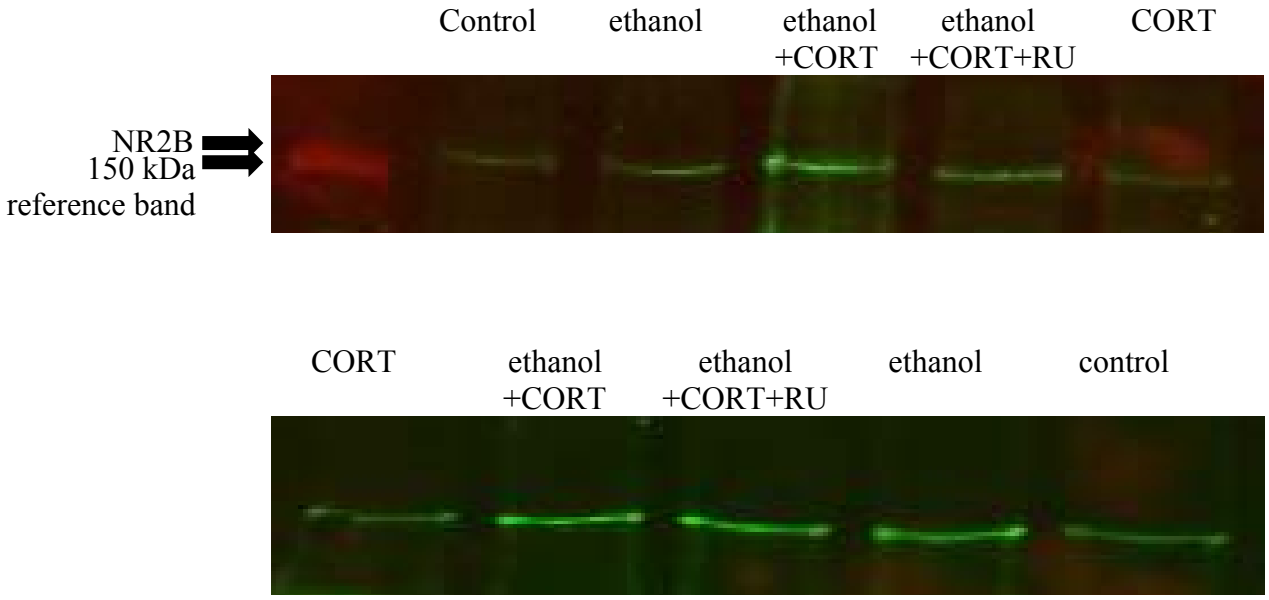

Figure 3.14. Representative images of Western blot immunoreactivity for the NMDA NR2B subunit.

BSA-Conjugated Corticosterone in Ethanol-Naïve and Ethanol-Withdrawn Cultures

An additional series of studies was conducted to examine the effect of 10 day exposure to BSA-conjugated CORT on PI uptake in male and female hippocampal cultures. Cultures were exposed to BSA-CORT (0.1 μM) for 10 days or co-exposed to BSA-CORT and ethanol for 10 days before continued exposure to BSA-CORT during 24 hour ethanol withdrawal. A two-way ANOVA (treatment × sex) was conducted for each hippocampal region. Within the CA1 region, there was a main effect of sex ($F(1, 171) = 9.542$, $P < 0.01$), such that PI uptake was greater in male cultures compared to female cultures. Subsequent one-way ANOVAs were conducted for male data and female data separately (factor: treatment); however, there was no effect of drug treatment on PI uptake for either male or female cultures. A similar pattern was observed in the CA3 region, such that there was a main effect of sex ($F(1, 171) = 6.610$, $P < 0.05$) due to greater PI uptake in male cultures compared to female cultures; however, one-way ANOVAs indicated that there was no effect of drug treatment on PI uptake in either male or female cultures. In the DG, a two-way ANOVA showed that there was no significant interaction, nor main effect of either sex or treatment. These data suggest that effects of CORT and ethanol co-exposure on PI uptake are not related to putative cell-membrane bound CORT receptors (Figure 3.15). Representative images of PI uptake are presented in Figure 3.16.

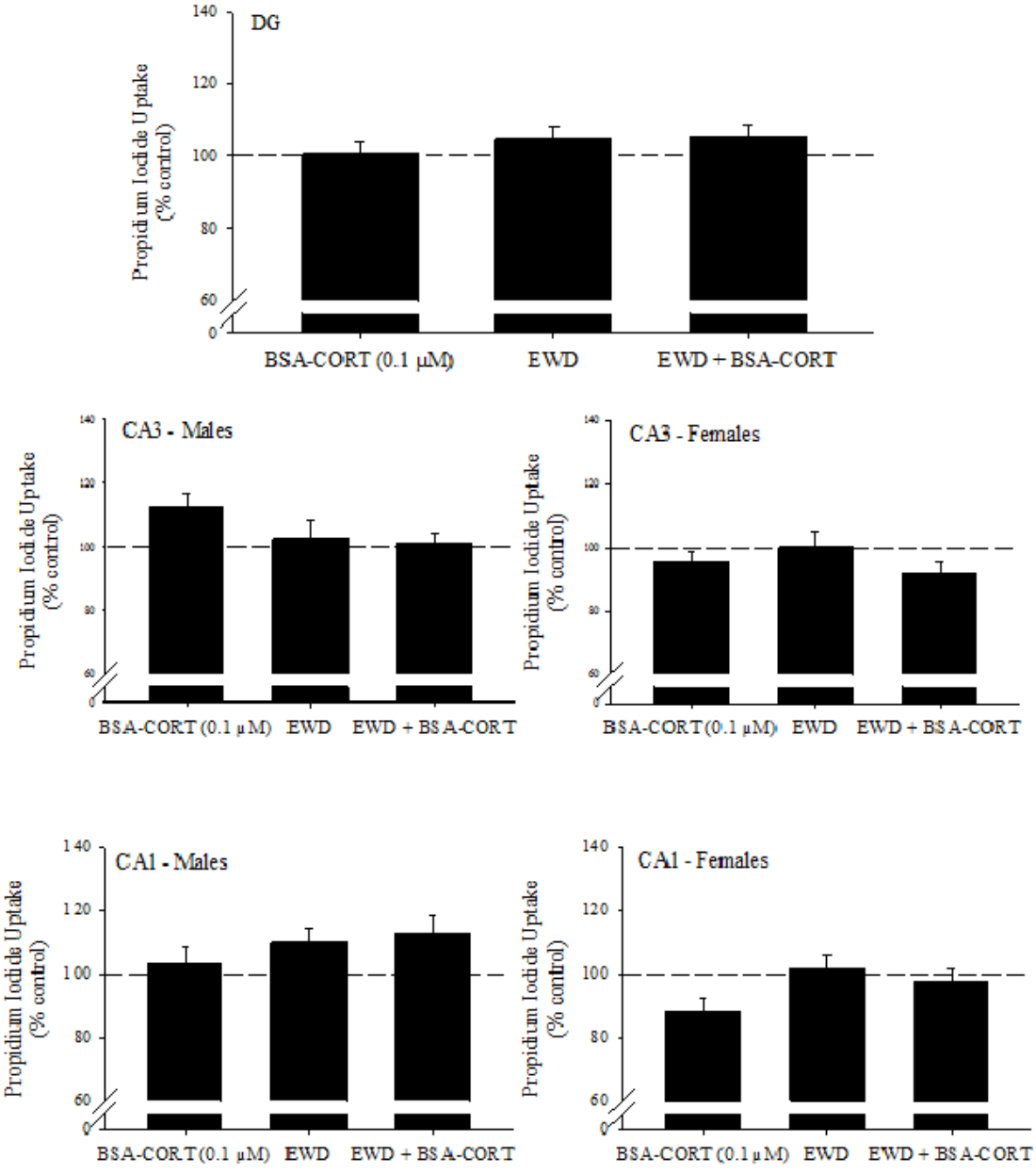

Figure 3.15. Propidium iodide uptake in hippocampal cultures exposed to BSA-CORT (0.1 µM) for 11 days or co-exposed to BSA-CORT and ethanol for 10 days followed by 24 hour ethanol withdrawal (EWD).

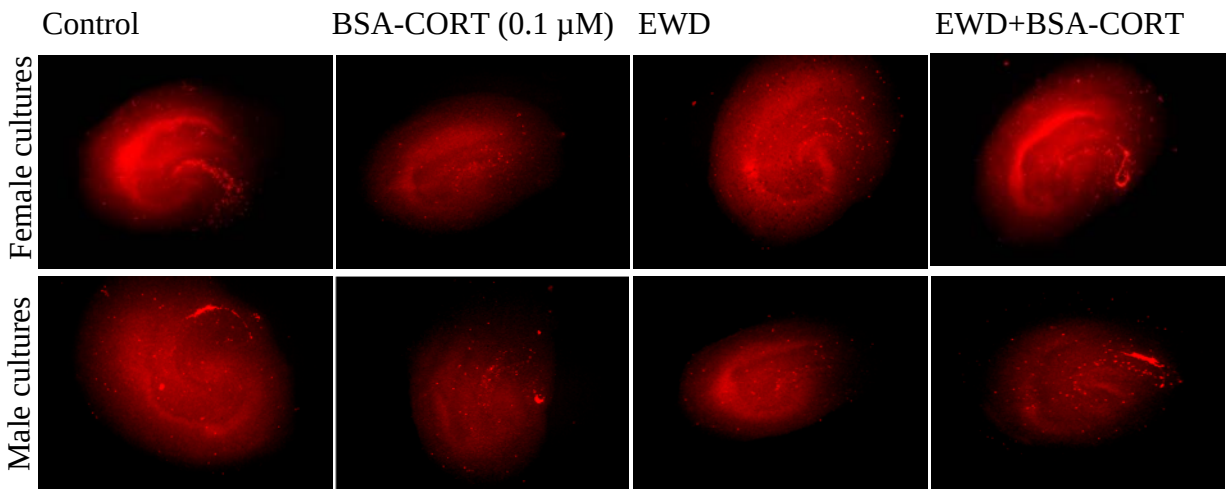

Figure 3.16. Representative images of PI uptake following 11 day BSA-CORT (0.1 µM) exposure in ethanol-naïve and ethanol-withdrawn (EWD) cultures.

