INTERACTIVE EFFECTS OF CHRONIC STRESS AND ALCOHOL INTAKE ON BEHAVIORAL, PHYSIOLOGICAL, AND NEURONAL COMPONENTS IN MALE AND FEMALE RATS

By

JUAN L. GOMEZ

A dissertation submitted to the Graduate Faculty in Psychology (Biopsychology and Behavioral Neuroscience subprogram) in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

2012

This manuscript has been read and accepted by the Graduate Faculty in Psychology, the subprogram in Biopsychology and Behavioral Neuroscience, in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy

Victoria Luine, Ph.D.
_____

_____ _____
Date Chair of Examining Committee

Maureen O'Connor, Ph.D.
_____

_____ _____
Date Executive Officer

Victoria Luine, Ph.D.
Michael J. Lewis, Ph.D.
Peter Serrano, Ph.D.
Scott Russo, Ph.D.
Cheryl Conrad, Ph.D.
Supervisory Committee

THE CITY UNIVERSITY OF NEW YORK

Abstract

INTERACTIVE EFFECTS OF CHRONIC STRESS AND ALCOHOL INTAKE ON BEHAVIORAL, PHYSIOLOGICAL, AND NEURONAL COMPONENTS IN MALE AND FEMALE RATS

By

Juan L. Gomez

Advisor: Dr. Victoria Luine

For many individuals, exposure to stress is frequently accompanied by increased substance use. However, studies on stress and alcohol consumption often only focus on anxiety and depression to assess the interaction between these two factors. Cognitive function has been implicated in acquisition of drug abuse and thus requires further investigation. In addition to anxiety and depression, the interactive effects of stress and alcohol were evaluated on memory function using the object placement, object recognition, and Y-maze tasks. Four experimental designs used male and female rats randomly assigned to one of 4-groups: No Stress / No Alcohol Control (CON), Alcohol alone (ALC), Stress alone (STR), or Stress plus Alcohol (STR+ALC). In Aim 1, we found that restraint stress increased voluntary alcohol consumption in males and that alcohol consumption after a stressor alleviated stress-induced memory impairments in the object placement task. To control for variability in voluntary drinking, Aim 2 administered alcohol via gastric gavage (2 g/kg). Males exposed to the combination treatment (STR+ALC) had intact working and spatial memory and reduced levels of anxiety on the elevated plus maze and depression in the forced swim task (Aim 2). In contrast, females showed impaired memory and increased depression following 7-days of alcohol intake after a stressor (Aim 3). Physiological measures from male rats exposed only to the 7-day treatment (Aim 4) were consistent with some of the behavioral findings. The STR+ALC group showed adaptation of the corticosterone response from day-1 to day-7 of stress, while the other groups showed consistent corticosteroid release. Additionally, neurotransmitter receptor expression of hippocampal GABAα4 and GluN2B was upregulated for the ALC and STR+ALC groups on the last day of stress, but not

3-days after stress. Overall, the interactive effects of exposure to stress and alcohol were evident in behavior in a sexually dimorphic manner. These effects may explain the increased use of psychoactive substances during times of stress and may contribute to the development of dependence. The opposing results between males and females in response to alcohol intake after a stressor suggests a need for sex specific prevention and treatment methods for chronic stress and alcoholism.

Acknowledgements

I am a man with a curiosity driven by the need to explore expressed in the art of science. The culmination of my work with a doctorate degree could not have been accomplished without the help of countless individuals. My mentor Dr. Victoria Luine is a fantastic scientist dedicated to the advancement of knowledge. Her efforts have provided me with grand opportunities to brainstorm, conduct, and disseminate our research endeavors. She exemplifies the diligence needed to excel and has become my model of a true academic.

I would like to thank my parents Santos and Josefina and my sister and brother Jarelly and Harvey for supporting and inspiring me to excel. Not having the opportunity themselves, my parents provided all they could so that my siblings and I could pursue our dreams. From my father and mother I learned that hard work leads to prosperity. Additionally, they instilled the value of community and charity for which I am grateful. My goals are shaped by these ideals and I hope that with my Ph.D. I will be able to provide the opportunity and encouragement for others to follow. My friends, of which there are too many to name, have helped and encouraged me throughout my academic venture. Their support made my years in graduate school both enjoyable and intellectually stimulating.

Finally, I would like to recognize my mentors Dr. Cheryl Conrad and Dr. Michael Lewis. Dr. Conrad first took me into her lab and provided me with the tools to become critical scientific thinker. Her encouragement imparted a drive that has motivated me to continue in the world of academics. Upon meeting Dr. Lewis I felt a rapport that lead to the generation of ideas and concepts essential for the crux of my scientific inquires. He has provided mentorship and insight, both academic and personal, for which I am very thankful.

This research was made possible by funding from the MBRS-RISE program NIH Grant number GM060665 and in part by grant number RR03037.

Table of Contents

List of Tables

List of Figures

General Introduction

According to an American Psychological Association survey, 35% of Americans report suffering from extreme stress, but despite recognizing the problem, few actually seek help (2007). Coping with everyday stress can often lead to the use of psychoactive substances. The "self-medication hypothesis" explains the increase in substance use as a way of mitigating the aversive effects associated with psychological or physically painful events (Khantzian, 1997). During rehabilitation, most alcoholics claim that stress is the largest factor that leads to their continued drinking or relapse (Sinha, 2001). The experiments presented here test the effects of stress, alcohol, and their combination on various behavioral, physiological, and neurological measures and also attempt to provide information on self-medication.

Effects of Stress Exposure

Stress has been defined as an emotional state caused by a perceived or real threat. Response to a stressor occurs in a consistent pattern of physical reactions that are similar across organisms (Selye, 1956). The general adaptation syndrome was proposed by Hans Selye to explain physiological stages used to combat and adapt to a stressor. However, if the stages are prolonged or exhausted the consequences could produce injury or death. Conversely, response to a stressor is essential for the survival of humans and animals alike. In response to a stressor, the body mobilizes energy in preparation to fight or flee, heightens awareness, facilitates memory retrieval, and preps the immune system (McEwen, 2008). The initial activation of the stress response helps an organism deal with the situation in a manner necessary for survival. The delayed effects of a stressor helps facilitate memory consolidation and recall as well as physiologically prepare an organism for future encounters with the stressful situation (Roozendaal et al., 1999). However, chronic stress has negative consequences, which include, but are not limited to, increases in depression, anxiety, and substance use/abuse (Belda et al., 2008; Caspi et al., 2003; Sinha, 2001). To alleviate the negative affects associated with stress, organisms have devised various ways of coping before, during, and following a stressor

(Anderson, 1976). Some coping mechanisms, such as exercise and social support networks, are effective in attenuating the harmful effects produced by exposure to chronic stress. Unfortunately, some coping techniques alleviate the unfavorable effects short term, but may themselves lead to other adverse long-term consequences. For example, it is well known that stress leads to the use of drugs, alcohol, or medication as potential solutions for dealing with stressors (Sinha, 2001). However, the reinforcing properties of such substances, and their increased use, may later lead an individual to substance abuse or dependence (Koob and LeMoal, 2008).

Under ideal circumstances, a stress response would only occur during a real threat to survival. However, human societies have created non-threatening situations that constantly trigger the stress response and constant activation leads to many deleterious effects on an organism's behavior and physiology (McEwen, 2008). Office workers under high workload and deadline demands report increases in muscle tension in various body areas including the neck, wrist, and lower back (Griffiths et al., 2011). A study of traffic stress found male and female bus drivers to have elevated levels of stress hormones (adrenaline, noradrenaline, and cortisol), increased blood pressure/heart rate, and greater body mass indexes compared to controls (Aronsson and Rissler, 1998; Roohi and Hayee, 2010). A product of urban living is noise, and increase noise levels over prolonged periods of time has negative consequences. Ising and Ishing (2002) found children living near busy highways have higher cortisol levels during the first half of the night, impaired sleep, and memory/concentration problems compared to children living in quiet areas.

Animal models focused on stress-induced memory effects corroborate the negative impact of chronic stress exposure. Specifically, chronic restraint stress for 6hr/day/21days was shown to impair spatial memory in male rats (Luine et al., 1994; Conrad et al., 1996; Wright et al., 2005; McLaughlin et al., 2007). Luine et al. (1994) found restraint stress impairs acquisition of learning on the radial arm maze, an appetitive task. Here, rats treated with stress made errors earlier and

made fewer correct choices on the eight-arm maze. If rats were treated with phenytonin or tianeptine prior to restraint stress, the stress-dependent memory impairments were abolished. Conrad et al. (1996) replicated the findings by Luine, and found chronically stressed rats were impaired on the spatial memory task, the Y-maze. The impairment was blocked if rats were injected with tianeptine (TIA) prior to each stress bout. A difference between these two studies was the Y-maze does not require food deprivation, which means the test can be administered immediately following the last day of stress. Using the radial arm maze requires that rats be food restricted and trained, which may take up to 2 weeks. The delay between the last day of stress and testing may mask or reduce the stress effects that may have appeared earlier. Thus, our study utilizes the Y-maze for assessing spatial memory.

Stress and its physiological components play a substantial role in drug use and abuse. Rassnick et al. (1993) has shown that corticotropin-releasing factor (CRF) increases with use or withdrawal from ethanol, opiates, cocaine, and Tetrahydrocannabinol (THC). The increase in CRF during withdrawal is thought to account for relapsing behavior when a chronic user becomes abstinent (Rassnick et al, 1993). Foot shock stress (increased CRF) has been shown to quickly reinstate alcohol-paired lever preference in rats previously exposed to alcohol-paired conditioning (Hansson et al., 2006). A genetic upregulation of the corticotropin-releasing hormone receptor 1 (*Crhr1*) gene in an alcohol preferring rat line (msP), was shown to be associated with relapse (Hasson et al., 2006). Blockade of the *Crhr1* receptor with antalarmin decreases reinstatement of alcohol preference seen after foot-shock (Hasson et al., 2006). In accordance, blocking the release of corticosteroids (by adrenalectomy) eliminates behavioral responsiveness to morphine, while replacing corticosteroids to physiological levels, via injection, returns behavioral responsiveness to morphine back to control levels (Deroche et al., 1992). As is often the case, the interaction between stress and drug efficacy has a bi-directional effect. Whereby, drugs can have an effect on stress responses and stressors can have an effect on drug responses. Bisagno et al. (2004) demonstrated chronic amphetamine treatment impairs object

recognition memory in female rats, but the effect was blocked when chronic stress was combined with amphetamine treatment. It was hypothesized that chronic stress was counteracting the anxiogenic effects associated with chronic amphetamine treatment, sparing cognitive function from impairment (Bisagno et al., 2004).

The mechanisms for chronic or acute stress impairments in memory and mood are continually being studied because of the enormous toll that stress exacts on our society. The economic impact of stress-related disorders has been estimated at $42 billion per year (Kalia, 2002). Thus the importance of understanding the mechanisms of the stress response and possible self-medication with alcohol are essential for the development of proper treatments. Chronic stress leads to suppression of neurogenesis and altered cell survival in the dentate gyrus and hippocampus by changing NMDA function (Galea et al., 1997). Manipulation of the GABA system also influences morphology in the hippocampus and may contribute to stress induced changes in neural function. For instance, intraperitoneal administration of a GABA agonist (adinazolam) was shown to block CA3 dendritic retraction in chronically restrained rats (Magariños et al., 1999). However, chronic stress boosts global GABAergic systems, but the increase in activity is region specific, and not sufficient to block dendritic retraction in memory areas (Conrad, 2006). The importance of GABAergic systems for memory function was shown in rats with altered of BDNF levels. Kuczewski et al. (2008) found the hippocampal GABAergic system was dependent on BDNF, and blocking BDNF led to suppression of electrophysiological GABAergic activity in the hippocampus. Furthermore, chronic stress reduces BDNF mRNA levels in the hippocampus (Murakami et al., 2005), which would lead one to infer that stress alters GABAergic function in the hippocampus via changes in BDNF levels. Thus, it is clearly evident that chronic stress alters mechanisms important for learning and memory.

Assessing Stress-Induced Memory Impairments

The type of apparatus used to test memory can influence experimental outcomes due to the demands of the task. Tasks such as the Morris water maze (MWM), radial arm maze (RAM), object

placement (OP), object recognition (OR), Y-maze, and fear conditioning have all been used to test memory following stress or alcohol treatment (Luine et al., 1994; Conrad et al., 1996; Luine et al., 2003; McLaughlin et al., 2007; Cagetti et al., 2004; Spinetta et al., 2008). Wright and Conrad (2005) found rats restrained for 6hr/day/21days made similar amounts of entries into both the novel and other arms on the Y-maze after a 4-hour intertrial delay. Also, stressed rats spent equal amounts of time in both arms suggesting their memory for the novel arm was impaired. However, if the demands of the Y-maze were reduced (4hr to 1min delay), rats exposed to the same stress paradigm do not show the same impairment (Kleen et al., 2006). These results suggest the effects of stress were dependent on the constraints of the task.

Along with sex differences, two main factors have been shown to influence the effects a stressor can cause: the type and the duration. Under acute or mild circumstances, stressors can facilitate learning and memory and under chronic or severe circumstances, stressors lead to impairments in learning and memory in male rats, but not in female rats (review Luine, 2002). In a recent experiment, Bowman et al. (2009) showed that following 7-days of restraint stress (6hr/day), male rats were impaired on the OR task, while females showed no difference from controls (the same result was found with 21 days of stress). However, the impairment in stressed males was not present in the object placement task and there was facilitation in stressed females. This influence of sex on stress responding found by Bowman et al. (2009) has been previously reported and replicated by a number of groups and will be discussed later. Environmental circumstances have a robust effect on mediating the stress response. Beck and Luine (1999) show chronic restraint stress impairs males on the OR task (4hr delay), but the impairment was alleviated if stressed males were also food deprived. Here, we see a new variable (food deprivation) can act as a mediator to negate the deleterious effects of stress.

There are various constraints associated with using appetitive or aversive tasks, mainly providing stress to the control group and adding additional stress to the stress group. Appetitive tasks often require food or water deprivation, which may alter stress effects (Beck and Luine, 1999).

Aversive tasks, given the nature of their procedure (water submersion, foot/tail shock, taste aversion, etc.), produce additional stress variables. Since the current study was focused on manipulating stress, we used the object placement and Y-maze tasks to probe the effects on spatial memory and the object recognition task to measure effects on working memory. Using these two tasks allowed the researchers to test two forms of memory using the rat's natural exploratory tendencies while minimizing stress attributed to the task itself (Luine, 2002). As stated earlier, stress can be beneficial to an organism and given situational influences on the response, it is important to see how drugs like alcohol (due to its use as a self-medicating substance) alter the cognitive and physiological effects attributed to chronic stress. A factor that needs consideration is the type of stress in relation to alcohol consumption. As mentioned earlier, foot-shock stress reinstates alcohol-seeking behavior (Hasson et al., 2006). In comparison, restraint stress is considered a psychological stressor, while foot-shock is more of a physical stressor, which can influence behavioral responding (Luine et al., 2007). In terms of alcohol intake, both types of stress have been shown to decrease, increase, or cause no change in voluntary consumption (review Becker et al., 2011). For our studies restraint stress was used in order to provide a better representation of what the average human might experience as a daily stressor (psychological). This allows for the researchers to determine the effects of alcohol intake following a psychological stressor, which provides face validity for extrapolating to human individual who attempt to self-medicate with alcohol after a stressful day.

Effects of Alcohol Consumption

As aforementioned, alcohol consumption is often used as a form of self-medication during times of stress and anxiety (Khantzian, 1997). Alcohol is a short chain lipid soluble compound, but, unlike other drugs, there are currently no known endogenous alcohol receptors. A distinguishing characteristic of alcohol, unlike other drugs of abuse, is that it contains calories (about 7 calories per gram) (Porter et al., 2009). The prevailing theory for alcohol's mechanism of action is that it is an indirect GABA agonist and an indirect NMDA antagonist (Koob and Le

Moal 2008; Morrow et al., 2009). Alcohol intake has negative effects on spatial memory, but the bulk of the research has been done using aversive or appetitive tasks. Matthews et al. (1999) found the effects of alcohol on memory were selective for hippocampal dependent spatial memory. Rats were trained on a radial arm maze that could be learned using spatial or non-spatial information and were then given four doses of alcohol. Intraperitoneal injections were given 30-minutes prior to behavioral testing. Rats given saline or 1.0 g/kg were biased toward using more efficient spatial information, while rats given doses of 1.5 or 2.0 g/kg were biased toward using non-spatial information. In a similar experiment, mice given alcohol at 1.75 or 2.25 g/kg had impaired spatial memory on the MWM (Berry et al., 2004). In the Berry experiment, however, the mice exposed to 2.25 g/kg also demonstrated impairments in non-spatial memory. In addition, when mice were allowed one day to recover from acute alcohol administration, all mice swam in the target quadrant suggesting recovery of spatial memory. The results suggest that while the impairments were immediate they were not long lasting (Berry et al., 2004). Both of the above studies demonstrate high doses of alcohol disrupt functional use of hippocampal dependent spatial information to navigate through a maze in a dose-dependent manner.

