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An Assessment of the Neurobiological and Behavioral Changes that Occur During Abstinence Following Chronic Alcohol Drinking

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A dissertation submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Neurobiology Curriculum.

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ABSTRACT

Jennifer Rice Stevenson: An Assessment of the Neurobiological and Behavioral Changes that Occur During Abstinence Following Chronic Alcohol Drinking

(Under the direction of Dr. Clyde Hodge)

Although many alcoholics experience periods of abstinence, most will relapse. Indeed, the inability to resist alcohol drinking is central to alcohol addiction. The emergence of negative affective states during abstinence is thought to be a key mediator of relapse behavior. The data presented here indicate that abstinence from voluntary alcohol drinking leads to the emergence of depression-like behavior and reductions in neurogenesis. C57BL/6J mice were allowed to self-administer ethanol (10% v/v) vs H2O in the home cage for 28 days. Alcohol was then removed for 1-d or 14-d, and mice were tested in the forced swim test to measure depression-like behavior. After 14 days, but not 1 day of abstinence from alcohol drinking, mice showed a significant increase in depression-like behavior. The significant increase in depression-like behavior during abstinence was associated with a reduction in neurogenesis. Chronic treatment (14-d) with the antidepressant desipramine during abstinence prevented both the emergence of depression-like behavior and the reduction in new neurons indicating that abstinence-induced depression is associated with structural plasticity in the hippocampus. Alterations in CREB expression and CREB activation, as measured by CREB and pCREB immunoreactivity, have been linked to changes in alcohol reinforcement, as well as depression; therefore, the changes in CREB in response to desipramine or vehicle treatment were investigated during the abstinence period. The most

significant changes noted were in the CA3 subregion of the hippocampus which showed significant reduction in CREB and pCREB immunoreactivity due to 14-d abstinence, and complete prevention of this reduction by desipramine treatment. Dysregulation of the HPA axis is examined in this dissertation not only because it can lead to depression, but also because alcohol drinking, alcohol withdrawal, and alcohol abstinence are all associated with HPA axis dysregulation. Changes in the HPA axis as a result of 14-d abstinence were not evident. Overall, the results of this study support the conclusion that profound functional (i.e., behavioral) and structural changes occur during abstinence from alcohol use and suggest that antidepressant treatment may alleviate some of these pathological neurobehavioral adaptations.

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CHAPTER I: INTRODUCTION

ALCOHOLISM

Alcoholism affects millions of people's lives every day. Over 7% of the United States population is alcoholic, leading to annual costs of more than $180 billion (Harwood 2000). Alcoholism is characterized primarily by an inability to control alcohol drinking. Alcoholics experience craving for alcohol, a lack of ability to stop drinking once drinking has begun, physical dependence (withdrawal symptoms), tolerance, preoccupation with alcohol consumption, lack of interest in other life activities besides drinking alcohol, and continued alcohol use despite physical and psychological problems. Withdrawal symptoms include autonomic hyperactivity, tremor, nausea or vomiting, anxiety, and seizure, while tolerance is defined as a need for more alcohol in order to obtain the same level of intoxication (Becker; NIAAA 1995).

Chronic consumption of alcohol leading to alcoholism produces many neuroadaptations and creates an allostatic state (Koob 2003). Among these changes that occur as a result of drinking are alterations in the brain reward and stress systems. These manifest themselves through neuroadaptations in the nucleus accumbens, amygdala, hippocampus, pre-frontal cortex, and many other regions. Neurotransmitter systems known to become dysregulated as a result of chronic drinking and alcohol abuse are dopamine, gamma-aminobutyric acid (GABA), glutamate, serotonin, and several neuropeptides (Koob et al. 1998). A crucial aspect of alcohol addiction is that alcoholics experience increased negative affect in the absence of alcohol (Koob and Le Moal, 1997. A major goal in the field

of alcohol research is understand these neuroadaptations that underlie the development of addiction.

ABSTINENCE FOLLOWING ALCOHOL DRINKING

Although many alcoholics experience periods of abstinence, most will relapse. Indeed, the inability to resist alcohol drinking is central to alcohol addiction. The psychological and neurobiological factors that lead to relapse are an area of active investigation, and are the focus of this dissertation (Lowman C. et al., 2000; Li TK, 2000). In addition to the neuroadaptations that occur during active drinking periods, further neuroadaptations occur upon cessation of alcohol drinking as a result of the removal of a chronic stimulus (alcohol). These abstinence-induced neuroadaptations may lead to increased vulnerability for relapse (Figure 1). While the neurobiological consequences of acute alcohol withdrawal have been studied extensively, the central nervous system changes that occur during protracted withdrawal are not as well understood (Martinotti et al. 2008). The emergence of negative affective states during abstinence is thought to be a key mediator of relapse behavior (Koob and Le Moal 1997). Surprisingly, very few pre-clinical studies have examined neurobiological and behavioral changes beyond the initial 72-hours of withdrawal. Moreover, the vast majority of studies examining the effects of cessation of alcohol drinking have done so in models of forced alcohol consumption. The effects of abstinence following free choice or voluntary drinking in a animal models are understudied. The primary goal of the work presented in this dissertation was to characterize the neurobiological and behavioral changes that occur during protracted abstinence following chronic voluntary alcohol drinking.

COMORBIDITY OF ALCOHOLISM AND DEPRESSION

Alcoholism and depression are two devastating diseases that often co-occur. Studies on depression among alcoholics show comorbidity rates as high as 42.2% (Schuckit et al. 1997b), alcohol abuse and/or dependence are associated with a 2- to 4-fold increase in the

occurrence of depression (Grant and Harford 1995; Kessler et al. 1997). Moreover, rates of depression are significantly elevated among people who seek treatment for alcoholism (Lynskey 1998) and depressive symptoms including sadness, despair, sleep disturbances, altered endocrine functions, decreased energy, mental slowing, loss of concentration, pessimism, ideas of guilt and self-deprecation are common in alcohol dependence and abstinence (Garbutt et al. 1999). Unfortunately, when these two diseases co-occur, relapse to alcohol use is more likely and remission from depression is less likely (Greenfield et al. 1998; Mueller et al. 1994). Importantly, depression that occurs during abstinence increases the likelihood of relapse in alcoholics, whereas prior-onset depression is not related to relapse (Hasin et al. 2002). Abstinence-related depression is thought to increase the chance of relapse because drinking may help people cope with negative emotions (Carpenter and Hasin 1998; Hasin and Grant 2002). From a treatment perspective, the efficacy of antidepressant medications in treating a number of forms of depression is well validated; however the use of antidepressants in the treatment of alcoholism needs further study. There are a limited number of well-controlled studies on the efficacy of antidepressant use for alcoholics. However, clinical studies have demonstrated that both fluoxetine and desipramine are effective in reversing depression comorbid with alcoholism, and have shown modest success in preventing relapse (Le Fauve et al. 2004).

While the clinical literature is rife with information regarding alcoholism and depression, preclinical studies examining the relationship between alcoholism and depression are lacking. A handful of studies have examined how genetic predisposition to high alcohol intake affects depression-like behavior and how genetic predisposition to depression-like behavior affects alcohol intake in rats, but the results have been inconsistent (Godfrey et al. 1997; Overstreet et al. 1992; Vengeliene et al. 2005). Surprisingly, the effects of chronic drinking on depression-like behavior have not been studied. The work presented here

attempts to answer the question, “What are the neuroadaptations that occur during abstinence following voluntary drinking, and do these neurobiological changes increase vulnerability to the emergence of negative affective states?” While this dissertation in no way examines every possible mechanism by which abstinence can promote negative affect, this work focuses on an integrated mechanistic pathway of potential neuroadaptations during abstinence including alterations in the HPA axis and monoamine neurotransmitter systems, and resulting changes in intracellular signaling and synaptic structure.

MONOAMINE HYPOTHESIS OF DEPRESSION

Monoamine dysregulation has long been hypothesized to underlie depression largely due to the efficacy of monoamine-reuptake inhibitors in treatment of depression (Delgado and Moreno 2000). Multiple classes of antidepressant drugs act to block the reuptake of monoamines including serotonin (5-HT) and norepinephrine (NE). While enhancement of monoamine neurotransmission promotes recovery for many depressed patients, the evidence supporting monoamine deficiency as the primary dysfunction in depression is lacking. Specifically, monoamine depletion techniques do not produce depression. Depletion of specific monoamines is pro-depressant only in patients currently taking a reuptake inhibitor for the depleted transmitter or in patients who are experiencing remission from depression after having taken a reuptake inhibitor for the depleted transmitter (Delgado and Moreno 2000; Stone et al. 2008). More recent hypotheses of mechanisms underlying depression cite neurotransmitter, neurotrophic, and neuroanatomical systems that can be modulated by monoamines and that may represent final common pathways of depressive agents and antidepressant treatments. The tricyclic antidepressant desipramine (a NE reuptake inhibitor) is administered during experiments described in the dissertation. While the primary mechanism of action of desipramine is inhibition of the reuptake of NE, our investigations into the mechanisms by which desipramine produces its positive behavioral

effects focused on examples of possible final common pathways such as gene transcription and structural plasticity, which are introduced below.

CREB

Cyclic AMP-response-element-binding (CREB) protein is a transcription factor that has been shown to regulate the expression of many genes leading to long-term changes in cell and synaptic function and structure. CREB-dependent transcription is primarily regulated by kinase-induced phosphorylation of CREB at serine residue 133 (Ser-133) (Johannessen et al. 2004). A large number of kinases (e.g., PKA, PKB, PKC, MEK, ERK, MAPK, CaMK, etc.) can induce phosphorylation of CREB at Ser-133 (p-CREB) (Johannessen et al. 2004), which indicates involvement of numerous neurotransmitter-signaling pathways. Changes in the level of p-CREB can in turn alter expression of any number of CREB target genes (i.e., genes with CRE sites in their promoter regions). CREB target genes are found in a variety of systems including: metabolism, transcription (e.g., EGR-1, c-FOS, JUND), neuropeptides (CRF, galanin, NPY, vasopressin, dynorphin), and growth factors (BDNF, TNFalpha) (Mayr and Montminy 2001). Data have shown that changes in BDNF expression in the hippocampus results in parallel changes in hippocampal neurogenesis (see below) and may be a mechanism of action of antidepressant treatments (Sairanen et al. 2005). Accumulating evidence also suggests that CREB (and its target genes) regulates alcohol dependence and withdrawal and may underlie anxiety associated with alcohol withdrawal (Pandey 2003). Alterations in CREB expression and CREB activation, as measured by CREB and pCREB immunoreactivity, in several brain regions have been linked to changes in alcohol reinforcement, as well as depression (Carlezon et al. 2005; Pandey 2004). Identifying specific conditions that alter CREB activity has fundamental importance for understanding a variety of neural functions and disorders including alcoholism and depression.

NEUROGENESIS

The discovery of new neurons in the adult brain has created a completely new area of neuroscience research. Neurogenesis, the proliferation of neural progenitor cells (NPC), in the adult brain was originally discovered in 1965 (Altman and Das 1965), but was not accepted until recently. Although NPC are found throughout the adult mammalian brain, the majority of adult neurogenesis appears to occur in the subventricular zone (SVZ) of the lateral ventricles and the dentate gyrus subgranular zone (DG-SGZ) of the hippocampus (Gage 2002; Ming and Song 2005). The function of adult neurogenesis has so far been implicated in learning and memory, mood, and association of sensory information (Duman et al. 2001; Gould 1999; Gross 2000; Kempermann et al. 1998; van Praag et al. 1999a; van Praag et al. 1999b). In general, evidence suggests that new neurons in the adult brain contribute to synaptic plasticity (Snyder et al. 2001) and the more general ability of the organism to adapt to changing circumstances (Kempermann 2002). Enriched environment and exercise have been associated with increased neurogenesis, while stress and alcohol exposure have been associated with decreased neurogenesis (Gage 2000; Gould 1999; Nixon and Crews 2002). Given that neural stem cells that have divided and differentiated into neurons produce 6 percent of the total granule cell population each month in the adult hippocampus, the survival and proliferation of these cells is crucial for proper hippocampal function (Cameron and McKay 2001).

ALCOHOL AND NEUROGENESIS

Alcohol has long been known to produce hippocampal neuropathy and hippocampal-associated cognitive deficits (Brandt et al. 1983; Collins et al. 1996; Obernier et al. 2002; Sullivan et al. 2000; Walker et al. 1980). Nixon and Crews (Nixon and Crews 2002) have seen a 53% reduction in neural stem cell proliferation in rats as a result of a 4-day binge producing extremely high blood alcohol levels, and Herrera et al. (Herrera et al. 2003) have seen decreases in neural stem cell proliferation as a result of lower blood alcohol levels. Additional data suggest that not only proliferation, but also survival of neural progenitor cells

is decreased by ethanol exposure. These data on neurogenesis are consistent with the cell loss and hippocampal deficits associated with alcoholism.

The majority of the studies on the effects of alcohol exposure on neurogenesis have used animal models of forced ethanol consumption in which animals either consume a liquid diet containing ethanol or are intra-gastrically injected with ethanol. Both of these methods of ethanol exposure can be quite stressful for the animal. Given that humans generally self-administer alcohol voluntarily, it is important to examine the effects of voluntary ethanol consumptions on neurogenesis in an animal model.

A full characterization of the effects of chronic voluntary ethanol drinking on neurogenesis can be achieved by evaluating different markers for neurogenesis and differentiation of neural progenitor cells at different time points following voluntary drinking in mice. Several different immunohistochemical markers have been validated for use in studying adult neurogenesis. A common way of measuring neurogenesis is with the thymidine analog bromo-deoxyuridine (BrdU). BrdU can be injected peripherally and will then be incorporated into DNA similarly to thymidine when cells are replicating their DNA and dividing. In this way, BrdU labels cells in the brain that have divided. When brains are studied shortly after BrdU is injected, BrdU can serve as a marker for proliferating cells. By studying brains several weeks after BrdU injections, one can evaluate the number of BrdU-labeled cells that have survived since the time of labeling. By 28-days after BrdU injection, BrdU-labeled cells have migrated into the granule cell layer of the hippocampus and have begun to express proteins that indicate whether they are differentiating into neurons or glia. Cells that co-express BrdU and neuronal-specific nuclear protein (NeuN) are neural progenitor cells that have survived and are differentiating into neurons, and cells that co-express BrdU and glial fibrillary acidic protein (GFAP) are neural progenitor cells that have survived and are differentiating into glial cells. Additionally, antibodies to proliferating cell nuclear antigen (PCNA) can be used to immunohistochemically label proliferating cells, and

antibodies to doublecortin (DCX) can be used to immunohistochemically label immature neurons. DCX labeling, therefore, can indicate the number of new cells that have differentiated into neurons. Using these immunohistochemical techniques at different time points following voluntary alcohol drinking, one can characterize ethanol's effects on neurogenesis.

DEPRESSION AND NEUROGENESIS

Changes in hippocampal neurogenesis have been demonstrated to be related to depression, making neurogenesis is an interesting candidate as a mechanism underlying alcohol-related depression (Duman et al. 2000; Jacobs et al. 2000). Decreased hippocampal volume has been noted in clinical studies of depression patients (Davidson et al. 2002), and it has been suggested that dysfunction of the hypothalamic-pituitary-adrenal (HPA) axis leads to increased glucocorticoid levels in the hippocampus thereby causing neuronal cell loss (Gold et al. 1988; Sapolsky 2000). Jacobs et al. (Jacobs et al. 2000) have suggested that decreased neurogenesis is responsible for changes in hippocampal volume. Stress is known to cause dysfunction of the HPA axis, to increase glucocorticoid production, and to precipitate depression. Huang et al. (Huang et al. 2004) identified the dentate gyrus of the hippocampus as a region that shows c-Fos activation following post-shock escape testing in mice with learned helplessness. Inescapable stress, stress caused by a predatory odor, and corticosterone have all been shown to reduce neurogenesis, and stress is thought to precipitate depression (Cameron and Gould 1994; Gregus et al. 2005; Malberg and Duman 2003; Tanapat et al. 2001). Additional evidence supporting the neurogenesis hypothesis for depression includes the effects of antidepressant therapy on neurogenesis. Chronic administration of antidepressants causes increases neurogenesis, as does electroconvulsive therapy (Madsen et al. 2000; Malberg et al. 2000). Moreover, antidepressant treatment is ineffective in the absence of neurogenesis (Santarelli et al. 2003).

HPA AXIS DYSREGULATION

The hypothalamic-pituitary-adrenal (HPA) axis is the primary effector limb of the physiological stress system. It is via this axis that perceived stressors are transduced into a physiological response. Briefly, when the brain perceives a stressor, the hypothalamus releases corticotropin-releasing factor (CRF), which stimulates the pituitary gland to release adrenocorticotropic hormone (ACTH). ACTH, then, stimulates the adrenal cortex to release glucocorticoids (cortisol is the primary glucocorticoid in humans; corticosterone is the primary glucocorticoid in rodents). Glucocorticoids circulate throughout the organism with widespread effects on peripheral organs, as well as in the brain. The stress response evolved as a homeostatic mechanism that allows organisms to properly respond to a stressor and then return to basal physiological functioning via inhibitory feedback mechanism. Feedback inhibition occurs when glucocorticoids bind to glucocorticoid type II receptors (GR) which inhibit release of CRF and ACTH (Tsigos and Chrousos 2002). Activity of the HPA axis is critical for the survival of an organism, yet over-activation of the HPA axis can lead to the emergence of severe physical and mental illness (Sapolsky 1996). Chronic activation of the HPA axis can lead to dysregulation of activating and inhibitory mechanisms at each level of the HPA axis. In addition to the inherent feedback inhibition, the HPA axis can be inhibited by extra-hypothalamic structures in the brain, including the cortex and the hippocampus (McEwen 2001; Sapolsky 2000). Because of this, disruptive hippocampal neuroadaptations in response to stress can potentiate HPA axis dysregulation. Disruption of HPA feedback inhibition can lead to a state known as hypercortisolemia, in which glucocorticoid secretion is increased and pituitary and adrenal gland sizes are increased. In response to elevated glucocorticoid levels, GR are downregulated, causing further impairment of feedback inhibition Stone et al., 2008). Chronic overactivation of the HPA axis can precipitate depression. In fact, hypercortisolemia in depressed patients is one of the most consistent findings in psychiatry (Nemeroff 1996). Moreover, one of the

mechanisms by which monoamine reuptake inhibitors treat depression is by increasing glucocorticoid receptor expression in the hippocampus (Het and Wolf 2007).

Dysregulation of the HPA axis is examined in this dissertation not only because it can lead to depression, but also because alcohol drinking, alcohol withdrawal, and alcohol abstinence are all associated with HPA axis dysregulation (Sillaber and Henniger 2004). The relationship between stress and alcohol is integral to alcohol addiction. Both alcohol and alcohol withdrawal can produce hyperactivity of the HPA axis, leading to enhanced sensitivity to stressors, while abstinence following chronic alcohol drinking is associated with hypoactivity of the HPA axis. Both over- and under-activation impair the ability to properly cope with stressors. The inability to cope with stress is frequently reported as a reason for initiation of alcohol drinking and relapses to drinking following periods of abstinence; thus, HPA dysfunction can greatly enhance one's vulnerability to alcohol addiction (Adinoff et al. 1998; Rivier et al. 1996). The HPA axis, therefore, may be a mechanism underlying the relationship between alcoholism and depression.

CONCLUSIONS AND RATIONALE

Although comorbidity of alcoholism and affective disorders is major clinical issue, little is known about the mechanisms that are responsible for this relationship. The work presented in this dissertation describes a model for investigating depression-like behavior that emerges during abstinence following chronic voluntary drinking in mice. Because of the known effects of alcohol on CREB and neurogenesis, these factors are examined as possible mechanisms by which alcohol-related depression develops. Additionally, the HPA axis is investigated as a system that may become dysregulated during abstinence following chronic alcohol drinking, creating a greater vulnerability to negative affective states.

CHAPTER II: ABSTINENCE FOLLOWING ALCOHOL DRINKING PRODUCES DEPRESSION-LIKE BEHAVIOR AND REDUCED HIPPOCAMPAL NEUROGENESIS IN MICE

INTRODUCTION

Alcoholism and depression are two of the most costly and widespread neuropsychiatric disorders worldwide. In the United States, the prevalence of a lifetime history of depression in alcohol dependent individuals has been reported to be about 42% (Miller et al. 1996; Schuckit et al. 1997b) but comorbidity rates from 5% to as high as 80% have been noted in some studies (Pettinati 2004). Alcohol abuse and/or dependence are also associated with a 2-4 fold increase in depression rates (Grant and Harford 1995; Kessler et al. 1997). Moreover, rates of depression are significantly elevated among people who seek treatment for alcoholism (Lynskey 1998) and depressive symptoms are common in alcohol dependence and abstinence (Garbutt et al. 1999). When alcoholism and depression co-occur, relapse to alcohol use is more likely and remission from depression is less likely (Greenfield et al. 1998; Mueller et al. 1994).

Emerging evidence indicates that features of both alcoholism and depression may be mediated by changes in adult hippocampal neurogenesis (Crews and Nixon 2003; Duman et al. 2000; Jacobs et al. 2000). For example, several antidepressant treatments increase neurogenesis (Malberg et al. 2000; Santarelli et al. 2003; Warner-Schmidt and Duman 2006), while stress, which is widely thought to precipitate depression, reduces neurogenesis (Cameron and Gould 1994; Gregus et al. 2005; Malberg and Duman 2003; Tanapat et al. 2001). In addition to stress, alcohol has been shown to reduce neurogenesis. Studies have shown that high levels of alcohol exposure can reduce survival and proliferation of neural

progenitor cells (NPC) (He et al. 2005; Herrera et al. 2003; Nixon and Crews 2002). Withdrawal from alcohol exposure is associated with time-dependent increases and decreases in proliferation of NPCs (Nixon and Crews 2002). These data led us to hypothesize that decreased hippocampal neurogenesis may be a neurobiological mechanism that contributes to the co-occurrence of alcoholism and depression.

Factors that reduce neurogenesis, such as stress and dysregulation of monoamines (Jacobs et al. 2000), have been shown to increase depression-like behavior (Hwang et al. 1999; Johnson et al. 2006; Plaznik et al. 1988; Prince and Anisman 1984) as measured by the forced swim test (Aberg et al.) in rodents (Porsolt et al. 1977). Major classes of antidepressants including tricyclic antidepressants, selective serotonin reuptake inhibitors, and monoamine oxidase inhibitors show efficacy in the FST (Cryan et al. 2005; Dalvi and Lucki 1999; Lucki et al. 2001) and increase hippocampal neurogenesis (Malberg et al. 2000). Moreover, antidepressant efficacy in rodent behavioral models may require hippocampal neurogenesis (Santarelli et al. 2003). It is not known if antidepressants reverse alcohol-induced changes in neurogenesis or depression-like behavior.

Important clinical evidence indicates that depression that emerges during abstinence following alcohol drinking has a greater negative impact on relapse rates than pre-existing depression (Hasin et al. 2002), yet preclinical studies on this topic are lacking. In this study, we report the development and validation of a novel model of alcohol abstinence-induced depression-like behavior in mice as measured by the FST (Porsolt et al. 1977). Using this model, we demonstrate that abstinence-induced changes in depression-like behavior are associated with reduced hippocampal neurogenesis, and that this deficit is reversed in association with the positive behavioral effects of antidepressant treatment.

METHODS

Animals

Adult male C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME) were singly housed in standard Plexiglas cages with Purina Rodent Chow and fluid always available. Mice were 9 weeks old at the start of all experiments, and weighed between 22-26g at the beginning of the experiments. At the end point of experiments, mice weighed between 25-31g. The vivarium was maintained at 27ºC on a reverse 12:12 light-dark cycle (lights on at 22:00). Animals were under continuous care and monitoring by veterinary staff from the Division of Laboratory Animal Medicine at UNC-Chapel Hill, and all procedures were also carried out in accordance with the *NIH Guide to Care and Use of Laboratory Animals* (National Research Council, 1996) and institutional guidelines.

Experimental Procedures

In the first set of experiments, mice were weighed and handled daily for 7 days prior to experiments to facilitate adaptation to the laboratory (Figure 3A). All mice were administered BrdU (see below) once per day for 3 days. Animals were then randomly assigned to experimental groups. Experimental groups were as follows: alcohol-drinking mice were allowed to voluntarily consume alcohol or water for 28 days using a two-bottle drinking procedure (see below) and were behaviorally tested after 1 (n=12) or 14 (n=12) days of abstinence; water-only controls (n=12) had access to two bottles of water for 28 days and then were behaviorally tested one day later. Behavior was evaluated in the forced swim test. Mice were returned to the vivarium after behavioral testing and brains were removed 24 hours later (Figure 3A).