Chapter 4

DISCUSSION

NMDA NR2B Subunit-Dependent Toxicity During Withdrawal Following Ethanol and Corticosterone Co-Exposure

Chronic alcohol exposure and withdrawal are related to numerous deleterious consequences. In particular, CNS-related damage is associated with persisting deficits in cognitive functioning, with a greater number of withdrawal episodes positively correlating with greater long-term neurologic function (Duka et al., 2003). Chronic alcohol intake and withdrawal is also related to perturbations in HPA axis functioning in humans and rodents. Exogenous administration of CORT and ethanol exposure and withdrawal have been independently shown to affect signaling of glutamatergic NMDA receptors, in such a way as to make the brain more vulnerable to injury. In particular, increased abundance and/or function of the polyamine-sensitive NMDA NR2B subunit has been shown to be an important mediator of ethanol withdrawal-related neuronal damage and excitability *in vitro*, and also an important mediator of ethanol-related learning deficits *in vivo* (Butler et al., 2010; Prendergast et al., 2000; Thomas et al., 2004). CORT exposure has also been shown to increase NMDA NR2B receptor subunit expression (Meyer et al., 2004). The NR2B subunit is unique from other NMDA receptor subunits in that its inclusion results in greater open channel time and Ca^{2+} influx (Chen et al., 1999). Also, the NR2B subunit contains a number of modulatory binding sites, including an N-terminal site for endogenous polyamine binding, that further potentiates

open channel time. Ethanol exposure and withdrawal, as well as CORT exposure, also result in increased polyamine abundance, thus providing a mechanism for potentiated excitotoxic damage during ethanol withdrawal that is mediated by the NMDA NR2B subunit following long-term ethanol and CORT co-exposure (Davidson & Wilce, 1998; Gibson et al., 2003). The current studies examined the effect of long-term CORT and ethanol co-exposure on hippocampal toxicity during ethanol withdrawal using an *in vitro* model of prolonged ethanol exposure and withdrawal. We hypothesized that the CA1 region would be most vulnerable to damage during ethanol withdrawal following 10 day co-exposure. Additionally, we hypothesized that neuroadaptations in polyamine-sensitive NMDA NR2B receptor subunits would occur following long-term ethanol and CORT co-exposure. A final series of studies examined the potential contribution of membrane-bound CORT receptors on damage during withdrawal resulting from ethanol co-exposure.

It is important to note that the concentrations of CORT used in the current studies parallel stress-relevant concentrations that have been measured in rodent plasma, brain tissue, hippocampal extracellular fluid, and human plasma, including CORT concentrations observed following ethanol exposure and withdrawal. However, CORT levels measured directly from brain tissue show differences compared to plasma CORT levels. Basal concentrations of free CORT measured in hippocampal dialysate of unstressed adult male rats approximate 0.01-0.1 μM (Penalva et al., 2003), thereby activating MRs (K_d ~ 0.005 uM) with little, if any, activation of GRs (K_d ~ 0.05 μM). Little et al. (2008) reported that in the hippocampus of male mice, 3 week ethanol diet

without withdrawal did not increase CORT compared to control mice. However, when the 3 week drinking period (~20-24 g/kg/day alcohol) was followed by 6 week ethanol withdrawal, hippocampal CORT levels were doubled in magnitude compared to control rats. Plasma CORT levels were also measured, and interestingly, neither free nor total plasma CORT levels differed between ethanol drinking and control mice at any timepoint (Little et al., 2008). In a paradigm of prolonged exposure to ethanol fluid (20 weeks; ~15-20 g/kg/day) using female C57/BL10 mice, ethanol drinking mice had a significantly greater CORT concentration in hippocampal tissue (up to 50 ng/g), as well as in total and free plasma CORT levels (total: 250 nM; free: 35 nM). Additionally, 6 day ethanol withdrawal resulted in greater CORT concentration in hippocampal tissue (~75 ng/g), but not plasma (back to baseline and equivalent to control, ~100 nM) (Little et al., 2008). In adult male rats, measurement of total plasma CORT after long-term ethanol drinking rats (3 weeks; BEC ~140 mg/dl) peaks at approximately 60 ng/ml (~0.2 μM) (Rasmussen et al., 2000). CORT administration is also able to significantly potentiate alcohol withdrawal seizures in mice (Roberts, Crabbe, & Keith, 1994). These data suggest that duration of ethanol exposure and withdrawal(s) may be critical in the degree of HPA axis adaptation measured by CORT. One caveat to CORT measurement in plasma is that free or total CORT levels may be reported. Total CORT levels include free-CORT and bound-CORT (CORT bound to CORT binding globulin). Bound-CORT, however, cannot cross the blood brain barrier, and MR and GR activation in the CNS is accomplished by free CORT. It has been reported that levels of CORT binding globulin are not changed after long-term ethanol intake in adult mice following an ethanol exposure regimen that does result in increases in CORT concentration during both ethanol exposure and withdrawal,

suggesting greater availability of CORT to bind its receptors (Tabakoff, Jafee, & Ritzmann, 1978). It is important to note that differences in brain and plasma CORT exist in preclinical literature; however, this suggests that difference in peripheral CORT levels likely represent robust group differences. This reinforces the potentially great impact of exaggerated CORT responses on neuronal function in alcohol dependent humans, as several studies have noted marked increases in salivary and plasma CORT levels in alcohol-dependent individuals compared to control subjects (Adinoff et al., 1991; Adinoff et al., 2003). When comparing adult male human alcoholics to control subjects, plasma CORT levels are approximately twice as great in alcoholic subjects during chronic ethanol intake and after 24 hours of ethanol withdrawal, though CORT levels decrease and match control subject CORT levels following 7 days of abstinence (Kutscher et al., 2002).

Regional Differences in Hippocampal Toxicity Produced by 11 Day Corticosterone Exposure in Ethanol-Naïve Cultures

The current data demonstrate that 11 day exposure to CORT (0.01 – 1 μM) results in slight, but significant, toxicity within all three hippocampal regions. Toxicity was most prominent in the pyramidal cell layer of the CA3 region, with exposure to all concentrations of CORT resulting in increased PI uptake by approximately 20-30% above control values. This was in contrast to the slight, but significant, toxicity observed in the granule cell layer of the female DG with exposure to CORT and the minimal toxicity observed in the CA1 region with exposure to CORT. Regional differences in PI

uptake following 11 day CORT exposure is not entirely surprising, as each hippocampal region has been noted for particular vulnerability to certain types of insult. For instance, CA3 region pyramidal cells are most vulnerable to damage with kainic acid exposure, perhaps because this region contains the greatest density of kainate receptors (Holopainen et al., 2004; Martens & Wree, 2001); DG granule neurons are most vulnerable to cell death following binge ethanol exposure (Obernier et al., 2002); and CA1 pyramidal neurons are most vulnerable to cell death in models of excitotoxicity, including ethanol withdrawal (Butler et al., 2010; Prendergast et al., 2004).

In regard to the current dataset, increased susceptibility to damage within the CA3 hippocampal region is in agreement with *in vivo* studies of long-term CORT exposure that have shown extensive damage to dendritic spines and reduced neuron size within the CA3 region compared to other hippocampal regions. These data demonstrate that high levels of CORT are detrimental to neuronal integrity by decreasing neuron number and by altering neuronal morphology (Woolley et al., 1990). Similarly to effects produced by long-term exposure to CORT, long-term exposure to repeated stress also results in atrophy of CA3 pyramidal neuron dendrites (Watanabe, Gould, & McEwen, 1992a; Watanabe et al., 1992b). Greater vulnerability of CA3 region neurons to damage and/or cell death with prolonged CORT exposure could be attributable to GR distribution among the hippocampal regions. GRs are least abundant in the CA3 region as compared to the CA1 and DG regions. It is possible that concentrations of CORT that saturate GRs in the CA3 region do not saturate GRs in the CA1 and DG regions (Sarabdjitsingh et al., 2009). It is not clear in the model used in the current studies how GR density may be affected in

each hippocampal region by long-term CORT exposure, though high doses of CORT *in vivo* (4 day exposure; ~245 ng/ml plasma CORT; ~0.7 μM) have been documented to reduce mRNA for GRs in the CA1 region (Herman & Spencer, 1998). No change in protein expression of GRs (Herman & Spencer, 1998) or downregulation of GR protein and mRNA has been noted in the hippocampus following long-term CORT treatment or stress *in vivo* or *in vitro* (Sapolsky et al., 1995). MR mRNA and protein expression has also been noted to decrease with prolonged CORT exposure (Hugin-Flores et al., 2004; Xu et al., 2010). Pyramidal neurons of the CA3 region also lack immunoreactivity for the Ca^{2+} binding proteins Calbindin-D28k and parvalbumin, perhaps increasing vulnerability to CORT-related damage by Ca^{2+} influx (Sloviter et al., 1989).