Alcohol consumption does not render an organism incapable of navigation through the world. When rats were trained only using spatial information, Devenport et al. (1989) confirmed all rats, regardless of alcohol dose (0, 0.75, 1.5, or 2.0 g/kg), eventually developed cognitive maps on the MWM, however, the effect was dose-dependent. All rats learned where the platform was located, but the rats injected with higher doses swim greater distances compared to rats receiving vehicle or low doses. Another effect became apparent when the platform was repositioned. Rats given alcohol had trouble switching strategies when the platform was made visible in a new location. Therefore, when the task was learned using spatial information, rats given alcohol were impaired in abandoning the established strategy in favor for a new one. Devenport et al. (1989) also found the same effect on the RAM, in that rats had difficulty switching strategies. Despite the

dependence on the cognitive mapping strategy, alcohol treated rats could become proficient at cued place learning.

Different aspects of memory may be affected by alcohol. Three basic components of memory are acquisition, consolidation, and retention (Abel and Lattal, 2001). Using alcohol-preferring mice (C57BL/6J), Ryabinin et al. (2002) looked at the effects of acute alcohol on object recognition. The experimenters injected mice with differing doses of alcohol (0, 1.6, or 2.4 g/kg) two minutes prior to training or immediately after training. The results showed only rats injected with 2.4 g/kg of alcohol, two minutes prior to training, show no discrimination between new and old objects. Ryabinin et al. (2002) suggests that the impairment of memory was based on alcohol's ability to interfere with acquisition and not consolidation. A question then begins to emerge about the time frame when alcohol may have its effects on memory.

Dose-time and duration of administration of alcohol are all critical factors that have differing effects on outcomes. Some of the variability between experimental studies on behavioral and physiological effects of alcohol can also be accounted for by the differing methods of administering alcohol. Looking at different durations of alcohol drinking, Steigerwald et al. (1997) found time-dependent changes in memory on the RAM and MWM. Rats exposed to 1 or 14wks of alcohol (liquid diet) took less time to complete the maze than rats exposed to 20 or 28wks. Rats exposed to 14wks had fewer memory errors on the RAM when compared to other durations. Rats in the 1 or 20wk exposure had fewer errors on the MWM than those exposed to 14 or 28wks. The differences caused by duration of exposure lead Steigerwald et al. (1997) to conclude that the effects of alcohol on memory are not all-or-nothing. Essentially, there were transient time-dependent effects of alcohol on memory.

A prior experience with alcohol before certain cognitive tasks can block some of the deleterious effects of alcohol compared with individuals with no prior experience (Boulouard et al., 2002). When rats were given an acute dose of alcohol (1.5 g/kg) 30-minutes prior to behavioral testing they were impaired in learning on the MWM. However, if rats were given access to alcohol

as their only liquid for 2-weeks prior to behavioral testing the impaired learning caused by acute alcohol administration was diminished. One may infer that rats with prior alcohol exposure were more tolerant and possibly not as affected (physiologically) by an acute injection. However, when blood alcohol levels were assessed, there was no difference between groups with and without prior alcohol experience. The results suggest that prior alcohol experience has no effect on metabolism of an acute alcohol injection (Boulouard et al., 2002). The term "functional tolerance" has been used to describe the effects found in the above example, but the mechanism by which functional tolerance occurs is still unclear (Matthews and Morrow, 2000). Based on the research presented above, alcohol impairs memory and impairment was dependent on the duration and dose of alcohol, as well as, the time of administration relevant to the memory task and the task itself. In addition, Matthews and Morrow (2000) have determined that the impairing effects of chronic alcohol on spatial learning and memory can persist even when animals were given long recovery periods (> 8-weeks). Thus, one must consider all of these factors when comparing studies of alcohol consumption and memory function.

Chronic exposures to stress or alcohol are both associated with increases in anxiety (Kushner et al., 1990; Valdez et al., 2002; Izidio and Ramos, 2007; Santucci et al., 2008) and depression (Berner et al., 1986; Pietraszek et al., 1991; Madden, 1993; Ukai et al., 2009). Changes in these emotional states are demonstrated in both human and animal studies. A model extensively used to test anxiety in rats is the elevated plus maze (EPM) (Pellow and File, 1986; Dawson and Tricklenbank, 1995; Lonstein, 2005; Macbeth and Luine, 2010). The maze consists of four arms, two open and two closed. Rats that are less anxious show greater exploration of the open arms compared to the closed arms. Substances such as, alcohol, benzodiazepines (diazepam), and opioids (morphine) are all associated with anxiolytic effects on the EPM, given the proper dose (Izidio and Ramos, 2007; Drapier et al., 2007; Zarrindast et al., 2008). Belda et al. (2008) found one stress session (immobilization) was enough to sensitize the stress response to additional stressors and increase anxiety like behaviors on the EPM. In male rats, 7-days of stress shows

anxiogenic effects on the EPM, while the same stress paradigm did not change anxiety levels in females (Bowman et al., 2009). Chronically consuming alcohol (26 days) produces long-lasting increases in anxiety, even after 4 months of recovery from alcohol treatment (Santucci et al., 2008). Conversely, acute alcohol administration has dose-dependent anxiolytic and anxiogenic properties on the EPM. Lewis and June (unpublished) have shown an inverted "U" response of alcohol on the EPM, with 0.75-1.0 g/kg having the most anxiolytic effects 20-30 minutes post-administration. Additionally, many of the anxiogenic effects of alcohol are often associated with withdrawal (Kliethermes et al., 2004). Thus, our use of the EPM was conducted a day after the final stress or alcohol treatment in order to test any possible increases in withdrawal associated with intake. Anxiety and depression generally show high comorbidity and each is associated with alcohol use and abuse (Sartorius et al., 1996).

Depression is a major emotional state influenced by the use of alcohol or in response to stress (NIMH, 2012). People exposed to a natural or man-made disaster were more likely to develop depression, have PTSD, and increased substance use (Galea et al., 2002; Vlahov et al., 2002). As stated earlier, some coping mechanisms can have short-term benefits, but lead to long-term problems. In a yearlong study, human alcohol use was shown to reduce depression levels (short-term), but after one year, those with persistent alcohol consumption reported an increase in depression levels (Aneshensel et al., 1983). Caspi et al. (2003) reported a positive correlation between the number of life stressors and self reported depression symptoms, probability of suicide, and probability of major depression episodes. Like stress, self-reported depression and anxiety are two major contributors that lead to increased alcohol abuse and potential relapse (Sinha, 2001).

Recently, the forced swim task (FST) was modified to test depression in rats (Cryan et al., 2002). The FST scores behaviors such as swimming, climbing, and immobility to determine depressive-like symptoms in rodents. Cryan et al. (2002) demonstrated administration of anti-depressants increased swimming behavior and decreased immobility. Rats administered fluoxetine (an SSRI) show an increase in swim behavior, which indicates reduced anxiety. Clearly, anti-

depressants have robust effects on depressive states. Stress and alcohol have both been implicated as mediators of depressive behaviors as well. Chronic stress has been shown to have depressive effects on the FST, with stressed mice swimming less than non-stressed controls (Prince and Anisman, 1984). In support, Molina et al. (1994) found stressed rats spent more time immobile on the FST, but the depressive effect was attenuated when stress was combined with the opioid inverse agonist, Naloxone. Other models of depression (anhedonia) show acute and chronic stress both lead to depressive behaviors, but acute stress only produces short-term depression (~effect gone after 24h) (Pucilowski et al, 1993).

Mechanisms of Stress and Alcohol Effects

It is clear that stress, both acute and chronic, leads to depressive behaviors in rats and humans, and studies have been conducted to determine the mechanisms for depression. A possible mediator of increased depression following alcohol is the alteration of allopregnanolone levels, a positive modulator of $GABA_A$ receptor function. Alcohol increases serum allopregnanolone concentrations in both rats and humans (Tokunaga et al., 2003; Torres et al., 2004). Administration of allopregnanolone was shown to decrease the amount of time rats spent immobile, suggesting less depressive-like behaviors (Zimmerberg et al., 2009). From this finding we can infer that an acute administration of alcohol has anti-depressant effects on the FST, but the effects of chronic stress and alcohol administration on depressive-like behaviors using the FST have yet to be determined.

Stress and alcohol both increase levels of the stress hormone corticosterone (cortisol in humans) (Ylikahri et al., 1980; Thayer et al., 2006; Adinoff et al., 2003; Pitman et al., 1988; Harbuz and Lightman, 1992; review McEwen, 2008). The changes in stress hormones are dependent on a number of factors, such as, prenatal exposure (Henry et al., 1994), duration of stress (McLaughlin et al., 2007), prior exposure (Nisenbaum et al., 1991; Marin et al., 2007), age (Sapolsky et al., 1983), and gender (Jezova et al., 1996; Galea et al., 1997). Rats with peripubertal exposure to stress respond with an increased stress response to a stressor in

adulthood (Bellani et al., 2006). Prenatal studies suggest that stress can cause organizational changes in the developing organism. Vallee et al. (1997) confirmed an increase in CORT following prenatal stress, but postnatal handling could attenuate the increase. We see that an environmental mediator (handling) has a robust ameliorating effect on prenatal stress. However, genetics plays a role in response to stress. Schuckit et al. (1987) found that sons of alcoholics mounted a lower CORT response than sons of non-alcoholics following an acute administration of alcohol. Much like the Boulouard et al. (2002) study, there appears to be a genetic "functional tolerance" of alcohol on the stress response. This tolerance may be responsible for the high probability of becoming an alcoholic if your father was an alcoholic.

The release of CORT and other stress hormones are not always increased in systematic ways by stress. Nisenbaum et al. (1991) demonstrated that norepenephrine release in the hippocampus increases in chronically stressed rats after an acute novel stressor. In support, Marin et al. (2007) showed pre-exposure to a stressor changes the CORT response to a novel stressor. In Marin's experiment, all rats exposed to a novel environmental stressor have an increase in CORT levels. However, the rats that were pre-exposed to restraint stress mount a greater increase when compared to controls (sensitization). Both of these experiments show that chronic stress leads to increased sensitivity to new stressors. We expect the same sensitization of the stress response to appear after rats pre-exposed to restraint stress are tested on the forced swim task. Basically, rats pre-exposed to stress should mount a greater CORT response after exposure to the FST (swim stress).

Similar to stress, alcohol increases stress hormones. Dhabhar et al. (1997) showed an adaptation of the HPA-axis response during exposure to stress, with rats showing higher CORT release on day-1 compared to day-10 of stress. When chronic alcohol was used as a stressor the same result was obtained, CORT levels were significantly less on the last day of alcohol than on the first day (Spencer and McEwen, 1990). The adaptation of the HPA-axis to stress also changes with age. In a follow up study, Spencer and McEwen (1997) show an impairment of the

HPA-axis to adapt to chronic alcohol stress in aged rats (24-months). These studies show that changes of the stress response were dependent on time and duration of stress or alcohol. Lee and Rivier (1997) showed pre-treatment with alcohol blunted the increase in ACTH and CORT following an acute alcohol injection. In addition, pre-treatment with a CRF antagonist also produced the same effects of pre-treatment with alcohol. Therefore, pre-treatment with alcohol was mediated by CRF modulation in response to acute alcohol challenge. However, the beneficial qualities of pre-treatment with alcohol did not transfer over to other stressors. Lee and Rivier (1997) showed no reduction in ACTH or CORT to foot-shock following pre-treatment with alcohol, suggesting no cross-tolerance effects for foot-shock stress. When measuring cross-tolerance, the type of stress is important. Pre-exposure to one stressor will usually lead to blunted responses to similar stressors (McEwen, 2008). However, if the novel stressor is significantly different, an elevated stress response is usually mounted (McEwen, 2008). The changes in stress response were adequately shown in Khisti et al. (2005). They found a reduction of both plasma and brain deoxycorticosterone (DOC, a precursor of CORT) levels following an acute alcohol injection in rats with prior alcohol experience, supporting the findings by Lee and Rivier (1997). The reduction in DOC shows a functional tolerance effect to alcohol with prior experience. Overall, we see a general pattern of adaptation to stressors over time. However, any adaptations to the stress-response are often short-lived and chronic stress and alcohol consumption will eventually lead to detrimental effects.

Neurological changes following stress or alcohol provide support for some of the behavioral findings. For example, following 5-months of alcohol, via liquid diet at 8.5%, there was a 15% loss of pyramidal neurons in the CA3 region of the hippocampus and dentate gyrus (Walker et al., 1980). It has been well established that the hippocampus is a crucial structure involved in memory function (Morris et al., 1982; Aggleton et al., 1986; review Squire, 1992; Rosenbaum, 2007). Dendritic atrophy has also been shown in the CA3 region of the hippocampus following 6hr/day/21day of chronic restraint stress (McLaughlin et al., 2007).

However, depending on the type of stressor and the duration, the impairments to morphology are reversible with appropriate recovery time (Conrad, 1999). Additionally, sex differences and hormonal states have found that reduction of dendritic arborization caused by stress was only apparent in males or ovariectomized females (McLaughlin et al. 2009). In congruence, only males and ovariectomized female rats show impairments in spatial memory on the Y-maze following chronic stress (McLaughlin et al., 2005). Therefore, some of the impairments in memory caused by chronic alcohol or stress may be due to dendritic retraction in hippocampal neurons.

Spine density is often used as a measure of learning and memory capabilities (review Leuner and Shors, 2004). McLaughlin et al. (2010) found chronic stress in OVX females with hormone replacement increases spine density in CA1 and facilitates spatial memory on the MWM. Also, rats chronically stressed but lacking estrogen showed a significant decrease in spine density and impairment of spatial memory. When examining stress studies the type of stressor being used is important because, Shors (2001) found that acute tail-shock stress increased spine density in males and decreased density in female CA1 neurons. The sexually dimorphic changes in spine density to tail-shock were congruent with an increase in associative learning (eye-blink conditioning) in stressed males compared to stressed females. Essentially, males and females react differently to physical stressors (foot-shock) compared to psychological stressors (restraint stress). Thus, our studies focused on psychological stress and used restraint stress in order to directly compare the results to others using the same paradigm.

A clear example of the effects of alcohol on hippocampal plasticity (specifically dendritic spines) lies in environmental enrichment studies. Environmental enrichment prevents stress-induced impairments in memory and increases dendritic spines (Leggio et al., 2005; Wright and Conrad, 2008). However, rats prenatally exposed to alcohol do not show an increase in spine density after enrichment (Berman et al., 1996). The changes in spine density were dependent on opportunity for enrichment, in that no difference was found between rats prenatally exposed to alcohol and non-enriched controls (Berman et al., 1996), suggesting that prenatal alcohol

hindered neuronal plasticity only when the opportunity for change was presented. To my knowledge only one study has shown a compensatory response following alcohol-induced changes in spine density. Carpenter-Hyland and Chandler (2006) found *in vitro* hippocampal slices exposed to chronic ethanol treatment showed increased spine length in pyramidal neurons. It was proposed that due to the loss of neurons, surviving neurons begin to extend their dendritic spines in search of new connections (Carpenter-Hyland and Chandler, 2006). However, to date no experiments have directly looked at how changes in spine structure, induced by alcohol, affect behavior. One can only hypothesize that the neuronal loss and lack of synaptic connections caused by alcohol would generally lead to impairments in learning and memory.

Neurotransmitter Mechanisms

Alcohol and stress act on GABAergic and glutamatergic function (Morrow et al., 2009). Alterations in these neurotransmitters may underlie alcohol and stress effects on behavior. Unlike other drugs of abuse, to date, alcohol has no specific known endogenous receptor sites. Specifically, alcohol acts as an indirect GABA agonist. Administration of $GABA_A$ agonist (muscimol) or antagonist (bicuculline) enhances or depresses (respectively) the behavioral effects of alcohol (Mattucci-Schiavone and Ferko, 1987; Givens and Breese, 1990). Similar effects of known $GABA_A$ agonists with the effects found with alcohol treatment further support alcohol's agonistic properties on GABAergic function. Treatment with chronic restraint stress reduces GABAergic activity, which appears necessary for reduction of the stress response mediated by the HPA-axis (Verkuyl et al., 2005). Administration of the $GABA_A$ agonist muscimol prior to a stressor blunts the stress-induced increase in corticosterone, while bicuculline potentiates stress-induced CORT release (Cullinan et al., 2008). Given that alcohol acts as a GABA agonist, a working theory regarding alcohol dependence is that dysregulation of hormone secretion via the HPA-axis during periods of stress are normalized by alcohol consumption (Koob and Le Moal, 2008). The reduction in the stress response mediated by

alcohol consumption was only effective under acute or sporadic administrations due to an increase in GABAergic chloride (Cl^-) influx. However, chronic alcohol intake eventually diminishes the GABAergic Cl^- influx to a point lower than control levels (Grobin et al., 1998). This disruption of ionic flow may lead to increased alcohol consumption to attain homeostatic balance. In the current studies, a subunit of the GABA receptor system (GABAα4) was quantified due to its association with the anxiolytic properties of alcohol (Gulinello et al., 2001). It was hypothesized that GABAα4 would be downregulated in rats exposed to alcohol as a compensatory response for the chronic activation of GABA function induced by alcohol.