In the second set of experiments, mice were allowed 7 days to adapt to handling and the laboratory environment (Figure 3B). Mice were randomly assigned to the following treatment conditions: two groups of alcohol-drinking mice voluntarily consumed alcohol or

water for 28 days using a two-bottle drinking procedure (see below); one group of water-only controls had access to two bottles of water for 28 days. Alcohol-drinking mice then received injections of either vehicle (n=12) or desipramine (n=12, see Drugs below) during a 14 day abstinence period. Water-only control mice received daily injections of either vehicle (n=12) or desipramine (n=12) in parallel to the alcohol-abstinent mice. Water-only mice that received vehicle injections served as a control for alcohol exposure and injection stress. In addition, this group allowed us to rule out potential effects of aging on neurogenesis. The first injection was given the same day that alcohol bottles were removed from the cages, so that the 14th injection occurred 13 days after removal of alcohol. Behavior of all mice was evaluated in the forced swim test 1 day after the final desipramine injection (Figure 3B). Desipramine was not administered on behavioral test days, in order to examine the effects of chronic, but not acute, antidepressant treatment. Animals were sacrificed 24-hr after behavioral testing (Figure 3B).

An additional experimental group was included in immunohistochemical studies in which animals were sacrificed at the end of their 28th day of alcohol drinking (0 days of abstinence). This group was not included in behavioral studies to avoid potential confounding effects of acute alcohol.

Two-Bottle Drinking Procedure

During the one week period of acclimation and handling, water was the only fluid available. All fluids were presented in the home cage via 50mL plastic centrifuge bottles with stainless steel sipper tubes. Alcohol-drinking mice were then given access to one bottle of water and one bottle of alcohol (10%v/v) as previously described (Hodge et al. 1999). Animals were weighed and the fluid levels in the bottles were monitored to the nearest 0.5 mL at 24-hr intervals to determine daily fluid intake (mL and g/kg). The position (left-right) of the alcohol and water bottle was changed each day to control for side preferences. Mice

were given access to alcohol for 28 days. Control animals had access to two bottles of water for the same duration (28 days). On day 23 of alcohol drinking, tail blood was collected and analyzed for blood alcohol level (File et al.). On the final day of drinking, the alcohol solution-containing bottle was removed from the cages of alcohol drinkers, and one water bottle was removed from the cages of controls; thus, during abstinence only one bottle was on each cage.

Blood Alcohol Determination

BAL was measured from tail vein blood samples taken between 2 and 3 hours into the dark cycle as an index of alcohol intake. Individual blood samples (approx. 20 μl) were centrifuged and 5 μl of plasma from each sample was used to determine alcohol concentration using an AM1 Alcohol Analyzer (Analox Instruments, Lunenburg, MA).

Forced Swim Test Procedure

Depression-like behavior was assessed in mice using the FST (Porsolt et al. 1977). Mice were placed in a 2000mL beaker containing 1300mL of water maintained at 23-25°C for 6 minutes. Behavior was videotaped for later analysis by two trained observers who were blind to the treatment conditions. The duration of immobility during the last 4 minutes of the FST was measured during a single session as an index of depression-like behavior. Mice were considered immobile if they were completely still except for small movements of the paw made only to keep the mouse afloat, and not producing any noticeable propulsion of the mouse.

Open-field anxiety testing

Spontaneous locomotor activity was measured in brightly lit (500 lux) chambers (43 × 43 cm) as described (Hodge et al. 2002). For assessment of activity in the center of the field, the chamber floor was divided post hoc into a center zone (21 × 21 cm; center equidistant from all four walls of the chamber) and a periphery zone (the remaining area of the floor).

Distance traveled and time spent in each area was calculated from the locomotor activity data. After each test session, the equipment was cleaned with acetic acid (2%) to limit animal odors.

Drugs

Bromo-deoxyuridine (BrdU) (Sigma-Aldrich, St. Louis, MO) (300 mg/kg, 15 mL/kg, dissolved in saline, IP), used here as a marker of surviving neural progenitor cells, was administered once a day for three days before the onset of alcohol drinking. Where indicated, mice were injected with desipramine-HCl (Sigma-Aldrich, St. Louis, MO) (15 mg/kg, 10 mL/kg, IP) or vehicle (0.9% saline, 10mL/kg, IP) once per day for 14 days during abstinence. The antidepressant desipramine was used in this study because it has been shown to be highly effective (more so than fluoxetine) in the FST in C57BL/6J mice (Lucki et al. 2001). This particular dose of desipramine was selected based on its efficacy in the FST and its ability to produce hippocampal neuroadaptive changes (Lucki et al. 2001; Thome et al. 2000).

Tissue Fixation and Sectioning

Animals were deeply anesthetized with pentobarbital (60 mg/kg, 10 mL/kg, IP) and perfused transcardially with 0.1 M phosphate buffered saline (PBS) followed by 4% paraformaldehyde. The brains were removed from the skull and placed in the same fixative solution for at least 24-hr before being washed with PBS and sliced on a Leica VT 1000S vibrating microtome into 40 µm sections. The free-floating sections were stored in cryoprotectant at -20°C until immunohistochemistry was performed.

Immunohistochemistry

Tissue was washed in PBS, and then treated with 0.6% hydrogen peroxide to block endogenous peroxidase activity. Tissue was incubated for 1-hr in a blocking solution (3% horse serum, 0.1% Triton-X, 0.1M PBS) before incubation in the primary antibody. Sections were incubated in the anti-PCNA antibody (EMD Biosciences Inc, San Diego, CA; dilution

1:400) overnight at 4°C or in the anti-doublecortin antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA; dilution: 1:1000) for 48 hrs at 4°C. The following day, tissue sections were rinsed in PBS before incubation in the appropriate secondary antibody (Vector Laboratories, CA) for 1-hr. Tissue was then processed with avidin-biotin complex (Vector ABC kit, Vector Laboratories, CA) and immunoreactivity was visualized with DAB (Polysciences, Inc, PA). Tissue sections were then rinsed in PBS and, in the case of PCNA immunohistochemistry, counterstained with toluidine blue before sections were mounted onto slides.

For BrdU immunohistochemistry, tissue was prepared according to the method of Nixon and Crews (Nixon and Crews 2002). Tissue was washed in PBS, and then treated with 0.6% hydrogen peroxide to block endogenous peroxidase activity. Next, tissue was incubated in 50% formamide/2X SSC for 2 hrs at 65°C, then in 2N HCl for 1 hr at 37°C. After neutralizing in 0.1M boric acid, tissue was rinsed in PBS, then incubated overnight at 4°C in primary antibody (anti-BrdU: Accurate Chemical and Scientific Corp., NY; dilution 1:400). The following day was performed as described above for PCNA immunohistochemistry. Antibody dilutions were chosen based on dilution curves, and antibody specificity was verified secondary only controls in all immunohistochemical assays.

Analysis of Immunohistochemistry

For BrdU and PCNA immunohistochemistry, immunopositive cells in the dentate gyrus were counted manually with an oil immersion lens (Plan Apo 60x oil; numerical aperture, 2.0; Olympus Optical, Melville, NY). Profile counting methodology was used as it has previously been shown that profile counting and stereological estimations show identical results in percentage change (Crews et al. 2004). Moreover, stereology may not be appropriate for quantifying BrdU + cells, because they are not homogeneously distributed throughout the dentate (Popken and Farel 1997). Cell counts are expressed as the number of immunopositive cells per dentate gyrus section. For doublecortin immunohistochemistry, image analysis software (Bioquant Nova Advanced Image Analysis; R&M Biometric,

Nashville, TN) was used to measure optical densitometry of the staining. Densitometry measurements are divided by the area of the section and expressed as pixels/mm^2. Every sixth section within stereotaxic range from Bregma -1.46mm to Bregma -2.54mm (Paxinos and Franklin 2001) was analyzed to generate an average cell count or pixel density for each subject. Experimenter performing immunohistochemical analyses was blind to the treatment condition of the subjects. Desitometry analyses were repeated during a subsequent Bioquant session to ensure that results replicated.

Immunofluorescence

Triple-label confocal microscopy was performed according to Nixon and Crews (2004). The colocalization of BrdU with neuronal-specific nuclear protein (NeuN) or glial fibrillary acidic protein (GFAP) proteins was used to characterize the fate of surviving NPCs. Tissue was washed in PBS, then incubated in a blocking buffer. Tissue was then incubated in a solution containing primary antibodies (rat anti-BrdU, Accurate, Westbury, NY; dilution 1:400; mouse anti-NeuN, Chemicon, Temecula, CA; dilution 1:500; rabbit anti-GFAP, Dako, Glostrup, Denmark; dilution 1:2000). The following day, tissue was rinsed in PBS, then incubated in the dark with fluorescent-coupled secondary antibodies appropriate for each primary antibody. Tissue was then rinsed in PBS and mounted onto slides, taking care to protect the sections from the light. Slides were coverslipped using ProLong Anti-Fade reagent (Molecular Probes, OR).

Visualization and Analysis of Immunofluorescence.

Triple-label immunofluorescence was visualized with a Zeiss (Oberkochen, Germany) Axiovert LSM510 confocal microscope on multitrack setting with a water immersion lens (C-Apochromat 40x/1.2 W corr). Colocalization was confirmed by confocal microscopy optimized for the analysis of tissue sections. For each subject, 50 BrdU-labeled cells were analyzed for colocalization with GFAP or NeuN. Percentages of BrdU-labeled cells that colabeled with each cell type were determined. The 50 cells were selected from

several fields in a minimum of five tissue sections. Z-plane section images (512 x 512 pixels) were collected at <1 μm thickness and then analyzed using LSM Image Examiner software (Zeiss). The criteria for co-labeling included appropriate morphology and more than two Z-plane images of co-positive staining.

Statistical Analyses

In the first set of experiments, between subjects one-way analysis of variance (ANOVA) were used to analyze changes in behavior and immunohistochemistry. Post-hoc Dunnett's tests were conducted to determine if treated groups were different from control. In the second set of experiments, between subjects two-way ANOVA were used to analyze changes in behavior and immunohistochemistry. Post-hoc Tukey tests were used to determine between-group differences. Statistical significance was always defined as $p<0.05$.

RESULTS

Abstinence following Alcohol Drinking is Associated with an Increase in Depression-Like Behavior

To determine if abstinence following alcohol drinking is associated with changes in depression-like behavior, FST performance of water-only control mice was compared to mice that consumed alcohol for 28 days followed by 1 or 14 days of abstinence. Alcohol exposed mice voluntarily consumed average of 17.8 ±1.0 g/kg/day of alcohol (10% v/v) in the home-cage for 28 days, which resulted in peak blood alcohol levels of 112.8±7.4 mg/dL. Total fluid intake did not differ between water and alcohol exposed mice (data not shown). One way ANOVA on FST data showed a significant main effect of exposure condition [$F(2,33)=3.53$, $p<0.05$] that was due to a 36% increase in immobility following 14 days of abstinence as compared to water-only controls ($p<0.05$, Dunnett's test). No significant increase in immobility was evident 1 day after cessation of alcohol drinking

(Figure 4). These data show for the first time that alcohol drinking, followed by abstinence, leads to an emergent increase in depression-like behavior.

Abstinence following Alcohol Drinking is Associated with a Transient Increase in Anxiety-Like Behavior

Anxiety-like behavior was evaluated by measuring the amount of time spent in the center of an open field. A one way ANOVA comparing water mice to mice that drank alcohol for 28 days followed by 1 or 14 days of abstinence demonstrated a significant difference in time spent in the center of an open field [$F(2,33)=13.33$, $p<0.001$] (Table 1). A significant decrease in the amount of time spent in the center of an open field was evident in mice after 1 day of abstinence ($p<0.05$, Dunnett's test) but not 14 days of abstinence ($p>0.05$, Dunnett's test). These data indicate a temporary increase in anxiety-like behavior after 1 day of abstinence that disappears by 15 days of abstinence. Overall locomotor activity was analyzed 1 or 14 days after the removal of alcohol access. A one way ANOVA showed an overall difference in locomotor activity [$F(2,33)=4.26$, $p=0.02$] (Table 1); however post hoc comparisons revealed that neither alcohol treatment differed from control ($p<0.05$, Dunnett's test).

Survival and Differentiation of Neural Progenitor Cells

Survival of hippocampal NPCs is reduced by exposure to forced alcohol liquid diet in rats that causes high blood alcohol levels (Herrera et al. 2003). However the effects of abstinence following chronic voluntary drinking on the survival of NPCs are not known. BrdU was injected before the onset of drinking in order to assess the number of dividing cells that survived until the brains were removed. There was no significant difference [$F(2,29)=1.67$, $p=0.207$] in the number of BrdU-labeled cells per dentate gyrus that remained after 1 or 14 days of abstinence following chronic voluntary drinking compared to water-only controls (Figure 5A). Representative images of BrdU immunohistochemistry in the dentate gyrus are shown (Figure 5B). These results indicate that neither short term nor protracted abstinence

affects the survival of NPCs in this model of alcohol consumption. Although no change in the number of surviving BrdU-labeled cells was evident, the number of surviving NPCs that became neurons or glia could have been affected. Triple label immunofluorescence, however, revealed that the remaining BrdU-labeled cells underwent neuronal and glial differentiation at the expected rates (Nixon and Crews 2004). Of the BrdU-labeled cells analyzed from alcohol drinkers (n=50 cells/animal) and water-only controls (n=50 cells/animal), 89 and 92% colocalized with NeuN in the dentate gyrus, respectively. Representative immunofluorescence in the dentate gyrus is shown (Figure 5C and 5D). These data indicate chronic voluntary drinking does not affect the percentage of neuronal differentiation of surviving NPCs.

Proliferation of Neural Progenitor Cells

Although survival of NPCs was unchanged by voluntary alcohol drinking and abstinence, it is possible that other stages of neurogenesis, such as proliferation, were affected. Previous studies have demonstrated biphasic alterations in NPC proliferation associated with alcohol exposure and withdrawal (Aberg et al. 2005; Crews et al. 2004; He et al. 2005; Nixon and Crews 2002). This study examined the effects of 0 days, 1 day, and 14 days of abstinence following chronic voluntary drinking on proliferation of NPCs, using immunohistochemical analyses of PCNA expression. PCNA is a protein expressed on the nuclei of cells in the G1/S phase, therefore, PCNA immunoreactivity can be used to determine the number of cells undergoing proliferation in the hippocampus. To determine if abstinence from chronic drinking (and depression-like behavior) is associated with changes in NPC proliferation, serial sections of hippocampus were immunolabeled with proliferating cell nuclear antigen (PCNA), which is a cell cycle specific nuclear protein belonging to the replication complex that permits DNA synthesis needed for G1 to S transition(Krude 1999; Yew et al. 2001) and correlates with the proliferative state of cells (Celis et al. 1987). One way ANOVA comparing PCNA cell counts from alcohol drinking mice to water-only control

mice revealed a main effect [$F(3,36)=6.5$, $p=0.001$]. Follow up comparisons showed that abstinence following alcohol drinking for 14 days produced a significant decrease in NPC proliferation ($p<.05$, Dunnett's test) as indexed by the number of PCNA labeled cells, while 0 days and 1 day of abstinence did not produce a change in PCNA immunoreactivity (Figure 6A). Representative photomicrographs illustrating PCNA immunoreactivity in the dentate gyrus are shown in Figure 6C. These results indicate that 14 days of abstinence following voluntary drinking leads to a decrease in the proliferation of NPCs in the dentate gyrus.

Number of Immature Neurons

Another approach to assessing neurogenesis is to examine the number of immature neurons in the dentate gyrus. Doublecortin immunohistochemistry can be used to determine the density of cells in the hippocampus that have proliferated, survived, differentiated into neurons, and begun to develop neurites (Couillard-Despres et al. 2005; Rao and Shetty 2004). A main effect was revealed by a one way ANOVA comparing alcohol drinking animals to water-only controls [$F(3,40)=8.2$,$p=0.001$]. Similar to results from PCNA immunohistochemistry, the density of doublecortin immunoreactivity was significantly decreased following 14 days ($p<0.01$, Dunnett's test), but not 0 or 1 day of abstinence following alcohol drinking relative to control (Figure 6B). Representative photomicrographs illustrating DCX immunoreactivity in the dentate gyrus are shown in Figure 6D. These data suggest that the number of new neurons in the dentate gyrus is reduced following 14 days of abstinence following voluntary drinking.

Antidepressant Treatment during Abstinence

To determine if the emergence of depression-like behavior during abstinence can be prevented by administration of antidepressant medication, mice were given a single injection of desipramine each day for 14 days beginning the day alcohol was removed from the home cage. The day after the last desipramine injection, mice were tested in the FST. Desipramine was also used here to validate that the increase in immobility in the FST was a

depression-related behavior. Results indicated that chronic desipramine treatment prevented the emergence of depression-like behavior in mice that were treated with the antidepressant during two weeks of abstinence following chronic drinking. Antidepressant treatment had no effect on the behavior of mice with no history of drinking. A two-way ANOVA found a significant interaction between alcohol drinking history and antidepressant treatment [$F(1,41)=9.74$, $p=0.003$]; there was no main effect of alcohol or desipramine alone. Post-hoc multiple comparison procedures showed that immobility was increased in ethanol drinking mice relative to water controls under the vehicle condition ($p=0.007$, Tukey test), indicating that abstinence following chronic drinking was associated with increased depression-like behavior. Within ethanol-drinking mice, desipramine treatment significantly decreased immobility in the FST ($p=0.008$, Tukey test), demonstrating that desipramine prevents increases in depression-like behavior following abstinence from chronic drinking (Figure 7A). Moreover, alcohol-exposed mice that received desipramine treatment did not differ from water controls, indicating that the depression-like behavior that emerged during abstinence was fully blocked by the antidepressant desipramine.

A two-way ANOVA revealed that anxiety as measured by time spent in the center of an open field did not differ between water and alcohol drinking groups [$F(1,41)=0.62$, $p=0.43$], or between vehicle of desipramine treated groups [$F(1,41)=0.40$, $p=0.53$]. Additionally, there was no interaction between drinking history and desipramine treatment [$(F1,41)=3.32$, $p<0.08$] (data not shown). These data indicate that neither 14 days of abstinence, nor desipramine treatment alter the amount of time spent in the center of an open field. Overall locomotor activity was also measured. A two-way ANOVA revealed a significant effect of alcohol [$F(1,41)=5.27$, $p=0.02$], and a significant effect of desipramine [$F(1,41)= 10.60$, $p=0.002$], but no interaction between drinking history and desipramine treatment [$F(1,41)=1.39$, $p=0.25$] (data not shown). These data suggest that 14 days of

abstinence from alcohol drinking leads to an overall decrease in locomotor activity, and that 14 days of desipramine treatment leads to an overall increase in locomotor activity.

Because studies have indicated that increasing neurogenesis may be a mechanism by which antidepressants produce their behavioral effects, we tested the ability of desipramine to block the decrease in neurogenesis associated with the emergence of depression-like behavior following two weeks of abstinence from chronic drinking. Immunohistochemical analyses of PCNA and DCX expression were performed to measure proliferation of NPCs and the number of immature neurons, respectively, 14 days after cessation of chronic drinking. Two-way ANOVA revealed no main effect of alcohol drinking or antidepressant treatment alone on the number of PCNA labeled cells. Importantly, however, there was a significant interaction between alcohol drinking history and desipramine treatment [$F(42,1)=4.71$, $p=0.036$]. Post-hoc multiple comparisons (Tukey test) showed that the interaction was due to a significant reduction in the number of PCNA labeled cells in alcohol drinking mice as compared to water controls in the vehicle condition ($p=0.012$, Figure 7B). The number of PCNA positive cells following desipramine treatment was not different from water controls (Figure 7B). These data indicate that abstinence following chronic drinking decreases the number of proliferating cells in the dentate gyrus, and that this decrease is blocked by administration of desipramine during the abstinence period. Similarly, a two-way ANOVA found only a significant interaction between alcohol drinking history and antidepressant treatment on DCX density [$F(41,1)=10.3$, $p=0.003$]. As shown in Figure 7C, DCX density was reduced in alcohol exposed mice within the vehicle condition as compared to water controls ($p=0.004$, Tukey test). Also, desipramine blocked the reduction in DCX density in alcohol exposed mice ($p=0.005$, Tukey test). Taken together, these data suggest that abstinence following chronic drinking leads to decreased neurogenesis in the dentate gyrus, and that desipramine treatment during abstinence prevents this decrease.

DISCUSSION

Clinical studies have noted high degrees of comorbidity between alcoholism and depression (Pettinati 2004) and shown that depression that emerges during abstinence increases the likelihood of relapse (Hasin et al. 2002). Although little is known about the factors that promote this comorbidity, converging evidence implicates reductions in hippocampal neurogenesis as a mechanism that may underlie pathologies associated with both alcoholism and depression (Nixon 2006; Warner-Schmidt and Duman 2006). In this study, we show that depression-like behavior is increased during abstinence following voluntary alcohol drinking in mice. This behavioral pathology is associated with a reduction in hippocampal neurogenesis and treatment with the antidepressant desipramine restores the behavior and measurements of neurogenesis to normal levels. From these results, we propose that reduced hippocampal neurogenesis reflects a neuroadaptive process that underlies depression-like behavior that emerges during abstinence following alcohol drinking.

Though alcoholism is commonly associated with human depression, a functional relationship between alcohol consumption and depression-like behavior in a preclinical model has not been demonstrated previously. Using a novel mouse behavioral model, this study establishes an important link between abstinence following moderate alcohol drinking and depression. In mice that voluntarily consumed alcohol for 28 days, increased depression-like behavior as measured by the FST was evident 14 days after alcohol was removed from the home cage. Although FST performance showed a trend after 1 day of abstinence, this increase in depression-like behavior was not significantly different from water controls. This suggests that the depression-like behavior was dissociated from any immediate effects of alcohol exposure or withdrawal. Further, the absence of withdrawal seizures, weight loss, or changes in overall locomotor activity suggests that the voluntary alcohol drinking procedure did not produce dependence. Overall, these results suggest that

neuroadaptive changes that occur during protracted abstinence following alcohol drinking lead to the emergence of depression-like behavior, even in moderate drinkers.

In addition to identifying the emergence of depression-like behavior during protracted abstinence, the results of this study complement numerous investigations that have established a link between alcohol withdrawal and anxiety. For example, withdrawal from alcohol liquid diet, chronic injections, vapor exposure, or consumption from a single drinking bottle increase anxiety-like behavior in mice that persists for up to 48-h after alcohol exposure (Costall et al. 1988; Joshi et al. 2005; Kliethermes et al. 2004; Prediger et al. 2006; Sparta et al. 2008). Similar results have been obtained in rat models showing increased anxiety-like behavior during acute and early withdrawal from an alcohol liquid diet (Moy et al. 1997; Pandey et al. 1999; Rassnick et al. 1993). In the present study, we observed increased anxiety-like behavior in non-dependent mice 1 day after voluntary alcohol drinking as measured by an open-field thigmotaxy assay. Alcohol exposed mice did not differ from water controls when anxiety-like behavior was measured after 2-wks of abstinence. Accordingly, other data show that withdrawal from alcohol vapor increases anxiety-like behavior in rats during acute (8 hr) withdrawal but the effect dissipates by 2 weeks (Zhao et al. 2007). However, studies have reported increased anxiety-like behavior at 4 (Rasmussen et al. 2001) and 12 weeks (Zhao et al. 2007) after withdrawal from alcohol liquid diet or vapor exposure, respectively. Together, these and other data (Kliethermes 2005) suggest that anxiety is a prominent feature of abstinence that may dissipate or cycle over time. Importantly, we found no evidence of increased anxiety-like behavior after 2 weeks of abstinence when depression-like behavior emerged, which suggests a time-dependent dissociation of these behavioral pathologies during abstinence.

A key finding of this preclinical study is that chronic treatment with the antidepressant desipramine during abstinence completely blocked the emergence of abstinence-induced depression as measured by the FST in mice. Although the FST is most widely used as a

behavioral screen for antidepressant efficacy, previous studies have also demonstrated that factors associated with human depression including chronic stress, inescapable stress, dysregulated monoamine function, and elevated glucocorticoids produce immobility in the FST (Hwang et al. 1999; Johnson et al. 2006; Plaznik et al. 1988; Prince and Anisman 1984). The efficacy of desipramine validates the FST as a measure of depression in the present study. Importantly, the dose of desipramine used had no effect on behavior in the FST in water-only control mice. Furthermore, the prevention of depression-like behavior with desipramine, a tricyclic antidepressant, suggests that abstinence following chronic drinking may lead to dysregulation of noradrenergic and other monoaminergic systems, and that desipramine may prevent this dysregulation. Additional mechanistic studies are required to test this hypothesis.