Minimally increased injury was observed in CA1 region pyramidal neurons with 11 day CORT exposure compared to control cultures. CA1 region damage has been demonstrated after long-term *in vivo* CORT treatment (40 mg/kg/day for 3 weeks) that is similar to the damage observed in CA3 region neurons, such that the CA1 pyramidal neurons had disrupted dendritic morphology, reduced spine number, and shorter dendrite length (Morales-Medina et al., 2009; Sapolsky et al., 1985). However it should be noted that *in vivo* paradigms have yielded inconsistent results in regard to CA1 neuronal damage across studies. This may be due largely to duration and dose of CORT treatment, with longer duration and/or higher dose or concentrations of CORT required for overt neuronal loss. The slight CA1 damage that was observed in this model could also be attributable to sustained stress-relevant CORT levels that directly affected CA3 neuronal integrity and synaptic signaling, leading to increased damage in CA1 and DG regions in

this model, as has been suggested by previous investigators (Morales-Medina et al., 2009). The current studies required cultures to remain *in vitro* for 16 days before measurement of cell death, and it is possible that network connectivity let to enhanced excitation that was propagated along the trisynaptic circuit without regulation by afferent or efferent connections to modulate synaptic transmission between the cell layers. The organotypic hippocampal cell culture model has the advantage of maintaining the trisynaptic circuitry of the hippocampal layers and synaptic transmission remains largely intact relative to the *in vivo* situation. However, it has been shown that, unlike the *in vivo* environment, CA1 pyramidal axons begin to make extensive connections with granule cells of the DG after only one week *in vitro* (Gutierrez & Heinemann, 1999).

High concentrations of CORT, thereby activating GRs, may also be detrimental due to increased NMDA receptor subunit mRNA and NMDA receptor abundance (measured by [^{3}H]MK-801 binding). Specifically, 10 day CORT exposure *in vivo* (pellet implants; 30-35ug/dl plasma CORT levels) increases mRNA for NR2A and NR2B receptor subunits, but not the NR1 subunit, in adult male rat hippocampus. Additionally, CORT administration via drinking water for 10 days results in significant increases in binding of [^{3}H]MK-801 in a hippocampal membrane preparation, suggesting an increase in the overall number of NMDA receptors (Weiland et al., 1997). Acute restraint stress in adult male rats also increases mRNA for NR1 and NR2B, but not NR2A, subunits in the CA3 hippocampal region. A similar pattern is noted in the CA1 region mRNA for the NR2B subunit (Bartanusz et al., 1995). GR activation with dexamethasone exposure or CORT injection *in vivo* is also able to upregulate ODC activity in the hippocampus of

adult male mice, resulting in increased polyamine biosynthesis and providing for increased allosteric potentiation of NMDA receptor activity (Cousin et al., 1982; González Deniselle et al., 1997). GR-dependent upregulation of ODC in peripheral tissue has been demonstrated in a model of early life stress by food deprivation/isolation in pre-weaned rat pups, showing that increases in serum CORT concentration precedes significant increases in ODC measured in intestinal mucosa. Importantly, administration of the GR antagonist RU-486 blocks this increase. Additionally, exogenous administration of hydrocortisone upregulates ODC mRNA in intestinal mucosa, and this effect is also blocked by co-administration of RU-486 or the ODC inhibitor α-difluoromethylornithine (DFMO) (Nsi-Emvo et al., 1996). ODC mRNA is significantly upregulated in rat pancreatic cells by administration of dexamethasone; an effect that is blocked by co-administration of GR-38486 or an inhibitor of protein synthesis (cycloheximide) (Rosewicz & Logsdon, 1991). Taken together, previous literature has shown a significant effect of CORT on polyamine biosynthesis, thus suggesting that the damage observed in the current studies following long-term CORT exposure may be due, in part, to increased polyamine content and potentiation of NMDA receptor activity.

Unexpectedly, the current data showed sex-selective damage in the DG, such that the female DG was more vulnerable than the male DG to damage by CORT exposure (0.01 – 1 μM). As discussed in the above paragraph, CA1 connections back to DG granule neurons may make the DG more vulnerable to injury *in vitro* than *in vivo*, though it is unclear if this differs between male and female cultures. Alternatively, the DG is uniquely susceptible to altered neuronal density by CORT-induced suppression of

neurogenesis via a reduction in cell proliferation (reviewed by Mirescu & Gould, 2006). Sex differences have been noted regarding effects of CORT on neurogenesis *in vivo* (Brummelte & Galea, 2010). After 21 days of daily CORT administration (40 mg/kg), adult female rats had significant suppression of cell proliferation (significantly fewer Ki67-labeled cells) in the ventral hippocampus compared to female control rats. No effect on cell proliferation was noted in male rats, whereas CORT-treated male rats showed significant reductions in survival of immature neurons compared to male control rats. These data highlight potential sex-dependent mechanisms of plasticity in the rodent hippocampus that may contribute to vulnerability to injury. A mouse model of organotypic hippocampal cultures has shown that 14 DIV is sufficient for visualization and quantification of proliferating cells. Further, though cultures were obtained from neonatal pups, sites of cell proliferation were similar to those observed in vivo in adult rodents, suggesting that sex-dependent effects on neurogenesis observed *in vivo* are relevant to studies in organotypic cultures (Raineteau et al., 2004).

Recent literature has also found marked sex differences in basal circulating CORT levels and GR expression. Ordyan et al. (2008) compared plasma CORT levels and expression of GR isoforms in hippocampal homogenates from Sprague Dawley rat pups ranging from PND 3-30. Their data showed that early in postnatal development (PND 3-15), males have a significantly greater level of plasma CORT than females. This pattern reverses, however, such that females have significantly higher CORT levels from PND 18-30, with CORT levels increasing across the period studied in both male and female rats. Sex differences were also detected in hippocampal GR expression such that

expression of the 94 kDa isoform of the GR receptor was significantly lower (nearly absent) in female hippocampi at PND5 compared to male hippocampi. Female hippocampi also had significantly lower levels of the 82 kDa GR isoform at PND 5-15 compared to male hippocampi. In consideration of the current data, it is not clear why the sex difference in toxicity was observed only in the DG, though the GR expression studies cited above did not compare GR expression among the different hippocampal regions. Sex differences in postnatal ontogeny of GR expression suggests that lower expression of GRs in the female brain may contribute to greater vulnerability to injury in neurons in female cultures. Lower expression of GRs and/or MRs in female cultures relative to male cultures would result in greater non-specific actions by CORT, and perhaps increase increase vulnerability to toxicity through multiple signaling pathways. It is unclear at this time what the functional differences are, if any, of GR isoforms in rat, as other rodent studies do not discuss separate isoforms. Studies of human GR isoforms suggest there may be as many as 16 different isoforms that are able to homo- and heterodimerize to produce tissue-specific actions of CORT, though much work is needed to understand the functional importance of isoform combinations and the relevance to preclinical models (Lu & Cidlowski, 2006).

10 Day Ethanol Exposure: Increased Vulnerability to Toxicity During Ethanol Withdrawal

Toxicity observed with CORT exposure in ethanol-naïve cultures was not completely unexpected given past literature on CORT-related hippocampal damage *in*

vivo. However, one of the primary hypotheses governing the current studies suggests that neuroadaptations occurring during the 10 day ethanol exposure period would render the hippocampus more vulnerable to injury during withdrawal following ethanol and CORT co-exposure. Previous data using this ethanol exposure model (10 days; 50 mM ethanol, ~230 mg/dl; Butler et al., 2008) has shown that this ethanol exposure regimen sensitizes the hippocampus to injury during ethanol withdrawal without producing overt toxicity (Butler et al., 2008; Butler et al., 2009; Self et al., 2004; Self et al., 2005). Using this model, various drugs or receptor ligands have produced significant toxicity during ethanol withdrawal, including the polyamine spermidine; a specific adenosine A1 receptor antagonist (DPCPX); the HIV-1 protein Tat; and beta-amyloid protein (Barron et al., 2008; Butler et al., 2008; Self et al., 2004; Self et al., 2005), thus modeling vulnerability to excitotoxicity that occurs during withdrawal from long-term ethanol exposure. Vulnerability to insult using this model has been shown to be largely dependent on alterations in NMDA receptor function, as toxicity from all of the drugs or ligands noted above was reduced by NMDA receptor antagonism. This model is distinct from that used in previous studies of long-term ethanol exposure in our laboratory with a higher concentration of ethanol (10 days; 100 mM, ~460 mg/dl; Prendergast et al., 2004) that resulted in overt toxicity during withdrawal. Injury during withdrawal from 10 day exposure to 100 mM ethanol was also shown to be NMDA-receptor dependent, and could be reduced by NMDA receptor antagonism, including antagonism of polyamine activation of the NMDA NR2B subunit (i.e. inhibition of polyamine synthesis with DFMO; polyamine-site antagonism with ifenprodil). Additionally, 10 day exposure to 100 mM ethanol significantly increased NR1 and NR2B subunit expression measured by

Western blot analysis (Harris et al., 2003). Therefore, it is clear that 50 mM ethanol exposure for 10 days increases functionality of the NMDA receptor system, resulting in increased vulnerability to insult during ethanol withdrawal, but alterations in NMDA receptor subunit expression that may also contribute to increased vulnerability to toxicity during ethanol withdrawal have yet to be investigated until now in this model of 10 day exposure to 50 mM ethanol followed by withdrawal.