The interaction between GABA and NMDA has been implicated in modulation of long-term potentiation, whereby; the function of $GABA_A$ activity directly influences the function of NMDA-R activity (Schummers and Browning, 2001). In the presence of alcohol, antagonizing $GABA_AR$ function via picrotoxin significantly enhances NMDA evoked potentials, while normal $GABA_AR$ function reduces NMDA evoked potentials in hippocampal CA1 cells (Schummers and Browning, 2001). This experiment shows NMDA function was dependent on maintaining steady state of GABA activity. Durand and Carlen (1984) found that rats exposed to 9-months of chronic alcohol developed a depression of long term potentiation (LTP) compared to controls. However, if rats were given 2 months to recover (following 7 months of alcohol) LTP readings were not different from controls. In a similar fashion, Pavlides et al. (2002) showed 6hr/day/21days of stress produces suppression of LTP compared to controls, which correlated with behavioral impairments in memory. Whitlock et al. (2006) found glutamate receptors (GluR1 and GluR2) are increased following an avoidance memory task in the hippocampus and hypothesized the increase was necessary for induction of LTP. The avoidance task required rats to avoid an area of the maze associated with a shock. If the shock was not present, hence no learning, glutamate receptors were not different than rats not exposed to the maze (Whitlock et al., 2006). Specifically, in the following studies, the GluN2B receptor was chosen for investigation because it is associated with the withdrawal effects caused by alcohol (Hu et al.,

1996). Others have found that one week of alcohol treatment upregulates GluN2B receptors and we expect to find a similar upregulation in rats treated with alcohol. Investigating the interaction between stress and alcohol on GABAergic and glutamatergic function will provide important understanding of behavioral effects.

Sex Differences of Alcohol Effects

Biologically speaking, alcoholic women show differences in metabolism (Frezza et al., 1990), brain impairments (Hommer et al., 1996), dependence (Becker and Hu, 2007), and nutritional deficiencies (Mancinelli et al., 2003) when compared to alcoholic men. Women are generally more prone to the adverse consequences of chronic drinking, which are mediated by age, hormonal state, and drinking history (review Mancinelli et al, 2007). While few studies focus on females, the sex differences in the response to alcohol and stress suggest this area is in need of investigation.

Animal models also show differences between sexes in how they are affected by alcohol or stress. Chronically stressed male rats display spatial memory impairments on the Y-maze, while female rats show facilitated memory (Conrad et al., 2003; review Luine, 2002). However, the same is not true for alcohol consumption. Female rats given alcohol prior to behavioral training on the MWM perform poorly on acquisition of spatial localization of the platform (Sircar et al., 2009). Despite the fact that women generally start to drink later in life, they rapidly reach dependence and show the negative consequences of drinking (Mancinelli et al., 2007). Even with studies demonstrating a clear sex differences, most research is still done using only male subjects. For example, depression in humans is 2-3 times more likely in women, but most research on depression is based on studies utilizing only male subjects (Kessler, 2003).

Hellemans et al. (2010) demonstrated a differential effect of prenatal alcohol exposure (PAE) between male and female rats. In this study, both male and females were exposed to alcohol prenatally and then to a chronic mild stressor as adults. PAE males showed increased basal CORT levels than control males, while there was no difference between female groups.

PAE males showed depressive behavior on an anhedonia test, increased locomotor activity, and social anxiety. In contrast, PAE females showed greater behavioral despair on the forced swim task and decreased social interactions than control females (Hellemans et al., 2010). The effects of PAE are not apparent until behavioral or physiological domains are challenged in the appropriate manner (in this case, with chronic mild stress as adults).

The goal of this study was to examine the interaction of stress and alcohol intake in young adult rats. Along with testing effects on anxiety and depression, it is of specific interest to determine the effects on memory. Test such as the object placement, object recognition, and Y-maze were chosen due to established stress effects. In addition, we examined the interaction of stress and alcohol in a sex specific manner to determine any sexually dimorphic effects.

Specific Aims

Aim 1. Investigate the effects of chronic restraint stress on voluntary alcohol intake and spatial memory in male rats.

Aim 2. Investigate the effects of alcohol intake, via gastric gavage, on stress-induced changes in memory, anxiety, and depression in male rats.

Aim 3. Investigate the effects of alcohol intake, via gastric gavage, on stress-induced changes in memory, anxiety, and depression in female rats.

Aim 4. Measure the physiological and neurological effects of stress and alcohol treatment immediately following the end of treatment without behavioral tests: Following up Aim 2.

Aim 1.

Introduction

Alcohol consumption and abuse are major problems with devastating social and economic consequences. Initial consumption may lead to one of four possibilities: abstinence, non-problematic use, abuse, or dependence. Chronic alcohol consumption often leads to various aversive consequences such as, memory impairments, anxiety disorders, depression, renal and liver failure, and teratogenic effects (Hoffman and Matthews, 2001; Santucci et al., 2008; Seeley, 1960; Berner et al., 1986; Pfefferbaum and Sullivan, 2005). Behavioral and physiological responses to alcohol vary and are often dependent on drinking and family history (Schuckit et al., 1988). Schuckit has shown that sons of alcoholic fathers have a reduced stress response to an alcohol intoxication challenge, and may predict future abuse potential. Furthermore, alcoholics in treatment claim that stress is the major factor for their continued drinking and relapse (Sinha, 2001). The relationship between life stressors and alcohol consumption is complex and warrants further investigation.

The "self-medication hypothesis" suggests that an individual may initiate or increase substance use as a way of relieving aversive daily experiences (Khantzian, 1997). Under stressful conditions, animal models of alcohol consumption show a variety of drinking patterns. Various researchers have found reinstatement of alcohol seeking behavior after extinction when foot-shock stress is applied (Liu and Weiss, 2003; Le et al., 2005; Matthews et al., 2008). Using a social model of stress, Pohorecky (2008) found that rats classified as subordinate (high-stress) drink more alcohol than dominant rats during a 23-hour probe period. Restraint stress has been sparsely studied in terms of voluntary drinking in rodent models. However, restraint stress has been extensively used to test cognitive function (Luine et al., 1994; Conrad et al., 1996; Bowman et al., 2009). Lynch et al. (1999) found that when rats were given a choice between alcohol and water following 15 minutes of restraint stress, the stress group drank more alcohol than the non-stressed.

Chronic restraint stress has been repeatedly shown to disrupt cognitive behaviors (Luine et al., 2007). Following restraint stress for 6hr/day/21days, male rats show impaired learning and memory on tasks such as the radial arm maze, object placement, and Y-maze (Luine et al., 1994; Conrad et al., 1996; Wright et al., 2005; McLaughlin et al., 2007). However, the negative effects of stress appear to depend on environmental, hormonal, and pharmacological factors. Wright and Conrad (2008) found that an enriched environment prevents the stress-induced memory impairments on the Y-maze and Morris Water Maze compared to stressed rats in standard housing. McLaughlin et al. (2010) showed that ovariectomized rats treated with 17β-Estrodial or cholesterol are spared from dendritic retraction in hippocampal areas caused by 3-weeks of restraint stress. Bisagno et al. (2004) found that chronic injection of amphetamine impairs memory on the object recognition task in female rats; however, when the rats were restraint stressed and given amphetamine the impairments were reversed. Thus, stress-induced impairments can be mediated when other variables are combined.

The purpose of this study was to investigate the effects of chronic restraint stress on alcohol consumption and possible interactive effects of stress and alcohol on spatial memory. In accordance with the self-medication hypothesis and Lynch et al. (1999), we predicted that stressed rats would voluntarily consume more alcohol than non-stressed rats. We also investigated whether chronic stress-dependent impairments in spatial memory would be alleviated by alcohol availability following a stressor as has been reported by other combinations.
This experiment has recently been published (Gomez et al., 2012)

Materials and Methods

Subjects

Male Sprague-Dawley rats (~250 g, N = 60) obtained from Harlan-Sprague Dawley were individually housed and kept on a 12h reverse light cycle with lights off at 10:00AM. Standard rat chow and water was available ad libitum. After arrival, rats were allowed to acclimate to the environment for one week before treatments were conducted. Rats were randomly assigned to

one of four groups; No stress-No Alcohol control (CON, n=10), Alcohol alone (ALC, n=10), Stress alone (STR, n=20), and a combination of Stress and Alcohol (STR+ALC, n=20). The stress paradigm began after reliable drinking was established (see below). All procedures were approved by Hunter College's Animal Care and Use Committee.

Procedures

Following arrival and acclimation, rats were trained to prefer alcohol via a two-bottle limited access paradigm (Martinetti et al., 2000). Ethanol was diluted with water to 1%, 3%, 5%, 7%, and 8% v/v. Each concentration was presented in a counterbalanced fashion with water for 1-hour a day for three days. Following the last training day of alcohol, stress treatment began and 8% v/v was available after the stressor for 1-hour for the ALC and STR+ALC groups. The stress and/or alcohol treatments continued for 10 consecutive days. Rats in the CON and STR groups received a two-bottle choice with both bottles containing only water (See Figure 1 for a timeline of the experimental treatments).

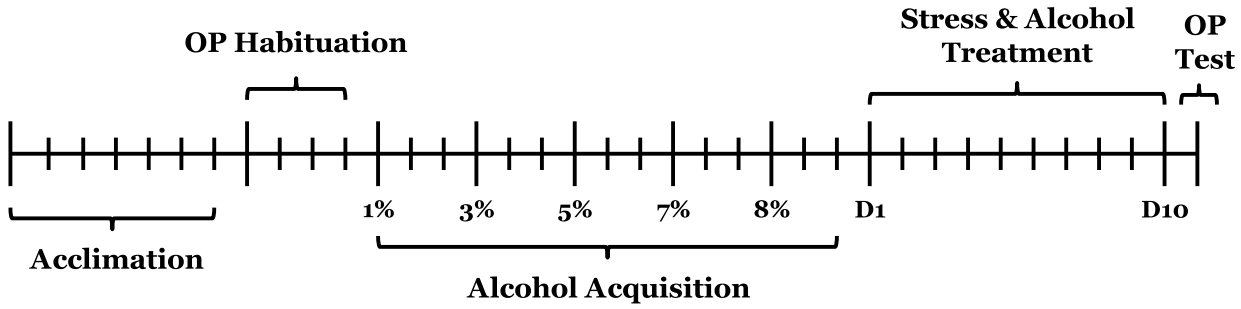

Figure 1. Time-line. Each tick mark indicates one calendar day.

After the stress and alcohol treatments were complete an object placement (OP) task was conducted. The OP task is a hippocampal dependent spatial memory test designed to measure memory of object location in an open field following a predetermined inter-trial delay (Bowman et al., 2009). Several studies have shown that performance of this task is compromised following hippocampal lesions (Ennaceur and Aggleton, 1994; Ennaceur et al., 1997; Broadbent et al., 2004). The OP task consists of a sample trial (T1), and a retention trial (T2) (Luine et al., 2003).

T1 requires two identical objects to be placed at one end of an open field (70 x 70 cm). The rat is allowed to explore the objects for 3-minutes and the amount of time spent exploring each object was recorded with stopwatches. Exploration was defined as whisking, sniffing, and looking at the object from no more than 2 cm away. Following T1, rats are placed back in their home cages left in the experimental room for a 4-hour inter-trial delay. After the delay (T2), one object is relocated to a new position across the open field. The locations of the objects were equidistant from the corners in a diagonal fashion and positioning was counterbalanced. The rat was allowed to explore for 3 minutes and the time spent exploring the objects in the old and new locations was recorded.

Drug Treatment

Rats were administered alcohol via a two-bottle choice limited access paradigm from 12:00 – 1:00PM daily (Martinetti et al., 2000). Ethanol (200 proof, Sigma-Aldrich) was diluted in water and introduced at a concentration of 1% v/v. The percentage of alcohol was gradually increased every three days until rats established reliable drinking of 8% v/v (Figure 1).

Restraint Stress

Restraint stress began after all rats showed stable consumption of ethanol. Rats were restrained, not immobilized, for 1hr/day/10days (11-12PM) in a cylindrical tube constructed of clear Plexiglas measuring 21.5cm long x 6.3cm internal diameter (Harvard Apparatus). One end has a clear, closed end piece containing ventilation holes. The other end has a sliding plastic plug that is secured in place by a screw and adjusted to fit the size of the rat. A slotted opening in the plug allows for free mobility of the tail. Restraint procedures are similar to Lynch et al. (1999) and Bowman et al. (2009). After the restraint stress period, the animals were returned to their home cage and ALC and STR+ALC rats were given a two-bottle choice of 8% ethanol or water for 1-hour (Figure 1).

Statistical Analyses

All statistics were conducted using PASWStatistics v.18 (IBM Corp., Somers, NY). A repeated measures ANOVA was used to analyze consumption rates within and between the ALC and STR+ALC groups, and regression analyses was used to determine overall consumption over the course of the stress treatment. A repeated-measures ANOVA was used to analyze exploration times on the object placement task. Significant main effects were further analyzed using paired-t tests (Bonferroni corrected) to determine group performances in ability to discriminate between objects in the New and Old location. A repeated-measures ANOVA was conducted to analyze body weight gain over the treatment period. Significant main effects were further analyzed using a one-way ANOVA and Fisher's Least Significant Difference (LSD) test based on day of treatment.

Results

Alcohol Consumption During Stress

Alcohol consumption was measured daily during the stress period (10-days) in rats treated with or without restraint stress. A repeated-measures two-way ANOVA showed no significant main effects of day [$F_{(9,252)}=1.14$, $p=0.34$] and treatment [$F_{(1,28)}=0.49$, $p=0.49$], but a significant interaction between day and treatment was found [$F_{(9,252)}=2.34$, $p=0.015$] (Figure 2A). Thus, a post-hoc examination of the data was made by linear regression. The regression analysis of drinking behavior over the 10-days of stress (Figure 2B) showed that chronic restraint stress predicts the increased alcohol consumption by the STR+ALC group compared to the ALC group [$b=0.495$, $t_{(18)}=8.54$, $p<0.0001$]. Chronic stress also accounted for the majority of variance associated with the differences between intake values [$R^2=0.245$, $F_{(1,18)}=5.85$, $p=0.026$] (Figure 2B).

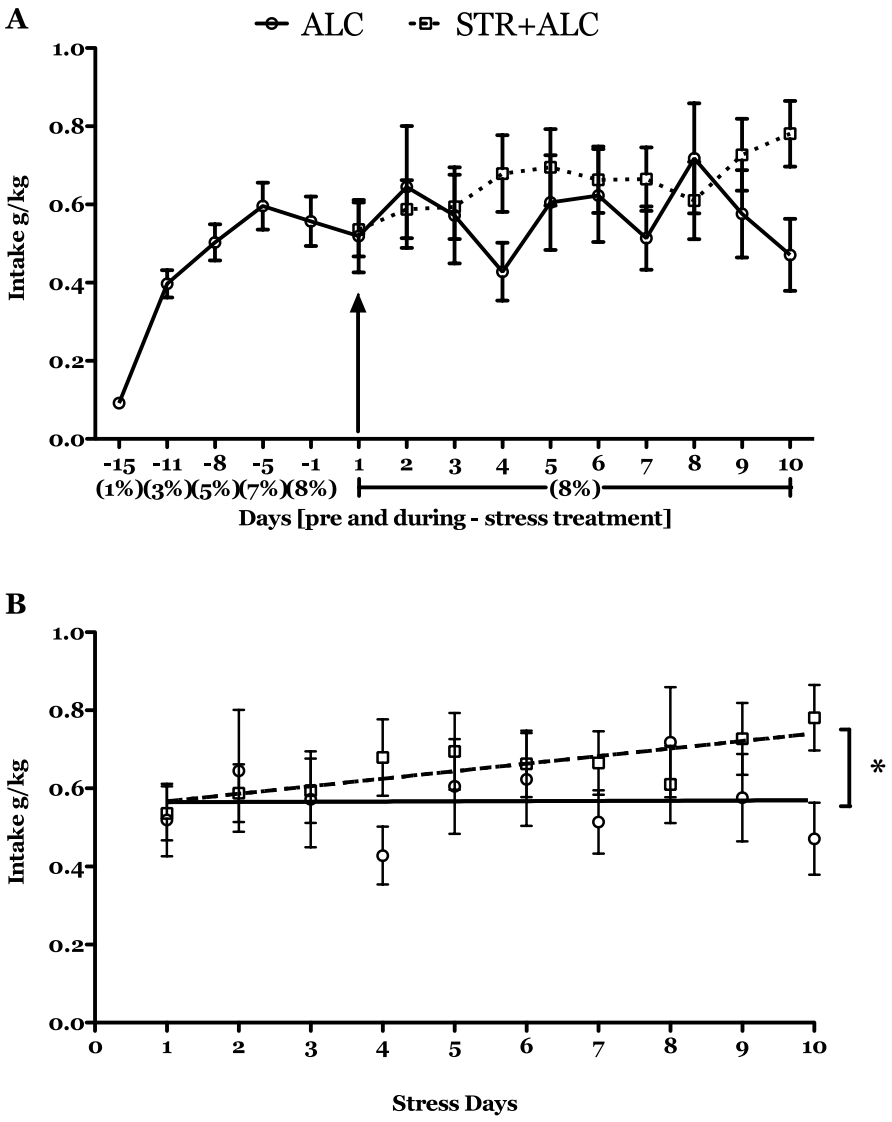

Figure 2. (A) Mean alcohol consumption pre- and during-stress treatment. Negative numbers designate days before stress and positive numbers designate days of stress. Numbers in parenthesis show concentration of alcohol (v/v) presented. Arrow denotes first day of stress. (B) Mean alcohol consumption over days of restraint stress. Dashed regression line plotted for STR+ALC group and solid regression line plotted for ALC group. Asterisk |*| indicates a significant difference (R^2=0.245, p=0.026) between group regression lines.