While the effectiveness of antidepressants in treating depression is well validated, potential efficacy of these compounds in the treatment of alcoholism needs further study. There are only a limited number of well-controlled clinical studies on the efficacy of antidepressant use for alcoholics (Ostacher 2007). Clinical studies have demonstrated that antidepressants are effective in reversing depression comorbid with alcoholism, and have shown modest success in preventing relapse (Le Fauve et al. 2004). As mentioned above, depression that occurs during abstinence has been linked to an increased risk of relapse (Hasin et al. 2002). When taken together with the present results, these findings suggest that antidepressant treatment that begins concurrently with cessation of drinking may be helpful in preventing the emergence of abstinence-related depression in the clinic. In addition, the beneficial effect of antidepressant treatment during abstinence provides further support for the conclusion that depression-like behavior may be functionally linked to abstinence-induced neuroadaptive changes.

The concept of adult neurogenesis has provided a new framework (e.g., the idea of *structural plasticity*) for understanding neuroadaptive changes in the adult brain, which

extends prevailing theories that regard changes in synaptic efficacy (Hebb 1949) as a solitary mechanism of neuroplasticity (Gage 2002). Accumulating evidence now indicates that NPCs exist in a variety of mammalian brain regions where they give rise to new neurons, astrocytes, and oligodendrocytes throughout life (Ming and Song 2005). Neurogenesis in the adult brain is a dynamic process that can be influenced by internal and external events that also influence behavior (Carlezon et al. 2005; Crews and Nixon 2003; Duman et al. 1999; Gage 2002; Henn and Vollmayr 2004; Kempermann 2002; Ming and Song 2005). In general, evidence suggests that new neurons in the adult brain contribute to synaptic plasticity (Snyder et al. 2001), as well as the general ability of the organism to adapt to changing circumstances (Kempermann 2002). Thus, neurogenesis is a structural event that may have functional consequences. Indeed, emerging evidence suggests that hippocampal neurogenesis may be functionally linked to stress and mood disorders (Dranovsky and Hen 2006; Malberg and Blendy 2005; Mirescu and Gould 2006; Warner-Schmidt and Duman 2006)cf.(Henn and Vollmayr 2004).

Generation of functional new neurons by neurogenesis requires proliferation, differentiation and survival of NPCs. In this study, we sought to determine if abstinence following voluntary alcohol drinking (and depression-like behavior) is associated with changes in adult hippocampal neurogenesis. To address this goal, serial sections of hippocampus were immunolabeled for PCNA, which is a cell cycle specific nuclear protein belonging to the replication complex that permits DNA synthesis needed for G1 to S transition (Krude 1999; Yew et al. 2001) and correlates with the proliferative state of cells (Celis et al. 1987). Results showed that 14 days of abstinence following alcohol drinking was associated with a 40% reduction in the number of PCNA positive cells (cells in the process of proliferating) in the dentate gyrus as compared to water-only control mice. Abstinence from alcohol drinking was also associated with a 40% reduction in immunoreactivity for DCX, which is a protein expressed by new cells that are in the process

of differentiating into mature neurons. However, no reductions in PCNA or DCX immunoreactivity were observed at 0 or 2 days after alcohol indicating that the reduction in proliferating and differentiating NPCs emerged during protracted abstinence. These results indicate that abstinence-induced depression-like behavior in mice is associated with a concomitant reduction in hippocampal neurogenesis.

In contrast to the effect of protracted abstinence, we found no effect of 28 days of voluntary alcohol drinking, or short-term abstinence (2 days), on adult hippocampal neurogenesis (Figure 6B). Several studies have demonstrated that exposure to high doses of alcohol has a detrimental effect on adult hippocampal neurogenesis. For example, exposure to alcohol through binge injection or forced liquid diet models in rats produces significant decreases in proliferating NPCs and differentiating neurons (He et al. 2005; Nixon and Crews 2002). In mouse models of voluntary alcohol drinking, high dose intake (>25g/kg/day) is also associated with a profound decrease (Crews et al. 2004), whereas lower dose intake (6g/kg/day) is associated with an increase in NPC proliferation (Aberg et al. 2005). In the present study, we found that 17-18 g/kg/day of voluntary alcohol intake produced no change in proliferating or differentiating NPCs in the dentate gyrus in mice. Together these studies suggest that alcohol produces dose-dependent effects on hippocampal neurogenesis.

Emerging studies have also shown that exposure to high doses of alcohol via binge injection or liquid diet reduces NPC survival in rats (He et al. 2005; Herrera et al. 2003; Nixon and Crews 2002). To determine if voluntary alcohol drinking and/or abstinence is associated with changes in NPC survival, mice were injected with BrdU just before the onset of 28 days of alcohol drinking, and the number of surviving cells was measured following 2 or 14 days of abstinence. Results showed that the number of BrdU positive cells in the dentate gyrus of alcohol exposed mice was unchanged following short-term or protracted abstinence as compared to water-only controls. Although the overall number of surviving

NPCs was unchanged, it is possible that there was a change in the number of surviving cells that became neurons rather than glia. However, the data from triple-label immunofluorescence experiments, found no difference in the percentage of BrdU positive cells that were co-labeled with either GFAP or NeuN in the dentate gyrus of alcohol exposed mice as compared to water-only controls. These data indicate that the abstinence-induced depression-like behavior in the present study was not associated with changes in the survival rate or phenotype of cells born before the onset of drinking. Thus, alcohol-induced changes in the survival of NPCs may require higher doses of alcohol than those that are achieved by voluntary drinking in mice.

Because it is known that factors that precipitate depression, such as stress and increased glucocorticoids, decrease hippocampal neurogenesis (Mirescu and Gould 2006) and that antidepressants increase hippocampal neurogenesis (Malberg and Blendy 2005; Malberg et al. 2000; Santarelli et al. 2003), we sought to determine if antidepressant treatment is effective in preventing abstinence-induced impairments in hippocampal neurogenesis that occur in parallel with depression-like behavior. Chronic treatment with the antidepressant desipramine during 14 days of abstinence following alcohol drinking prevented the decrease in the number of immature neurons, as well as the number of proliferating cells in the dentate gyrus. While the decrease in proliferating cells was smaller in the second experiment relative to the first, this effect was due to lower raw data control values in the second experiment. We hypothesize that decreased proliferation in the control animals in the second experiment could be due to the effects of daily injections. It is worth noting that 14 days of desipramine administration had no effect on hippocampal neurogenesis in water-drinking mice as measured by PCNA and DCX immunohistochemistry. These data suggest that chronic desipramine treatment did not simply cause an overall increase in neurogenesis, but rather prevented abstinence-induced decreases in hippocampal neurogenesis.

The concomitant increase in DCX and PCNA immunoreactivity suggests that blockade of abstinence-induced depression-like behavior by desipramine may have been mediated by changes in multiple phases of hippocampal neurogenesis. Evidence indicates that NPCs express PCNA during and up to 4 days after the S phase of the cell cycle (Mandyam et al. 2007); however, DCX expression peaks in the dentate gyrus between 1 and 21 days after the S phase when NPC differentiation is occurring (Brown et al. 2003). Immunohistochemical studies of PCNA and DCX performed on a single day, therefore, are likely labeling mostly distinct populations of new cells. Thus, it is not clear from the present study if antidepressant treatment specifically protected against the abstinence-induced reduction in NPC proliferation or enhanced survival of post-proliferative cells born during the abstinence phase, either or both of which would lead to an increase in new neurons. Alternatively, it is also plausible that protection against the abstinence-induced reduction in the number of new neurons by desipramine may have been conferred by a compensatory burst in NPC proliferation, which has been reported to occur 7 days after withdrawal from binge injection of alcohol (Nixon and Crews 2004). These potential alternatives could be addressed in future studies that further elucidate the timecourse of changes in various phases of neurogenesis during abstinence from alcohol drinking. In either case, it appears from the present study that chronic antidepressant treatment protects against abstinence-induced reductions in both NPC proliferation and development of new neurons.

Although there is no single neurobiological mechanism that accounts for the behavioral pathologies associated with alcoholism or depression, converging evidence suggests that alcoholism and depression (and perhaps their co-occurrence) may share common molecular mechanisms of action. For example, chronic alcohol exposure reduces cAMP response element-binding protein (CREB) activity in the dentate gyrus (Bison and Crews 2003). Reductions in CREB activity and function can occur during acute and protracted alcohol withdrawal and are associated with negative mood states, such as anxiety (Pandey 2003)).

By contrast, a variety of antidepressant drugs increase levels of CREB mRNA in the hippocampus (Nibuya et al. 1996). Viral over-expression of CREB in the dentate gyrus of the hippocampus also results in less depression-like behavior in both the FST and learned helplessness tests (Chen et al. 2001). Accordingly, alcohol withdrawal is associated with reduced expression of CREB-target genes including brain-derived neurotrophic factor (BDNF) in the dentate gyrus (Tapia-Arancibia et al. 2001) whereas several different antidepressant drugs, including desipramine which was used in the present study, increase BDNF mRNA in the hippocampus (Nibuya et al. 1995). Moreover, chronic alcohol reduces hippocampal neurogenesis (Nixon and Crews 2002) but the behavioral effects of antidepressant medications may require hippocampal (Santarelli et al. 2003). These and other findings have led to emerging theories that implicate a CREB – BDNF – neurogenesis pathway in both the etiology of depression and antidepressant efficacy (Duman et al. 2001; Malberg et al. 2000; Thomas and Peterson 2003). Overall, CREB and BDNF activity in the dentate gyrus appear to be important mediators of antidepressant efficacy and raise the hypothesis that decreases in p-CREB, such as those that occur after chronic alcohol, have the potential to induce behavioral despair and reductions in neurogenesis as seen in the present study.

Several limitations to the present study should be discussed. First, although the data presented in this study suggest that depression-like behavior and related neuroadaptations emerge during abstinence following chronic drinking, it is worth mentioning that no single animal model of depression is sufficient to characterize the human condition of depression. In these experiments, we sought to characterize changes in affective behavior at a single time point during abstinence, which limited the number and type of behavioral measures we were able to conduct. Thus, it will be important in future studies to utilize other validated rodent behavioral tests of anxiety- and depression-like behavior to confirm and extend the

present results. Second, it is worth noting that a 300 mg/kg dose of BrdU was chosen to examine NPC survival based on a published method (Cameron and McKay 2001), which suggested that this dose labels all cells in the S phase in the rat. However, a recent publication (Mandyam et al. 2007) has demonstrated that 150 mg/kg is sufficient to label all cells in the S phase in the mouse. Thus, we cannot rule out the possibility that our BrdU injection regimen caused toxicity to dividing cells, especially in combination with subsequent alcohol consumption; however, as no differences were found in the number of BrdU-labeled cells between any groups, we feel this probably did not contribute significantly to our results. Finally, our results showing that desipramine did not alter FST performance in or neurogenesis in control mice are somewhat surprising. Another study demonstrated that chronic desipramine treatment increases neurogenesis and decreases depression-like behavior in control mice; however, this study used utilized twice-daily administration of 12.5 mg/kg desipramine (Gur et al. 2007), indicating that a higher dose may have produced changes in our control animals. Additionally, while acute desipramine treatment has been shown to reduce immobility in the FST (Lucki et al. 2001), we did not administer desipramine on the day of the test. Our results are similar to those found by MacQueen et al., (2003), showing that that 14 days administration of desipramine at the same dose used in the current study (15 mg/kg) and not administered on the day of testing reduced immobility in the FST in mice in which depression had been induced, but not in control mice.

In conclusion, alcoholism and depression are two devastating disorders that commonly co-occur. Emerging evidence indicates that alcohol has detrimental effects on molecular pathways that may be involved in the pathophysiology of depression. Important clinical evidence indicates that depression that emerges during abstinence has a greater negative impact on relapse rates than pre-existing depression. Preclinical studies on this topic are lacking. To address this need, we have developed and validated a novel model of alcohol drinking-induced depression-like behavior in mice. The data presented here lend

further support to the hypothesis that hippocampal neurogenesis is related to depression, as well as antidepressant efficacy. We show that abstinence following chronic alcohol drinking leads to a decrease in adult hippocampal neurogenesis at a time when depression-like behavior is also evident. Antidepressant treatment during abstinence prevents both the decrease in new neurons and the depression-like behavior, suggesting a mechanistic link. Given that new neurons constitute 6% of the total granule cells in the hippocampus, neurogenesis is crucial for hippocampal function (Cameron and McKay 2001) and of potential importance to the etiology and treatment of alcoholism and co-morbid depression.

CHAPTER III: THE EFFECTS OF DESIPRAMINE ADMINISTRATION DURING ABSTINENCE FOLLOWING ALCOHOL DRINKING ON CREB EXPRESSION AND ACTIVATION

INTRODUCTION

Alcoholism and depression are two of the most costly and widespread neuropsychiatric disorders worldwide. In the United States, the prevalence of a lifetime history of depression in alcohol dependent individuals has been reported to be about 42% (Miller et al. 1996; Schuckit et al. 1997b) but comorbidity ranges from 5% to as high as 80% have been noted in some studies (Pettinati 2004). When alcoholism and depression co-occur, relapse to alcohol use is more likely and remission from depression is less likely (Greenfield et al. 1998; Mueller et al. 1994) Importantly, depression that occurs during abstinence increases the likelihood of relapse in alcoholics, whereas prior-onset depression is not related to relapse (Hasin et al. 2002). Abstinence-related depression is thought to increase the chance of relapse because drinking may help people cope with negative emotions (Carpenter and Hasin 1998; Hasin and Grant 2002).

Converging evidence suggests that alcoholism and depression (and perhaps their co-occurrence) may share common molecular mechanisms of action. Our previous work indicates a direct mechanism by which abstinence following alcohol drinking leads to depression-like behavior, and suggests that alterations in neurogenesis could represent such a mechanism, though no causal relationship has been demonstrated (Stevenson et al.). Recent studies show that alcohol and antidepressant drugs produce opposite changes in second messenger systems in the hippocampus that can alter activity of transcription factors such as cAMP response element-binding protein (CREB). CREB activity influences

neurotrophic pathways and, depending on the direction of change, can increase or decrease hippocampal neurogenesis (D'Sa and Duman; Malberg and Blendy 2005). Interestingly, decreased CREB function in the nucleus accumbens has been shown to have an antidepressant effect (Pliakas et al. 2001), indicating that CREB may have regionally distinct effects on behavior. We hypothesized that CREB activation may be a molecular mechanism by which desipramine prevents changes in depression-like behavior and neurogensis produced by abstinence from chronic alcohol drinking.

The transcriptional regulator cAMP-response-element-binding protein (CREB) is widely and abundantly expressed in the mammalian brain. Transcription factors such as CREB transduce changes in membrane receptor activity and signaling pathways into alterations in gene expression. By regulating changes in gene expression, CREB activation can lead to long-term changes in the function of single neurons, brain regions, and neural circuits. Indeed, many of the plasticity-related neural events associated with addiction (Nestler 2004) and depression (Duman et al. 1997) are thought to involve changes in CREB activity. CREB-dependent transcription is primarily regulated by kinase-induced phosphorylation of CREB at serine residue 133 (Ser-133) (Johannessen et al. 2004). Changes in the level of p-CREB can in turn alter expression of any number of CREB target genes (i.e., genes with CRE sites in their promoter regions). CREB target genes are found in a variety of systems including: metabolism, transcription (e.g., EGR-1, c-FOS, JUND), neuropeptides (CRF, galanin, NPY, vasopressin), and growth factors (BDNF, TNFalpha) (Mayr and Montminy 2001). Identifying specific conditions that alter CREB activation has fundamental importance for understanding a variety of neural functions and disorders including alcoholism and depression.

Accumulating evidence also suggests that CREB (and its target genes) regulates alcohol dependence and withdrawal and may underlie anxiety associated with alcohol withdrawal

(Pandey 2003). Studies have shown that CREB activation is altered due to chronic and acute alcohol exposure. Furthermore, CREB activation in the amygdala, nucleus accumbens, and cortical structures is altered during the first 72 hours of withdrawal from chronic alcohol drinking. (Li et al. 2003; Misra et al. 2001; Pandey et al. 1999). Interestingly, CREB haplodeficient mice have a higher preference for ethanol, resulting in greater ethanol consumption in a two-bottle choice method of drinking (Pandey et al. 2004).

Our laboratory has developed a mouse model in which depression-like behavior emerges after two weeks of abstinence following chronic voluntary alcohol drinking (Stevenson et al.). We have shown that the emergence of depression-like behavior occurs in parallel with decreases in both proliferating and differentiating hippocampal neurons. In addition, this study showed that administration of the antidepressant desipramine for the duration of the abstinence period (14 days) completely prevented the increase in depression-like behavior, as well as the decrease in hippocampal neurogenesis.

In the current study, we examined the effects of abstinence following chronic alcohol on CREB activation. In addition, we studied the brains of mice that received chronic desipramine treatment during the abstinence period in order to investigate the role of CREB activation in the prevention of abstinence-induced depression-like behavior and decreases in neurogenesis. The nucleus accumbens, amygdala, and hippocampus are all brain regions that are implicated in emotionality as well as addiction; therefore we examined changes in CREB activation in the core and shell of the nucleus accumbens and the central and basolateral amygdala, in addition to the denate gyrus, CA1, and CA3 regions of the hippocampus.

METHODS

Animals

Adult male C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME) were singly housed in standard Plexiglas cages with Purina Rodent Chow and fluid always available. Mice were 9 weeks old at the start of all experiments. The vivarium was maintained at 27ºC on a reverse 12:12 light-dark cycle (lights on at 22:00). Animals were under continuous care and monitoring by veterinary staff from the Division of Laboratory Animal Medicine at UNC-Chapel Hill, and all procedures were also carried out in accordance with the *NIH Guide to Care and Use of Laboratory Animals* (National Research Council, 1996) and institutional guidelines.

Animals were handled and treated as previously reported (Stevenson et al.). Mice were allowed 7 days to adapt to handling and the laboratory environment. Mice were randomly assigned to the following treatment conditions: two groups of alcohol-drinking mice voluntarily consumed alcohol or water for 28 days using a two-bottle drinking procedure (see below); one group of water-only controls had access to two bottles of water for 28 days. Alcohol-drinking mice then received injections of either vehicle (n=12, saline) or desipramine (n=12, once per day, 15 mg/kg, I.P.) during a 14 day abstinence period. Water-only control mice received daily injections of either vehicle (n=12) or desipramine (n=12) in parallel to the alcohol-abstinent mice. Water-only mice that received vehicle injections served as a control for alcohol exposure and injection stress. After the 14th day of abstinence, animals were sacrificed and their brains were removed for immunohistochemical studies.

Two-Bottle Drinking Procedure

During the one week period of acclimation and handling, water was the only fluid available. All fluids were presented in the home cage via 50mL plastic centrifuge bottles with

stainless steel sipper tubes. Alcohol-drinking mice were then given access to one bottle of water and one bottle of alcohol (10%v/v) as previously described (Hodge et al. 1999). Animals were weighed and the fluid levels in the bottles were monitored to the nearest 0.5 mL at 24-hr intervals to determine daily fluid intake (mL and g/kg). The position (left-right) of the alcohol and water bottle was changed each day to control for side preferences. Mice were given access to alcohol for 28 days. Control animals had access to two bottles of water for the same duration (28 days).

Tissue Fixation and Sectioning

Animals were deeply anesthetized with pentobarbital (60 mg/kg, 10 mL/kg, IP) and perfused transcardially with 0.1 M phosphate buffered saline (PBS) followed by 4% paraformaldehyde. The brains were removed from the skull and placed in the same fixative solution for at least 24-hr before being washed with PBS and sliced on a Leica VT 1000S vibrating microtome into 40 µm sections. The free-floating sections were stored in cryoprotectant at -20°C until immunohistochemistry was performed.

Immunohistochemistry

Tissue was washed in PBS, and then treated with 0.6% hydrogen peroxide to block endogenous peroxidase activity. Tissue was incubated for 1-hr in a blocking solution (3% horse serum, 0.1% Triton-X, 0.1M PBS) before incubation in the primary antibody. Sections were incubated in the anti-phospho CREB antibody (Upstate Biotechnology Inc, Lake Placid, NY; dilution 1:2500) overnight at 4°C or in the anti-CREB antibody (Cell Signaling Technology, Danvers, MA; dilution: 1:4000) for 48 hrs at 4°C. The following day, sections were washed in PBS and incubated in secondary antibody for one hour (Dako EnVision Kit, Dako). Immunoreactivity was detected with nickel-enhanced diaminobenzidine (Dako EnVision Kit) as a chromagen. Sections stained for pCREB were counterstained with toluidine blue. All sections were then mounted onto slides, dried and coverslipped with

Cytoseal. For consistency of staining across subjects, brain tissue from all groups was processed simultaneously.

Immunoreactivity was quantified using Bioquant image analysis software (Bioquant Nova Advanced Image Analysis; R&M Biometric, Nashville, TN). The image was background corrected and normalized to preset light levels to ensure consistent data collection. Pixel count measurements were calculated from a brain region and divided by the area of the region and expressed as pixels/mm^2. All data was acquired by a researcher blind to group condition from every sixth section of a whole brain and averaged to obtain a single value per subject.

RESULTS

CREB expression and or activation has been shown to be involved in addiction, depression, antidepressant activity, and neurogenesis (Chen et al. 2001; Gur et al. 2007; Malberg and Blendy 2005; Nestler 2004; Pandey 2004; Tardito et al. 2006; Thome et al. 2000). In the current study, we performed immunohistochemical analyses of CREB and phospho-CREB in the brains of mice that drank alcohol vs. water or water only for four weeks, followed by two weeks of abstinence. During the abstinence period, mice received either desipramine or vehicle treatment.

Two-bottle choice drinking. A two-way repeated measures ANOVA revealed that mouse weight significantly increased over the six week period [$F(5,110)=118.161$, $p<0.001$], but that the weights of alcohol and water drinking mice were not different. A two-way repeated measures ANOVA comparing total fluid intake in alcohol versus water drinking animals over the six week experiment showed that, during the four weeks of two-bottle choice drinking, alcohol-drinking mice consume an significantly less fluid per day than water-drinking mice [$F(1,108)=15.397$, $p<0.001$]; however the difference in means is 0.451 mL. Additionally, there was a main effect of time during the experiment, with fluid consumption during the final two weeks (when only one bottle was present on the cages) being

significantly lower than the first four weeks (when two bottles are present on each cage) [$F(5,108)=3.566$, $p=0.005$], again with a difference of means of less than 0.5 mL. We believe this decrease in fluid intake over the final two weeks is probably due to smaller error (water dripping from the bottle) associated with reading fluid levels on one bottle instead of two.

CREB activation in the hippocampus. Evidence suggests that CREB activity in the hippocampus may be involved in depression, antidepressant activity, and neurogensis (Carlezon et al. 2005; Chen et al. 2001; Gur et al. 2007; Malberg and Blendy 2005; Nakagawa et al. 2002b; Nibuya et al. 1996; Thome et al. 2000). We measured pCREB immunoreactivity (IR) in the dentate gyrus, CA1, and CA3 regions of the hippocampus to determine if alcohol abstinence or desipramine treatment produced changes in CREB activation. A two way ANOVA revealed that both alcohol [$F(1,31)=7.1$, $p=0.012$] and desipramine [$F(1,31)=10.0$, $p=0.003$] significantly increased pCREB IR in the dentate gyrus. Additionally, there was a significant interaction between alcohol drinking and desipramine treatment [$F(1,31)=4.62$, $p=0.039$]. Post-hoc multiple comparisons demonstrated that desipramine treatment significantly increased pCREB IR in the dentate gyrus in W+D mice compared to W+S mice ($p<0.001$, Tukey test), and in E+S mice compared to W+S mice ($p=0.003$, Tukey test). These data indicate that both abstinence following alcohol drinking and treatment with the antidepressant desipramine promote CREB activity in the dentate gyrus. Desitometric analysis within the CA1 region of the hippocampus showed that neither abstinence following alcohol drinking nor desipramine treatment produced significant changes in pCREB IR. In contrast to the CA1, two way ANOVA of pCREB IR in the CA3 region of the hippocampus revealed a main effect of abstinence from alcohol drinking [$F(1, 31)=4.205$, $p=0.049$], as well as an significant interaction between alcohol drinking history and desipramine treatment [$F(1, 31)=4.216$, $p=0.049$]. Post-hoc multiple comparisons demonstrated a significant decrease in CREB activation in E+S mice compared to W+S mice ($p=0.008$, Tukey test), and compared to E+D mice ($p=0.018$, Tukey test),

demonstrating that desipramine treatment completely blocked or reversed abstinence-induced decreases in pCREB in the CA3.