Increased Vulnerability of the CA1 Region to Excitotoxic Insult

Our laboratory demonstrated in a series of studies using this organotypic hippocampal cell culture model that increased vulnerability of CA1 neurons to excitotoxic insult is due, at least in part, to greater density of NMDA NR2B-containing neurons (Butler et al., 2010). This is true in cultures aged to 5 DIV or in cultures exposed to ethanol for 10 days followed by 24 hour ethanol withdrawal (aged to 16 DIV). To demonstrate the importance of polyamine-sensitive NR2B subunits in NMDA toxicity, these studies showed that ifenprodil significantly reduced toxicity from NMDA exposure, and co-exposure to the polyamine spermidine and a sub-toxic concentration of NMDA resulted in markedly enhanced toxicity in cultures (Butler et al., 2010). These data are highly relevant to the current studies, as they reinforce the vulnerability of the CA1 region to NMDA-receptor mediated excitotoxic damage that is modulated by the polyamine binding site located on the NR2B subunit.

Excitotoxic Insult During Ethanol Withdrawal Following Ethanol and Corticosterone Co-Exposure: Polyamine-Sensitive NR2B Subunit-Mediated Toxicity

We hypothesized that toxicity measured after 24 hour ethanol withdrawal would be greatest in the CA1 region following ethanol and CORT co-exposure, and markedly enhanced as compared to ethanol-naïve cultures. Indeed, in the CA1 region, CORT (0.01 – 1 μM) and ethanol co-exposure resulted in significantly greater injury during ethanol withdrawal than injury observed with ethanol withdrawal alone or CORT (0.01 – 1 μM) exposure in ethanol-naïve cultures. We further hypothesized that, as in the excitotoxicity studies cited above, NR2B subunit antagonism at the polyamine binding site would attenuate excitotoxic insult during ethanol withdrawal from ethanol and CORT co-exposure. In addition to increases in function and/or number of NMDA receptors after long-term ethanol exposure that make the hippocampus more vulnerable to injury during withdrawal, increased polyamine abundance (spermidine) and glutamate content has been observed in multiple models of excitotoxicity, including during ethanol withdrawal (Gibson et al., 2003). This may be critical in NMDA-mediated hyperexcitiability and toxicity during ethanol withdrawal via allosteric potentiation of NMDA receptor channel function at the polyamine site located on the N-terminus of the NR2B subunit (Williams et al., 1994). And in accord with this hypothesis, the current data support a role for polyamine-sensitive NR2B subunits in hippocampal injury during withdrawal after 10 day co-exposure to ethanol and CORT, as the addition of ifenprodil during the withdrawal period significantly reduced toxicity produced by ethanol and CORT (0.1 μM) co-exposure. The enhanced damage from ethanol and CORT co-exposure during

ethanol withdrawal in the CA1 region and the preferential expression of polyamine-sensitive NMDA NR2B receptor subunit in this region (Butler et al., 2010) supports the hypothesized importance of the NR2B subunit in mediating damage during ethanol withdrawal after CORT and ethanol co-exposure.

CORT and ethanol co-exposure produced the greatest toxicity in the CA1 region following withdrawal, although significantly greater injury during ethanol withdrawal was noted in DG granule neurons in male cultures (0.01 and 1 μM) that was greater than injury observed following ethanol withdrawal alone and CORT exposure in ethanol-naïve cultures. CA3 region injury produced by CORT exposure was not made worse by co-exposure to ethanol and withdrawal, perhaps due to the significant toxicity observed following CORT exposure in the CA3 region of ethanol-naïve cultures. Damage to DG granule cells in female cultures was also not made worse by co-exposure to ethanol and withdrawal. It is likely that subtle alterations reported after CORT exposure (i.e. changes in dendritic integrity) have major implications for neuronal signaling that contribute to profound neurodegeneration over long-term treatment. Taken together, these results support a hypothesis in which CORT enhances disruptions in glutamatergic signaling produced by long-term ethanol exposure that results in CORT-related neuronal atrophy during ethanol withdrawal. It is unclear why injury was not concentration-dependent in any hippocampal region, though biphasic effects of CORT on neuronal integrity have been noted. For instance, both adrenalectomy and administration of high concentrations of CORT (~.310-.650 μM) potentiate toxicity produced by NMDA exposure in cholinergic neurons (Abraham et al., 2000).

Glutamatergic Signaling Involved in Ethanol and Corticosterone-Related Damage During Ethanol Withdrawal

Previous literature has noted in models of hypoxia/ischemia that even slightly increased CORT levels that do not injure neurons independently increase vulnerability of neurons to NMDA-receptor mediated excitotoxic cell death (Sapolsky & Pulsinelli, 1985). Thus, though high levels of long-term CORT exposure can result in damage on their own, sub-threshold concentrations of CORT can also increase susceptibility to neuronal damage, just as long-term ethanol exposure can increase susceptibility to neuronal damage during ethanol withdrawal; together, ethanol and CORT likely have profound effects on glutamatergic signaling. Converging lines of evidence suggest the importance of glutamatergic signaling in CORT related damage and GR-dependent changes in NMDA receptor function and/or number. Competitive NMDA receptor antagonism ameliorates stress-induced hippocampal dendritic atrophy to a similar degree as inhibition of steroid biosynthesis in adult male Sprague-Dawley rats (Magarinos & McEwen, 1995). Suppression of glutamate release by the Na^+ channel inhibitor phenytoin reverses the reductions in the number of apical dendritic branching points and the total dendritic length produced by chronic restraint stress or chronic CORT administration in the CA3 region of adult male rats (Watanabe et al., 1992b). Of particular relevance to the current studies, previous data using the same co-exposure paradigm in organotypic hippocampal cultures have shown that ethanol and CORT co-exposure results in significant increases in cytosolic Ca^{2+} during ethanol withdrawal that is significantly reduced by GR antagonism with RU-486. These same studies also demonstrated that both

MK-801 and RU-486 significantly reduced toxicity associated with ethanol and CORT co-exposure during withdrawal (Mulholland et al., 2005). Little et al. (2008) noted that though GR membrane expression was reduced after long-term ethanol exposure *in vivo*, nuclear localization of GRs was increased; thus suggesting that long-term ethanol exposure increases GR-related gene transcription. This is highly relevant to the current hypothesis, as these data suggest that damage from ethanol and CORT co-exposure is related to both NMDA receptor activation and GR-dependent effects.

The NR2B Subunit, Polyamines, and Ifenprodil

NMDA receptors are heteromeric ion channels composed of an obligatory NR1 subunit and some combination or NR2 and/or NR3 subunits. NR2A and NR2B subunits are the most abundant NR2 subtypes found in the hippocampus, with the presence of the NR2B subunit allowing greater open-channel time and Ca^{2+} influx (Chen et al., 1999). Polyamines have been noted to have stimulatory and inhibitory actions on NMDA receptor currents, with stimulation resulting in potentiation of open channel time and increased binding of the NMDA receptor channel blocker MK-801 (Ransom & Stec, 1988). Stimulatory effects of polyamines at NMDA receptors expressed in oocytes are mediated by NR1A/NR2B heteromers, but not by NR1 homomers or NR1A/NR2A or NR1/NR2C heteromers (Williams et al., 1991; Williams et al., 1994). Seemingly contradictory actions of polyamines on channel function may be attributable to the model being studied; however it seems clear that polyamine concentrations increase during period of excitotoxicity as an adaptive protective response and likely become cytotoxic

given the right conditions (i.e. increased NR2B abundance from ethanol co-exposure) (Davidson & Wilce, 1998). Models of excitotoxicity (e.g. ischemia) have shown changes in extracellular pH such that the environment becomes more acidic and results in greater extracellular proton concentration (Giffard et al., 1990). It has been shown that protons in the extracellular environment tonically inhibit NMDA receptor channels at a site located on the NR1 subunit (Traynelis & Cull-Candy, 1990; Traynelis et al., 1995), with NR1a/NR2B-containing NMDA receptors displaying the highest pH sensitivity relative to receptors containing other NR2 subunits (IC_{50} ~ pH 7.4; Traynelis et al., 1995). Polyamines are believed to have stimulatory actions at the NR2B subunit by relief of inhibition by extracellular protons (Traynelis, Hartley, & Heinemann, 1995).

It is pertinent to note that polyamines do not act selectively at NR2B subunits, but they also modulate activity of inward-rectifying K^+ channels (Yan et al., 2005); L-type Ca^{2+} channels (Herman et al., 1993); perhaps bind to a distinct site on the NMDA NR1 subunit (Stoll et al., 2007); and activate α1-adrenergic receptors (Chenard et al., 1991). Ifenprodil is also non-specific for the NMDA receptor, as it is able to inhibit 5-HT_3 receptors (IC_{50} value ~ 1.3 μM; McCool & Lovinger, 1995); inhibit sigma receptors (Karbon et al., 1990); inhibit G-protein activated inwardly rectifying potassium channels (GIRKs; Kobayashi et al., 2006); and inhibit of high voltage-activated Ca^{2+} channels (IC_{50} value ~ 17 μM; Church et al., 1994). However, in regard to NMDA receptor antagonism by polyamines, ifenprodil antagonizes spermidine enhancement of NMDA receptor channel blocker binding (Carter, Rivy, & Scatton, 1989) and only inhibits NR2B-containing NMDA receptors (Williams et al., 1991; Williams et al., 1994).