Spatial Memory on the Object Placement Task

Treatments with alcohol, restraint stress, or the combination of stress and alcohol influenced performance on the object placement task. During the sample trial (T1), an ANOVA

showed no significant difference of object exploration time between groups [$F_{(3,56)}$=1.51, p=0.22] (Figure 3A). However, repeated-measures within subjects analysis of the retention trial (T2) showed a main effect of object [$F_{(1,56)}$=7.70, p=0.008] and an object by group interaction [$F_{(3,56)}$=3.42, p=0.02]. A Bonferroni corrected paired-t test was conducted for each group to test for differences in exploration of objects in the old and new locations. Rats in the CON and STR+ALC groups explored the objects in the new location significantly more than objects in the old location [$t_{(9)}$=3.43 p=0.007; $t_{(19)}$=2.83, p=0.011, respectively] (Figure 3B). Rats in the ALC and STR groups did not significantly discriminate between objects in the old and new locations [$t_{(9)}$=1.48, p=0.17; $t_{(19)}$=0.86, p=0.40, respectively] (Figure 3B). Thus, treatment with ALC or STR impairs spatial memory, but the combination of STR+ALC does not.

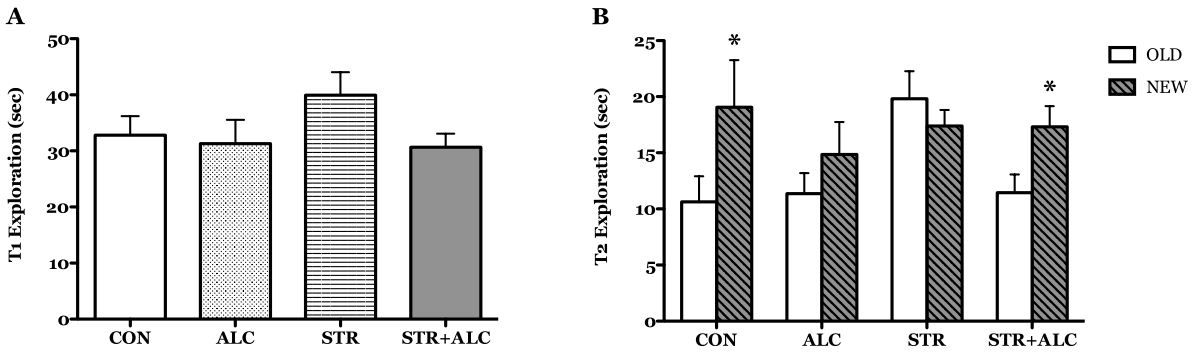

Figure 3. (A) Mean exploration time for sample trial (T1) on the object placement task. There was no significant difference between groups (mean=33 sec.). (B) Following a 4-hour delay T2 shows mean exploration time of objects in the Old and New locations. Asterisk |*| indicates a significant difference (p<0.05) between exploration of the objects in the Old and New locations.

Stress Effects on Body Weight Gain

Body weights were analyzed by two-way ANOVA (group x day). There was no significant main effect of group [$F_{(3,56)}$=2.07, p=0.12], but a significant main effect of day [$F_{(2,112)}$=320.53, p=0.0001] and significant interaction between day and group [$F_{(6,112)}$=2.94, p=0.01]. A post-hoc LSD found that by day-5 the CON group weighed more than the STR group (p=0.04) and by day-10 both the CON and STR+ALC groups weighed more than the STR group (p=0.015)

(Figure 4). On day-1 of stress, no weight differences between groups were found (mean ≈ 395 g), and all rats gained weight over the 10-day stress period (mean ≈ +35 g) (Figure 4). Despite the added calories present in alcohol, rats treated with alcohol did not differ in weight compared to other groups.

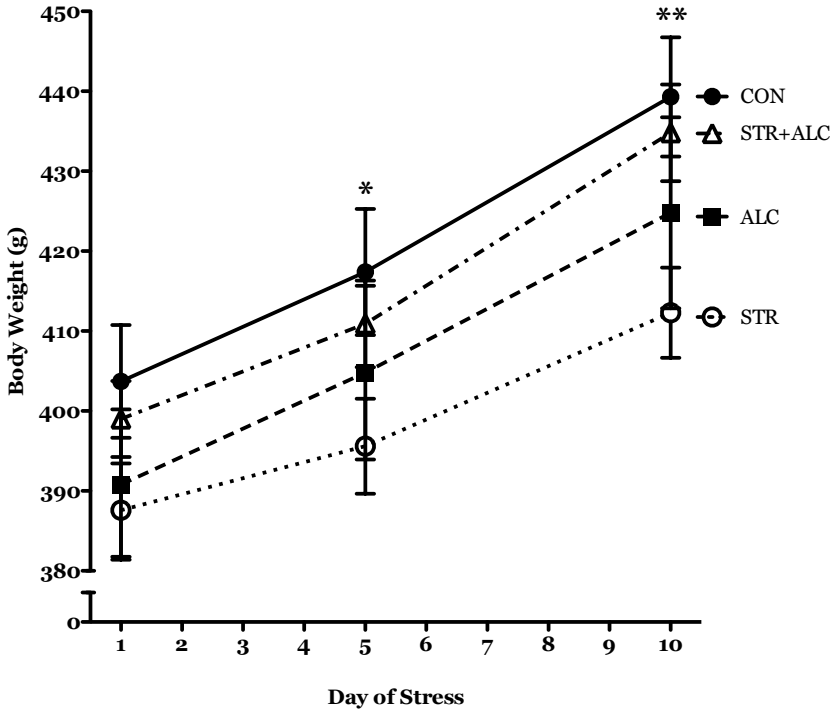

Figure 4. Mean body weights in grams presented at 5-day intervals. Asterisk |*|, significant difference between the CON and STR group (p=0.04). Double Asterisk |**|, significant difference between the CON/STR+ALC groups and the STR group (p=0.02).

Discussion

The current results show that during the stress treatment, rats in the STR+ALC group consume more alcohol than those not stressed. Our findings support others who have found an increase in alcohol self-administration following stress treatment (Lynch et al., 1999; Ploj et al., 2003; Roman et al., 2004) and shows support for the "self-medication" view of increased substance use during times of stress. With respect to spatial memory, alcohol availability after each exposure to stress blocked the impairing effects of treatment with alcohol or stress alone,

which is a novel finding and provides rationale for self-medicating. The reduction in weight gain, which is a well-known effect of chronic stress, was observed in the STR but not in the STR+ALC group. Despite these findings, it is not entirely clear whether restraint stress is affecting the actions of alcohol or whether alcohol is affecting the stress response.

Alcohol Consumption

Our analyses show that rats in the STR+ALC group significantly increase their alcohol consumption during stress treatment compared to the no-stress ALC group. To elucidate this finding we have to consider previous research using restraint stress and alcohol consumption. Studies using animal models of voluntary consumption have a high amount of variability between results. Previous research on the effects of restraint stress and alcohol consumption has shown a decrease (Sprague and Maickel, 1994; Chester et al., 2004; Boyce-Rustay et al., 2008), increase (Lynch et al., 1999; Pohorecky 2008; Yaroslavsky and Tejani-Butt 2010) or no change (Roske et al., 1994; Rockman and Glavin, 1984; Sillaber et al., 2002) in alcohol intake following stress treatments. Reasons for the high variability between studies may be due to species, type of stress, duration of stress, and availability to alcohol. Even with the few studies that found increased alcohol consumption, there were considerable differences in methodology. Compared to the present study, Ploj et al. (2003) and Roman et al. (2004) used the same alcohol dose, but added maternal separation to their 4-day restraint stressor. Lynch et al. (1999) found an increase in consumption on days where alcohol was given via two-bottle choice, however, when rats were forced to drink alcohol (i.e. only fluid), both the stress and non-stressed rats had equal consumption rates. Our data indicate that restraint stress alone increased alcohol consumption. This behavior is often seen in stressed humans and may be associated with the development of dependence over time (Sinha, 2001).

Given the stress-induced increase in alcohol consumption found in our experiment, it is pertinent to test whether blood alcohol content (BAC) was altered as well. However, due to a loss of blood samples (defective freezer), we could not directly measure BAC in our study. Future

studies will measure BAC to determine if rats undergoing stress altered BAC. This information will help clarify and interpret the current results (the importance of BAC is further discussed below). Additionally, measuring stress hormone levels will provide more information on alcohol's effects on the stress response. Galea et al. (1997) has noted that restraint stress causes a significant increase in plasma corticosterone (CORT) after 30-minutes from day-1 to day-21 of restraint. Our study used restraint for 1-hour per day, so it is reasonable to assume that rats in the stress treated groups had higher CORT levels.

Spatial Memory

Numerous experiments have shown that following chronic stress, male rats exhibit impaired memory in tasks such as the radial arm maze, Y-maze, object placement, and water maze (Luine et al., 1994; Conrad et al., 1996; Beck and Luine, 2002; Kitraki et al., 2004). Chronic alcohol consumption has also been shown to impair memory in tasks like the radial arm maze, object recognition, and water maze (Steigerwald and Miller, 1997; Ryabinin et al., 2002; Matthews and Morrow, 2000). Thus, our findings are consistent with previous work showing that chronic stress or alcohol impairs spatial memory on the object placement task. However, when the two treatments are combined (STR+ALC), no deficits in spatial memory were present. Thus, the results do not support additive effects of stress and alcohol on memory. To our knowledge no study has examined spatial memory using low-stress tasks when alcohol is accessible for voluntary consumption following the stressor. In addition, other studies examining stress/alcohol interactions have utilized different spatial memory tasks than employed here. Sircar and Sircar (2005) utilized the Morris Water Maze and Maier and Pohorecky (1986) utilized the radial arm maze. These tasks expose subjects to stressful circumstances (forced swim and food deprivation), which may have confounded testing the interaction between stress and alcohol (Engelmann et al., 2006). Object placement does not use positive or negative reinforcements, and rats undergo habituation trials so that stress is minimal when memory is assessed. We know that chronic exposure to corticosterone impairs spatial

memory in male rats (Luine et al., 1993; McLay et al., 1998; Wright et al., 2006). Therefore, it is important to measure corticosterone levels following stress and alcohol treatments to determine the effect on the stress response. There may be a value in interpreting some of these findings such as the possibility that alcohol consumption reduces the negative effects of chronic stress on spatial memory.

Body Weight Gain

As a physiological measure of stress effects, rats were weighed at the beginning, middle and end of the stress treatment. During chronic stress, rats show a reduction of their weight gain, which rebounds once the stressor is terminated (Baran et al. 2005; Conrad et al, 2007). Our data are consistent with these observations; the STR group gained weight at a lower rate than those in the CON or STR+ALC groups. Hence, when alcohol is available after a stressor, the effect of stress on weight gain is normalized to that of rats in the CON group. Here, we should consider the possibility that alcohol, which has calories, is responsible for the added weight. However, this possibility appears unlikely because rats in the ALC group did not differ from CON, STR, or STR+ALC groups in body weight. Thus, it appears that the intake of alcohol reduced stress-induced reduction in weight gain.

Conclusions

We have found that combination of chronic restraint stress and voluntary alcohol consumption in male rats is associated with increases in alcohol intake, reduced impairment of spatial memory produced by each treatment alone, and inhibition of stress-induced weight loss. These findings are novel and may provide some insight into the complex mechanisms that motivate alcohol abuse and lead to improved treatment for alcohol dependence. However, the mechanisms responsible for the maintenance of spatial memory function at control levels in STR+ALC treated rats are unknown. One explanation is that stress enhances alcohol metabolism, which leads to a decrease in alcohol's effects (Ryabinin et al., 1995; Ryabinin et al., 1999). In our study it is unknown whether alcohol metabolism was affected by stress because

alcohol levels were not measured in the subjects; however, preliminary data from ongoing experiments has shown that blood alcohol levels are not altered following 7-days of chronic restraint stress (Gomez et al., 2011). Another mechanism, which may be responsible for the interactive effects of the treatments on spatial memory, is that alcohol may be dampening the stress response (Spencer and McEwen, 1990; Lee and Rivier, 1999; Richardson et al., 2008). We have found some support for this mechanism in that STR+ALC treated rats showed a significant reduction of corticosterone levels from day-1 to day-7 of restraint stress (Gomez et al., 2011) whereas rats in the other groups (CON, ALC, and STR) showed no change in corticosterone levels.

These data show that restrained stressed rats seek alcohol solutions and that alcohol intake can reduce the consequences of the chronic stress on weight gain and memory function. Therefore, it appears likely that alcohol consumption may reduce the aversive effects of stress on measures of anxiety, depression, and stress hormone levels. Our current research focuses on these measures to understand how the combination of two impairing manipulations can result in a return to normal memory function. The physiological and neural mechanisms underlying these changes were not testable in this experiment, but are the focus of future research.

Aim 2.

Introduction

Alcohol consumption has long been seen as a common way of coping with stressful life events (Sinha, 2008). However, research has focused mainly on aspects involving emotional states, such as anxiety or depression. Few studies have examined the effects of alcohol consumption after a stressor on cognition. The immediate response to a stressor is beneficial and leads to adaptive functions like mobilization of energy, improved memory recall, and suppression of non-essential processes (Sapolsky, 1997). However, chronic stress is detrimental and many negative behavioral effects have been attributed to the constant activation of the stress response system (McEwen, 2008). After 21-days of stress, male rats show impairments in tasks assessing learning and memory such as object recognition, Y-maze, water maze, and radial arm maze (review Luine et al., 2007). Altering the stress response via enriched housing or drug treatment can mitigate the deleterious effects of chronic stress on memory (Conrad et al., 1996; Wright and Conrad, 2008; Hutchinson et al., 2012) and lessen or block stress-induced memory impairments. Therefore, the link between stress mediation and memory function may emerge as an important factor associated with alcohol intake during times of stress.

Changes in anxiety or depression are often associated with increased or prolonged stress and alcohol consumption. The initiation or continuance of psychoactive substances is often attributed to an attempted reduction of negative affect (Koob and LeMoal, 2008). However, with prolonged use of alcohol, levels of anxiety and depression rise. Thus, in alcoholics it is difficult to determine if dependence is due to self-medication of a pre-existing mood disorder or if excessive drinking is maintained by negative reinforcement of alcohol-induced changes in mood (Allan, 1995). Studies using animal models have found a range of effects of restraint stress on alcohol consumption. Cases report decreased (Sprague and Maickel, 1994), increased (Lynch et al., 1999), and no change (Roman et al., 2004) in voluntary alcohol intake following stress treatment. In Aim 1 we reported that 1-hour of restraint stress followed by 1-hour access to

alcohol increases consumption as compared to that of non-stressed rats. In addition, deficits in spatial memory associated with either the alcohol or stress treatment alone were alleviated in rats with access to alcohol after the stressor (Gomez et al., 2012). Some studies have supported our finding and have shown that alcohol can block stress-induced impairments in memory by altering the stress response system (Thatcher-Britton and Koob, 1986; Liu and Weiss, 2002; Scullion et al., 2009). In order to elucidate these behavioral findings, a main area of focus for the current study will be the effects of stress and alcohol neurotransmitter receptor function. Examining the agonistic GABAergic and antagonistic glutamatergic activity caused by stress or alcohol (Crabbe et al., 2011; Orchinik et al., 1995; Martin and Wellman, 2011), provides a framework for expounding the interactive effects on behavior. Due to the analogous effects of chronic stress and chronic alcohol on memory, anxiety, and depression, it is no surprise that the effect of chronic stress on neurotransmission is the same as chronic alcohol use (Calvo et al., 1998).

The focus of the present study was to determine the behavioral, physiological, and neuronal effects of gavage alcohol after a stressor. Controlling the dose of alcohol, via gastric gavage, allows for a direct cause and effect measure of the actions of intoxication after a stressor. In contrast to Aim 1, the stressor in Aim 2 was changed to 6hr/day/7days of restraint in order to correspond with work showing stressed-induced changes in memory and anxiety (Bowman et al., 2009). Additionally, the dose of 2 g/kg of alcohol was chosen because it seemed like the most common for causing memory impairments without leading to dependence or withdrawal following one week of administration (Devenport et al., 1989; Matthews et al., 1999; Ryabinin et al., 2002; Berry et al., 2004). The procedure in Aim 2 provides face validity in that individuals who drink generally consume alcohol after a stressful event. As previously stated, most studies center around anxiety and depression measures. In addition to mood assessment, our study added memory function as a factor not frequently considered when examining substance use during times of stress.

Methods and Materials

Subjects

Male Sprague-Dawley rats (~230 g, N = 32) obtained from Harlan Sprague-Dawely, Inc. (USA) were pair-housed and kept on a 12hr light cycle with lights *on* at 09:00. Standard rat chow and water were available *ad libitum*. Rats were randomly assigned to one of four conditions (n=8 per group): No Stress / No Alcohol Control (CON), Alcohol alone (ALC), Stress alone (STR), or combination of Stress and Alcohol (STR+ALC). All procedures were approved by Hunter College's Animal Care and Use Committee.