CREB expression in the hippocampus. We examined expression of CREB using anti-CREB immunohistochemistry in dentate gyrus, CA1, and CA3 regions of the hippocampus. In the dentate gyrus, abstinence following alcohol drinking is associated with a small increase in CREB expression [$F(25,1)=4.431$, $p=0.046$]. This is in accordance with the parallel abstinence-induced increases in CREB activation in the dentate gyrus. While CREB activity in the CA1 was unchanged by abstinence following alcohol drinking and desipramine, this region did show significant changes in CREB expression. A two-way ANOVA revealed that desipramine caused a significant increase in CREB expression in the CA1 [$F(25,1)=9.735$, $p=0.005$], and a significant interaction between abstinence following alcohol drinking and desipramine treatment was also found [$F(25,1)=17.706$, $p<0.001$]. Post-hoc pairwise comparisons revealed that W+D mice had significantly higher levels of CREB expression compared to W+S mice ($p<0.001$, Tukey test), and E+S mice showed increased CREB expression compared to W+S mice ($p<0.001$, Tukey test). These data demonstrate that desipramine treatment increases CREB expression in the CA1, but does not cause a further increase in CREB expression in alcohol-abstinent animals. In the CA3 region of the hippocampus, abstinence following alcohol drinking was associated with a significant decrease in CREB expression [$F(23,1)=5.776$, $p=0.025$]. A two-way ANOVA also found an interaction between abstinence from alcohol drinking and treatment with the antidepressant desipramine [$F(23,1)=7.069$, $p=0.014$]. Post-hoc pairwise multiple comparisons revealed that a large decrease in CREB expression in E+S mice compared to W+S mice ($p=0.003$, Tukey test), and a large increase in E+D mice compared to E+S mice ($p=0.005$, Tukey test), indicating that desipramine treatment completely prevented alcohol-abstinence induced decreases in CREB expression in the CA3. due to alcohol abstinence was completely prevented by desipramine treatment These data demonstrate that CREB expression and

CREB activation in the CA3 are altered in a parallel manner in response to alcohol abstinence and desipramine treatment.

CREB activation in the nucleus accumbens. Activity of the transcription factor CREB in the nucleus accumbens has been implicated in drug addiction, alcohol exposure, and depression-like behavior (Carlezon, 2005; Li, 2003; Misra, 2001; Nestler, 2004; Pandey, 2004; Pliakas, 2001; Yang, 1998). In the present study we examined the effects of two weeks of abstinence following alcohol drinking on CREB activation in the nucleus accumbens core and shell. Additionally, we investigated if CREB activation is altered in the nucleus accumbens in response to desipramine treatment during the abstinence period. Both the core and the shell of the nucleus accumbens showed similar responses. Abstinence following alcohol drinking was not associated with a change in pCREB IR in the core [$F(1,30)=2.221$, $p=0.147$] or the shell [$F(1,30)=0.0359$, $p=0.851$] of the accumbens. Desipramine treatment, however, resulted in a significant overall increase in pCREB IR in both the core [$F(1,30)=5.096$, $p=0.031$] and the shell [$F(1,30)=8.150$, $p=0.008$]. These data suggest that two weeks of abstinence following chronic alcohol drinking is not associated with changes in CREB activity in the nucleus accumbens, but that desipramine treatment, regardless of drinking history, significantly upregulates CREB activity in the accumbens.

CREB expression in the nucleus accumbens. No changes were evident in CREB expression in the core or the shell of the nucleus accumbens in any group of animals.

CREB activation in the amygdala. CREB has been shown to be activated in the amygdala in response to alcohol and alcohol withdrawal (Pandey 2003; Pandey et al. 2003; Pandey et al. 2004; Pandey et al. 1999). In the current study, we sought to determine if CREB activity in the central (CeA)and basolateral (BLA) nuclei of the amygdala was altered in response to two weeks of abstinence following chronic alcohol drinking, as well as if desipramine treatment during the abstinence period affected CREB activity in these amygdalar nuclei. The CeA showed results similar to the nucleus accumbens. Desipramine

treatment during the abstinence period was associated with a significant overall increase in pCREB IR [F(1,31)=5.07, p=0.032]. Two weeks of abstinence from alcohol drinking did not produce any change in pCREB IR in the CeA. No changes in pCREB IR were evident in the BLA as a result of abstinence from alcohol drinking or desipramine treatment during the abstinence period. These data indicate that desipramine upregulates CREB activity in the CeA, but has no effect on CREB activity in the BLA.

CREB expression in the amygdala. Lastly, we examined CREB expression in the CeA and BLA. The CeA showed a significant increase in CREB activation in response to desipramine treatment, but no change in CREB expression; whereas the BLA showed no change in CREB activation, but did show a significant increase in CREB expression in response to desipramine treatment (F(28,1)=5.544, p=0.026). Abstinence following alcohol drinking was not associated with changes in CREB expression in either nucleus of the amygdala.

DISCUSSION

In the current study we demonstrate that abstinence following chronic voluntary alcohol drinking is associated with changes in CREB activation and expression in the hippocampus. Additionally, we show that desipramine, when administered for two weeks following chronic alcohol drinking or water drinking is also associated with changes in CREB activity and expression in the hippocampus and amygdala. CREB activation, but not expression in the nucleus accumbens is altered by chronic desipramine treatment in animals with or without a history of alcohol drinking. Together, these data suggest that CREB-dependent transcription is affected by both abstinence following alcohol drinking and desipramine treatment, and that these effects are brain region specific.

CAMP-response-element-binding protein is a transcription factor that, when phosphorylated at serine 133, is activated and can bind to CRE sites in the promoter region

of many genes. The binding of CREB is one of the requirements for activation of transcription of CRE site-containing genes. Phosphorylation of CREB can be accomplished via several kinases that are downstream of G-protein-coupled receptors, receptor tyrosine kinases, and calcium-dependent signaling cascades, all of which are implicated in alcohol addiction and depression (Mayr and Montminy 2001; Nestler 2004; Pandey 2004). CREB signaling, therefore, may represent a final common mechanism underlying both of these diseases. Abstinence following alcohol drinking has been shown to produce depression-like behavior, and this depression is prevented by antidepressant treatment (Stevenson et al.). We, therefore, wanted to determine the effects of abstinence following chronic alcohol drinking and antidepressant treatment on CREB signaling in brain regions known to be involved in alcoholism and mood.

The transcription factor CREB has been suggested to play an important role in the behavioral efficacy of antidepressants. Changes in CREB expression and activation are seen only after chronic, but not acute, administration of antidepressants (Nibuya et al.; Thome et al.). CREB, therefore, could be a mechanism underlying the delayed behavioral efficacy of antidepressants. Additionally, virally mediated expression of CREB of in the hippocampus of rats has been shown to have an antidepressant effect (Chen et al.). Other studies, however, have demonstrated that the behavioral effects of chronic antidepressant treatment may occur in a CREB-independent manner and be enhanced by CREB down-regulation (Conti et al.; Gur et al.). The role of CREB in the therapeutic efficacy of antidepressant drugs, therefore, remains unclear.

The role of CREB in the hippocampus has been the target of many studies on antidepressant efficacy (Blom et al.; Gur et al.; Nibuya et al.; Thome et al.; Tiraboschi et al.); therefore, a primary goal of this study was to evaluate the role of CREB activation and expression in the hippocampus in our study of antidepressant treatment during alcohol abstinence. We found subregionally distinct changes in both activation and expression of

CREB in the hippocampus, as measured by pCREB and CREB IR, respectively. Two weeks of abstinence following chronic alcohol drinking was associated with increased pCREB IR in the dentate gyrus, and increased CREB IR in the CA1. We were surprised to see increases in CREB and pCREB IR in the CA1 and dentate after two weeks of abstinence from alcohol drinking, as these data suggest that CREB expression and activation are higher in the hippocampus, however our previous data demonstrate that depression-like behavior emerges during this abstinence period (Stevenson et al.). These increases were also not altered by desipramine treatment, which we have shown prevents abstinence-induced depression-like behavior. These data are consistent with the finding that CREB-deficient mice show a baseline antidepressant phenotype and are less sensitive to desipramine (Gur et al.). Chronic desipramine administration increased pCREB IR in the dentate gyrus, and CREB IR in the CA1 of water drinking animals only. In contrast, we found a large decrease in CREB and pCREB IR in the CA3 due to abstinence, and desipramine treatment completely prevented these changes, while having no effect on CREB or pCREB IR the CA3 of water drinking animals. These changes in CREB perfectly parallel the behavioral changes we have previously reported using this model of alcohol-related depression-like behavior (Stevenson et al.); therefore, alterations in CREB expression and activation indicated by decreased CREB and pCREB IR in the CA3 may represent an anatomically specific molecular mechanism underlying depression-like behavior that emerges during abstinence following chronic alcohol drinking, and a target of desipramine treatment. The subregional dissociation of the changes observed in CREB may explain some of the inconsistent findings regarding the role of CREB in the hippocampus.

Several studies have demonstrated that antidepressants of different classes promote hippocampal neurogenesis (Malberg and Blendy 2005). Researchers have also postulated that the beneficial effects of antidepressants require hippocampal neurogenesis (Malberg and Schechter 2005; Santarelli et al.). Treatment with antidepressants increases CREB and

neurogenesis in a parallel fashion (Malberg and Blendy 2005), and mice with a dominant negative form of CREB in the hippocampus show decreased neurogenesis (Nakagawa et al.). Furthermore, pCREB is expressed in new neurons (Nakagawa et al.). Because mice show a significant decrease in neurogenesis after two weeks of abstinence from alcohol drinking (Stevenson et al.), we hypothesized that we would also see an abstinence-induced decrease in CREB or pCREB IR in the dentate gyrus. The fact that we observed an increase in CREB IR in the dentate after two weeks of abstinence, a treatment shown to reduce neurogenesis, again supports the finding that CREB deficient mice have increased basal levels of hippocampal neurogenesis (Gur et al.).

Desipramine treatment and abstinence following chronic alcohol drinking have opposing effects on CREB expression and activation in the CA3 subregion of the hippocampus in the current study. In fact, the CA3 is unique among the brain regions we have examined in showing an interaction between abstinence and desipramine treatment on changes in CREB. This finding suggests that CREB signaling in the CA3 may play an important role in mediating the emergence of depression-like behavior during alcohol abstinence (Stevenson et al.). The CA3 subregion of the hippocampus is known to be especially sensitive to stress and high levels of glucocorticoids (Arbel et al.; De Kloet et al.; Fuchs et al.; Magarinos et al.). Pyramidal cells in the CA3 show dendritic retraction in response to high levels of corticosterone (McEwen 1999), and it has been suggested that this dendritic remodeling may underlie increased vulnerability of the brain following chronic stressors (McEwen 2001). Interestingly, increased brain-derived neurotrophic factor (BDNF), a target gene of CREB, has been shown to be upregulated in the CA3 of rats that are resilient to chronic mild stress (Bergstrom et al.). Taken together, these data suggest that CREB-dependent transcription of trophic factors such as BDNF may protect against stress-induced structural changes in the CA3. Furthermore, CREB-dependent transcription

in the CA3 may protect against the vulnerabilities created by abstinence following chronic alcohol drinking.

Because the amygdala is known to be involved in stress, emotional reactivity, and addictive behavior, we investigated CREB expression and activation in this region by measuring CREB and pCREB IR. CREB, a molecular mechanism known to be involved in long-term potentiation, has been linked to consolidation of emotional memory in the amygdala (Rodrigues et al.). The amygdala has been shown to be involved in allostasis and negative affective states associated with the development of alcoholism has been demonstrated (Koob 2003; Pandey 2004). In particular, CREB is decreased in the amygdala following 24 hours of withdrawal from chronic alcohol drinking, when anxiety-like behavior is increased (Pandey et al. 2001a; Pandey et al. 2003). Furthermore, inhibition of CREB phosphorylation in the amygdala is anxiogenic and increases preference for alcohol (Pandey et al. 2003).

We examined CREB IR and pCREB IR in the basolateral and central nuclei of the amygdala following desipramine or vehicle treatment during abstinence from alcohol drinking. In general, it is thought the basolateral nucleus of the amygdala receives information from sensory and association cortices. The basolateral nucleus has projections to the central nucleus, as well as the nucleus accumbens among other targets. The central nucleus is considered to be the major output region of the amygdala (LeDoux 2007). Two weeks of abstinence following chronic alcohol drinking did not produce changes in CREB or pCREB IR in either nucleus of the amygdala. As this abstinence period has been shown to promote depression-like behavior (Stevenson et al.), these data suggest that this particular negative affective state is not associated with decreased CREB in the amygdala. Desipramine treatment, however, increased pCREB IR in the central nucleus, and CREB IR in the basolateral nucleus, suggesting that a treatment that promotes positive affective states also increases CREB in the amygdala. While increased phosphorylation of CREB, as

in the central nucleus following desipramine treatment, indicates an upregulation of CREB-dependent transcription, increased CREB expression in the absence of increased CREB activation, as in the basolateral nucleus is more difficult to interpret. One possible interpretation is that increased expression of CREB is a compensatory response to decreases in one of the CREB signaling pathways that could have occurred earlier in abstinence or during the alcohol drinking phase of the experiment. Indeed, previous work in rats does supports the idea that CREB activity is downregulated in the amygdala due to chronic alcohol drinking and short-term withdrawal (Pandey 2004; Pandey et al. 2001a; Pandey et al. 2001b; Pandey et al.). One limitation of this study is that we were only able to measure changes in CREB at the end of the two-week abstinence period. Further studies of the effects of chronic voluntary alcohol drinking and short-term withdrawal in the mouse are warranted.

The nucleus accumbens plays an important role in reward and addiction. Most drugs of abuse, including alcohol, cause dopamine release in the nucleus accumbens, and this neurotransmission has been shown to be crucial for the rewarding effects of drugs of abuse (Di Chiara and Bassareo 2007). Furthermore, dopaminergic, GABAergic, and neuropeptidergic signaling the nucleus accumbens have all been shown to influence alcohol reinforcement (Hodge and Alken 1996; Hodge et al.; Olive et al.; Slawecki et al.). CREB is downstream of multiple neurotransmission pathways and may represent a convergent mechanism for altering gene transcription (Pandey 2004). Decreased CREB activity in the nucleus accumbens is associated with increased alcohol drinking, while increased CREB is associated with reduced rewarding properties of drugs of abuse (Carlezon et al.; Misra et al. 2001). Moreover, over-expression of CREB in the nucleus accumbens has been shown to have a pro-depressant effect (Pliakas et al.), indicating that CREB signaling in the nucleus accumbens may play a role in positive and negative affective states, as well as drug abuse and addiction.

In the present study, we investigated possible changes in CREB in the nucleus accumbens after two-weeks of abstinence following alcohol drinking with or without desipramine treatment during the abstinence period. Similar to the amygdala, abstinence following alcohol drinking was not associated with changes in CREB or pCREB IR in the nucleus accumbens. Desipramine treatment, however, was associated with increased pCREB IR in the nucleus accumbens, regardless of the animal's drinking history. These data indicate that chronic desipramine treatment increases CREB phosphorylation in the nucleus accumbens. Based on the studies demonstrating that CREB overexpression in the nucleus accumbens increases depression-like behavior (Pliakas et al. 2001), we expected to see decreased CREB or CREB activation in the nucleus accumbens following desipramine treatment. The fact that we did not may suggest dissociation between the role of CREB signaling in the behavioral response to or coping strategy for an acute stressor (eg. FST) and the role of CREB signaling in mediating the effects of chronic antidepressant treatment.

Abstinence following alcohol drinking and antidepressant treatments have opposing effects of depression-like behavior (Stevenson et al.). We, therefore, sought to examine the role of CREB in mediating these opposing effects. The current study indicates that CREB expression and activation are altered both by abstinence following chronic alcohol drinking and by desipramine during the abstinence period. Our data suggest that the CA3 subregion of the hippocampus may be a brain region that is vulnerable to abstinence following alcohol drinking, and a target of antidepressant drugs. CREB, particularly in the CA3 subregion in the hippocampus, may represent a common mechanism underlying abstinence- and antidepressant-related neuroadaptations.

CREB expression and activation are altered in a regionally specific manner in response to abstinence following alcohol drinking and treatment with the antidepressant desipramine. In our studies, the effects of abstinence following alcohol drinking on CREB

are limited to the hippocampus, where pCREB IR is increased in the dentate gyrus, CREB IR is increased in the CA1, and both CREB and pCREB IR are robustly decreased in the CA3. Desipramine treatment, on the other hand, caused increases or prevented decreases in CREB or pCREB IR in all three regions that we examined. These data would indicate that desipramine has an overall effect of increasing CREB-related signaling mechanisms. Importantly, desipramine treatment completely prevented the most significant decrease in CREB expression and activation due to abstinence, which occurred in the CA3 subregion of the hippocampus. Because of the central position of CREB as a downstream effector molecule of several signaling pathways, this molecule may represent a convergence point for the opposing effects of alcoholism and antidepressants treatments.

CHAPTER IV: ASSESSMENT OF THE HPA AXIS DURING ABSTINENCE FOLLOWING CHRONIC ALCOHOL DRINKING

INTRODUCTION

Alcoholism is a disease that is characterized by periods of abstinence followed by relapse. The counter-adaptations that occur during abstinence as a result of chronic alcohol drinking and cessation of chronic alcohol drinking may enhance one's vulnerability to relapse (Ahmed and Koob 1998; Koob and Le Moal 2008). Among the counter-adaptations known to occur are alterations of the hypothalamic-pituitary-adrenal (HPA) axis. It has been hypothesized that dysregulation of the HPA axis during withdrawal and abstinence may contribute to altered sensitivity to stress and negative affective states, which are characteristics that can enhance the probability of relapse (Adinoff et al. 1998; Adinoff et al. 2005b; Costa et al. 1996; Koob 2000; Sillaber and Henniger 2004).

The HPA axis is a critical system by which the brain activates the physiological response to stressors. The physiological stress response is an adaptive mechanism that allows organisms to react to harmful or potentially harmful stimuli and to return to a homeostatic state following reaction to a stressor. When the brain perceives a stressor, the hypothalamus secretes corticotropin-releasing factor (CRF), which stimulates the pituitary gland to release adrenocorticotropic hormone (ACTH). ACTH, in turn, activates the adrenal cortex to release glucocorticoids (cortisol in humans, corticosterone in rodents). Glucocorticoids have myriad central and peripheral effects, including providing negative feedback via the glucocorticoid receptor type II (GR) to inhibit the release of CRH and ACTH (Tsigos and Chrousos 2002). Dysregulation can occur at each level of the axis producing

varied effects, and evidence suggests that alcohol is capable of producing dysregulation of the HPA axis via several mechanisms (Sillaber and Henniger 2004).

While many people report stress-relief as a motivation for drinking alcohol, the effects of alcohol consumption on the stress response are unclear (Pohorecky 1991). Both human and animal studies have shown inconsistent results regarding the effects of acute and chronic alcohol consumption on stress hormones. While the majority of studies on interactions between stress and alcohol have focused on the effects of stress on active alcohol consumption (Sillaber and Henniger 2004), alterations of the HPA axis during abstinence have been demonstrated in human alcoholics. It is generally thought that alcohol and acute alcohol withdrawal produce hyper-reactivity of the HPA axis, and that abstinent alcoholics have a hypo-reactive HPA axis, as indicated by decreased responsivity to stimulation at each level of the axis (Adinoff et al. 2005a; Adinoff et al. 2005b). In rats, chronic daily consumption of alcohol-containing liquid diet and withdrawal produced suppression of the HPA axis during abstinence that persisted for up to 3 weeks (Rasmussen et al. 2000). The current literature, however, is lacking studies on the effects of abstinence following alcohol consumption in non-dependent subjects. Further studies of HPA axis alterations during abstinence are necessary to gain a more complete understanding of the mechanisms and consequences of abstinence-induced HPA axis dysregulation, including the effects of abstinence following moderate voluntary alcohol.

In the present study, we investigated HPA axis basal functioning and responsivity during abstinence following chronic voluntary drinking. Mice were allowed to self-administer alcohol vs water in the home cage following a two-bottle drinking procedure for 28 days. At the end of the drinking period, alcohol bottles were removed, and mice experienced a 14 period of forced abstinence. We examined 24-hr corticosterone rhythmicity on the 14th day of abstinence, sensitivity of the HPA axis to dexamethasone following 14 days of abstinence, and the behavioral and stress hormonal response to chronic exogenous

corticosterone administration during abstinence. These studies were designed to determine if the HPA axis is dysregulated during abstinence following moderate voluntary alcohol drinking.

METHODS

Animals

Adult male C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME) were singly housed in standard Plexiglas cages with Purina Rodent Chow and fluid always available. Mice were 9 weeks old at the start of all experiments, and weighed between 22-26g at the beginning of the experiments. At the end point of experiments, mice weighed between 25-31g. The vivarium was maintained at 27ºC on a reverse 12:12 light-dark cycle (lights on at 22:00). Animals were under continuous care and monitoring by veterinary staff from the Division of Laboratory Animal Medicine at UNC-Chapel Hill, and all procedures were also carried out in accordance with the *NIH Guide to Care and Use of Laboratory Animals* (National Research Council, 1996) and institutional guidelines.

Experimental Procedures

24-hr corticosterone rhythmicity. (Figure 16) In the first set of experiments, mice were weighed and handled daily for 7 days prior to experiments to facilitate adaptation to the laboratory. Animals were then randomly assigned to either alcohol- or water-drinking groups. Alcohol-drinking mice (n=18) were allowed to voluntarily consume alcohol or water for 28 days using a two-bottle drinking procedure (see Two-Bottle Drinking section below) followed by a 14 day period of forced abstinence, during which only one bottle of water was on each cage; water-drinking mice (n=18) had access to two bottles of water for 28 days followed by a 14 day period when they had access to only one bottle of water. At the end of the 13th day of abstinence, mice were assigned to one of three subgroups (n=6 alcohol and

n=6 water per group). Tail blood was collected every 2-hr for 24-hr, but the subgroups were staggered such that tail blood was collected from each mouse every 6-hr (Dalm et al. 2005). Serum was analyzed for CORT levels to determine the diurnal CORT rhythmicity as a function of abstinence following alcohol drinking.

Sensitivity to dexamethasone following the abstinence period. (Figure 16)In the second set of experiments, mice were again allowed to acclimate to the vivarium conditions then assigned to alcohol- or water-drinking groups. Mice drank alcohol vs. water or water vs. water for 28 days, and then had access to only one bottle of water for 14-d. On the night before the 14th day of abstinence, mice were injected with dexamethasone (n=12 water, n=12 alcohol), or vehicle (n=12 water, n=12 alcohol). 6 hrs later (morning of day 14), trunk blood was collected to determine CORT suppression in response to the dexamethasone injection.

Response to corticosterone administration during abstinence. (Figure 16) The thrid set of experiments followed the same procedures as the first set through the 28 days of alcohol or water drinking. At the end of 28th day of drinking, all bottles were removed from both alcohol- (n=36) and water drinkers (n=36). Mice were then randomly assigned to one of three CORT administration groups. Beginning the same day that alcohol/water bottles were removed, mice were given one bottle of CORT-containing water (0 µg/mL, 2.5 µg/mL, and 5.0 µg/mL; n=12 alcohol-drinkers and n=12 water-drinkers per dose) as their only available fluid for the 14 day abstinence period. On day 11 of CORT administration, tail blood was collected from all mice to establish a pre-stressor CORT level. On day 14 of CORT administration, mice were subjected to the forced swim test (FST) for six minutes, and then trunk blood was immediately collected for analysis of post-stressor CORT levels.

Two-Bottle Drinking Procedure

During the one week period of acclimation and handling, water was the only fluid available. All fluids were presented in the home cage via 50mL plastic centrifuge bottles with

stainless steel sipper tubes. Alcohol-drinking mice were then given access to one bottle of water and one bottle of alcohol (10%v/v) as previously described (Hodge et al. 1999). Animals were weighed and the fluid levels in the bottles were monitored to the nearest 0.5 mL at 24-hr intervals to determine daily fluid intake (mL and g/kg). The position (left-right) of the alcohol and water bottle was changed each day to control for side preferences. C57Bl/6J mice were given access to alcohol for 28 days. We have previously reported that, using this method of alcohol administration, mice consume an average of 15 mg/kg/day and achieve peak blood alcohol concentrations of 100-150 mg/dL (Stevenson et al., 2008). Control animals had access to two bottles of water for the same duration (28 days). On the final day of drinking, the alcohol solution-containing bottle was removed from the cages of alcohol drinkers, and one water bottle was removed from the cages of controls; thus, during abstinence only one bottle of water was on each cage.