Ifenprodil is a non-competitive inhibitor of polyamine binding, and has been suggested to confer inhibition by stabilizing an inactive receptor formation (Reynolds & Miller, 1989). Reynolds and Miller (1989) reported that an ifenprodil concentration of 300 nM (0.3 μM) was necessary to produce consistent inhibition of Ca^{2+} influx resulting from NMDA and glycine exposure in rat cortical neurons, suggesting that although polyamines and ifenprodil have mechanisms of action that are not specific to NMDA receptors, the concentration of ifenprodil used in the current studies (100 μM) was appropriate given the desired effect of reducing NMDA receptor-mediated excitotoxic cell death during ethanol withdrawal.

NMDA NR receptor subunits have distinct developmental profiles. NR2B subunit mRNA is present in the embryonic rat brain and expression levels are high after the first week of birth. At approximately PND 7-10, NR2B subunit expression declines whereas NR2A subunit expression rises, such that NR2A subunits are more abundant relative to NR2B subunits (Laurie et al., 1997; Monyer et al., 1994). However, *in vitro* ethanol exposure during this developmental period disrupts this change in expression of NR2A and NR2B such that NR2B subunit abundance does not decrease and NR2A subunit abundance does not increase, thus resulting in expression of NR2B subunits that remains greater than that of NR2A subunits (Snell et al., 2001). This is important because NR2B subunits relative to NR2A subunits result in greater Ca^{2+} influx, and therefore the presence of NR2B subunit increases vulnerability to excitotoxic insult. The developmental shift in NR subunit expression presumably occurred in our slices before ethanol exposure was initiated, as NMDA receptor subunit aging *in vitro* is similar to *in*

vivo NMDA receptor subunit expression in adult rats (Martens & Wree, 2001). Polyamine potentiation of MK-801 binding also changes throughout development; however, adult-like polyamine potentiation is observed by PND 10 (Williams et al., 1991). Using the same organotypic hippocampal culture model as in the current studies, Barron et al. (2008) demonstrated that cultures taken from PND 2 rats were much more sensitive to cell death following 10 day ethanol exposure and withdrawal than cultures taken from PND 8 rat pups. This effect was likely due, at least in part, to greater polyamine-sensitive NR2B subunit expression at PND 2 compared to PND 8. Toxicity observed in PND 2 cultures after ethanol withdrawal was significantly potentiated by the polyamine spermidine, with ifenprodil co-exposure reversing the effect of spermidine. These data further support the importance of polyamine-sensitive NR2B receptors in excitotoxic hippocampal damage.

In addition to reducing toxicity *in vitro* via antagonism of the NR2B subunit, *in vivo* antagonism of the NR2B subunit reduces ethanol withdrawal beahviors in rodents. Malinowska et al. 1999 showed that administration of ifenprodil during withdrawal from chronic ethanol exposure (13 days) significantly reduces the severity of handling induced withdrawal seizures in mice without affecting ethanol levels measured in plasma or brain tissue. Similarly, Narita et al. (2000) showed that ifenprodil administration during withdrawal from 5 days ethanol drinking (5% ethanol solution) significantly reduced seizure activity 6-12 hours following the removal of ethanol. Western blot analysis also revealed a significant upregulation of NR2B protein expression immediately after cessation of ethanol intake and following 9 hours of ethanol withdrawal. Administration

of another NR2B-polyamine site antagonist similar in structure to ifenprodil (eliprodil) during ethanol withdrawal also reduces seizures (Kotlinska & Liljequist, 1996) and reverses the impairing effects of ethanol on spatial learning (Thomas et al., 2004).

Polyamines are essential in cell growth and differentiation (Slotkin & Bartolome, 1986), and polyamine concentrations increase during excitotoxic events (Adibhatla et al., 2002). The increase in polyamine content during excitotoxicity is likely an adaptive response to alleviate cell damage (Davidson & Wilce, 1998). However, in a model of chronic ethanol exposure in which NR2B subunits are upregulated, polyamine potentiation at the NR2B subunit may result in the opposite of an adaptive response by allowing for pathological levels of Ca^{2+} influx and neurotoxicity via allosteric potentiation of NMDA receptor function. The rate-limiting enzyme in polyamine synthesis is ornithine decarboxylase (ODC). CORT exposure has been documented to increase the expression of ODC in the brain (Cousin et al., 1982). ODC expression is also increased progressively over 15 day ethanol exposure regimen that results in dependence in male rats, thus resulting in a two-fold increase in polyamine content within the hippocampus. Blockade of ODC activity also significantly reduces ethanol withdrawal seizures (Davidson & Wilce, 1998). Given these previous studies, the current data showing that ifenprodil reduces toxicity during ethanol withdrawal produced by CORT and ethanol co-exposure suggests that toxicity is specifically mediated by a polyamine-sensitive NR2B receptor, whether by increased NR2B expression or increased polyamine content, or both.

Corticosterone and Ethanol Co-Exposure: Potential Neuroadaptations in NR2B Subunit Immunoreactivity

Sapolsky et al. (1985) noted that 2 week CORT administration in adult male rats (5 mg/day) was enough for HPA axis adaptation (i.e., downregulation in the number of CORT receptors). However, 3 month CORT exposure was necessary for concomitant decreases in CORT receptor-containing hippocampal neuron number; suggesting that important neuroadaptations may be occurring that precede neuronal loss. The initial studies established the main finding in regard to the hypothesis that increased neuronal damage during ethanol withdrawal would result from ethanol and CORT co-exposure, particularly in the CA1 region. Following studies supported the importance of the polyamine-sensitive NR2B subunit in toxicity with the finding that ifenprodil significantly reduced toxicity, and most markedly in the CA1 region. Amelioration of CA1 region toxicity during ethanol withdrawal by NMDA receptor antagonists suggests that toxicity is attributable to either changes in NMDA receptor function or expression. However, it has not been shown in this model of 10 day exposure to 50 mM ethanol (~230 mg/dl) whether this is mediated by increases in NMDA receptor expression, and in particular, the NR2B subunit. Previous data using this model but 10 day exposure to 100 mM ethanol (~430 mg/dl) have shown increased abundance of NR1 and NR2B subunit protein with western blot analysis (Harris et al., 2003). Taken together, previous findings of potentiated injury during ethanol withdrawal that are NMDA-receptor dependent suggest that an increase in NR2B subunit expression is possible following 50 mM ethanol exposure for 10 days.

Initial immunohistochemical studies tested whether chronic ethanol exposure (10 days; 25, 50, or 100 mM) would result in increased immunoreactivity of NR2B receptor subunit protein (method after Butler et al., 2010). The data showed, however, that 10 day exposure to ethanol alone (25, 50, or 100 mM) resulted in a few slight changes in immunoreactivity that were statistically significant, though the functional significance of those changes are difficult to interpret. The statistically significant effects that were noted were very small in magnitude; smaller than expected if the ethanol exposure had produced neuroadaptations that were postulated to account for the significant toxicity observed with exposure to ligands during ethanol withdrawal. Further, no changes were observed with the concentration of relevance for all other studies (50 mM), nor were changes observed in the CA1 region where toxicity was most prominent following ethanol and CORT co-exposure. Details of the method may have contributed to these results, including the primary antibody used or the age-related thickness of the tissue. The primary NR2B subunit antibody used in these studies binds to the C-terminus of the subunit. Therefore, these data allow inferences to be made regarding changes in NR2B subunit number, but the use of a C-terminus antibody does not address changes that may occur in localization or functional state of NMDA receptor proteins. For instance, this antibody cannot distinguish between receptors that are inserted in the plasma membrane or those that are in the intracellular compartment; nor can this antibody distinguish whether the NR2B subunits labeled are primarily synaptic or extrasynaptic. This may be of critical importance in synaptic functioning, as NR2 subunits in the synaptic compartment stimulate signaling pathways for neuronal survival, whereas NR2 subunits in the extrasynaptic compartment stimulate signaling pathways leading to neuronal

demise (Hardingham & Bading, 2010). Regarding the age-related thickness of the tissue, this immunohistochemistry method has been used to reliably detect changes in immunofluorescence in tissue aged for less than one week (5-10 DIV). However, original studies of this model by Stoppini et al. (1991) demonstrated that significant spreading of the cell layers occurs over time, with significant spreading occurring after the first week. This may result in more diffuse labeling in older cultures (16 DIV in the current studies) than is observed in immunoreactivity in cultures kept for fewer days *in vitro*.

Despite the lack of significant changes in NR2B subunit immunoreactivity after 10 day ethanol exposure (25-100 mM), we wanted to determine whether changes in NR2B immunoreactivity were present after 10 day ethanol and CORT co-exposure, which was hypothesized to result in more robust increases in immunoreactivity as compared to changes after ethanol exposure only for 10 days. Changes in NR2B immunoreactivity were not detected in either the DG or CA3 hippocampal regions following 10 day CORT and ethanol co-exposure. In the CA1 region, however, disparate effects were noted in male and female cultures. In male cultures, 10 day ethanol and CORT co-exposure significantly increased NR2B immunoreactivity, but to the same level as cultures only exposed to CORT for 10 days. These results suggest that this effect was driven only by the presence of CORT and do not rule out the possibility that CORT activation of GRs alone may account for increased transcription and/or expression of NMDA NR2B receptor protein following 10 day exposure. Co-exposure to the GR antagonist mifepristone and an MR antagonist would be necessary to confirm this suggestion.