Procedure

After acclimation to the environment, habituation for object recognition (OR) was conducted for one week. At two points during OR habituation, rats were administered 1cc of saline via gastric gavage to reduce stress associated with the procedure. Alcohol and stress treatment started after the last OR habituation trial. For seven consecutive days rats in the stress groups were restrained for 6-hours each day from 10:00-16:00. Each day, post-stress, alcohol (ALC and STR+ALC) or saline (CON and STR) was administered via gastric gavage at a dose of 2 g/kg. Following treatments, rats were put through a battery of behavioral tests. Rats were tested on the elevated plus maze for anxiety and OR task for visual memory on day-8, the Y-maze for spatial memory on day-9, and the forced swim task for depression on day-10. Following the completion of the FST (30-40min. post-swim) brain and blood samples were collected for analysis. The order of behavioral testing was deemed to provide optimal information regarding the interactive effects of stress and alcohol. Thus, the EPM was conducted on day-8 not only to test for anxiety, but also test for any possible withdrawal effects of alcohol. It was expected that conducting the OR task immediately after the EPM would have no effect on performance. To confirm, correlation analyses were run and no significant correlation was found between activity on the EPM and performance on the OP task. The Y-maze was done alone on day-9, as it was the task that took the longest (~6-hours). To test for depression and limit any possible confounding

effects of stress-induced changes in memory, the FST was run last. Additionally, the FST provided a novel stressor to assess hormone release between groups.

Stress and Alcohol Administration

Rats were restrained, not immobilized, for 6hr/day/7days (10:00-16:00) in a Plexiglas restrainer measuring 21.5cm long x 6.3cm internal diameter (Harvard Apparatus). The closed front end was clear and contains ventilation holes, while the back end has a sliding plastic plug, which was adjusted to fit the size of the rat. Pure ethanol (200 proof, Sigma-Aldrich) was diluted in saline (0.9% NaCl, Fisher Scientific) to produce a concentration of 20% v/v ethanol. Gavage was performed using a feeding needle (18G x 2") affixed to a 10cc syringe (VWR). All rats were gavaged with a dose of 2 g/kg of alcohol or saline in a counterbalanced fashion at the same time every day (~16:15). To measure blood alcohol levels, 30-minutes after the final alcohol administration (day-7), blood samples were taken via tail-nick and blood alcohol content (BAC) was analyzed using 5µl of sera in an Analox GM7 micro-stat machine. Using the Analox GM7, after calibration with alcohol reagents (GMRD-113, Analox Instruments LTD), ethanol was oxidized by alcohol oxidase. Thus, the Analox GM7 measures the rate of oxygen used by samples as a direct measure of BAC.

Memory Tasks

The object recognition task (OR) is a pre-frontal cortex/hippocampal working memory task comprised of a sample trial (T1) and a retention trial (T2). The sample trial and a retention trial were separated by a 4-hour intertrial delay and the task was recently described in detail by Bowman et al. (2009). During T1, two identical objects were placed in corners of an open field (Plexiglas: 70 x 70 x 30 cm) and the rat was allowed to explore for 3-minutes. Exploring was determined as touching, whisking, or sniffing the objects from no more than 2cm away. Following the intertrial delay, the objects and field were cleaned and one of the objects was replaced with a new object and the rat was allowed to explore for 3-minutes. Increased exploration/preference for the new object is indicative of intact recognition memory (Ennaceur

et al., 1997). Placement and new/old objects were presented in a counterbalanced fashion to reduce possible confounding effects of place or object preference.

The Y-maze is a hippocampal dependent–spatial memory task that requires rats to use external maze cues to navigate the identical internal arms. In contrast to Aim 1 where the object placement task was used to test spatial memory, the Y-maze was chosen in Aim 2 to reduce habituation time and provide a different measure of spatial memory with known stress effects. The maze was constructed of wood coated with high-gloss black paint and placed in a room with high-contrast external cues (bullseyes, checkerboards). The arms were 56cm long by 19cm wide by 36cm high and connected at 60° angles creating an equilateral triangle at the center. The floor was covered with cage bedding that was thoroughly mixed before every trial to reduce any potential odor cue trails (Conrad et al., 2003). During the training trial, one arm was blocked and the rat was allowed to freely explore the *start* and *other* arm for 15-minutes. During the 4-hour intertrial delay, the Y-maze was rotated and all arms were open. Memory was assessed during the 5-minute probe trial as percent entries into the *novel* versus the *other* arm. An entry was designated as the rat crossing the threshold of the arm with both forepaws and half its torso. The *novel*, *other*, and *start* arms were counterbalanced between rats to reduce any confounding effects of place. All behavior was videotaped and analyzed off-line by an experimenter blind to the conditions.

Anxiety and Depression

The elevated plus maze (EPM) is designed to test general anxiety levels in rodent models (Pellow et al., 1985). The EPM consists of two open (50 x 12cm) and two closed arms (50 x 12 x 40cm). Equal total entries into the open arms are associated with equal motivation to explore and increased exploration time is associated with reduced anxiety. An entry was designated as the rat crossing the threshold of an arm with both forepaws and half its torso. The rats were allowed to explore the arms for 5-minutes and all behavior was videotaped and scored off-line.

One animal from the ALC group was removed due to lack of exploration (only one entry into each arm).

The modified forced swim task (FST) is designed to measure general depressive-like behaviors in a rat model. Originally developed by Porsolt et al. (1977), the FST measures swimming and immobile behaviors with increases in immobility associated with increases in depressive-like symptoms. Rats were placed in a Plexiglas cylinder (30cm diameter) filled with water (~25°C) to a level of 30cm. Rats were tested for 5-minutes and behavior was videotaped and scored off-line. For our experiments the FST was modified from traditional use which uses 10-minutes or conducts the test over two days. We used 5-minutes because previous work in our lab found rats struggled to stay afloat if exposed past 5-minutes (data not shown). We also ran the behavior only on one day to test for general depressive levels and not learn helplessness, which is tested over two days (Huynh et al., 2011). Behaviors consisted of swimming (horizontal movement), climbing (upward thrashing), and immobility (erect floating). Behaviors were counted at 5-second intervals over the 5-minutes of testing for a total of 60 counts, and scores were converted into percentages and analyzed within groups (Cryan et al., 2002).

Western Blots and Spine Density

After the final gavage administration, rats were rapidly decapitated and brains were removed and split down the mid-sagittal line and one hemisphere was fast frozen on dry ice. Whole hippocampal tissue was later dissected, homogenized, and separated into membrane and post-synaptic density fractions (as detailed in Sacktor et al., 1993). Gels were run using an Invitrogen XCell Mini Electrophoresis system. Fractions were mixed in sample buffer and dye to a final concentration of 50µg of protein for all rats. Gels were loaded with 10µg of sample protein and run for 1.5-hours. Gels were then trans-blotted to membranes and incubated in primary anti-bodies for GABAα4 (Anti-GABA A Receptor alpha 4 antibody, 66 kD – no. ab4120, Abcam Inc., Cambridge, MA), GluN2B (Anti-NMDAR2B antibody, 148 kD – no. ab81271, Abcam Inc., Cambridge, MA), and GAPDH (Anti-Glyceraldehyde-3-Phosphate Dehydrogenase

Antibody, 38 kD – no. MAB374, Millipore Co., Temecula, CA) for 24-hours. GABAα4 and GluN2B primaries were diluted to 1:500 and GAPDA was diluted to 1:1000 using a hemoglobin based blocker. Membranes were incubated in secondary anti-bodies for 2-hours (Rabbit for GABAα4 and GluN2B – no. A3687 and Mouse for GAPDH – no. A9316; Sigma-Aldrich Co., St. Louis, MO). After secondary incubation, membranes where developed using BCIP/NBT phosphatase substrate for colorimetric detection (KPL Inc., Gaithersburg, MD). Developed blots were scanned and analyzed using Image-J software provided by the National Institutes of Health.

The hemisphere not used for western blots, alternating right and left between each rat, was used for spine density analysis. A rapid Golgi staining kit was purchased from FD NeuroTechnologies (Columbia, MD) and tissue was prepared as directed. Following incubation in Solutions A & B for 2-weeks and Solution C for 3-days, brains were frozen at -80° C. Brains were sectioned into 100μm slices using a Leica CM3050S cryostat set to -30°C and placed on gel coated slides. The prefrontal cortex was sectioned roughly from Bregma (+)5.00 to (+)2.50 mm and the hippocampus was sectioned from Bregma (-)2.00 to (-)4.20 mm (Paxinos and Watson, 2005). One day after sectioning, brains were stained in Solutions D & E and cover slipped with paramount. Pyramidal neurons in the medial prefrontal cortex (mPFC) and CA1/CA3 areas of hippocampus were located using a Nikon Eclipse E400 microscope set at 100x magnification. For each rat, six tertiary apical and six secondary basal dendrites were measured and averaged using SPOT RT Software v3.5 (Sterling Heights, MI) on a computer connected to the scope. Spines were manually counted by a trained individual and total spine count was divided by length and multiplied by 10 to get a density equal to spines/10μm.

Corticosterone

Blood samples were taken from trunk blood 30-40 minutes after the forced swim task was complete. Samples were centrifuged at 3000g in 4°C for 5-minutes and sera was collected. Using a corticosterone ELISA kit (Neogen Corp., Lexington, KY), 100μl of plasma was dissolved

in diethyl ether to separate the organic phase and allowed to evaporate for 48-hours. Samples went through a series of washes and incubations as directed by the kit instructions. Samples and standards were placed in a kit-provided 96-well plate and read in a microplate reader at 650nm. Output was converted into corticosterone levels at ng/ml via logarithmic equations provided by Neogen Corp.

Statistics

An independent samples t-test was used to test BAC between the ALC and STR+ALC groups. One-Way ANOVAs followed by post-hoc LSDs for significant differences were used for corticosterone, object recognition, elevated plus maze, and western blot analyses. Two by three way ANOVAs were used for the Y-maze and forced swim task with post-hoc LSDs. Repeated-measures ANOVA was used to determine body weight differences between groups. Finally, a 4x3 ANOVA was used to analyze behavior on the forced swim task. All statistical analyses were run on PASWStatistics v.18 (SPSS IBM, USA).

Results

Physiology Effects

Circulating alcohol (BAC) was measured from tail-nick blood sampled 30-40 minutes after the last alcohol administration on day-7. No difference was found between the ALC and STR+ALC groups [$t_{(13)}$=0.82, p=0.43] (Figure 5A).

Corticosterone levels were analyzed 30-40 minutes post-forced swim. Compared to the CON, ALC, and STR groups, the STR+ALC group mounted the greatest CORT response [$F_{(3,28)}$=5.59, p=0.004; LSD, p=0.02]. All other groups had lower levels and did not differ from each other (Figure 5B).

With and without alcohol treatment, stressed rats showed a reduction in weight gain over the 7-day stress period. A main effect of weight [$F_{(6,168)}$=37.91, p=0.0001] and a weight by group interaction [$F_{(18,168)}$=10.25, p=0.0001] was found. Post-hoc analysis revealed that by day-4, rats in the CON and ALC groups weighed more than rats in the STR and STR+ALC groups

[$F_{(3,28)}$=4.28, p=0.01]. The difference in weight gain continued until the final day of stress (Figure 5C).

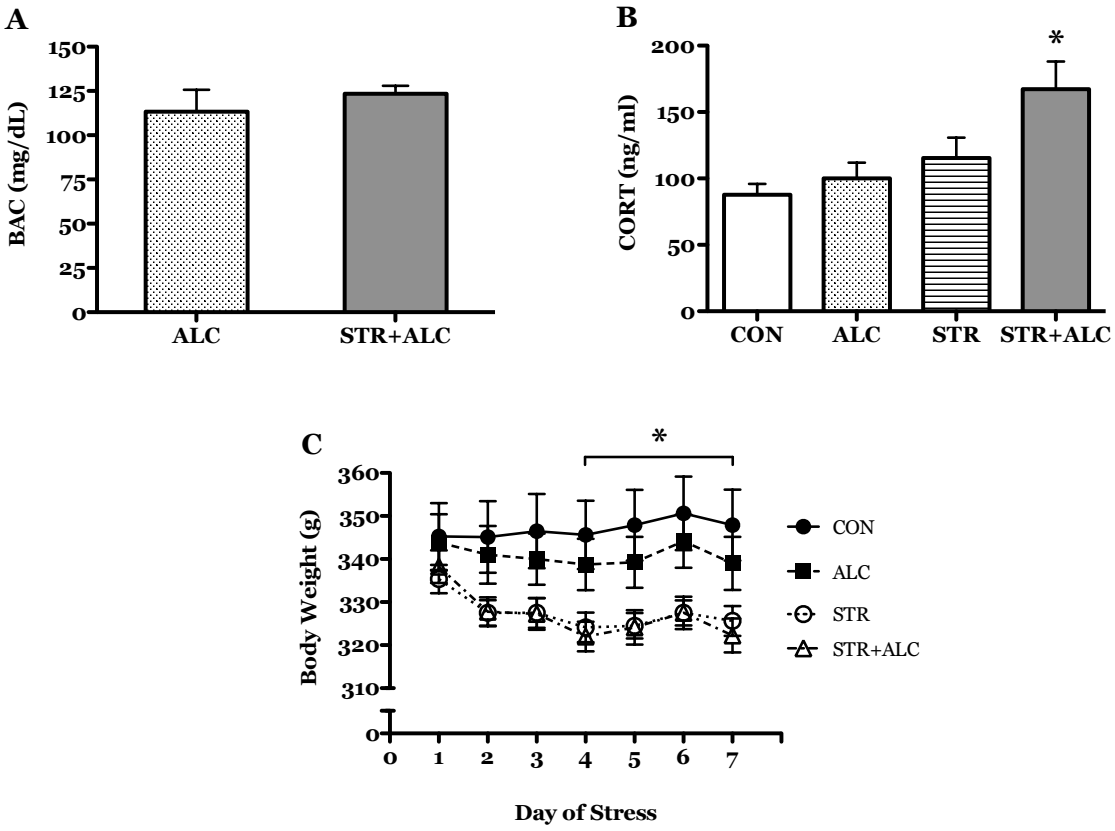

Figure 5. Physiological Effects: (A) Comparison of blood alcohol concentration, presented as means±SEM. (B) Stress responses 30-40 minutes after forced swim task. Corticosterone levels presented as means±SEM. The STR+ALC group had a significantly greater stress response to the swim task (Asterisk |*| p<0.05). (C) Body weights over days of stress treatment in means±SEM. By day-4 through day-7, the STR and STR+ALC groups had significantly lower weight compared to CON and ALC (Asterisk |*| p<0.05).

Working Memory Using Object Recognition

During the initial sample trial (T1) of the object recognition memory test, there was no difference in exploration times between groups [$F_{(3,28)}$=0.23, p=0.88] (Figure 6A). For the retention trial (T2), exploration ratios were calculated (time with new / total time). Treatment altered the exploration ratios between groups [$F_{(3,28)}$=13.97, p=0.0001] (Figure 6B). Post-hoc

LSD revealed that ratios for the CON and STR+ALC groups were significantly greater than the ratios for the ALC and STR groups. Only the CON and STR+ALC groups significantly discriminated between the old and new objects, suggesting intact recognition memory.

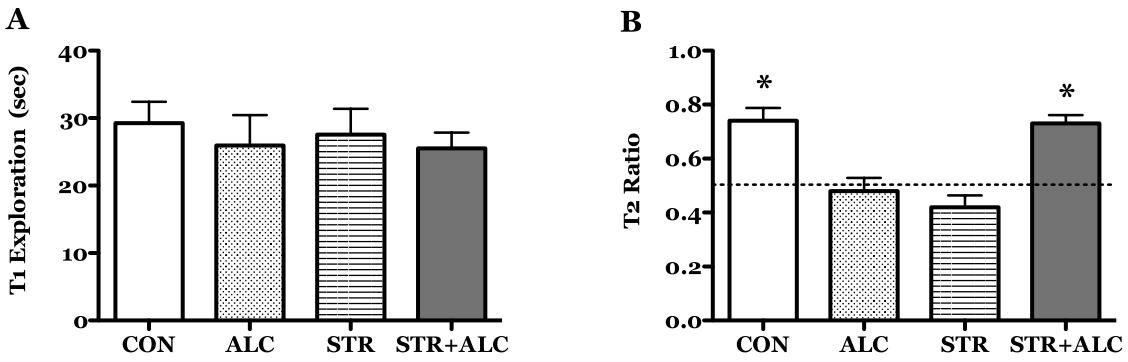

Figure 6. Object Recognition: (A) Exploration (means±SEM) of identical objects during the sample trial (T1). No differences were found between groups. (B) Exploration ratios (means±SEM) determined by time spent with new object divided by total time spent with both objects. The dotted line represents chance performance (50%). Both CON and STR+ALC groups were significantly different from the ALC and STR groups, showing intact memory for the new and old objects (Asterisk |*| $p<0.05$).