Dexamethasone Suppression Test

On the 14th day of abstinence, mice were given a subcutaneous injection of dexamethasone (0.1 mg/kg, 10 mL/kg dexamethasone 21-phosphate disodium salt, Sigma-Aldrich, Inc, St. Louis, MO) or vehicle (saline) at 2:00 am, and immediately returned to the home cage. 6-hr later at 8:00 am, mice were rapidly decapitated and trunk blood was collected to determine post-dexamethasone injection CORT levels. This method is based on Groenink et al., 2002.

Corticosterone Administration during Abstinence

Mice were given access to one bottle containing 0, 2.5, or 5.0 μg/mL CORT in the home cage *ad libitum* for 14-d beginning as soon as alcohol and water bottles were removed. Corticosterone (4-pregnen-11β, 21-diol-3, 20-dione 21-hemisuccinate, Steraloids, Inc, Newport, RI) was dissolved in distilled de-ionized water (2.5 μg/mL, and 5.0 μg/mL). Solution was brought up to a pH of 12.0 and allowed to stir for 1 hour, and then the pH was

brought down to 7.0-7.4. Mice were given fresh water or CORT solution every three days (method developed by S.Gourley).

Tail-bleeding

On day 11 of abstinence/CORT administration, tail blood was collected to assess pre-stress levels of CORT. Blood collection was performed between 4:00 and 5:00 pm. Each mouse cage was taken into a procedure room, then the mouse was placed into a restrainer, and blood collection was started within 45 seconds of first touching the cage. 1-2mm of the distal tail end was removed using a scalpel blade, and 100 µL of blood was collected using heparinized capillary tubes, and then placed on ice until blood collection from all animals was completed. As soon as the capillary tube was full, light pressure was applied to the end of the tail using sterile gauze, and the mouse was returned to the home cage and brought back to the vivarium. When blood collection from all animals was completed, samples were spun in a centrifuge for 5 minutes at 3,000 rpm, and then frozen at -20ºC until CORT assay was performed.

Forced Swim Test Procedure

On the 14th day of abstinence/CORT administration, mice were subjected to the forced swim test (FST) to measure depression-like behavior, as well as the CORT response to the swim stressor (Porsolt et al. 1977). The FST was performed between 4:00 and 5:00 pm. Mice were placed in a 2000mL beaker containing 1300mL of water maintained at 23-25°C for 6 minutes. Behavior was videotaped for later analysis by two trained observers who were blind to the treatment conditions. The duration of immobility during the last 4 minutes of the FST was measured during a single session as an index of depression-like behavior. Mice were considered immobile if they were completely still except for small movements of the paw made only to keep the mouse afloat, and not producing any noticeable propulsion

of the mouse. Immediately following the FST, mice were brought into a procedure room and trunk blood was collected.

Trunk Blood and Brain Tissue Collection

At the endpoint of each experiment, mice were rapidly decapitated and trunk blood was collected into heparin-coated microfuge tubes. Blood samples were placed on ice until blood collection from all animals was completed. Immediately after decapitation, brains were removed, snap frozen in isopentene cooled to -40°C by dry ice, wrapped in aluminum foil, and placed on dry ice until all brains had been collected. After blood and brain collection from all animals was completed, brain tissue was stored at -80°C, and blood samples were spun (5 min at 3,000 rpm) and serum was removed and frozen at -20°C.

Assessment of Serum Corticosterone

Serum was analyzed for CORT content using a radioimmunoassay (MP Biomedicals, Irvine, CA) exactly according to kit instructions. All data are expressed as ng/mL.

Statistical Analyses

Data were analyzed using two-way ANOVA . Post-hoc Tukey tests were used to determine between-group differences. Statistical significance was always defined as $p<0.05$.

RESULTS

24-hr corticosterone rhythmicity is not altered by abstinence following chronic alcohol drinking.

Serum CORT levels were measured every 2-hr for 24-hr to determine the circadian rhythm and to evaluate the effects of abstinence following alcohol drinking on CORT rhythmicity. A two-way ANOVA revealed that there was a main effect of time [$F(13,134)=9.75$, $p<0.0001$] on serum corticosterone levels, but no main effect of treatment. Serum CORT, therefore, did not differ between alcohol-drinking and water-drinking mice at any time point over the course of 24-hr (Figure 17). These data indicate that the circadian

rhythm of CORT is not altered by two weeks of abstinence following chronic voluntary ethanol drinking.

Sensitivity to dexamethasone is not altered by abstinence following voluntary drinking.

The response of the HPA axis to administration of the exogenous glucocorticoid, dexamethasone, was determined at the end of the 14-d abstinence period. A two-way ANOVA showed a main effect of dexamethasone on serum CORT [$F(1,43)=19.508$, $p<0.001$] (Figure 18). A history of 28-d voluntary alcohol drinking had no effect on sensitivity to dexamethasone.

14-d corticosterone administration alters basal serum corticosterone, post-stressor serum corticosterone, and behavior in the forced swim test.

Corticosterone (0, 2.5, or 5.0 μg/mL) was administered to mice via the drinking water for 14-d. Mice consumed an average of 5.79 ± 0.08 mL of fluid, and fluid consumption did not differ based on dose of corticosterone or drinking history. A two-way ANOVA revealed a main effect of dose of CORT in drinking water on the amount of CORT (but not the amount of fluid) consumed [$F(1,44)=255.3$, $p<0.001$], but there was no main effect of drinking history on CORT consumption. This indicates that mice consumed a higher amount of corticosterone only because the dose of CORT in their drinking water was higher (data not shown). These data indicate that total fluid intake was not altered by a history of alcohol drinking or by the dose of corticosterone administered and that consumption of corticosterone was not affected by drinking history.

Basal serum corticosterone levels were measured following 11 days of CORT consumption via the drinking water. A two-way ANOVA showed a main effect of dose of CORT in drinking water on basal serum CORT levels [$F(2,30)=16.401$, $p<0.001$], but no main effect of drinking history (Figure 19a). Follow up comparisons revealed that basal serum CORT was significantly higher in mice that drank 5.0 μg/mL solution compared to 0.0

μg/mL CORT solution ($p<0.001$, Tukey test) and 2.5 μg/mL CORT solution ($p=0.038$, Tukey test). Also, mice that drank a 2.5 μg/mL CORT solution had significantly higher basal CORT levels compared to mice that drank 0.0 μg/mL CORT solution ($p=0.011$, Tukey test). These data indicate that basal serum CORT levels were increased in a dose-dependent manner by consumption of corticosterone in the drinking water, and were not affected by a history of alcohol drinking.

Stress-induced serum corticosterone levels were also measured on day 14 of CORT administration following the FST. A two-ANOVA showed a main effect of dose of CORT in drinking water on stress-induced serum CORT levels [$F(2,65)=14.43$, $p<0.001$], but no main effect of drinking history (Figure 19b). Follow up comparisons revealed that stress-induced serum CORT was significantly lower in mice that drank 5.0 μg/mL CORT solution compared to 0.0 μg/mL and 2.5 μg/mL CORT solution ($p<0.001$, and $p=0.021$, respectively, Tukey test). Also mice that drank a 2.5 μg/mL CORT solution had significantly lower stress-induced CORT levels compared to mice that drank 0.0 μg/mL CORT solution ($p=0.034$). The data suggest that consumption of CORT via drinking water blunts stress-induced CORT levels in a dose-dependent manner, and that this effect is not altered by a history of alcohol drinking.

The response to stress was measured by comparing the change in serum CORT between basal levels and post-stress levels. The two-way ANOVA showed only a main effect of the dose of CORT in the drinking water [$F(2,30)=11.234$, $p<0.001$] (Figure 19c). The difference between pre- and post-stressor serum CORT levels was significantly smaller in mice that drank 5.0 μg/mL CORT solution compared to 0.0 μg/mL CORT solution ($p<0.001$, Tukey test). The change in pre- and post-stressor serum CORT levels showed a strong trend toward a decrease in mice that drank 2.5 μg/mL CORT solution compared to 0.0 μg/mL CORT solution ($p=0.067$, Tukey test) and in mice that drank 5.0 μg/mL CORT solution compared to 2.5 μg/mL CORT solution ($p=0.057$, Tukey test), but these decreases were not significant. Again, there was no main effect of drinking history on the change in

CORT. These data indicate a dose-dependent decrease of the stress response, as indicated by change in CORT pre- and post-stressor, as a result of 14-d of CORT consumption via the drinking water.

Depression-like behavior in the FST was measured on the 14th day of abstinence following chronic drinking/CORT administration. A two-way ANOVA showed a main effect of dose of CORT in the drinking water on immobility in the FST [$F(2,64)=3.708$, $p=0.03$], but no main effect of drinking history on immobility (Figure 19d). Follow up comparisons indicated that immobility was significantly increased in mice that drank 5.0 μg/mL CORT solution compared to 0.0 μg/mL CORT solution ($p=0.027$, Tukey test). These data indicate that consumption of 5.0 μg/mL CORT solution for 14-d increases immobility in the FST, suggesting that low dose chronic CORT consumption can produce increased depression-like behavior.

DISCUSSION

The current study investigated the HPA axis of two-week abstinent mice that had voluntarily consumed alcohol for 28 days. Dysregulation of the HPA axis is known to occur in both actively drinking and abstinent alcoholics. It is not known, however, what level of consumption or dependence is required to produce alterations of the HPA axis during abstinence. The only studies investigating the HPA axis during abstinence have examined human subjects that were already alcoholic, and rats that were made dependent via chronic liquid diet alcohol consumption (Adinoff et al. 2005b; 2005c; Costa et al. 1996; Rasmussen et al. 2000). The effects of abstinence following voluntary moderate alcohol consumption on the HPA axis have not been studied. The experiments presented in the current study were designed to model early stage alcohol abuse to determine if abstinence following chronic moderate alcohol drinking contributes to alcohol-related HPA dysfunction.

Our data indicate that abstinence following chronic voluntary drinking is not associated with a shift in the circadian rhythm of CORT levels. Because CORT levels change so drastically throughout the 24-hr period, it was important to establish that the diurnal rhythm of CORT was not altered so that blood samples collected at the same time point in abstinent and control mice could be compared without accounting for circadian rhythm shifts. Alcoholism is associated with significant alterations in sleep and the circadian rhythm-dependent biological processes including the rhythmicity of melatonin and glucocorticoids, and core temperature (Brower 2001; Fonzi et al. 1994; Rosenwasser et al. 2005). Studies have also demonstrated that alterations of these circadian rhythm-dependent biological processes are associated with an increased risk for relapse (Brower 2001; 2003; Fonzi et al. 1994; Gillin et al. 1994; Rosenwasser et al. 2005). Our data demonstrate that abstinence following 28-d of voluntary drinking does not produce shifts in the circadian rhythm of CORT, nor does it produce changes in basal levels of CORT at any point throughout the 24-hr period. This suggests that alterations of basal CORT and CORT rhythmicity may not occur until later stages of alcohol abuse/dependence.

While changes in basal CORT production were not evident in two-week abstinent mice, we wanted to determine if abstinence following chronic moderate drinking produced vulnerabilities in the HPA axis. To investigate this possibility, we administered corticosterone to the mice throughout the abstinence period via their drinking water and then measured their CORT response following to an acute stressor. Our findings demonstrate that alcohol-abstinent mice did not differ from control mice in their basal response to chronic CORT administration (as measured by serum CORT following 11 days of CORT consumption), nor did they differ in their post-stressor CORT levels. Furthermore, the level of depression-like behavior exhibited in the forced swim test was not different in mice with a history of alcohol drinking. Repeated CORT administration may be a model of chronic stress, and has been shown to produce depression-like behavior at higher doses (Gourley et al. 2008). The data

presented here indicate that abstinence following chronic voluntary alcohol drinking does not produce altered vulnerability to chronic challenge of the HPA axis.

Though no differences were found in the HPA axis response between control and alcohol-abstinent mice, our data do suggest that even very low dose CORT consumption (2.5 μg/mL and 5.0 μg/mL) for two-weeks produces robust changes in basal CORT, post-stress CORT, and the expression of depression-like behavior. It has been previously demonstrated that repeated CORT administration increases immobility in the FST in rats and mice, and that these effects are dose-dependent (Gregus et al. 2005; Hill et al. 2003; Zhao et al. 2008). These previous studies, however, used significantly higher doses of CORT (20-40 mg/kg), and administered CORT via injection. Our method of CORT administration produced physiologically relevant serum CORT levels similar to those achieved via models of chronic mild stress (100-130 ng/mL) (Grippo et al. 2005). The findings of the current study also demonstrate that chronic low dose CORT administration via the drinking water produces dose-dependent elevations in basal serum CORT, dose-dependent reductions of CORT secretion in response to a stressor, and increased depression-like behavior. Elevated basal CORT with a decreased response to HPA activation is common in depressed people (Gillespie and Nemeroff 2005), indicating that administration of low dose chronic CORT administration via the drinking water in mice may be useful as an animal model of HPA dysfunction during depression.

In contrast to our findings, it was recently reported that administration of low dose CORT via the drinking water produced reductions in immobility in the FST (Stone and Lin). In these experiments, however, CORT was administered for only 2-4 days before the FST. Taken together with the findings presented here these data support a hypothesis previously suggested by other researchers, that low dose CORT administration has significantly different effects when given subchronically and acutely, rather than chronically. Low dose

and or acute glucocorticoid administration may be antidepressant, while chronic glucocorticoid administration, even if given at low doses, may have pro-depressant effects.

When dexamethasone was administered at the end of the abstinence period, we found robust suppression of serum CORT 6-hr later. These data are similar to the findings of Groenink et al. (2002), except that we did not find complete suppression of CORT at a dose of 0.1 mg/kg dexamethasone, but rather suppression of 60-75% of control values. There was no difference in the amount of suppression of CORT exhibited by control and alcohol-abstinent mice. These data again suggest that dysregulation of the HPA axis does not occur after two-weeks of abstinence following chronic moderate alcohol drinking.

In this study, however, we chose to focus our investigation of stress-related adaptations during abstinence on the HPA axis, although important extra-hypothalamic stress responses exist within the central nervous system, and are known to be affected by alcohol and abstinence following chronic alcohol consumption. These mechanisms include alterations in CRF in the amygdala, a region known to be involved in emotionality and fear processing. Changes in gene transcription and synaptic plasticity and structure in the hippocampus, nucleus accumbens, and other brain regions have also been reported in response to both stress and alcohol (Koob and Le Moal 2008; Pandey 2004; Stevenson et al.). Future studies could examine these extra-hypothalamic changes during abstinence following chronic voluntary drinking.

Because alterations of the HPA axis in abstinent alcoholics are associated with increased risk for relapse (Adinoff et al. 1998), it is important to establish why and when these alterations occur in the disease course of alcoholism. Data in rats suggest that alterations of the HPA axis in abstinence can occur as the result of alcohol abuse itself in addition to genetic predisposition to HPA axis dysregulation (Rasmussen et al. 2000); however these studies do not indicate how much alcohol drinking is required to produce HPA axis dysfunction in abstinence. Our data suggest that a greater level of dependence

than was achieved after one month of voluntary drinking in mice is required before abstinence in order for the HPA axis to become dysregulated. It is possible that a longer course of drinking (more than 28-d) would lead to HPA axis dysfunction upon cessation of drinking. Similarly, it is possible alcohol intake could be increased to levels that produced dependence using an intermittent or limited access alcohol drinking procedure, and that this would cause alterations of the HPA axis upon cessation of alcohol drinking. Furthermore, if we had evaluated the HPA axis earlier in the abstinence period, we might have seen measurable dysregulation. Additionally, as several studies have indicated that genetics may predispose alcoholics to HPA axis dysregulation (Gianoulakis 1998; Schuckit and Monteiro 1988; Schuckit et al. 1997a; Schuckit et al. 1997b; Waltman et al. 1994), it could be the case that HPA axis dysregulation occurs more readily in a subset of alcoholics who are genetically or environmentally predisposed to dysregulation of the HPA axis. Future studies including multiple alcohol drinking procedures, longer durations of alcohol drinking and abstinence, as well as studies using multiple strains of mice are warranted to better understand the processes by which alcohol consumption and abstinence produce HPA axis dysregulation.

CHAPTER V: DISCUSSION

CONCLUSIONS

Neuroadaptations occur not only during active alcohol drinking, but also during abstinence following chronic alcohol drinking. It is thought that these abstinence-induced changes can enhance the vulnerability to relapse. The studies presented here examined the emergence of negative affect during abstinence, and found that mice exhibit depression-like behavior 14-days after cessation of chronic voluntary alcohol drinking. In addition, decreased hippocampal neurogenesis is evident following 14-days of abstinence. Both of these abstinence-induced changes are prevented if the antidepressant desipramine is administered chronically throughout the abstinence period. In several brain regions, the expression and phosphorylation of the transcription factor CREB are altered in response to abstinence and desipramine administration. These abstinence-induced alterations occur in the absence of measurable HPA axis dysfunction. Understanding the behavioral and neuroadaptive changes occurring during abstinence may help develop proper treatments for alcohol abusers who are in the process of discontinuing alcohol use.

Our studies demonstrating the emergence of depression-like behavior after 14-days of abstinence provide a model for investigating how alcohol consumption (or cessation of alcohol consumption) is related to depression. Given the large percentage of patients who suffer from comorbid alcoholism and depression (Schuckit et al. 1997a), it is essential that we have a better understanding of the neurobiological mechanisms underlying this comorbidity. These data indicate that alcohol-related depression may emerge as a consequence of the process of chronic drinking followed by cessation of chronic drinking, and does not appear to require a pre-existing or genetic condition. The efficacy of

desipramine, a NE reuptake inhibitor, in preventing the emergence of depression-like behavior as well as change in neurogenesis validates these conditions as depression-related, and suggests that both conditions can be modulated by enhancement of monoaminergic neurotransmission. Accordingly, we investigated changes in CREB that result from abstinence and desipramine treatment, as CREB is downstream of monoamine receptor activation.

Analysis of CREB and pCREB immunoreactivity (IR) revealed subregionally distinct changes in response to both abstinence following alcohol drinking and chronic desipramine treatment. CREB alterations resulting from abstinence following chronic alcohol drinking were limited to the hippocampus. CREB and pCREB were slightly increased in the dentate gyrus and the CA1, but robustly decreased in the CA3 following 14-days of abstinence. Desipramine treatment alone, on the other hand, increased CREB and pCREB IR in the dentate gyrus, CREB IR in the CA1. While desipramine treatment did not affect CREB in the CA3, it did completely prevent abstinence-induced changes. These data highlight the hippocampus as a brain region that is sensitive to abstinence following chronic alcohol drinking and that can be protected by antidepressant treatment.

Decreased neurogenesis is another example of hippocampal neuroadaptations that result from abstinence following chronic alcohol drinking. Our data indicate that chronic moderate alcohol drinking itself does not lead to changes in neurogenesis, but abstinence following chronic moderate alcohol drinking produces decreased neurogenesis. As decreased neurogenesis has been proposed as a mechanism underlying depression, abstinence-induced decreased neurogenesis may be a neuroadaptation which promotes negative affect in alcoholics who try to stop drinking.

Changes in CREB and pCREB IR in other brain regions such as the amygdala and the nucleus accumbens occur in response to desipramine treatment, but are unrelated to abstinence following alcohol drinking. This does not necessarily indicate that the amygdala

and the nucleus accumbens are not affected by abstinence, but it does suggest that any changes that may occur are not mediated by the transcription factor CREB at the time point we examined. It is possible, however, that CREB-related changes occurred in these brain regions earlier in abstinence and/or during alcohol drinking, and that these changes precipitated the hippocampal adaptations that occur following 14-days of abstinence. In particular, it would be expected that dysregulation might occur in the amygdala early in abstinence. As indicated in the first set of experiments, mice exhibit anxiety-like behavior following 24-hours of abstinence, but this behavioral effect dissipates by the 14th day of abstinence. These data suggest and are in agreement with many previous studies indicating dysregulation of the brain stress system in early abstinence. In particular, alterations in CRF expression and CREB phosphorylation in the CeA have been demonstrated to produce withdrawal-related anxiety (Becker 2000; Koob and Le Moal 2008; Pandey 2004). It is possible that activation of such extra-hypothalamic stress circuits during the initial phase of abstinence is responsible for the presence of anxiety-like behavior one day after cessation of alcohol drinking in our experiments. It is also possible that activation of extra-hypothalamic stress circuits promotes the hippocampal and behavioral deficits that are evident after two weeks of abstinence, but further studies are warranted to evaluate this hypothesis. Our investigations of the HPA axis during abstinence, however, would suggest that the HPA axis is not responsible for the increased depression-like behavior and decreased hippocampal neurogenesis after 14 days of abstinence.

It is unclear from the present studies if decreased neurogenesis in the dentate gyrus and decreased CREB in the CA3 region of the hippocampus are directly related. Previous studies have demonstrated that certain types of stressors can lead to both decreased neurogenesis in the dentate gyrus and dendritic retraction in the CA3 (McEwen 2001), but it is not known if these changes share a parallel or a causal relationship. Because new neurons in the dentate gyrus project to the CA3 region (to make up the mossy fiber

pathway), it is conceivable that a decrease in this input to the CA3 could be responsible for the decrease in an activity-dependent signaling molecule such as CREB. It remains unknown, however, what the consequences of decreased neurogenesis are to CREB signaling in other subregions of the hippocampus.

Although two weeks of abstinence following a month of moderate voluntary alcohol drinking is not sufficient to promote dysregulation of the HPA axis, it is sufficient to produce significant hippocampal and behavioral adaptations. These include the emergence of negative affect, as well as impairment of structural and transcription-related mechanisms. These deficits warrant further investigations into the consequences of abstinence following even moderate drinking, and suggest that alcohol abusers may be at risk if they attempt to stop drinking, even when they have not become severely dependent.

LIMITATIONS AND FUTURE DIRECTIONS

While the research presented in this dissertation highlights some of the important cellular and behavioral changes that can occur in abstinence following chronic alcohol drinking, there were limitations to these studies that we plan to address in future experiments. First, the FST data presented in chapter 2 indicates the emergence of negative affective states beyond the initial withdrawal phase of abstinence, but a more complete characterization of these behavioral changes is warranted. We chose to focus of the FST because it is an assay of depression-like behavior that can be tested at a discrete time-point and that does not require a training period. Additional measures that will be important to conduct include assessing vulnerability to development of learned helplessness in the shock avoidance procedure and development and expression of anhedonia, as well as characterization of novelty exploratory behavior, as these are all measures that can assess a shift from positive motivational states to negative stress system-driven states (Stone et al. 2008). Such behavioral test will help define the extent and nature of abstinence-induced negative affective states.

Similarly, the time course for emergence of negative affect during abstinence needs further characterization. It will be important to define when in during abstinence these behaviors first emerge and how long they persist following cessation of drinking. Additionally, the studies presented here indicate that even moderate alcohol, when consumed chronically, can produce these behavioral and cellular deficits in abstinence; thus the duration and dose of alcohol sufficient to produce these changes should be explored. In addition, a more detailed time course of abstinence-induced changes in neurogenesis would be helpful in understanding the parameters of vulnerability for the hippocampus following cessation of alcohol drinking.

While the alterations in CREB and pCREB IR in multiple brain regions following abstinence and desipramine treatment indicate changes in gene transcription, further studies examining expression of some of the CREB target genes will be useful in determining the consequences of these CREB-related changes. It would be especially interesting to measure expression of BDNF (thought to be involved in neurogenesis and overall healthy neuronal functioning), and CRF and dynorphin which may play important roles in abstinence-induced negative affective states (Carlezon et al. 2005; Koob and Le Moal 2008). Furthermore, studies of CRF and CRF receptor expression during abstinence following chronic moderate alcohol drinking are warranted as studies have suggested that alterations in these proteins may be associated with the anxiety and stress-related changes in early abstinence and withdrawal, and these changes may precipitate the emergence of depression-like behavior later in abstinence.

Ultimately, the work presented in this dissertation provides initial evidence of neuroadaptations in abstinence following drinking in mice that are not severely dependent. These data provide the foundation and the impetus for further studies of behavioral and cellular adaptations that may contribute to the development of alcohol addiction.

Table 1. Anxiety-like behavior and locomotor activity.

Treatment Group	Time in center (sec)	Total locomotor (cm)
control	190.2 ± 17.6	4668.9 ± 245.6
alcohol+1day	90.75 ± 10.2*	5402.8 ± 242.9
alcohol+14days	147.9 ± 12.1	4503.5 ± 204.7

Table 1. *Anxiety-like behavior and locomotor activity.* Mice spent significantly less time in the center of an open field following 1 day of abstinence compared to control animals, indicating increased anxiety-like behavior following 1 but not 14 days of abstinence following chronic voluntary alcohol drinking. Total locomotor activity did not differ between any of the groups.