Previous literature has shown changes in NR subunit immunoreactivity following CORT exposure. For instance, in adult male lizards (*Anolis carolinensis)* that show a similar neuroendocrine response to mammals, increased immunoreactivity of NR2A and NR2B subunits is observed in response to social stress (24 hours) or CORT administration, with a much greater increase in NR2B subunits than NR2A subunits compared to control. Increased immunoreactivity of NR2A and NR2B subunits was greater following stress than CORT injections (Meyer et al., 2004). Though not statistically significant, Western blot analysis in the current studies indicated a slight increase in NR2B subunit expression following 10 day ethanol and CORT co-exposure that was greater than ethanol or CORT-exposed cultures alone and reduced by RU-486. It is possible that this slight increase could contribute to the potentiated toxicity observed with ethanol and CORT co-exposure, though firm conclusions cannot be made. However, Western blot analysis did not permit for analysis of NR2B expression in each hippocampal region, so it is possible that greater NR2B subunit upregulation occurred in the CA1 region as hypothesized, but pooling of hippocampal regions did not allow for a significant increase to be detected.

In the CA1 region of female cultures, however, while 10 day CORT increased NR2B immuoreactivity similarly to male cultures, 10 day ethanol reduced NR2B immunoreactivity compared to control, and ethanol and CORT co-exposure did not alter NR2B immunoreactivity from control levels. These data are contrary to the hypothesis in that marked increases were expected in NR2B immunoreactivity after ethanol and CORT co-exposure. The only increase in NR2B subunit immunoreactivity observed was driven

by CORT. This pattern of data suggests sex-specific hippocampal adaptations after concurrent 10 day ethanol and CORT exposure, or as noted above, methodological difficulties may preclude definitive conclusions within the current dataset. It should be noted, however, that *in vivo* data following ethanol exposure have shown differential effects on NMDAr subunit expression in male and female rats, such that prolonged ethanol liquid diet results in a slightly greater increases in hippocampal NR1, NR2A, and NR2B receptor subunit expression in male rats as compared to female rats (Devaud & Alele, 2004; Devaud & Morrow, 1999). Previous literature has not addressed sex differences that may exist in neuroadaptations following prolonged co-exposure to ethanol and CORT.

Effects of Ethanol and Corticosterone Exposure on NMDA Receptor Expression/Function and Localization

Ethanol and CORT co-exposure potentiated neuronal injury in the CA1 region during ethanol withdrawal, but co-exposure did not increase NR2B immunoreactivity as hypothesized. This data is not without precedent, because although various *in vivo* and *in vitro* models have noted increases in NR2B protein after ethanol exposure, this is not a consistent finding across laboratories and may vary by the method of ethanol administration/exposure (Carpenter-Hyland et al., 2004; Cebere et al., 1999; Rudolph et al., 1997). Importantly, withdrawal is still apparent in some *in vivo* models that do not report changes in NMDA receptor expression (Rudolph et al. 1997), suggesting that changes in NMDA receptor function occurred without discrete changes in receptor

number. Long-term ethanol exposure has been shown to alter function and/or trafficking of NMDA receptor subunits after long-term ethanol exposure, thus allowing for greater injury during ethanol withdrawal following ethanol and CORT co-exposure without discrete changes in the amount of NR2B subunit expressed. Phosphorylation of NMDA receptor subunits is another important mechanism in mediating NMDA receptor channel kinetics, with phosphorylation generally conferring greater channel activity. Specific to the NR2B subunit, the tyrosine kinase Fyn phosphorylates tyrosine residues on the C-terminal of the subunit, resulting in enhanced channel activity (Nakazawa et al., 2001; reviewed by Ron, 2004; Suzuki & Okumura-Noji, 1995). Increased phosphorylation of the NR2B subunit by Fyn has been correlated with induction of LTP in hippocampal CA1 synapses (Nakazawa et al., 1996), thus correlating NR2B phosphorylation with increased synaptic strength. Tyrosine phosphorylation of the NR2B subunit follows the same developmental trajectory as NR2B subunit expression in the brain, with high levels of expression until approximately PND 10, at which time NR2A expression increases and is the predominantly expressed NR2 subunit in the hippocampus. Ethanol exposure (25-100 mM) has been noted to result in a population of NMDA receptors that primarily contain the NR2B subunit because of internalization of NR2A subunits in hippocampal neurons (but not NR1 or NR2B) that is mediated by relief of Src phosphorylation (Suvarna et al., 2005). The resulting NR2B subunits that may be phosphorylated by Fyn result in greater channel activity that is thought to contribute to ethanol tolerance. Interestingly, this mechanism is unique to dendrites in CA1 pyramidal neurons. In CA1 hippocampal neurons, application of 100 mM ethanol decreases channel activity, but Fyn-mediated phosphorylation of the NR2B subunit that also occurs during exposure results in a

rebound in excitability upon ethanol washout (Yaka et al., 2003), suggesting that the ethanol exposure predisposes neurons for injury via excess excitation during withdrawal. Of relevance for the current studies, acute stress also results in dampened CA1 region excitability (Yamada et al., 2003). Though these data are only related to acute treatment, it is possible that this mechanism drives altered CA1 neuron excitability during withdrawal from long-term exposure (Yaka et al., 2003).

Long-term ethanol exposure also alters NMDA receptor-mediated synaptic transmission by lateral movement of NR2B subunits from extrasynaptic locations to synaptic localization and subsequent co-localization with PSD proteins, thus making NR2B subunits more integral to synaptic transmission after long-term ethanol exposure. NMDA receptor signaling pathways have been suggested to differ based on whether the activated receptors are synaptic or extrasynaptic, such that pathways related to cell survival are initiated by synaptic NMDA receptor activation and pathways related to cell death are initiated by extrasynaptic NMDA receptor activation (Hardingham, 2009; Hardingham & Bading, 2010). In cultured hippocampal neurons taken from PND 1 pups, seven day exposure to approximately 80 mM ethanol increased the co-localization of the NR1 subunit with synaptophysin and co-localization of the NR2B subunit with PSD-95. This effect on NR1 co-localization was mimicked by long-term exposure to the NMDA receptor antagonist AP-5, though alterations in co-localization were rapidly reversed back to control levels after 30 minutes of ethanol withdrawal. Long-term ethanol exposure also increased surface expression of NR2B subunits without changing total immunoreactivity that was not reversed after 4 hour ethanol withdrawal. However, the increased co-

localization of NR2B with PSD-95 was reversed after 4 hour ethanol withdrawal, suggesting that NR2B subunits moved back from the synaptic to the extrasynaptic compartment. Surface immunoreactivity was reduced back to control level by 24 hours of ethanol withdrawal (Clapp et al., 2010). Similar changes in localization of the NR2B subunit in hippocampal neurons are observed after 4 day exposure to the same ethanol concentration used in the current studies (50 mM), such that there is an increase in the size and density of NR2B clusters on dendrites, as well as increased co-localization with the synaptic protein synapsin. These changes were positively correlated with enhanced NMDA-dependent synaptic transmission in electrophysiology studies (Carpenter-Hyland et al., 2004). The current studies hypothesized that potentiated insult during withdrawal from ethanol and CORT co-exposure was due to increased protein expression for the NR2B subunit, though in accord with the studies cited above, changes in phosphorylation state, localization, or increased NR2B expression relative to NR2A expression resulting from internalization can all occur during prolonged ethanol exposure to contribute to vulnerability to excitotoxicity during withdrawal.

Corticosterone and Ethanol Co-Exposure: [^{3}H]Ifenprodil Autoradiographic Binding

Following studies of NR2B immunoreactivity, autoradiography studies were designed to measure changes in density of polyamine-sensitive NMDA receptor subunits by measuring [^{3}H]ifenprodil binding. Immunohistochemical studies have the advantage of allowing densitometric quantification of fluorescence among separate hippocampal regions. However, the signal acquired after [^{3}H]ifenprodil exposure did not allow for

clear differentiation between the hippocampal regions, though we allowed for eight weeks of exposure of radiolabeled cultures to film. Thus, CA1, CA3, and DG regions were combined into one measurement of [^{3}H]ifenprodil binding per slice. Previous studies in our lab have used autoradiography with organotypic slices in which visualization of all of the hippocampal regions was possible. However, those studies used [^{125}I]-labeled ligands. This difference in the radiolabeled isotope likely accounts for the ability to visualize cell layers using [^{125}I]-labeled ligands versus [^{3}H]-labeled ligands. [^{125}I] is a higher energy isotope compared to the [^{3}H] isotope. The cultures used in the current studies are relatively thick (200 microns at the start). A lower energy-emitting isotope will result in greater diffraction as it passes through the tissue to contribute to the image on the film, thus not allowing for visualization of cell layers. [^{125}I]ifenprodil was not commercially available for these studies. The current data show that 10 day CORT treatment (0.1 μM) resulted in significantly lower [^{3}H]ifenprodil binding in hippocampal slices. These data are contrary to the hypothesis that suggested an upregulation of ifenprodil-sensitive binding sites in CORT and ethanol co-exposed slices above what was hypothesized to occur in CORT treated slices. However, past data have shown using whole hippocampal membrane preparation from adult male rats given CORT via drinking water for 10 days that while the CORT treatment resulted in an increase in B_{max} of [^{3}H]MK-801 binding, the addition of ifenprodil did not alter [^{3}H]MK-801 binding. Had ifenprodil increased [^{3}H]MK-801 binding, this would have suggested a significant proportion of NMDA receptor subunits measured were polyamine sensitive NR2B subunits. However, the lack of an ifenprodil-effect suggests that this was not the case using whole membrane hippocampal tissue. As in the current autoradiography studies,