Spatial Memory Using the Y-maze

The Y-maze showed the same pattern of results as the OR task. Total entries were analyzed and no significant differences were found [$F_{(3,28)}=0.55$, $p=0.65$] (Figure 7A). Factorial analysis found a main effect of arm entry [$F_{(1,56)}=36.04$, $p=0.0001$]. To determine differences between percent entries into the novel and other arms, Bonferroni corrected t-tests were conducted. The CON and STR+ALC group significantly explored the novel more than the other arm [$t_{(14)}=4.19$, $p=0.001$; $t_{(14)}=4.59$, $p=0.0001$, respectively] (Figure 7B). No difference in exploration was found in the ALC or STR groups [$t_{(14)}=1.43$, $p=0.18$; $t_{(14)}=1.96$, $p=0.07$, respectively]. Thus, only the CON and STR+ALC groups significantly discriminated between the novel and other arms, suggesting intact spatial memory.

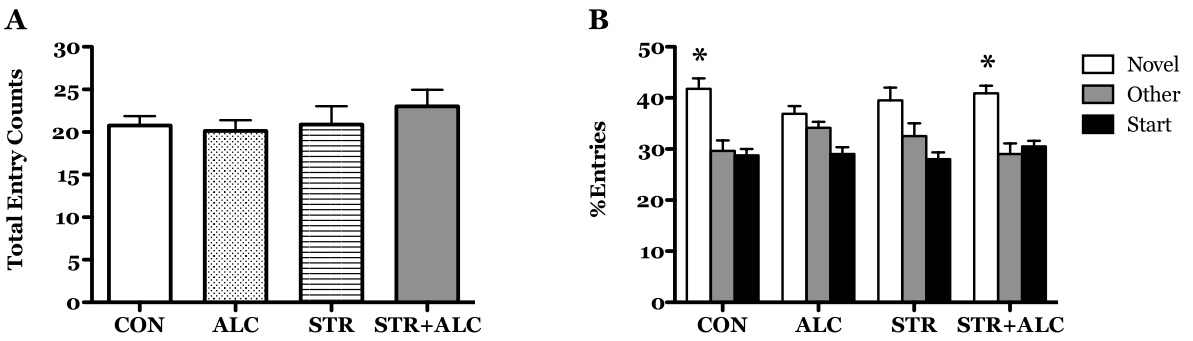

Figure 7. Y-Maze: (A) Total entries into the Start, Other, and Novel arms (means±SEM). No significant difference was found between groups. (B) Percent entries into each arm (means±SEM). The CON and STR+ALC groups significantly explored the Novel arm more than the Other arm, suggesting intact memory (Asterisk |*| $p<0.01$). No differences in arm exploration were found in the ALC or STR groups.

Anxiety and Depression Measures

On the elevated plus maze, the number of entries and amount of time spent in the open arms were converted into open arm ratios and analyzed. No significant difference in percentage of open arms entries was found between groups [$F_{(3,27)}=2.69$, $p=0.07$] (Figure 8A). The percentage of time spent in the open arms was significantly different between groups [$F_{(3,27)}=4.64$, $p=0.01$]. A post-hoc LSD found that the STR group spent significantly less time in the open arms compared to the other groups ($p<0.05$), an indication of increased anxiety (Figure 8B).

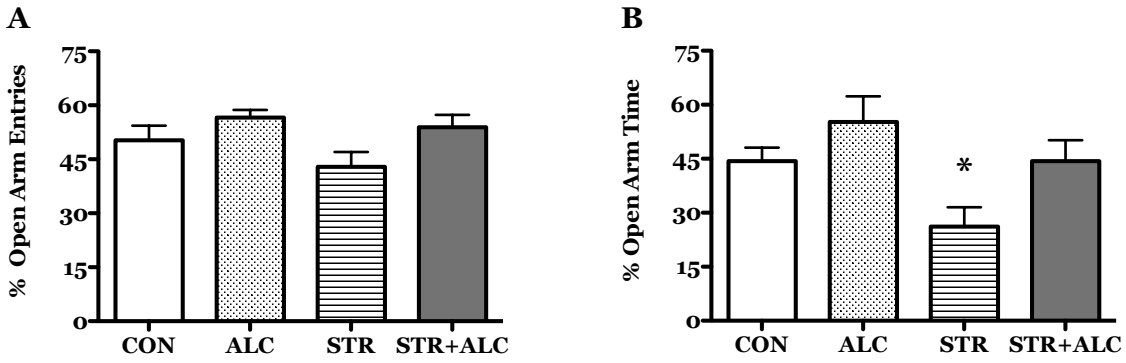

Figure 8. Elevated Plus Maze: (A) Percent of entries into the open arms (means±SEM). No significant difference was found between groups. (B) Percent of time spent in the open arms (means±SEM). The STR group spent significantly less time in the open arms, suggesting increased anxiety (Asterisk |*| $p<0.05$).

During the forced swim task swimming, climbing, and immobile behaviors were measured at 5-second intervals for 5-minutes. Behavioral counts were converted to percentages and analyzed between groups and post-hoc analyses were conducted within groups. A significant main effect of behavior was found [$F_{(2,84)}=65.13$, $p=0.0001$] (Figure 9). Post-hoc analyses found the CON and STR+ALC groups spent more time swimming than immobile [LSD; $p=0.005$ and $p=0.01$, respectively]. No difference between swim and immobile behavior was found in the ALC and STR groups [LSD; $p=0.68$ and $p=0.28$, respectively]. Climbing behavior was significantly greater than swimming for the CON, ALC, and STR groups [LSD; $p<0.05$], but no difference was found in the STR+ALC group [LSD; $p=0.08$].

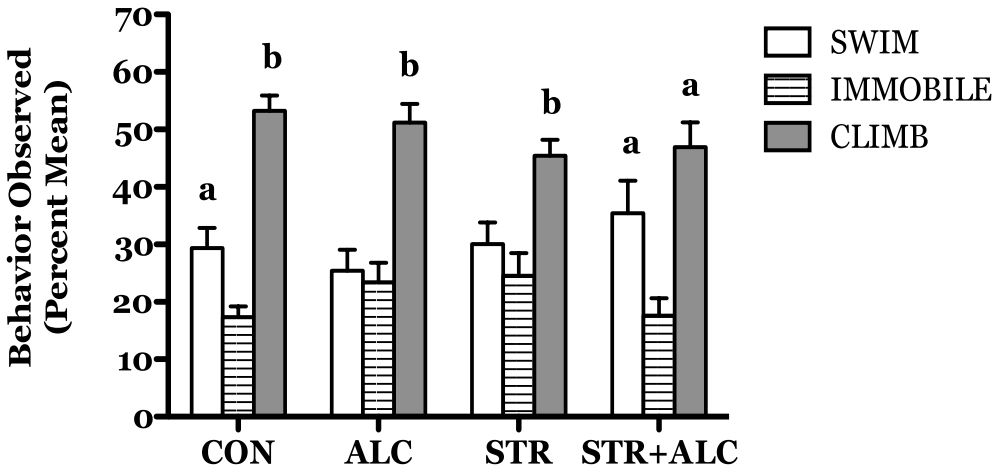

Figure 9. Forced swim task measuring swimming, immobile, and climbing behavior as mean±SEM. (a) indicates behavior greater than immobile ($p<0.05$). (b) indicates behavior greater than both swim and immobile ($p<0.05$).

Neurological Effects

Hippocampal tissue was fractionated and analyzed using western blots to determine the effects of treatment on neurotransmitter receptor expression. The membrane fraction was used for GABAα4 analysis and the post-synaptic density fraction was used for GluN2B analysis. No significant between group differences were found in levels of GABAα4 or GluN2B [$F_{(3,27)}=0.50$, $p=0.68$; $F_{(3,28)}=0.49$, $p=0.69$, respectively] (Figure 10).

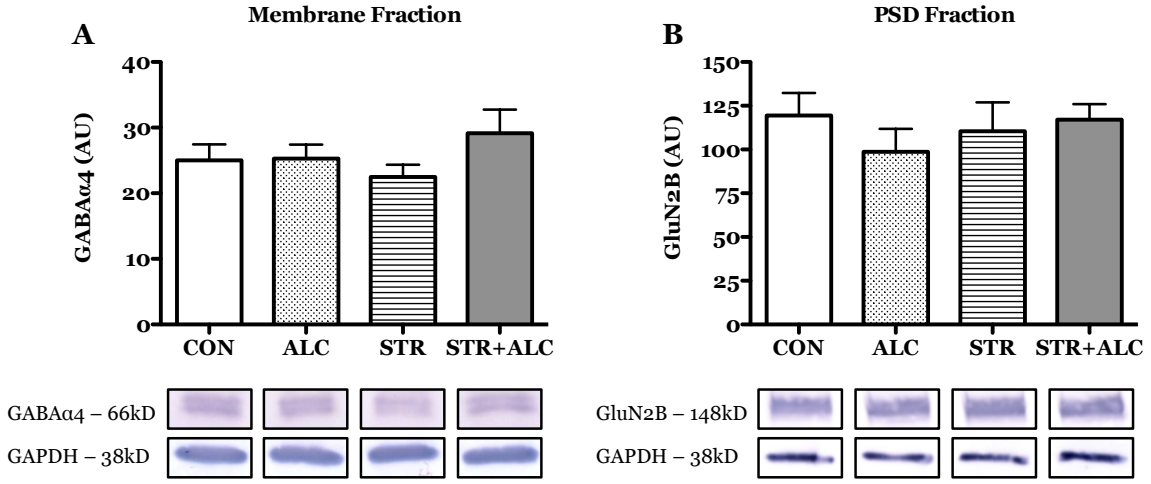

Figure 10. Neurotransmitter Receptor Expression: (A) GABAα4 receptor expression found in the membrane fraction of the hippocampus (AU: Arbitrary Units). (B) GluN2B receptor expression found in the post-synaptic density (PSD) fraction. No significant differences were found between groups.

Dendritic spine density was measured in pyramidal neurons of the medial prefrontal cortex and hippocampus (CA1 and CA3). Both tertiary apical and secondary basal dendrites were counted for the mPFC and CA1 areas, but only tertiary apical dendrites were counted for the CA3 region. Between groups, treatment had no effect on spine density in the mPFC, CA1, or CA3 areas [$p>0.05$ for all] (Table 1).

Table 1. Spine density of pyramidal neurons in the medial prefrontal cortex and hippocampus.

Pyramidal Neurons Six dendrites per area	Treatment			
	CON	ALC	STR	STR+ALC
mPFC – Apical	11.49 ± 0.48	10.73 ± 0.39	11.41 ± 0.44	11.34 ± 0.54
mPFC – Basal	9.65 ± 0.32	8.83 ± 0.45	10.06 ± 0.35	8.97 ± 0.65
CA1 – Apical	16.61 ± 0.87	15.33 ± 1.42	15.16 ± 0.94	13.79 ± 1.04
CA1 – Basal	14.74 ± 1.19	12.50 ± 0.98	14.44 ± 0.94	13.09 ± 1.00
CA3 – Apical	9.03 ± 1.10	9.12 ± 0.16	10.62 ± 0.68	10.42 ± 0.72
Brain Regions: Medial Prefrontal Cortex (mPFC) and Hippocampus (CA1 and CA3)				
Data Represent: Spine Density per 10μm – means±SEM				

Discussion

The results show that alcohol intake after stress exposure alters behavioral responses compared to each treatment alone. Memory function was impaired by stress or alcohol, but performance of rats receiving both treatments was equal to control rats. This finding supports our previous work showing that alcohol consumption after a stressor alleviates spatial memory impairments (Gomez et al., 2012). Alcohol also reversed the stress-induced increase in anxiety shown on the plus maze. In the forced swim task, depressive-like behaviors were lower in control rats and those treated with the combination of stress and alcohol. Thus, as is predicted by the self-medication hypothesis, alcohol consumption may increase during times of anxiety or depression as a form of reducing these negative effects and our results show that memory function may be involved in mediating the effect.

Previous work in our lab has shown that chronic stress impairs non-spatial working memory on the object recognition task in male rats (Bowman et al., 2003). Other forms of stress (foot shock, novel cage exposure) and durations (7-days vs. 21-days) have also been shown to cause memory impairments on object recognition tasks (Baker and Kim, 2002; Bowman et al., 2009; Scullion et al., 2009). Chronic ethanol administration shows the same impairment on non-spatial memory as chronic stress (Ryabinin et al., 2002). The Y-maze is a low-stress task measuring memory without stressors such as, food deprivation or forced swim (Conrad el al., 1996). Chronic stress impairs spatial memory on the Y-maze by reducing exploration of the novel arm (Conrad et al., 1996). Using various methods of alcohol administration (injection, voluntary consumption, chronic intermittent exposure, liquid diet), chronic alcohol studies have also shown a significant impairment of spatial memory (Matthews and Morrow, 2000). To our knowledge, few evaluated cognitive effects when stress and alcohol are combined. Some indirect evidence supports our results and may explain our findings. Ryabinin et al. (1995) found a significant decrease of hippocampal c-fos expression when rats were given alcohol after a stressor, suggesting an alcohol-induced amelioration of functional hippocampal activity.

Moreover, knockout models or rats with defective CRH receptor systems show changes in alcohol sensitivity and reinstatement of alcohol seeking behavior caused by stress (Hansson et al., 2006). Others have used different drugs of abuse and have found similar results. Nicotine and opiates (naloxone) administered during times of stress, block impairments on memory tasks and have even been shown to reestablish normal LTP function (Alkadhi et al., 2011; Schneider et al., 2009). Hence, it is speculated that the results found in our study may be associated with altered reward and pleasure pathways leading to stress reduction. It has been recently suggested that memory function plays a key role in drug relapse (Williams et al., 2011). The above suggestion may be associated with the behavioral effects found in the current study where connections between reduction of anxiety and depression with alcohol after stress may be strengthened during initial use.

A well-established observation is that alcohol is associated with decreases in anxiety (review Lewis, 1996). On the elevated plus maze, time spent exploring the open arms was affected only in the STR group, suggesting a stress-induced increase in anxiety. Alcohol treatment after a stressor ameliorates stress-induced anxiety on the plus maze. The effects of alcohol intake after a stressor are dependent on alcohol dose, duration, and stressor. Previous reports using various chronic stress paradigms, have found increased anxiety on the plus maze in male rats (Vyas et al., 2002). However, the effects of alcohol consumption on anxiety are mixed. Generally, alcohol is an anxiolytic; however, the anxiolytic properties are usually associated with low to moderate doses given over acute settings. Testing the anxiolytic properties of alcohol has shown an inverted-U shaped curve, with moderate doses having the greatest effects on anxiety reduction (Lister, 1987). Studies testing long-term alcohol intake (moderate to high doses) show increases in anxiety during immediate and prolonged alcohol withdrawal (Rasmussen et al., 2001). However, the anxiogenic effects have been shown to be transient. Zhang et al. (2007) found that after 3-days of high dose alcohol treatment, rats

displayed anxiety on the plus maze 9-15 hours post last injection, but the effects were no longer present after 24-hours.

Alcohol administration after a stressor results in anxiety levels that are either equal to controls (benefit) or increased (detriment) (Boyce-Rustay et al., 2007; Pohorecky, 2008). However, increases in anxiety associated with co-administration have been blocked by treatment with corticotropin releasing factor antagonists (Valdez et al., 2003), suggesting that anxiety associated with alcohol and stress is mediated by release of stress hormones. When an NMDA antagonist (AP7) was administered during the stressor, the stress effect was blocked (Padovan et al., 2000). Alcohol is a known NMDA antagonist, which may explain the results obtained in the present study. In our paradigm, we need to investigate the levels of stress hormones during alcohol and stress treatment to clarify the results.

The FST is a reliable test for measuring depressive-like responses in rodent models by observing escape directed behaviors like swimming, and comparing them to helplessness behaviors like immobility (Porsolt et al, 1977). The behaviors observed in the FST were analyzed similarly to that seen in Cryan et al. (2000). Swimming, climbing, and immobility behaviors were compared within groups to determine if treatment altered depressive-like symptoms. Regardless of group, a high amount of climbing behavior was observed. However, climbing was considered to be a non-escape motivated behavior, thus the more important interaction was that between swimming and immobility (review Cryan et al., 2002). Like the effects found in the OR and Y-maze tasks, compared to CON the ALC and STR groups showed greater depressive-like behaviors by showing no difference between swim and immobility. The STR+ALC group, not only shows an increase in swim compared to immobility, but also no difference between escape and non-escape motivated behaviors. Other studies have shown a reduction of stress-induced depression when alcohol is administered during or after a stressor (Sacharczuk et al., 2009). Our results are in agreement with the latter. Rats treated with alcohol after a stressor show fewer depressive behaviors when compared to rats only exposed to restraint stress or alcohol

alone. These effects are present three days after the final treatment, which suggests enduring behavioral effects after a week of stress and alcohol consumption.