Figure 1. ***Periods of abstinence in the disease course of alcoholism.***

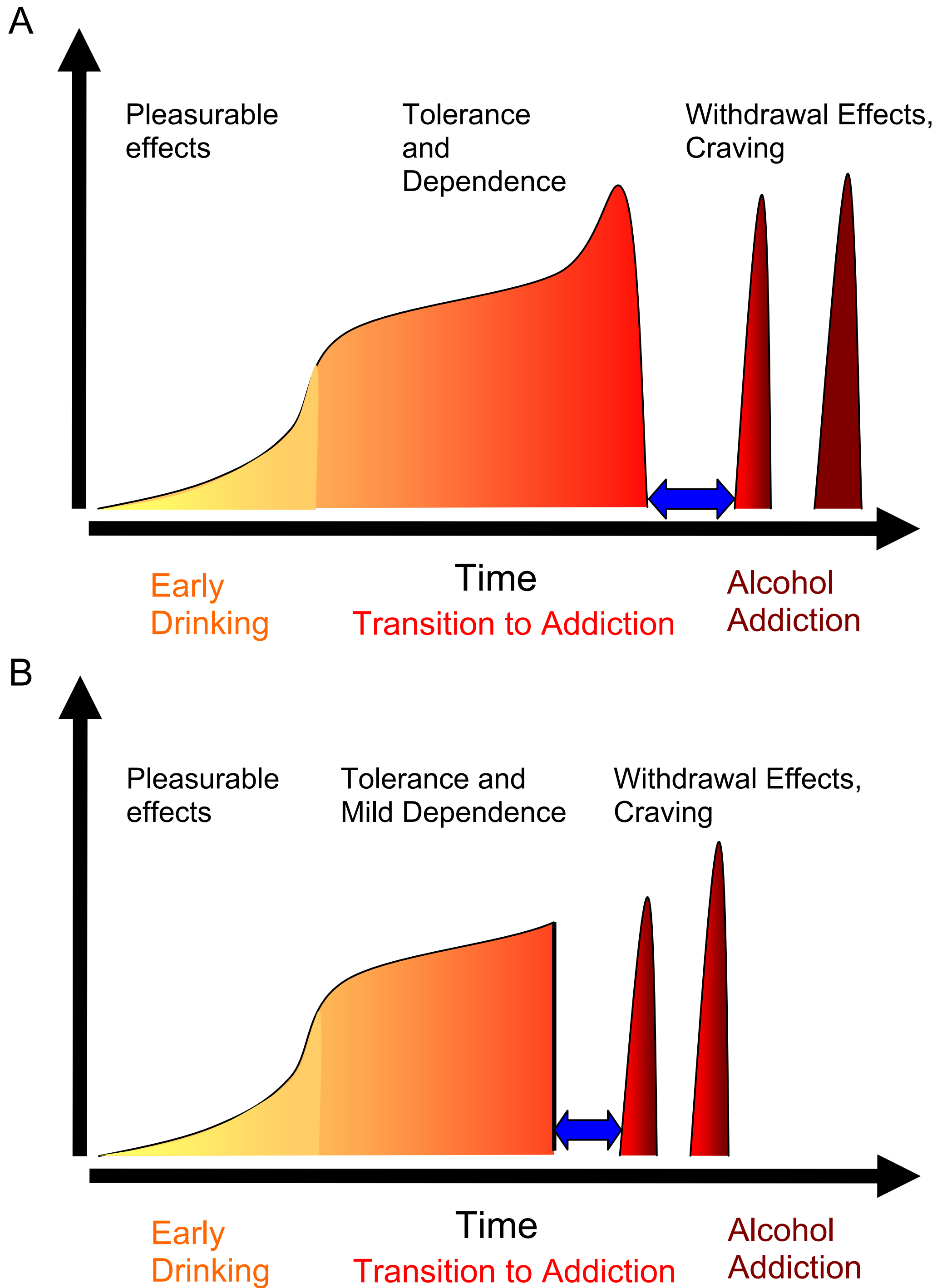


Figure 1. *Periods of abstinence in the disease course of alcoholism.* **a**. The neuroadaptations that occur during abstinence following chronic drinking may increase the risk for subsequent relapse. The blue arrow indicates a period of withdrawal and abstinence in a dependent alcoholic. The characteristics of early withdrawal are fairly well characterized and include overactivation of the autonomic nervous system and the HPA axis and anxiety. The changes that occur during more protracted abstinence are not as well understood. **b**. The neuroadaptations that occur during abstinence following chronic drinking in subjects that are not severely dependent have not been characterized. The blue arrow indicates a period of protracted abstinence in a non-dependent alcoholic. This period of abstinence is the focus of the experiments described in this dissertation.

Figure 2. ***Intracellular signaling pathways activated by alcohol and antidepressants.***

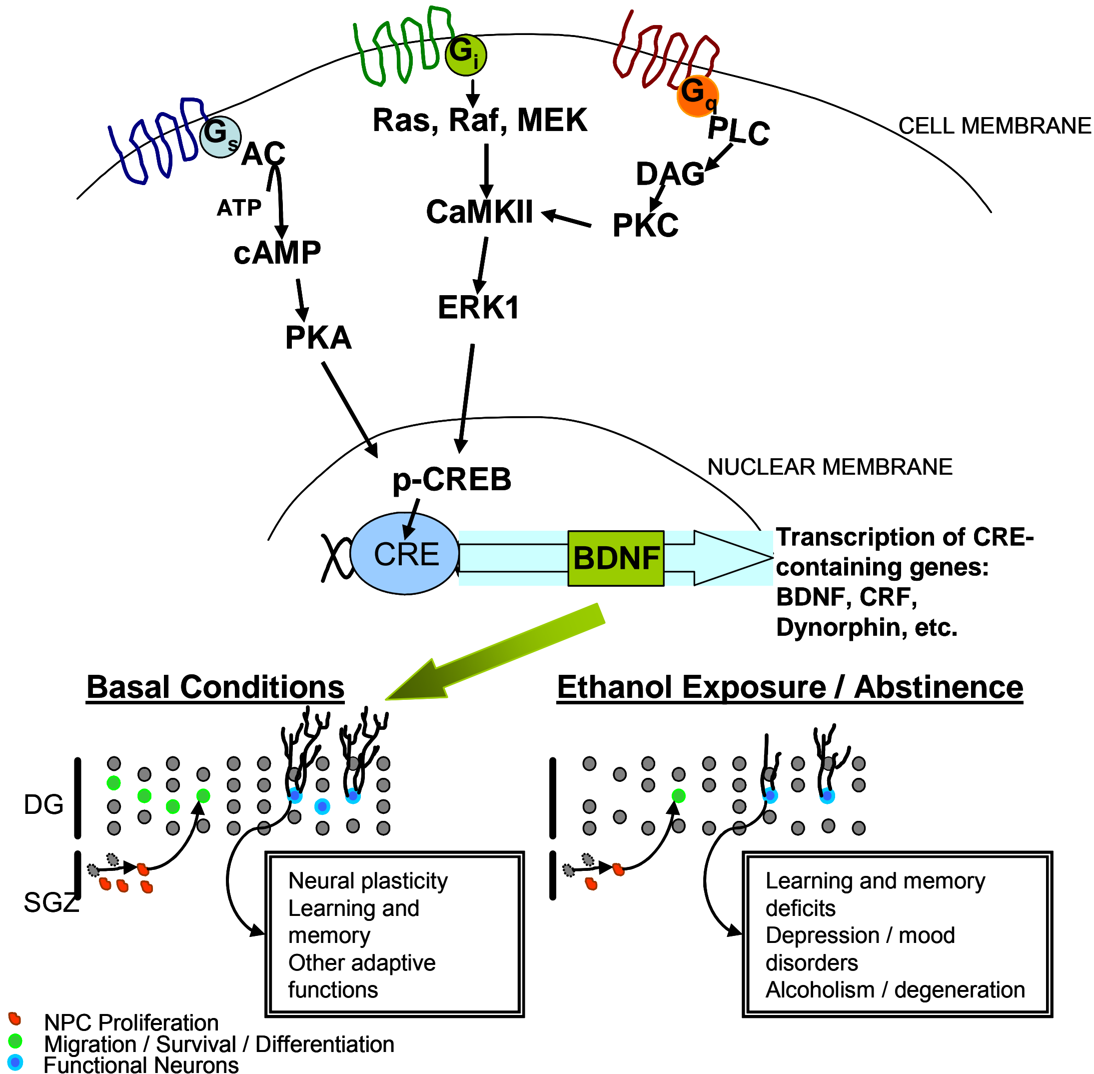
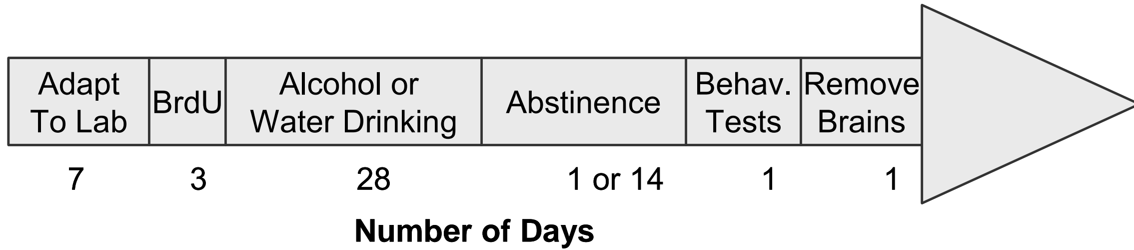


Figure 2. *Intracellular signaling pathways activated by alcohol and antidepressants.* Both alcohol and antidepressants produce changes in intracellular signaling pathways by activating or inhibiting G-protein-coupled-receptors (GPCRs). These signaling changes and alter the level of CREB-dependent transcription to produce long term changes in gene expression. Moreover, changes in the expression of trophic factors, such as brain-derived-neurotrophic-factore (BDNF) are thought to lead to parallel changes in neurogenesis.

Figure 3. ***Timeline of experimental procedure.***

A. Effects of abstinence on depression-like behavior and neurogenesis



B. Antidepressant treatment during abstinence

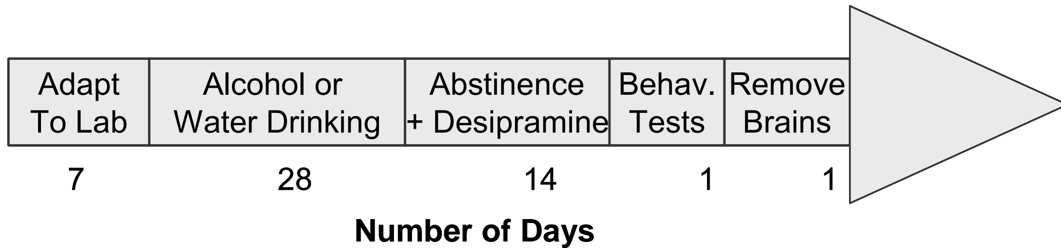


Figure 3: *Timeline of experimental procedure.* Each stage of the experiment is labeled as a separate section in the arrow. The duration of each stage (Number of Days) is shown under the arrow. (**A**) After 7 days of adaptation to the lab, mice received daily injections of BrdU (300 mg/kg, I.P.) for 3 days. The following day, the two-bottle drinking procedure began. Mice voluntarily self-administered alcohol versus water in the home cage for 28 days. On the 28th day, alcohol bottles were removed from the cage. One day later or 14 days later, locomotor activity and forced swim tests were perfomed. The following day, mice were sacrificed, and brains were removed. (**B**) After 7 days of adaptation to the lab, mice began 28 days of a two-bottle drinking procedure. On the 28th day, alcohol bottles were removed and mice received their first injection of either vehicle or desipramine (15 mg/kg, I.P.). Injections were given once daily for 14 days. 1 day after the last injection, locomotor activity and forced swim tests were performed. One day later, mice were sacrificed, and brains were removed.

Figure 4. ***Abstinence from alcohol drinking increases depression-like behavior.***

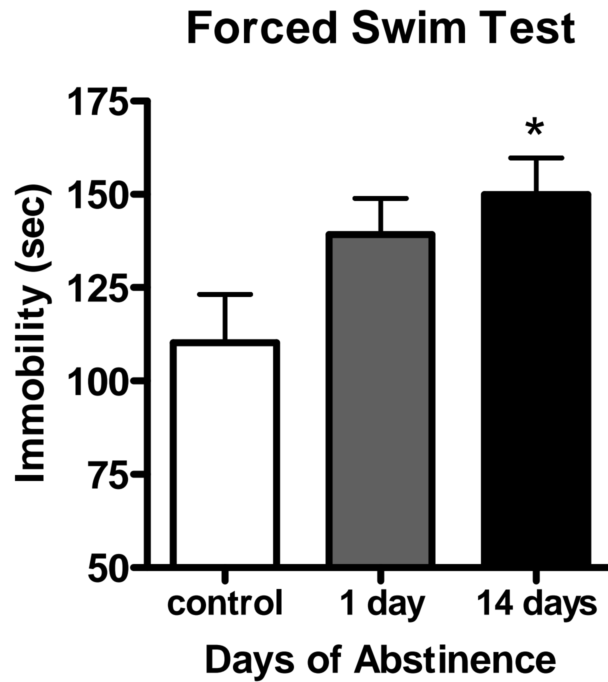


Figure 4: *Abstinence from alcohol drinking increases depression-like behavior.* Mean immobility (sec) in the Forced Swim Test (FST) increased as a function of days of abstinence from alcohol drinking as compared to water-drinking controls. Data are plotted as mean ± SEM from n=12 mice in each condition. * - significantly different from water-drinking control, $p<0.05$ (Dunnett's t-test).

Figure 5. ***No changes in survival of NPCs.***

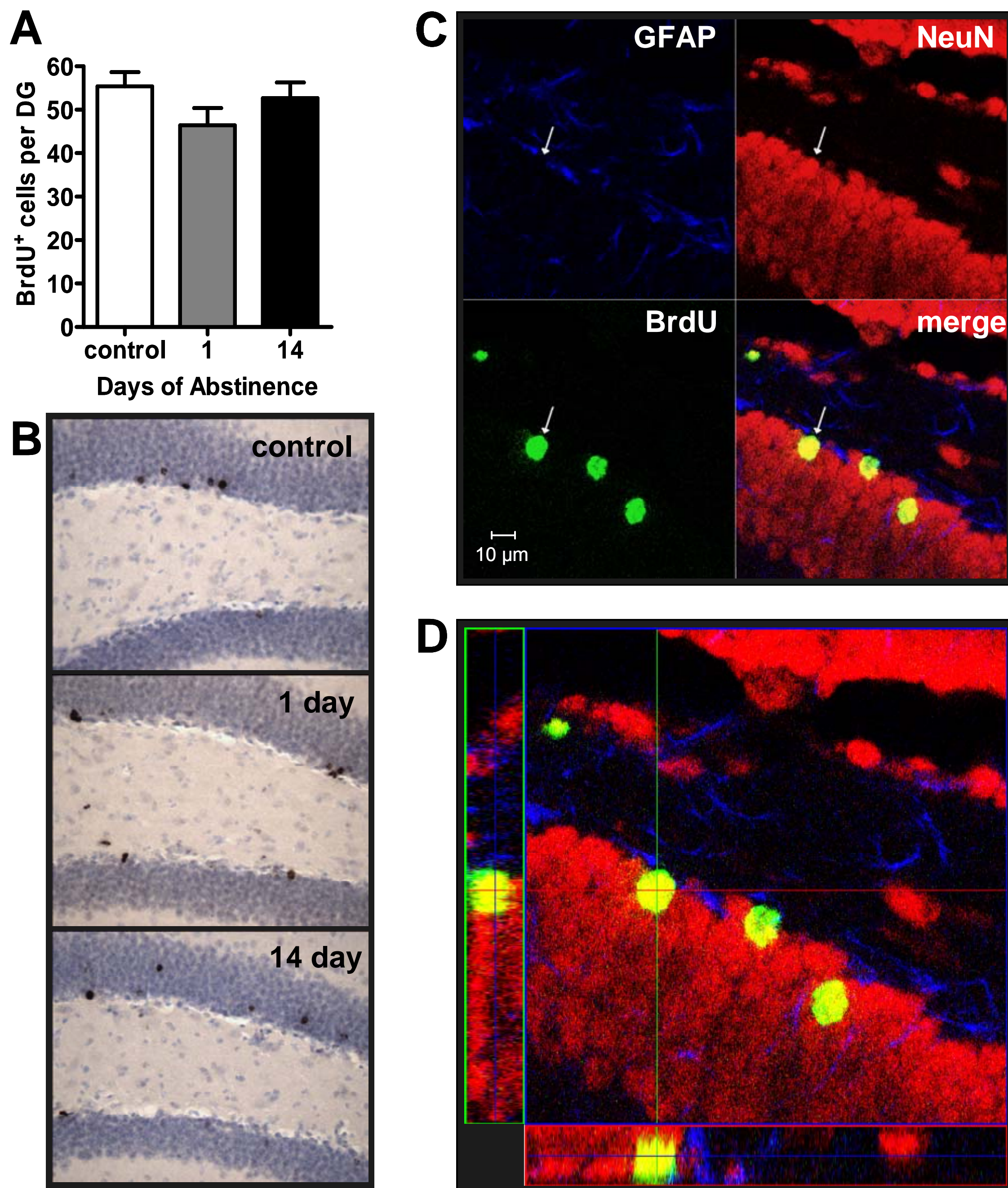


Figure 5: *No changes in survival of NPCs.* (**A**). The mean number of BrdU-labeled cells that survive is unchanged in the dentate gyrus following voluntary drinking and 1-d or 14-d abstinence. (**B**) Representative images showing BrdU-labeled cells in the dentate gyrus from control, 1 day of abstinence, and 14 days of abstinence. (**C**) Triple-label immunofluorescence was used to evaluate differentiation of surviving NPCs. A representative dentate gyrus is shown. GFAP-labeled glial cells are shown in blue, NeuN-labeled neuronal nuclei are shown in red, BrdU-labeled cells are shown in green. (**D**) An orthogonal view of a representative cell showing colocalization of BrdU and NeuN. The number of surviving BrdU-labled cells that co-labeled with the neuronal marker NeuN was unchanged in ethanol-treated brains. Data are plotted as mean
± SEM from n=9-12 mice in each condition.

Figure 6. ***Abstinence from alcohol reduces adult hippocampal neurogenesis. PCNA and DCX immunohistochemistry.***

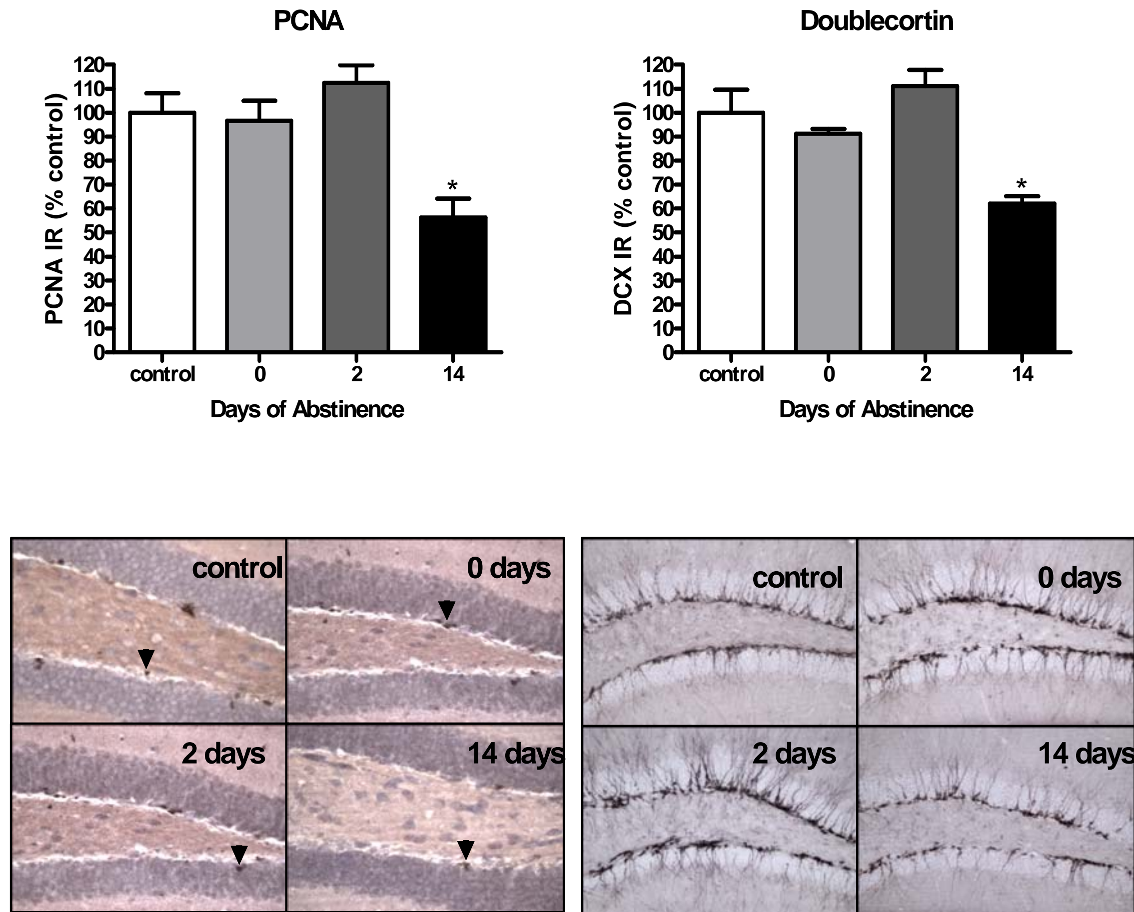


Figure 6: *Abstinence from alcohol reduces adult hippocampal neurogenesis. PCNA and DCX immunohistochemistry*. (**A**) Relative change in PCNA immunoreactivity (IR) in the dentate gyrus showing a reduction in NPC proliferation as a function of 0, 1, or 14 days of abstinence from alcohol drinking. (**B**) Relative decrease in DCX IR showing that the number of new neurons in the dentate gyrus is reduced as a function of abstinence from alcohol drinking. (**C – D**) Representative images of dentate gyrus from control, 0 days, 2 days, and 14 days of abstinence for PCNA (C) and DCX (D). Arrow heads point to representative PCNA-labeled cells. Mean data were obtained from averaging results from 4 tissue sections per mouse and plotted as percent change from water-drinking control. * - significantly different from water-drinking control, p<0.05 (Dunnett's t-test).