these data did not address possible region-specific effects of CORT on [^{3}H]MK-801 binding (Weiland et al., 1997). Taken into consideration with the PI data after 10 day CORT treatment in the current studies, it is possible that the treatment protocol resulted in significant losses in dendritic processes similar to what has been observed in hippocampus from *in vivo* studies, and therefore loss of a significant proportion of polyamine-sensitive NMDA receptors after CORT treatment alone. NMDA NR2B subunits are located predominantly on dendrites and dendritic spines in the postsynaptic density, as compared to the soma (Kohr, 2006). In summary, immunohistochemistry, autoradiography, and Western blot data suggest that the increased toxicity observed with PI measurements did not result from discrete changes in NR2B subunit number or expression of polyamine-sensitive NR2B receptors.

Long-Term Corticosterone Neurotoxicity is Independent of Effects at Membrane-Bound Corticosterone Receptors

We hypothesized that increased NMDA NR2B receptor subunit expression would contribute to the increased neuronal injury observed during ethanol withdrawal following CORT and ethanol co-exposure, and many previous studies have shown GR-dependent increases in NMDA receptor mRNA or subunit protein. However, CORT is also known to have multiple effects at the cell membrane that are independent of GR binding. Concurrent NMDA and CORT exposure significantly increases Ca^{2+} transients above NMDA alone in cultured rat hippocampal neurons, and this potentiation of CORT was suggested to occur at the cell membrane and not the GR (Takahashi et al., 2002). In

accord with the suggestion that CORT potentiates NMDA receptor function independently of the GR, previous data using the organotypic hippocampal cell culture model has shown that toxicity produced by co-exposure to NMDA and CORT (the same concentrations of CORT used in the current studies, 0.01 – 1 μM) is not ameliorated by co-exposure to a GR antagonist (RU-486), an MR antagonist (spironolactone), nor an inhibitor of protein synthesis (cycloheximide). However, toxicity produced by NMDA and CORT co-exposure was reduced by the NMDA receptor antagonist MK-801 (Mulholland et al., 2006). The findings cited above in regard to increased Ca^{2+} transients and toxicity with NMDA and CORT co-exposure could be due, in part, to increased glutamate levels measured by microdialysis, which have been observed upon acute CORT administration (Venero & Borrell, 1999), thus increasing GR-independent glutamatergic signaling with acute CORT exposure. In this model of long-term ethanol and CORT co-exposure it is not clear to what extent changes in gene expression versus changes in cell signaling at the level of the cell membrane would have on neuronal integrity given that GR activation, nuclear translocation, and changes in gene expression can occur *in vitro* as quickly as within 30 minutes. We hypothesized that increased NMDA NR2B receptor subunit expression, perhaps due to GR-mediated transcription, would contribute largely to neuroadaptations during ethanol and CORT co-exposure that would lead to greater toxicity during ethanol withdrawal. However, a separate series of studies was designed to consider whether toxicity during ethanol withdrawal that was observed following ethanol and CORT co-exposure could be due to effects at the cell membrane. Therefore, effects observed following ethanol and CORT co-exposure would be paralleled by 11 day exposure to a membrane impermeable form of CORT (BSA-

CORT) with and without ethanol co-exposure (Figures 15 and 16). To date, no studies have considered long-term effects of BSA-CORT to address whether toxicity occurs independently of MR and GR activation following prolonged CORT exposure, though effects on neuronal signaling have been noted following acute exposure to BSA-CORT. For example, it has been suggested that termination of HPA axis activation (or increased levels of CORT) is mediated by rapid inhibition via a membrane-bound receptor for CORT (Evanson et al., 2010). Using the same experimental design, hippocampal cultures were exposed to BSA-CORT for 11 days with or without ethanol and ethanol withdrawal, but did not result in alterations in PI uptake compared to control cultures. Therefore, the current data suggest that neurotoxic effects of long-term CORT are not attributable to actions at the membrane, but must also include actions of CORT binding to intracellular MRs and GRs. Additionally, from the data that is available that has probed the role of acute CORT on the membrane using BSA-CORT, it has become clear that actions of CORT at the cell membrane are dependent on the cell type being studied. For example, acutely (as quickly as within 5 minutes), CORT and BSA-CORT (.01 – 1 μM) similarly inhibit Ca^{2+} influx in a manner dependent upon pertussis-toxin sensitive G protein activation and subsequent PKC activation in PC12 cells (Qiu et al., 1998), though BSA-CORT exposure in hippocampal neurons has been shown to result in increased intracellular Ca^{2+} content. As no studies have considered exposure to BSA-CORT for a prolonged time period as used in the current studies (11 days), it is unclear what neuroadaptive changes could be occurring in number and/or affinity of membrane-bound CORT receptors and how that may influence the resistance of hippocampal neurons in this model to damage from BSA-CORT exposure.

Implications and Future Directions

Neuroadaptations that occur during ethanol exposure predispose the brain for excitotoxic insult during ethanol withdrawal. Increased neuronal and behavioral excitability that is observed during ethanol withdrawal is mediated, at least in part, by NMDA receptor signaling. The current studies investigated the role of polyamine-sensitive NR2B subunits in mediating neuronal damage during ethanol withdrawal following prolonged ethanol and CORT co-exposure that was hypothesized to be related to adaptive upregulation of NR2B subunits. While it is unclear whether neuronal death correlates with disrupted long-term cognitive function in humans, repeated withdrawal episodes are correlated with worse long-term cognitive outcome (Duka et al., 2003), thus highlighting the importance of targeting the reduction of ethanol withdrawal expression for pharmacological intervention in alcohol dependent individuals. To date, benzodiazepines are administered to prevent or reduce ethanol withdrawal seizures, though they do not prevent the long-term cognitive deficits (Duka et al., 2003; Duka et al., 2004). The NR2B subunit has previously been identified as a target for treatment of alcohol dependence because modulation of the NR2B subunit has been implicated in mediating multiple aspects of ethanol intoxication and withdrawal behaviors (Nagy, 2004). Importantly for clinical utility, NR2B receptor subunit ligands have been shown to be efficacious in the treatment of other neurological disorders (Chizh et al., 2005). Acamprosate is one of three drugs indicated for the treatment of alcohol dependence and has been shown to significantly reduce relapse in alcohol dependent individuals (Paille et al., 1995). Acamprosate has multiple mechanisms of actions, including reversal of

polyamine potentiation of NMDA receptor currents (Popp & Lovinger, 2000), thus further supporting a role for NR2B antagonism in ethanol withdrawal.

The current data show that toxicity in response to long-term ethanol and CORT co-exposure was inhibited by antagonism of polyamine-sensitive NR2B receptor subunits; however, the measures used in the current studies failed to show significant changes in NR2B subunit immunoreactivity measured with Western blots and immunohistochemistry, as well as a changes in the number of ifenprodil binding sites with autoradiography. Multiple interpretations are possible that do not preclude changes occurring at the NR2B subunit, including increased phosphorylation of NR2B subunits and therefore greater NR2B subunit function or NR2B subunit trafficking (Chazot et al., 2004). Future studies will be necessary to determine if these are viable alternate mechanisms of action mediating ethanol withdrawal toxicity following ethanol and CORT co-exposure.

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the *N*-methyl-D-aspartate receptor to polyamines. Mol Pharmacol 40: 774–782.

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Vita

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PERSONAL
Place/Date of birth: Beloit, WI; 2-17-84

EDUCATION
B.A., Wittenberg University (2006)
Psychology, Magna cum Laude

RESEARCH EXPERIENCE
University of Kentucky
F31 Predoctoral National Research Service Award Fellowship, NIAAA
"Adenosine Receptors and Ethanol Withdrawal" (August 2009- August 2011)
Mentor: Mark A. Prendergast, Ph.D.

University of Kentucky
Graduate Student/Research Assistant (July 2006- August 2009)

Husson University/University of Maine
Invited laboratory visit/organotypic cell culture setup; in the laboratory of Leslie L. Devaud, Ph.D. (January 2009)

National Institutes of Health/National Institute of Neurological Disorders & Stroke, Medical Neurology Branch, Movement Disorders section
Summer Student/Research Assistant (May 2005 – Aug. 2005), (May 2006 – Aug. 2006)
Mentor: Valerie Voon, M.D.

Wittenberg University
Department of Psychology, Senior Honors Thesis (Sept. 2005 – May 2006)
Department of Psychology, Research Lab Assistant (Sept. 2004 – May 2006)
Mentor: Josephine F. Wilson, D.D.S., Ph.D.

PUBLICATIONS
Butler TR & Prendergast MA. Review: Neuroadaptations in adenosine receptor signaling following long-term ethanol exposure and withdrawal. Submitted 1-31-11 to *Alcoholism: Clinical and Experimental Research* (accepted pending minor revisions, 3-3-11).