An underlying mechanism that may mediate our findings on memory and mood alterations following stress is the effect of alcohol on synaptogenesis. Reduction in synaptogenesis is associated with decreased memory function and increased depression levels (Zhang et al., 2010). Both chronic alcohol consumption and exposure to stress are known to reduce synaptogenesis and alter neurotrophic factors that are responsible for spine growth and maintenance (Carpenter-Hyland and Chandler, 2006; Russo et al., 2009). The link between stress hormones and neurotrophic factors is thought to be responsible for spine density and neuronal differentiation (Schaaf et al., 1998). As a means of examining the actions of alcohol on stress effects, we investigated spine density in the hippocampus and medial prefrontal cortex (mPFC), areas associated with memory function. Our results do not support previous findings showing that chronic restraint stress reduces spine density in the mPFC (Radley et al., 2006) or in the hippocampus (Magariños et al., 1996). However, previous studies use stress paradigms lasting 3-4 weeks and generally collect tissue samples shortly after the final stressor. Our stress was for 1-week and tissue samples were collected 3-days after the last stress/alcohol treatment. Spine density is a dynamic process and the 3-day window may have allowed for spine density to normalize between groups. Recent reports have shown that behavioral testing may alter spine density (Conrad et al., 2012; Eilam-Stock et al., 2012), thus our results may have been influence by the 3-days of behavioral testing. Future studies will measure spine density immediately after the last treatment to determine the composition of hippocampus and frontal cortex.

Alcohol is different from most other drugs of abuse in that it has no known endogenous receptors (Bates et al., 2002). Alcohol indirectly acts on various neurotransmitter (NT) functions, but its main effects appear to be as a GABA agonist and glutamate antagonist. The role of GABA and glutamate in memory processes has been shown by extensive research (review McEwen et al., 2002). However, we did not find any changes in GABA alpha-4 levels between

groups following the FST. The interaction between GABA and NMDA has been implicated in modulation of long-term potentiation; whereby, the function of $GABA_A$ activity directly influences the function of NMDA-R activity (Schummers and Browning, 2001). In the presence of alcohol, antagonizing $GABA_AR$ function with picrotoxin, enhances NMDA evoked potentials, while normal $GABA_AR$ function reduces NMDA evoked potentials in hippocampal CA1 cells. In addition, Pavlides et al. (2002) showed that 6hr/day/21days of stress produces suppression of LTP compared to controls, which correlated with behavioral impairments in memory. Again, no changes in GluN2B levels were found between groups. As indicated previously, the delayed time of our tissue collection as well as the stress of FST may have contributed to the non-significant results obtained. In further studies we plan to collect samples at specific time points throughout the experiment. This approach will allow for examination of how neurotransmitter function correlate with the behavior results observed here.

Exposure to chronic stress for 7-days caused physiological alterations which were consistent with results in previous studies using longer restraint durations (Conrad et al., 2003; McLaughlin et al., 2007). First, stressed rats gain weight at a lower rate than those in non-stress situations, and our results show that 7-days of restraint stress treatment alters weight gain, which replicates Bowman et al. (2009). In response to the forced swim task all rats mounted a corticosterone response. However, rats in the STR+ALC group mounted a response nearly double that of the CON group. Thus, based on work showing that non-stressed male rats have a mean daily CORT level of 60 ng/ml (Girotti et al., 2007), it was concluded that the combination of stress and alcohol created a sensitization to a novel stressor. When compared to those receiving either stress or alcohol alone the results for the STR+ALC group supports previous research showing that chronic stress sensitizes rats to novel stressors (Lee and Rivier, 1997). These results may have relevance to the development of alcohol dependence. Following the initiation of alcohol consumption and the switch from drinking for its positive reinforcing effects to drinking for negative reinforcing effects (Koob and LeMoal, 2008) may be a process linked to

stress sensitization. An unexpected increase in corticosterone may lead an alcohol abuser to increase consumption, which in turn may lead to dependence if the stressor persists. In order to test this hypothesis, measuring voluntary alcohol consumption after the FST would be valuable. If our proposed hypothesis was correct, then we would expect to see a greater and continued increase in alcohol consumption by rats in the STR+ALC group after the FST. Ryabinin et al. (1995) has shown that stress has the potential to alter alcohol metabolism, and found lower BACs in rats treated with both stress and alcohol compared to alcohol alone. Our results show that restraint stress did not alter alcohol metabolism as measured by BAC on the final day of treatment. Others have found a stress hormone tolerance effect after continued alcohol administration (Spencer and McEwen, 1990), suggesting an interactive effect that deserves further study. Current studies are examining BAC and corticosterone levels on the first and last days of treatment to determine any influence of tolerance.

In conclusion, we found that although alcohol intake generally adversely affects memory, anxiety, and depression, it also mitigates the behavioral effects of stress. These data may have implications for alcohol use and abuse during times of stress, and may provide an intermediate time point of focus that may be of value in understanding the interaction between alcohol and stress. Further research is necessary to determine the neural changes that underlie the effects of alcohol or stress, and their combination, on behavior.

Aim 3.

Introduction

Female organisms react and respond differently to the use of drugs and under stressful conditions. It has been reported that, compared to men, women are more susceptible to the effects of alcohol due differences in metabolism, hormones, and physiology (Mancinelli et al., 2007). When tested with the same dose (0.6 g/kg) women had higher blood alcohol levels than men (Frezza et al., 1990). In addition, the time from onset of alcohol use to acquiring chronic drinking behaviors or entering treatment due to complications is shorter in women when compared to men (Hernandez-Avila et al., 2004). However, alcohol is often used as a form of self-medication for coping with a stressor (Khantzian, 1997), but attempts of self-medication are often gender specific. Berger and Adesso (1991) tested alcohol on groups of depressed and non-depressed men and women. They found only depressed men and non-depressed women reported reduction in depressive feelings after consuming alcohol. A comparison of stressed versus non-stressed individuals found, in men not women, stress was a mediating factor for the amount of alcohol consumed, with greater stress associated with higher intake (Cooper et al., 1992). Despite the differences between genders, neurological consequences are often similar. Men and women show comparable cortical shrinkage and ventricle enlargement after chronic alcohol abuse (Mann et al., 1992). However, a comparison of hospitalized alcoholic women and men found the magnitude of corpus callosum shrinkage was greater in women than men even after correction for cranial size (Hommer et al., 1996), suggesting greater sensitivity to alcohol-induced brain damage in women.

Animal models confirm some but not all sexual dimorphisms in response to alcohol and stress in humans. After 20-weeks of ethanol treatment, both male and female rats show the same frontal cortex shrinkage (Savage et al., 2000). An acute administration of alcohol dose-dependently reduces anxiety on the plus maze in both sexes, but corticosterone was elevated in females (Wilson et al., 2004). Thus, there appears to be an interactive effect of alcohol use and

stress hormone response. Much like the effects of chronic alcohol intake, chronic mild stress decreases sucrose preference and increases anxiety in male and female rats (Dalla et al., 2005). Yet Dalla et al. found mild stress did not alter corticosterone levels in males, but it was elevated in females. Chronic stress impairs memory in male rats but either has no effect or enhances performance in female rats (Bowman et al., 2002; Beck and Luine, 2002; Conrad et al., 2004; McLaughlin et al., 2005). However, the effects of stress on memory are dependent on the type of stressor and the type of memory tested. Shors et al. (1998) found variable stress facilitates eye-blink conditioning in males and impairs conditioning in females. Unlike the no-effect or facilitated memory results found under chronic stress conditions in females, chronic alcohol consumption leads to memory impairments in both sexes. Memory impairment caused by ethanol depends on dose and duration, but as short as 6-days (2 g/kg/d) impairs performance on the Morris water maze and 6-weeks (0.7 g/kg/d) impairs performance on the Barnes maze in female rats (Sircar et al., 2009; Ranney and Petro, 2009).

The combination of stress and alcohol has been rarely studied in female subjects. Studies using other drugs find that stress increases self-administration of most drugs including cocaine (Haney et al., 1995), amphetamine (Piazza et al., 1990), morphine (Marks-Kaufman and Lewis, 1984), and alcohol (Lê et al., 1999). Bisagno et al. (2004) found amphetamine impairs memory in female rats, but the impairment is not present in rats also exposed to chronic restraint stress.

The present study examined the effects of chronic stress, alcohol, and their combination in female rats. The experiment was conducted as previously described in male rats, in order to determine the extent of sexually dimorphic effects to stress and alcohol. Tests were conducted to measure memory, anxiety, depression, physiological responses, and neurological changes. Compared to males in Aim 2, it was expected that female rats exposed to the combination treatment would show impaired memory and increases in anxiety and depressive-like behaviors. Based on previous research, females in the stress alone condition should show no effect or a facilitation in memory function.

Methods and Materials

Subjects

Intact cycling female Sprague-Dawley rats (~180 g, N = 32) obtained from Harlan Sprague-Dawely, Inc. (USA) were pair-housed and kept on a 12hr light cycle with lights *on* at 09:00. Standard rat chow and water was available *ad libitum*. Rats were randomly assigned to one of four conditions (n=8 per group): No Stress / No Alcohol Control (CON), Alcohol alone (ALC), Stress alone (STR), or combination of Stress and Alcohol (STR+ALC). All procedures were approved by Hunter College's Animal Care and Use Committee.

Procedure

All procedures and materials were identical to those performed in male rats. Please refer to Aim 2 for details.

Results

Physiological Effects

Blood samples were collected via tail-nick 30-40 minutes after the final alcohol administration, and alcohol concentration was measured in sera. No differences in alcohol metabolism were found between groups [$t(14)=0.70$, $p=0.49$], suggesting stress had no effect on alcohol metabolism (Figure 11A).

Corticosterone was measured as a stress response marker to a novel stressor 30-40 minutes after the forced swim task. All groups elicited a stress response to forced swim, but the ALC, STR, and STR+ALC groups had higher levels as compared to the CON group [$F_{(3,28)}=3.49$, $p=0.03$] (Figure 11B).

Body weight was used as a marker to determine the physiological effects of stress. An ANOVA revealed a main effect of group [$F_{(3,196)}=16.29$, $p=0.001$]. Weight differences were tested between groups on days-1, -4, and -7, and no difference was found on day-1, but by day-4, rats in the CON group weighed more than those in the ALC, STR, and STR+ALC groups [$F_{(3,28)}=3.04$,

p=0.045]. The difference in weight between groups was still present on day-7 [$F_{(3,28)}$=4.47, p=0.01] (Figure 11C).

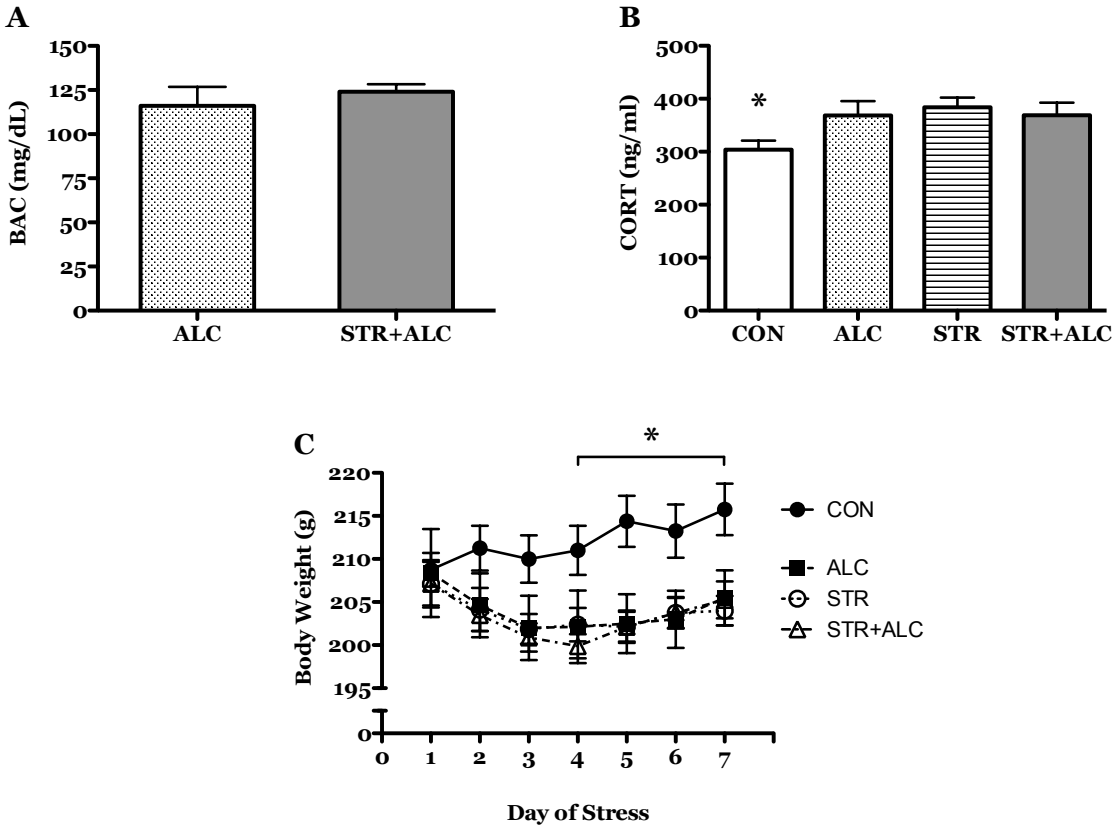

Figure 11. Physiological Effects: (A) Comparison of blood alcohol concentration, presented as means±SEM. No significant difference was found between groups. (B) Stress responses 30-40 minutes after forced swim task. Corticosterone levels presented as means±SEM. The CON group had a significantly lower response to the swim task compared to all other groups (Asterisk |*| $p<0.05$). (C) Body weights over days of stress treatment displayed in means ±SEM. By day-4 through day-7, the ALC, STR, and STR+ALC groups had significantly lower weight compared to the CON group (Asterisk |*| $p<0.05$).

Working Memory Using Object Recognition

The object recognition task tested working memory. During the sample trial (T1), no significant difference was found in exploration of identical objects between groups [$F_{(3,28)}$=1.48, p=0.24] (Figure 12A). In the probe trial (T2), exploration times were converted into exploration

ratios by dividing time exploring new object by total exploration time. One-way ANOVA found a significant difference between groups [$F_{(3,28)}$=6.98, p=0.001]. Post-hoc LSD analysis found the CON and STR groups had greater novel object exploration ratios than the ALC and STR+ALC groups ($p<0.05$), suggesting impaired memory attributed to alcohol treatment (Figure 12B).

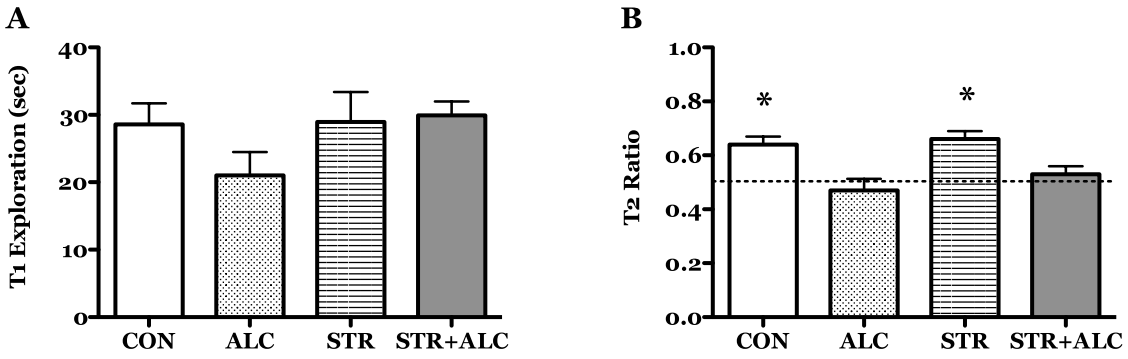

Figure 12. Object Recognition: (A) Exploration (means±SEM) of identical objects during the sample trial (T1). No differences were found between groups. (B) Exploration ratios (means±SEM) determined by time spent with new object divided by total time spent with both objects. The dotted line represents chance performance. Both CON and STR groups were significantly different from the ALC and STR+ALC groups, showing intact memory for the new and old objects (Asterisk |*| $p<0.05$).

Spatial Memory Using the Y-maze

The Y-maze assesses hippocampal dependent spatial memory by analyzing exploration of familiar and novel arms. No significant difference in total entries was found between groups [$F_{(3,28)}$=2.30, p=0.10], suggesting treatment did not alter locomotion or motivation (Figure 13A). Percent entries were analyzed between groups using factorial analysis. A main interaction effect of group by arm entry was found [$F_{(3,56)}$=2.78, p=0.05]. To examine preference for the novel arm, Bonferroni corrected t-tests were conducted. The CON group had greater exploration of the novel arm compared to the other arm [$t_{(14)}$=2.85, p=0.01] (Figure 13B). No difference in arm exploration was found in the ALC, STR, or STR+ALC groups, suggesting impairment in spatial memory caused by stress or alcohol treatments.

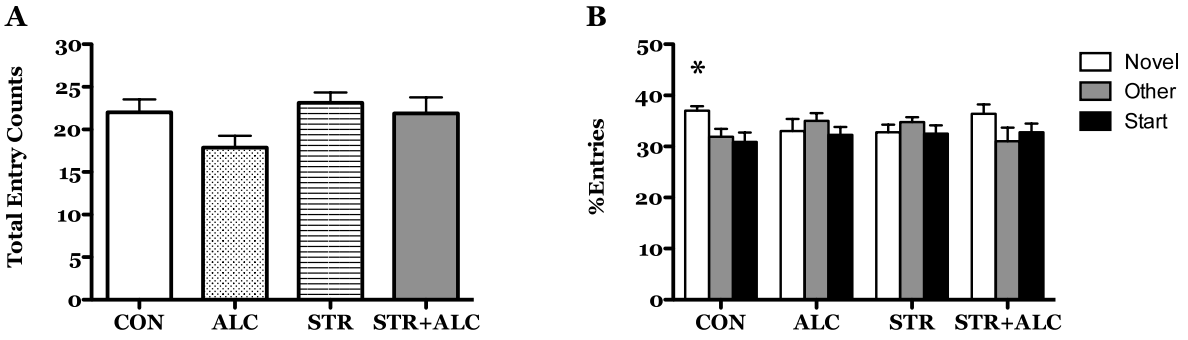

Figure 13. Y-Maze: (A) Total entries into the Start, Other, and Novel arms (means±SEM). No significant difference was found between groups. (B) Percent entries into each arm (means±SEM). The CON group significantly explored the novel more than the other arm, suggesting intact memory (Asterisk |*| $p<0.01$). No differences in arm exploration were found in the ALC, STR, or STR+ALC groups.