Figure 7. ***Desipramine treatment prevents abstinence-induced changes in depression-like behavior and neurogenesis.***

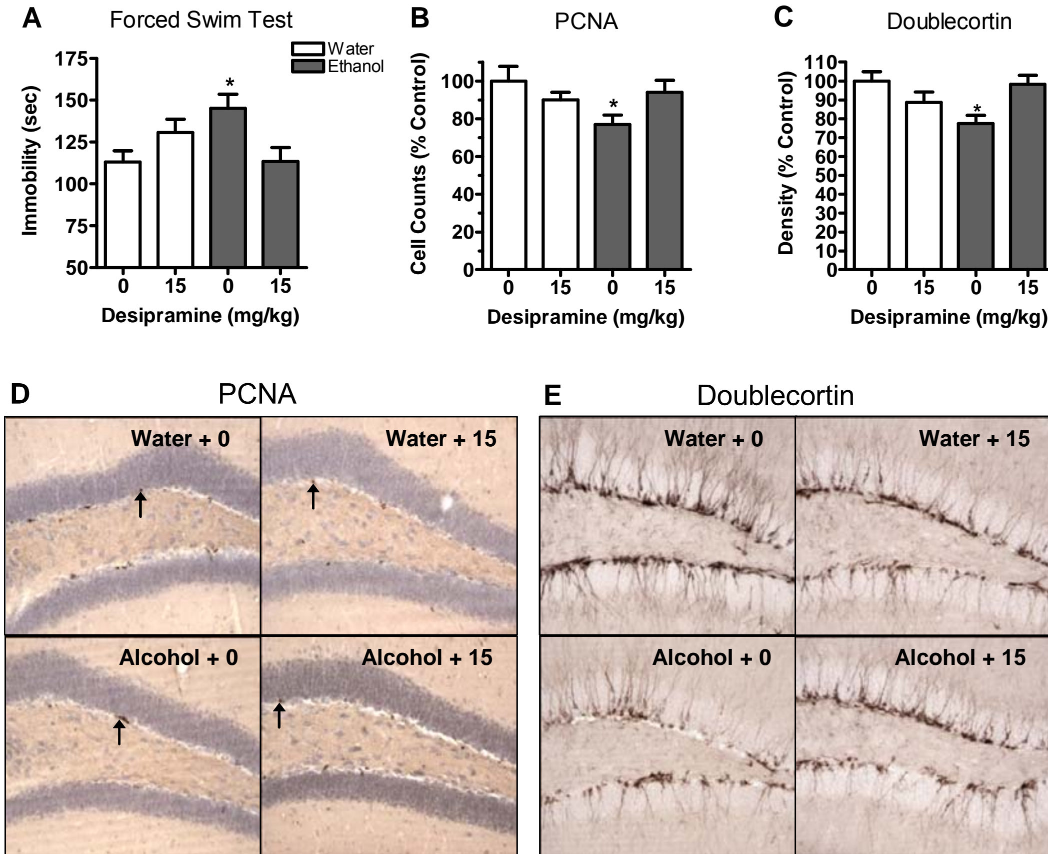


Figure 7: *Desipramine treatment prevents abstinence-induced changes in depression-like behavior and neurogenesis.* (**A**) Abstinence-induced increase in immobility (sec) in the Forced Swim Test (FST) was completely blocked by treatment with desipramine during 14 days of abstinence (n=12 per group). *-significantly different from water-drinking mice that received vehicle (desipramine, 0 mg/kg), p=0.007, and significantly different from ethanol-15 mg/kg, p=0.008 (Tukey test). (**B**) Desipramine treatment (14 days) prevented the abstinence-induced decrease in PCNA immunoreactivity in the dentate gyrus (n=12 per group). *-significantly different from water-drinking mice that received vehicle (desipramine, 0 mg/kg), p=0.012, (Tukey test). (**C**) Abstinence-induced reduction in DCX density was completely blocked by desipramine during 14 days of abstinence (n=12 per group). *-significantly different from water-drinking mice that received vehicle (desipramine, 0 mg/kg), p=0.004, and significantly different from ethanol- 15 mg/kg, p=0.005 (Tukey test). Mean ± SEM data for PCNA and DCX IR were obtained by averaging results from 4 tissue sections per mouse and plotted as percent change from water- 0 mg/kg controls. (**D**) Representative photomicrographs of PCNA IR (10X). Black arrows indicate PCNA-labeled cells. (**E**) Representative photomicrographs of DCX IR (10X).

Figure 8. ***Two-bottle drinking procedure.***

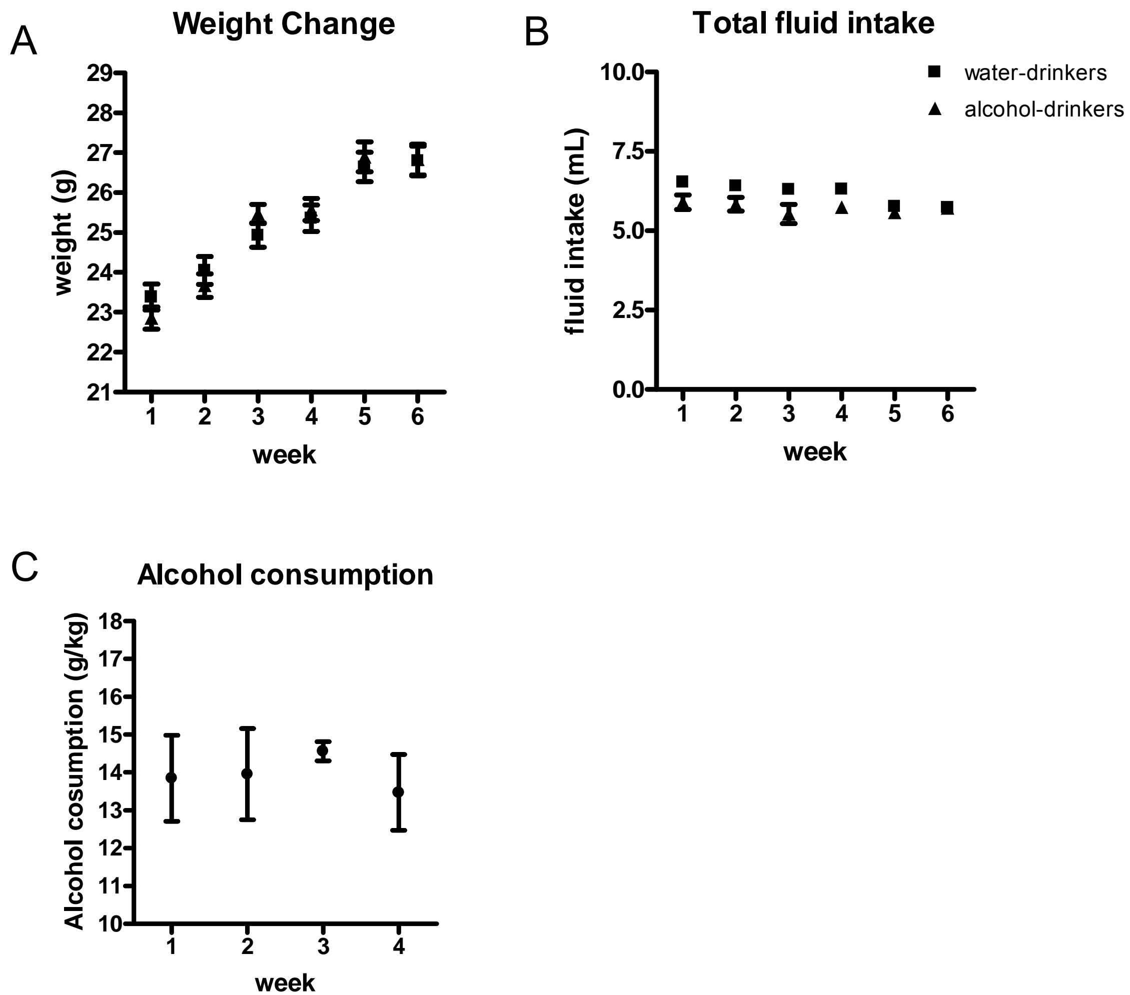


Figure 8: *Two-bottle drinking procedure.* (**A**)The weights of the mice increased significantly over the course of 28 days of drinking according to a two-bottle drinking procedure followed by two weeks of abstinence, and mouse weights did not differ at any point between water drinking and alcohol drinking mice. (**B**) Total fluid intake was significantly different between water and alcohol drinking mice during the two-bottle choice procedure with alcohol drinkers consuming an average of 0.5mL of fluid less per day ($p<0.001$). Mice also consumed significantly less total fluid when there was one bottle present on the cage during the two weeks of abstinence, probably due to less water dripping from the bottle ($p<0.001$). (**C**)Alcohol consumption by alcohol drinking mice did not differ over the 28 days of two-bottle drinking.

Figure 9. ***CREB changes in the dentate gyrus.***

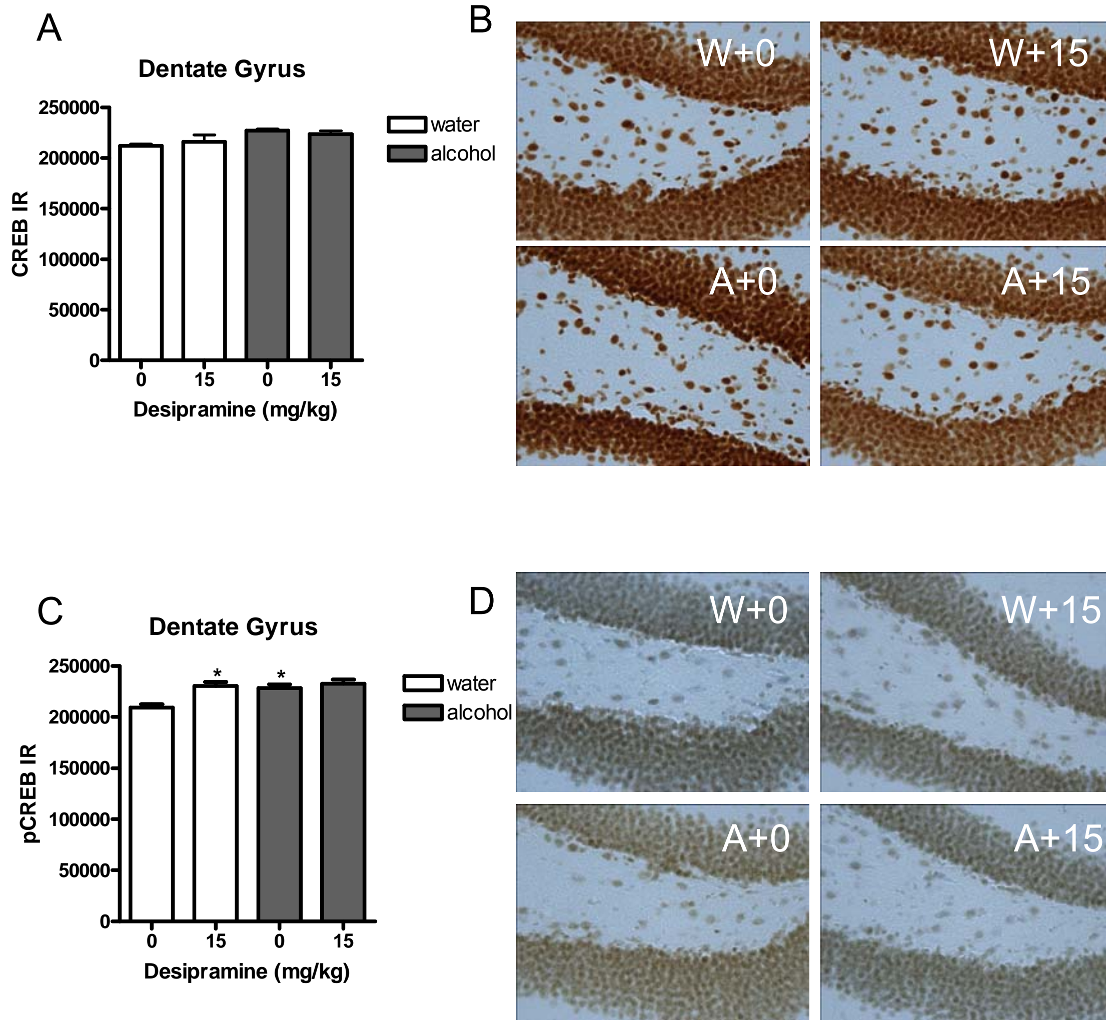


Figure 9: *CREB changes in the dentate gyrus.* (**A**) CREB IR was not significantly different in the dentate gyrus following abstinence or desipramine treatment. (**B**) Representative photomicrographs of CREB IR (40X). (**C**) pCREB IR was significantly increased by 14 days of desipramine administration compared to vehicle (p=0.003),and by 14 days of abstinence from alcohol drinking (p=0.12), with an interaction between alcohol abstinence and desipramine treatment (p=0.39). pCREB IR is significantly increased in W+15 compared to W+0 (p<0.001) and in A+0 compared to A+15 (p=0.003). (**D**) Representative photomicrographs of pCREB IR (40X).

Figure 10. ***CREB changes in the CA1.***

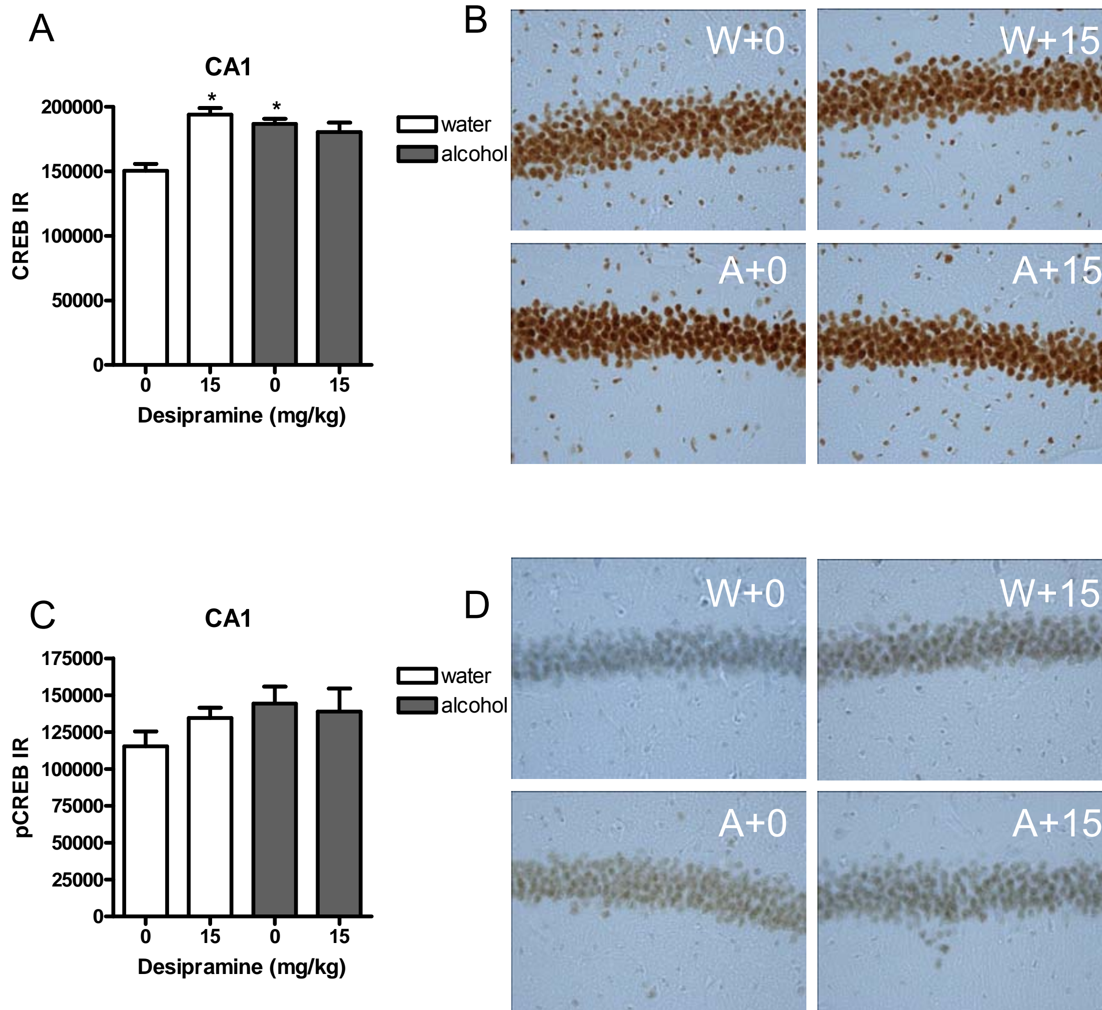


Figure 10: *CREB changes in the CA1.* (**A**) Desipramine treatment significantly increased CREB IR in the CA1 ($p=0.005$), with a significant interaction between abstinence and desipramine treatment ($p<0.001$). W+15 were significantly increased compared to W+0 ($p<0.001$), and A+0 were significantly increased compared to W+0 ($p<0.001$), indicating that desipramine and abstinence increased CREB in the CA1, but desipramine was not able to increase CREB IR beyond abstinence-induced increases. (**B**) Representative photomicrographs of CREB IR (40X). (**C**) Neither abstinence nor desipramine treatment altered pCREB IR in the CA1. (**D**) Representative photomicrographs of pCREB IR (40X).

Figure 11. ***CREB changes in the CA3***

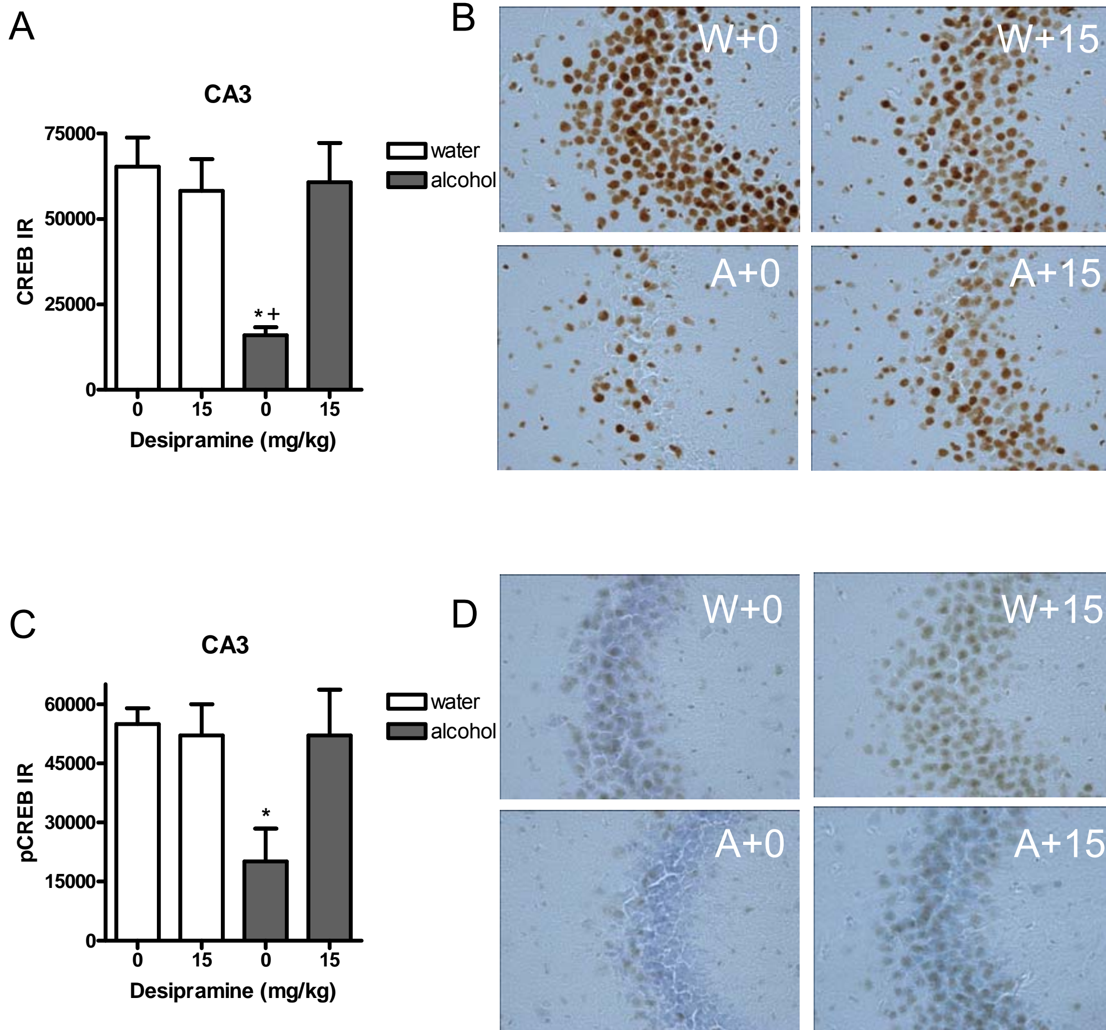


Figure 11: *CREB changes in the CA3.* (**A**) CREB expression was significantly decreased by abstinence following chronic alcohol drinking (p=0.003), and that this decrease was completely prevented by desipramine treatment (p=0.005). (**B**) Representative photomicrographs of CREB IR (40X). (**C**) pCREB IR was significantly decreased by abstinence following chronic alcohol drinking (p=0.008), and that this decrease was completely prevented by desipramine treatment (p=0.018). (**D**) Representative photomicrographs of pCREB IR (40X).

Figure 12. ***CREB changes in the Nucleus Accumbens Core.***

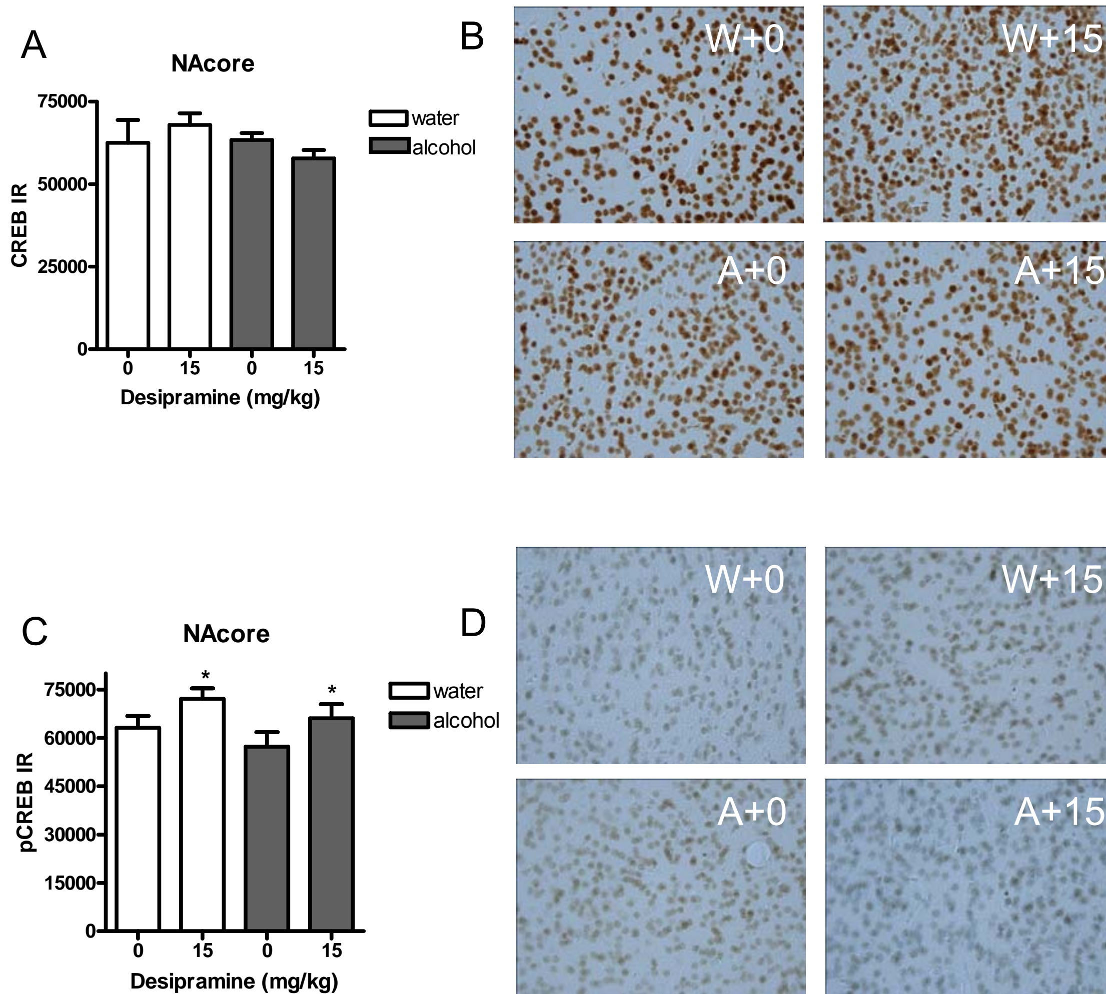


Figure 12: *CREB changes in the Nucleus Accumbens Core.* (**A**) CREB IR was not altered in the NA core by abstinence or desipramine. (**B**) Representative photomicrographs of CREB IR (40X). (**C**) pCREB IR was significantly increased by desipramine treatment in the core of the nucleus accumbens (p=0.031). (**D**) Representative photomicrographs of pCREB IR (40X).