1) **Butler TR**, Self RL, Smith KJ, Braden BB, & Prendergast MA**.** Neurodegenerative Effects of Recombinant HIV-1 Tat (1-86) are Associated with Inhibition of Microtubule Formation and Oxidative Stress-Related Reductions in Microtubule-Associated Protein-2(a,b). *Neurochemical Research,* 36(5): 819-828.

2) Voon V, **Butler TR**, Ekanayake V, Gallea C, Ameli R, Murphy DL, & Hallet M. (2010). Psychiatric symptoms associated with focal hand dystonia. *Movement Disoders*, 25(13): 2249-2252.

3) Smith KJ, **Butler TR**, & Prendergast MA. (2010). Inhibition of sigma 1 receptor reduces *N*-methyl-D-aspartate induced neuronal injury in methamphetamine-exposed and -naive hippocampi. *Neuroscience Letters* 481(3): 144-148.

4) **Butler TR**, Self RL, Smith KJ, Sharrett-Field LJ, Berry JN, Littleton JM, Pauly JR, Mulholland PJ, & Prendergast MA. (2010). Selective vulnerability of hippocampal cornu ammonis 1 pyramidal cells to excitotoxic insult is associated with the expression of polyamine-sensitive N-methyl-D-asparate-type glutamate receptors. *Neuroscience* 165: 525-534.

5) **Butler TR**, Smith KJ, Berry JN, Sharrett-Field LJ, & Prendergast MA. (2009). Sex differences in caffeine neurotoxicity following chronic ethanol exposure and withdrawal. *Alcohol and Alcoholism* 44(6): 567-574.

6) Self RL, Smith KJ, **Butler TR**, Pauly JR, & Prendergast MA. (2009). Intra-cornu ammonis 1 administration of the human immunodeficiency virus-1 protein trans-activator of transcription exacerbates the ethanol withdrawal syndrome in rodents and activates N-methyl-D-aspartate glutamate receptors to produce persisting spatial learning deficits. *Neuroscience* 163(3): 868-876.

7) **Butler TR**, Smith KJ, Self RL, Braden BB, & Prendergast MA. (2008). Sex differences in neurotoxic effects of adenosine A1 receptor antagonism during ethanol withdrawal: Reversal with an A_1 agonist and NMDA receptor antagonist. *Alcoholism: Clinical and Experimental Research* 32(7): 1260-1270.

8) Smith KJ, **Butler TR**, Self RL, & Prendergast MA. (2008). Potentiation of *N*-Methyl-D-Aspartate receptor-mediated neuronal injury during methamphetamine withdrawal requires co-activation of IP_3 receptors. *Brain Research* 1187: 67-73.

9) Smith KJ, Self RL, **Butler TR**, Mullins MM, Ghayoumi L, Holley RC, Littleton JM, Bardo MT, & Prendergast MA. (2007). Methamphetamine exposure antagonizes N-methyl-D-aspartate receptor-mediated neurotoxicity in organotypic hippocampal slice cultures. *Brain Research* 1157: 74-80.

10) Voon V, Fox S, **Butler TR**, & Lang AE. (2007). Antidepressants and psychosis in Parkinson disease: a case series. *International Journal of Geriatric Psychiatry* 22(6): 601-604.

11) Prendergast MA, Self RL, Smith KJ, Ghayoumi L, Mullins MM, **Butler TR**, Buccafusco JJ,Gearhart DA, Terry Jr. AV. (2007). Microtubule-associated targets in chlorpyrifos oxon hippocampal neurotoxicity. *Neuroscience* 146(1): 330-339.

BOOK CHAPTERS

Butler TR & Prendergast MA. Caffeine neurotoxicity and implications for tea. In Preedy VR (Ed.) Tea in Health and Disease Prevention Academic Press (In Progress)

POSTERS AND PRESENTATIONS

Poster: "NMDA NR2B subunit-mediated toxicity following Corticosterone exposure during ethanol exposure and withdrawal in organotypic hippocampal cultures"
Research Society on Alcoholism (Submitted for June 2011 meeting)

Poster: "Neurodegenerative Effects of recombinant HIV-1 Tat (1-86) are associated with inhibition of microtubule formation and oxidative stress-related reductions in microtubule-associated protein-2(a,b)"
Society for Neuroscience (November 2010)

Symposium Organizer/Participant: "Gender/Sex Differences in Alcohol-Related Behaviors and Neuronal Injury: A Graduate Student Perspective"
International Society for Biomedical Research on Alcoholism (September 2010)

Poster: "Sex differences in CA1 hippocampal region cell death following antagonism of GIRK channels during ethanol withdrawal"
Research Society on Alcoholism (June 2010)

Poster: "Hippocampal CA1 region vulnerability to neurotoxic insult: Involvement of polyamine-sensitive NMDA receptors"
Society for Neuroscience (October 2009)

Poster: "Sex differences in caffeine neurotoxicity following chronic ethanol exposure and withdrawal"
Research Society on Alcoholism (June 2009)

Poster: "Sex differences in neurotoxic effects of adenosine receptor antagonism following chronic ethanol exposure and withdrawal"
Bluegrass Chapter of the SfN Spring Neuroscience Day (March 2009)

Poster: "Sex differences in neurotoxic effects of adenosine A_1 receptor antagonism is potentiated by ethanol withdrawal in vitro: Reversal by NMDAr antagonism"
Bluegrass Chapter of SfN Spring Neuroscience Day (March 2008)
Research Society on Alcoholism (June 2008)

Poster: "Alterations in adenosine A_1 receptor activity produces neurotoxicity in developing hippocampal slice cultures and sensitizes cultures to ethanol withdrawal-induced neurotoxicity"
Society for Neuroscience (November 2007)

Poster: "Stop-signal task performance in patients with Psychogenic Movement Disorder: A pilot study"
NIH Summer Student Poster Day (August 2006)

Presentation: "Hemispheric lateralization of emotion, sociotropy, and autonomy: Effects of an achievement and interpersonal experience on drawing placement and affect in college students"
20th Annual Ohio Undergraduate Psychology Research Conference (April 2006)

Poster: "Hemispheric lateralization of emotion, sociotropy, and autonomy: Effects of an achievement and interpersonal experience on drawing placement and affect in college students"
Midwest Psychological Association Conference (May 2006)

Poster: "Obsessive-Compulsive Disorder in Focal Hand Dystonia"
NIH Summer Student Poster Day (August 2005)

PROJECTS IN PROGRESS

1. Sex differences in neuroadaptations following long-term ethanol-exposure and mechanisms mediating ethanol-withdrawal related toxicity. Methods: Organotypic hippocampal slice cultures, Western blots, immunohistochemistry.

2. Glucocortiocoid receptor involvement in potentiation of ethanol-withdrawal neuronal injury in vitro and ethanol withdrawal in vivo. Methods: Organotypic hippocampal slice cultures, intragastric binge ethanol exposure.

TEACHING EXPERIENCE

PSY 312: Brain and Behavior, Dr. Mark Prendergast
Lectures: Learning and Memory; Thirst and Hunger; Behavioral Neuroscience of Psychiatric Illness Fall 2010

PSY 565: Advanced Topics in Neuroscience: Biology & Motivation, Dr. Mark Prendergast
Lectures: Thirst and Hunger; Physiology of Motivation Spring, 2009, 2010

HONORS/AWARDS

NIAAA	International Travel Award for travel to 2010 ISBRA Congress	Sept. 2010
Research Society on Alcoholism	Student Merit Award	June 2010
Guze Symposium 2010	Meeting Travel Award	February 2010
Society for Neuroscience	Student Chapter Travel Award	October 2009
Research Society on Alcoholism	Student Merit Award	June 2009
Research Society on Alcoholism	Enoch Gordis Award Finalist	June 2008
Research Society on Alcoholism	Student Merit Award	June 2008
Bluegrass Chapter of the SfN, Spring Neuroscience Day	Graduate Student Poster Award	March 2008
NIH/NINDS	Exceptional Summer Student	August 2006
Wittenberg University Department of Psychology	Professional Potential in Psychology	May 2006
Wittenberg University Department of Psychology	Departmental Honors	May 2006

Wittenberg University Department of Psychology	Honors Thesis Project McGregor Grant recipient	Sept. 2006
Wittenberg University	University Honors Program	2002 – 2006

PROFESSIONAL SERVICE/OUTREACH ACTIVITIES

Brain Awareness Week High School Visit, Lexington, KY	March 2009
Fayette Co. Schools Annual Science Fair Neuroscience Exhibit	February 2009, 2010
Society for Neuroscience Bluegrass Chapter: Student Representative	2008-2009
Neuroscience Workshop Volunteer: Girls Enjoying Math & Science (sponsored by Girl Scouts of Kentucky)	Fall 2008, 2009

NATIONAL HONORARIES

Phi Beta Kappa	Member	Spring 2006
Psi Chi	Chapter President	Fall 2005 – Spring 2006
Mortar Board	Member	Spring 2005
Alpha Lambda Delta	Member	Fall 2002

SCIENTIFIC SOCIETY MEMBERSHIPS

International Society for Biomedical Research on Alcoholism,
Student Member (Fall 2009)

Society for Neuroscience,
Student Member (Spring 2007)

Research Society on Alcoholism,
Student Member (Fall 2007)