Figure 13. ***CREB changes in the Nucleus Accumbens Shell.***

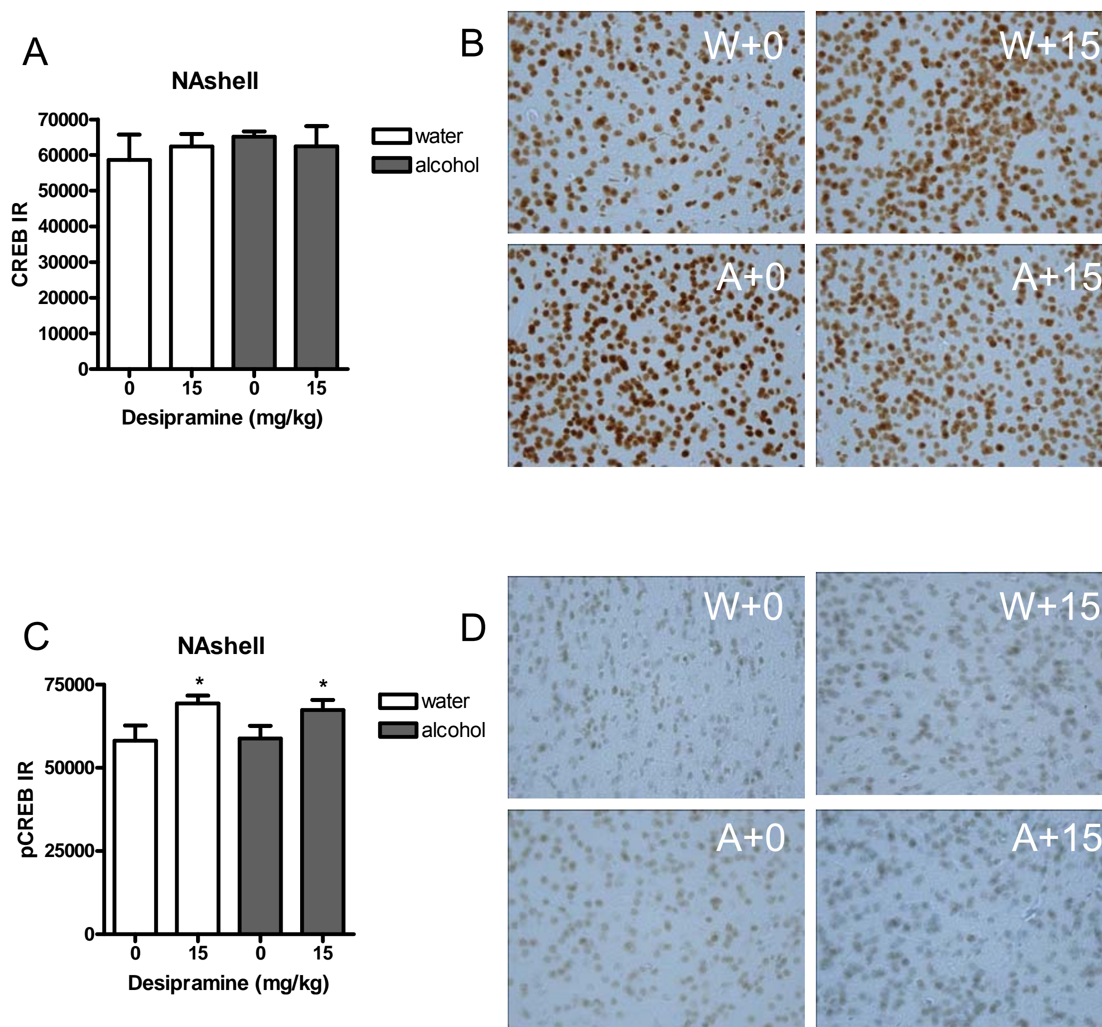


Figure 13: *CREB changes in the Nucleus Accumbens Shell.* (**A**) CREB IR was not altered in the NA shell by abstinence or desipramine. (**B**) Representative photomicrographs of CREB IR (40X). (**C**) pCREB IR was significantly increased by desipramine treatment in the shell of the nucleus accumbens ($p=0.008$). (**D**) Representative photomicrographs of pCREB IR (40X).

Figure 14. ***CREB changes in the Central Nucleus of the Amygdala.***

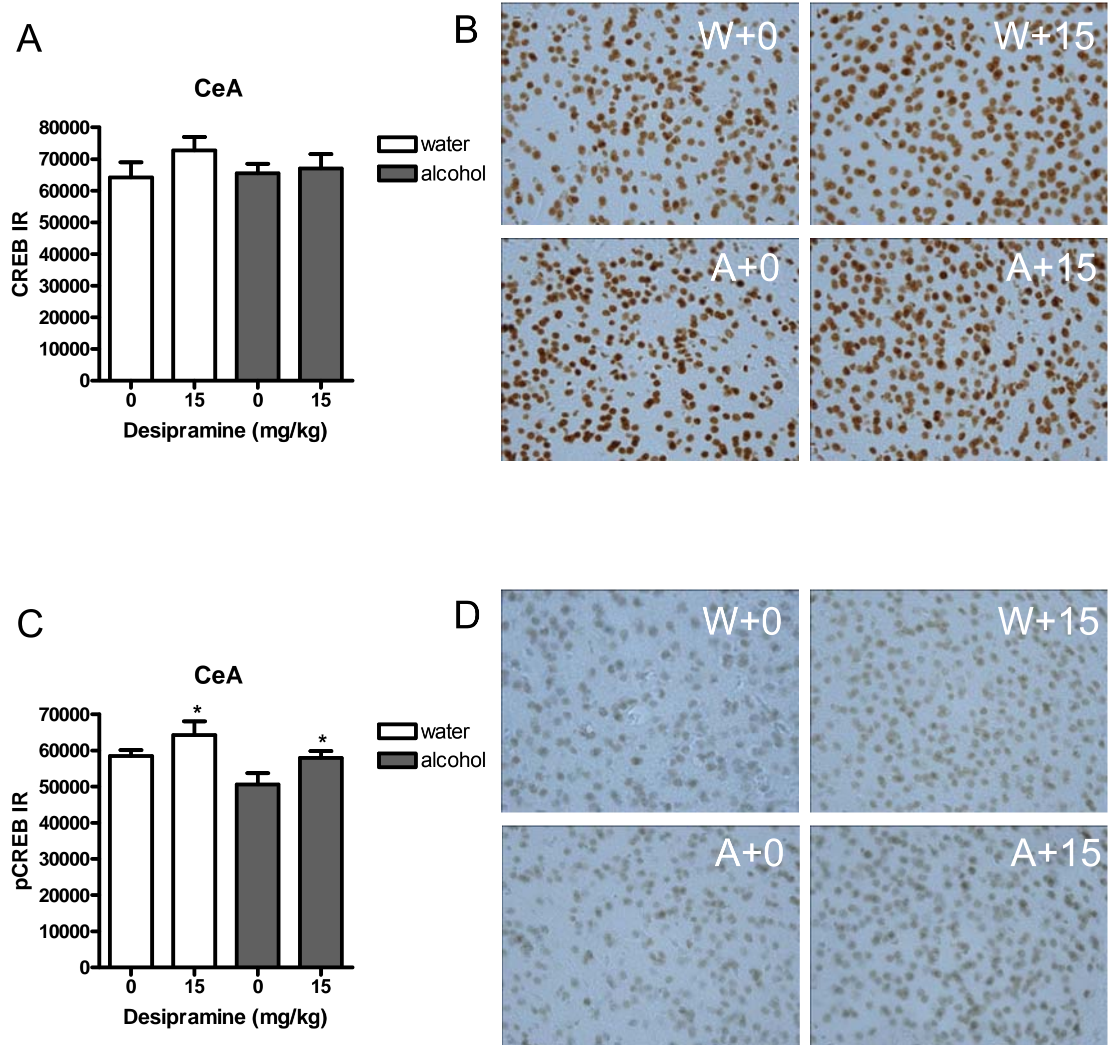


Figure 14: *CREB changes in the Central Nucleus of the Amygdala.* (**A**) CREB IR was not altered in the CeA by abstinence or desipramine. (**B**) Representative photomicrographs of CREB IR (40X). (**C**) pCREB IR was significantly increased by desipramine treatment in the CeA ($p=0.032$). (**D**) Representative photomicrographs of pCREB IR (40X).

Figure 15. ***CREB changes in the Basolateral Nucleus of the Amygdala.***

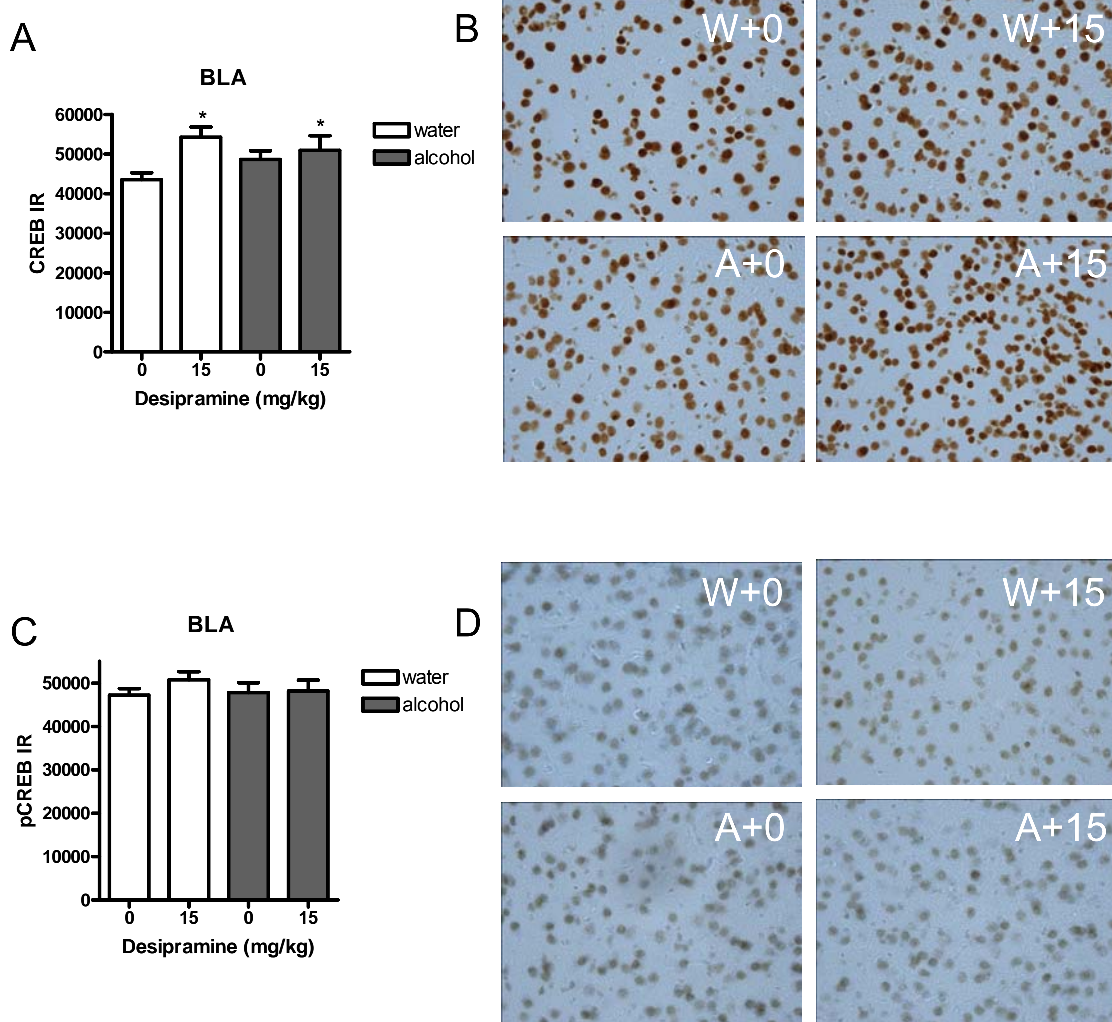
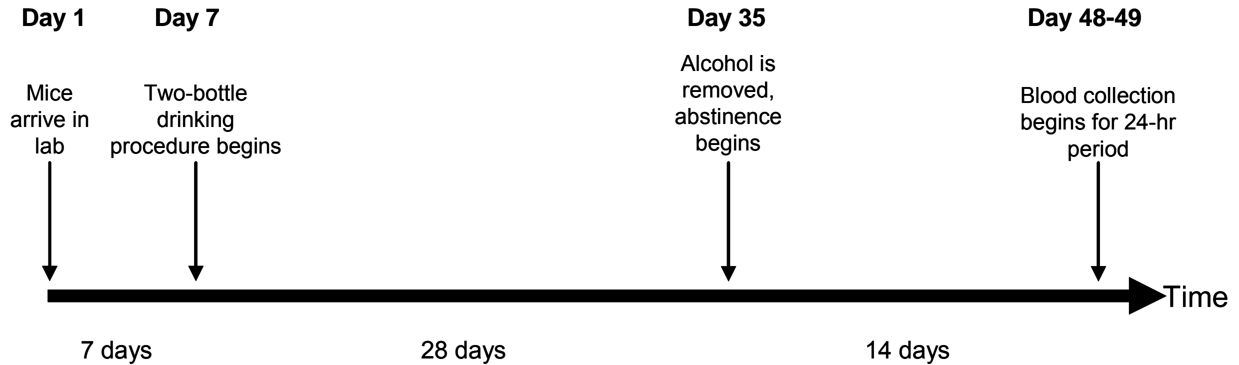
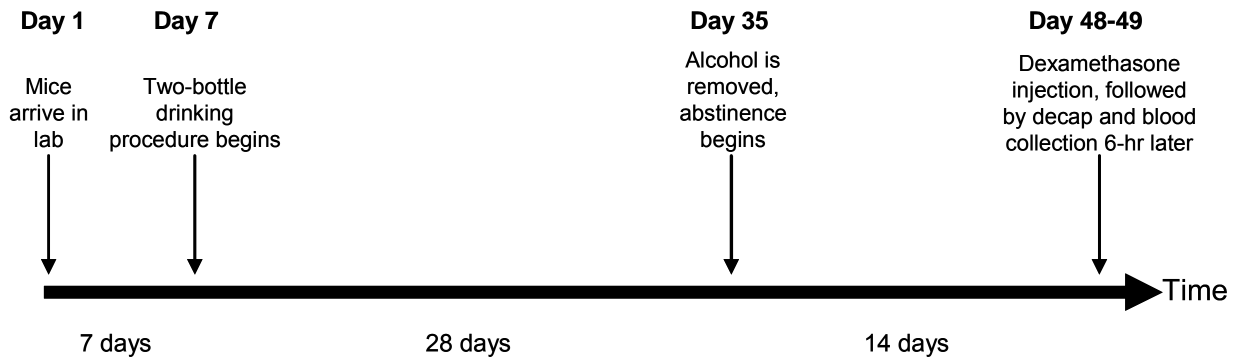


Figure 15: *CREB changes in the Basolateral Nucleus of the Amygdala.* (**A**) CREB IR was significantly increased by desipramine treatment in the BLA (p=0.026). (**B**) Representative photomicrographs of CREB IR (40X). (**C**) pCREB IR was not altered in the BLA by abstinence or desipramine. (**D**) Representative photomicrographs of pCREB IR (40X).

Figure 16. *Timeline of experimental procedures.*

Experimental Timelines





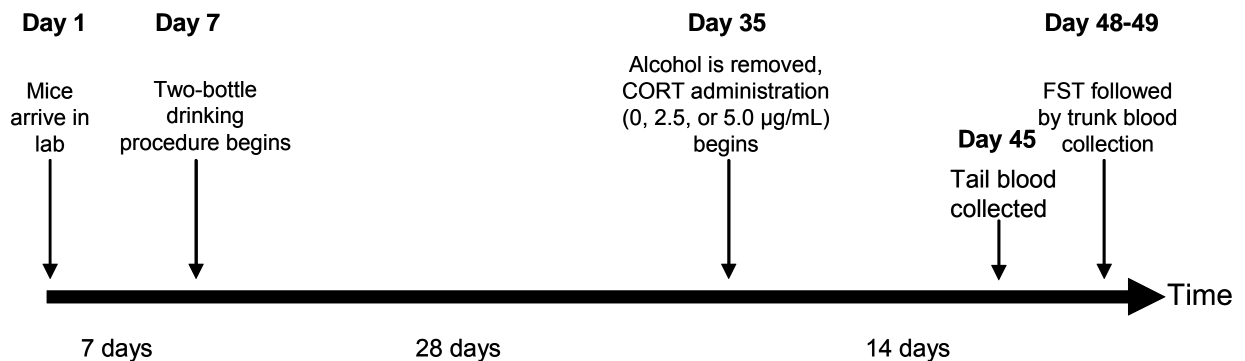


Figure 16: *Timeline of experimental procedures.* (**A**) Mice were allowed 7 days to acclimate to lab conditions before beginning the two bottle drinking procedure. After 28-days of two-bottle drinking, animals began 14-d of abstinence. At the end of the abstinence period, mice were assessed for 24-hr CORT rhythmicity. (**B**) Mice were allowed 7 days to acclimate to lab conditions before beginning the two bottle drinking procedure. After 28-days of two-bottle drinking, animals began 14-d of abstinence. At the end of the abstinence period, mice were assessed for sensitivity to dexamethasone. (**C**) Mice were allowed 7 days to acclimate to lab conditions before beginning the two bottle drinking procedure. After 28-days of two-bottle drinking, animals began 14-d of administration of CORT via the drinking water. On the 11th day of CORT administration, baseline serum CORT levels were measured. At the end of 14 days of abstinence/CORT administration, mice were assessed in the FST and post-stressor serum CORT levels were measured.

Figure 17. ***24-hour serum corticosterone rhythmicity is not altered by abstinence following alcohol drinking.***

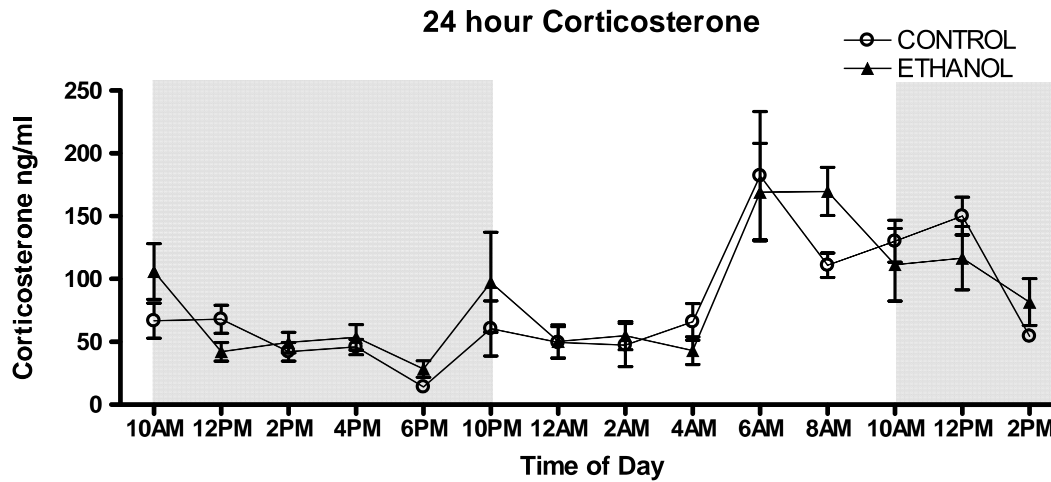


Figure 17: *24-hour serum corticosterone rhythmicity is not altered by abstinence following alcohol drinking.* Serum CORT levels did not differ between alcohol-drinking and water-drinking mice at any time point over the course of 24-hours of measurement. There was a main effect of time on serum CORT levels ($p<0.0001$). Shaded area shows lights off period.

Figure 18. ***Sensitivity to dexamethasone is not altered by abstinence following alcohol drinking.***

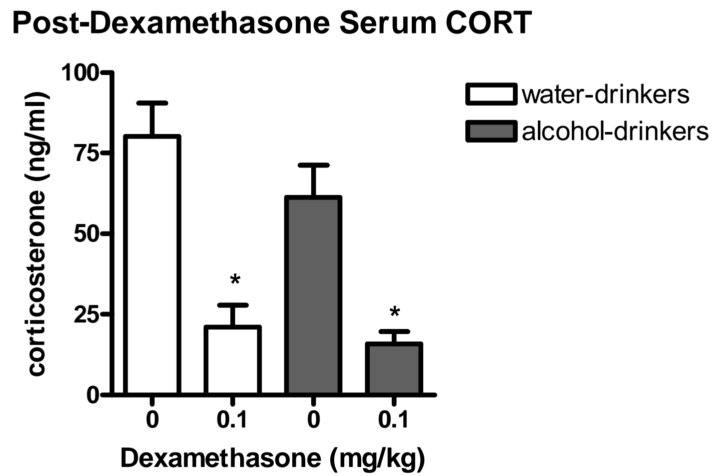


Figure 18: *Sensitivity to dexamethasone is not altered by abstinence following alcohol drinking.* Serum CORT levels were significantly suppressed 6 hours after an injection of dexamethasone (0.1mg/kg) ($p<0.001$), but sensitivity to dexamethasone was not different between alcohol-drinking and water-drinking mice.

Figure 19. ***14-d corticosterone administration alters basal corticosterone, post-stressor corticosterone, and behavior in the forced swim test.***

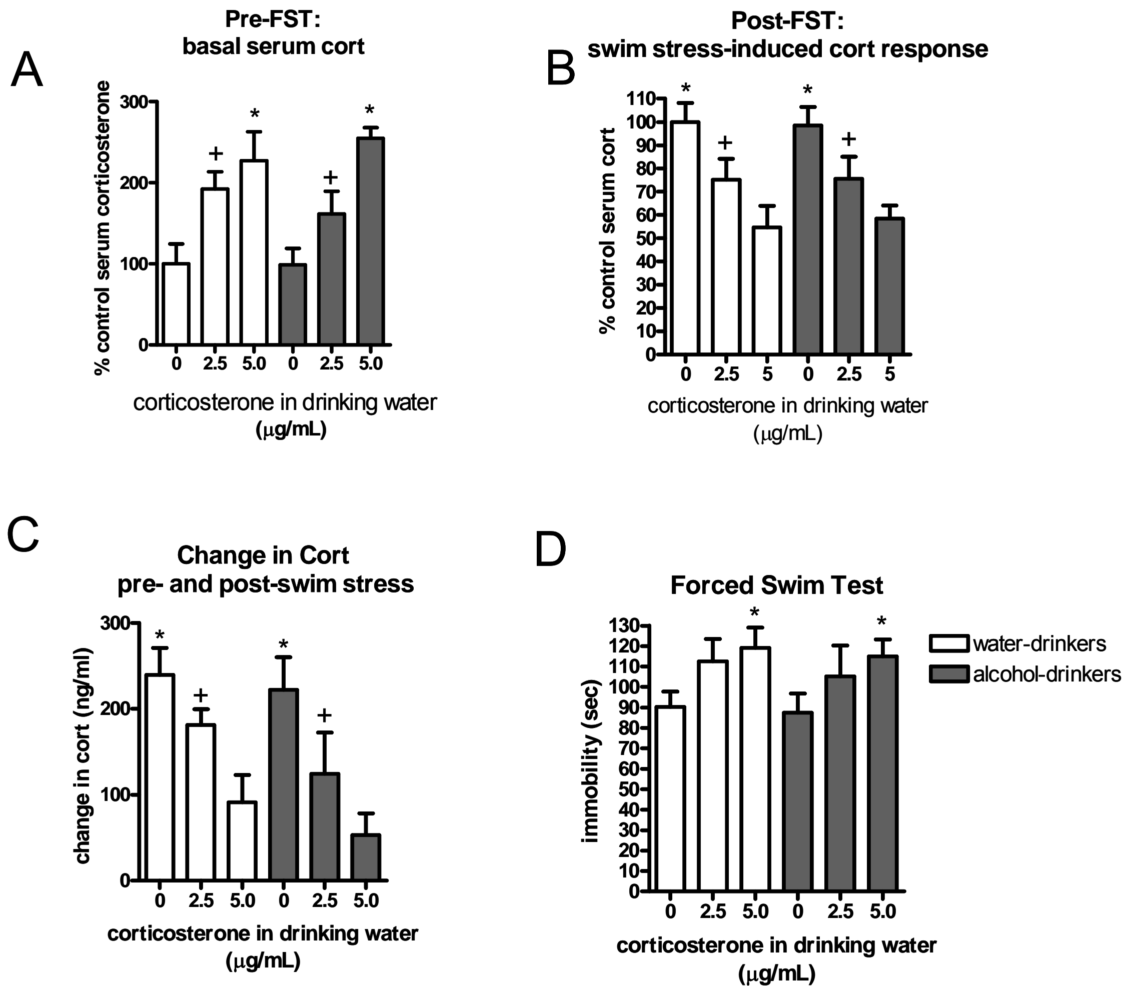


Figure 19: 14-d corticosterone administration alters basal corticosterone, post-stressor corticosterone, and behavior in the forced swim test. (**A**) Pre-stressor serum CORT was significantly increased by 14-d consumption of 5.0 μg/mL CORT solution compared to 0.0 μg/mL ($p<0.001$), and by 2.5 μg/mL CORT solution compared to 0.0 μg/mL ($p=0.038$). (**B**) Post-stressor serum CORT was significantly increased by 14-d consumption of 5.0 μg/mL CORT solution compared to 0.0 μg/mL ($p<0.001$), and by 2.5 μg/mL CORT solution compared to 0.0 μg/mL ($p=0.021$). (**C**)The stress response (as measured by the change in serum CORT from pre-stressor to post-stressor levels) was significantly decreased by 14-d consumption of 5.0 μg/mL CORT solution compared to 0.0 μg/mL ($p<0.001$). (**D**) Immobility in the FST was significantly increased by 14-d administration of 5.0 μg/mL CORT solution ($p=0.03$). Pre-and post-stressor serum CORT levels, stress response, and FST behavior were not different in alcohol-drinking and water-drinking mice.

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