Anxiety and Depression Measures

We examined open arm exploration on the plus maze to assess anxiety. No difference in percent of open arm entries or percent of time spent in the open arms was found between groups [$F_{(3,28)}=0.035$, $p=0.99$; $F_{(3,28)}=0.89$, $p=0.46$, respectively]. Alcohol and/or stress treatment had no effect on anxiety (Figure 14).

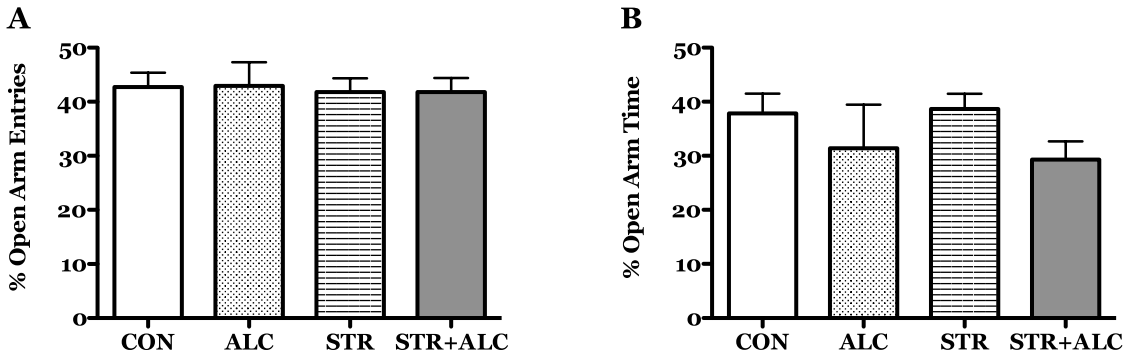

Figure 14. Elevated Plus Maze: (A) Percent of entries into the open arms (means±SEM). (B) Percent of time spent in the open arms (means±SEM).

To measure depressive-like symptoms, we assessed swimming, immobile, and climbing behaviors on the forced swim task. Behavioral counts were converted into percentages and analyzed between groups with post-hoc analyses within groups. A significant main effect of behavior was found [$F_{(2,84)}$=48.49, p=0.001] (Figure 15). Post-hoc analyses found the CON and STR groups displayed more swimming than immobile behavior [LSD; p=0.001 and p=0.0001, respectively]. No difference between swim and immobility was found in the ALC and STR+ALC groups [LSD; p=0.71 and p=0.06, respectively], suggesting an alcohol-induced increase in depressive-like behaviors (Figure 15).

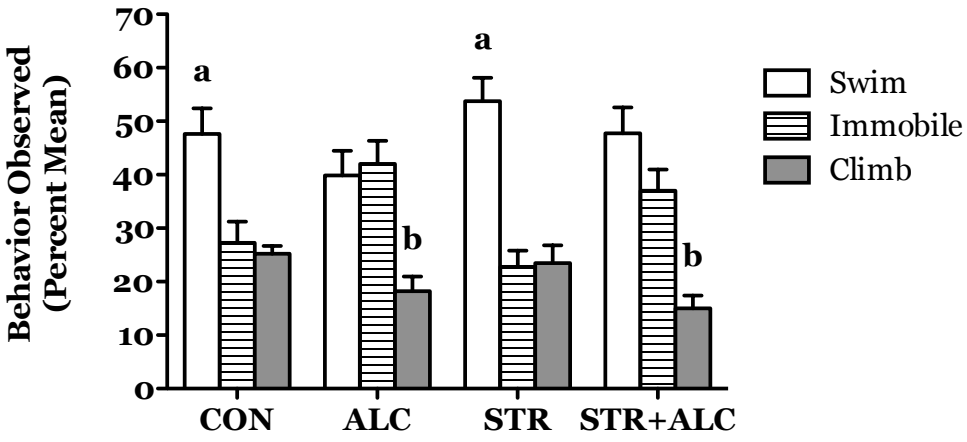

Figure 15. Behavior observed over a 5-minute trial counted at 5-second intervals. (a) indicates Swim greater than Immobile and Climb (p<0.05). (b) indicates Climb less than both Swim and Immobile (p<0.05).

Neurological Effects

Western blots were conducted to measure receptor expression for GABAα4 in the membrane fraction and GluN2B in the post-synaptic density fraction. No significant difference was found in GABAα4 [$F_{(3,28)}$=1.01, p=0.40] or GluN2B [$F_{(3,28)}$=0.25, p=0.86] between groups (Figure 16).

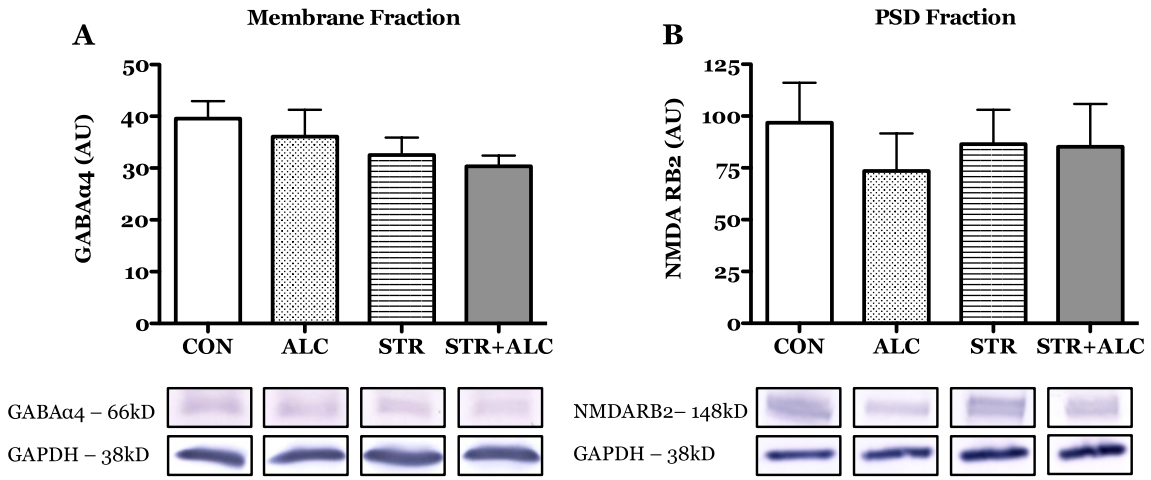

Figure 16. Western blot analysis (means±SEM). (A) levels of GABAα4 as arbitrary units (AU) in the membrane fraction. (B) levels of GluN2B as arbitrary units (AU) in the post-synaptic density fraction.

In the CA1 region of the hippocampus, spines were counted in tertiary apical and secondary basal dendrites of pyramidal neurons. No significant difference was found between groups in either the apical [$F_{(3,27)}$=0.74, p=0.54] or basal [$F_{(3,27)}$=1.06, p=0.38] areas (Table 2).

Table 2. Spine density of pyramidal neurons in CA1 of the hippocampus

Pyramidal Neurons Six dendrites per area	Treatment			
	CON	ALC	STR	STR+ALC
CA1 – Apical	20.67 ± 2.11	19.24 ± 1.56	20.64 ± 1.12	22.62 ± 1.22
CA1 – Basal	20.31 ± 1.14	18.78 ± 1.33	21.49 ± 1.17	21.41 ± 1.32
Brain Region: Hippocampus (CA1)				
Data Represent: Spines per 10μm – means±SEM				

Discussion

The present experiment tested the effects of alcohol intake after a stressor on female rats. Physiologically, stress did not alter metabolism as measured by blood alcohol content. In response to the forced swim task, all groups elicited a stress response, but the ALC, STR, and

STR+ALC groups had a sensitized response compared to controls (i.e. demonstrating a ~20% increase in serum CORT than in controls). The same pattern of the stress response was reflected in body weight, where by day-4 and continuing to day-7, the ALC, STR, and STR+ALC groups gained weight at a lower rate. Memory on the object recognition task was intact in the CON and STR groups and impaired in the ALC and STR+ALC groups. In the Y-maze, only the CON group displayed intact spatial memory by exploring the novel arm more than the other arms. Treatment had no effect on anxiety measures in the elevated plus maze, but alcohol did increase depressive-like behaviors in the forced swim task. Neurochemically, alcohol and stress treatment did not alter receptor expression of GABAα4 or GluN2B in the hippocampus. Additionally, spine density in the CA1 region was unaltered by treatment in apical or basal sections of pyramidal neurons.

Previous studies in our lab have shown that chronic stress has no effect or enhances memory on the object recognition task in female rats (Beck and Luine, 2002; Bowman et al., 2002). The present study supports the stress effects on object recognition; however, when alcohol was given after each stressor, memory was impaired on the OR task. Additionally, chronic treatment with alcohol alone also impaired memory, suggesting a non-specific impairing action of alcohol intake in female rats. With regards to stress effects on spatial memory, female subjects tend to show no effect or an enhancement in performance following chronic stress (Conrad et al., 2004; McLaughlin et al., 2005). Our results did not support the previous findings between stress and Y-maze performance because memory was impaired for the experimental groups. Only the control group showed a preferences and increased exploration of the novel arm, suggesting intact memory for the unexplored arm. Differences in effects of stress on memory in the current study may be due to the shorter duration of treatment, 7-days, as compared to 21-days in previous experiments (Conrad et al., 2004; McLaughlin et al., 2005). Also, McLaughlin et al. (2008) has suggested females habituate or lose interest in the Y-maze task quickly, implying the task is more sensitive in assessing spatial

memory in male rats. Thus, the memory tasks, usually associated with the prefrontal cortex (OR) and hippocampus (Y-maze) are differentially altered by stress and alcohol both within and between sexes.

A cornerstone of alcohol research involves the effects of consumption on anxiety and depression. Acutely, low to moderate doses of alcohol act as anxiolytics and increase open arm exploration on the elevated plus maze while high doses are anxiogenic (Wilson et al. 2004). Chronic intake leads to increases in anxiety and depression, and effects are often greater in females (Weinberger et al., 2009). There are multiple findings on anxiety as pertaining to stress or alcohol. McCormick et al. (2008) reports decreases in anxiety by social stress exposure, but only in females during high estrogen phases and no effect when estrogen is low. Immobilization and variable stress shows no change in behavior on the plus maze in female rats (Mitra et al., 2005), while restraint stress has shown increases in anxiety (Huynh et al., 2011). Usually, only after chronic intake and during ethanol withdrawal, does one see increases in anxiety (Kliethermes et al., 2004). Our results support studies showing no difference between control and experimental groups. Withdrawal may have been a factor; however, the dose and duration used was believed not to be sufficient to cause withdrawal-induced increases in anxiety.

Compared to the plus maze, a different pattern of results was found on the forced swim task. Rats treated with alcohol had an increase in depressive behaviors by showing equal swim and immobility. The result supports others who have found an increase in depression following alcohol consumption (Olivier et al., 2008). The behavior in the forced swim task reflects that found on the OR task. The relation may be mediated by function of the prefrontal cortex in that the OR task is PFC dependent and depression and alcohol are known to alter PFC function (Venzala et al., 2012; Li et al., 2009). Morphological and neurochemical investigation of the PFC may help confirm the involvement of the PFC on memory and depression.

Neuronal morphology and spine density show direct influence of stress, hormone levels, and alcohol use (Gould et al., 1990; Berman and Hannigan, 2000). Thus far, only the CA1 region

of the hippocampus has been analyzed and treatment had no affect on altering spine density between groups. McLaughlin et al. (2010) found CA1 spine density was only affected by estrogen treatment, via silastic capsule implantation, and not by restraint stress. The female rats in the current study were not ovariectomized, thus, hormone levels may have influenced the non-significant results found in spine density. In addition to spine density, neurotransmitter receptor levels were measured in hippocampal tissue. Again no significant differences were found between groups in expression of GABAα4 or GluN2B. The time of tissue collection may have accounted for the non-significant effect. Brain tissue was taken 3-days after the final stress and alcohol treatment, which may have allowed for recovery of receptor levels and spine density to normal levels. Future experiments are planned to test spine density and receptor expression immediately after the final treatment with stress or alcohol.

The levels of alcohol were measured in blood samples and stress did not alter BAC 30-40 minutes after administration. Studies have reported differences in blood alcohol content in response to an alcohol challenge between men and women (Breslin et al., 1994), but rats often show similar BACs between male and females (Savage et al., 2000; Wilson et al., 2004). These results suggest that the effects found in our study may be due to alcohol altering the effects of stress and not *vice versa*. In response to a novel stressor (forced swim), corticosterone was measured to determine if sensitization occurred in rats exposed to stress. Females generally show an elevated CORT response to both stressors and alcohol consumption (Kant et al., 1983; Rivier, 1993), but non-stressed female rats generally have a mean daily CORT level of 50 ng/ml (Windle et al., 1998). The levels of CORT were elevated in the experimental groups compared to the controls, showing that exposure to chronic alcohol, stress, or both increases the stress response to a new stressor. The significance of this finding needs further examination to determine if an increased CORT response may increase alcohol consumption when subjects are exposed to a new stressor. As seen in the CORT response, body weight was altered by treatment, with stress and alcohol as well as the combination showing a decrease in weight gain over the 7-

days of treatment. The difference in body weight supports others who show a decrease in weight gain over periods of stress treatment (McLaughlin et al., 2010; Conrad et al., 2012; Huynh et al., 2011). The current study found female rats have different responses compared to males. Given that effects on memory, anxiety, and depression were sexual dimorphic only strengthens the notion that sex specific prevention methods and treatments are needed when dealing with chronic stress or alcohol abuse.

Aim 4.

Introduction

The use of alcohol has been linked to subjective and objective reduction of heart rate, blood pressure, anxiety, and tension following a stressor (Levenson et al., 1980; Kushner et al., 1994). The physiological effects of alcohol consumption are fairly generalized, but depend on sex, age, weight, and drinking history. Studies have shown that alcohol is metabolized faster during times of stress (Mezey et al., 1979; Ryabinin et al., 1995) and the blood alcohol curve is altered with stressed individuals showing a later peak in BAC and a faster decline to baseline (Breslin et al., 1994). Intoxicating doses of alcohol increase stress hormone levels in human and animal models (Sinha, 2001; Patterson-Buckendahl et al., 2005). This rise of corticosterone by alcohol intake is sometimes enhanced when combined with a stressor (Trudeau et al., 1990, 1991). However, the enhancement or reduction is dependent on the type of stressor experienced and alcohol dose. Brick and Pohorecky (1982) found that ethanol treatment (low-dose 0.5 g/kg) before foot-shock stress reduces corticosterone response but does not alter response to restraint stress. Much like a psychological stressors, chronic alcohol exposure leads to tolerance, both in stress hormone response and in behavioral effects (Spencer and McEwen, 1990; Koob and LeMoal, 2008). Neurologically, alcohol is known as GABA agonist and an NMDA antagonist. Chronic use causes changes in receptor expression to compensate for the constant influx or suppression of these neurotransmitter functions (Grant et al., 1990; Trevisan et al., 1994).

In the present study we examined the effects of alcohol intake, via gastric gavage, on physiological measures. In Aim 2, we found various behavioral effects associated with stress and alcohol treatment. However, it was believed that the physiological and neurological changes normalized following 3-days of behavioral testing. It has been recently shown that simply being exposed to a behavioral task can alter spine density (Conrad et al., 2012; Eliam-Stock et al., 2012). In order to elucidate the previous findings, using the same treatment methods, we measured blood alcohol content and corticosterone levels on the first and final day of treatment

and neurotransmitter receptor expression after 7-days of treatment. The adapted paradigm allows for an understanding of how alcohol and stress treatment alter the physiological and neurological composition of the organism immediately following the treatment and before possible return to homeostasis. Aim 4 was intended to provide a foundation to help elucidate the behavioral results found in Aim 2.

Methods and Materials

Subjects

Male Sprague-Dawley rats (~250 g, N = 32) obtained from Harlan Sprague-Dawely, Inc. (USA) were pair-housed and kept on a 12hr light cycle with lights *on* at 09:00. Standard rat chow and water was available *ad libitum*. Rats were randomly assigned to one of four conditions (n=8 per group): No Stress / No Alcohol Control (CON), Alcohol alone (ALC), Stress alone (STR), or combination of Stress and Alcohol (STR+ALC). All procedures were approved by Hunter College's Animal Care and Use Committee.

Procedure

Rats were allowed to acclimate upon arrival for one week. After the acclimation period, rats were randomly assigned to groups and treated for seven consecutive days. On day-1, rats in the STR and STR+ALC groups were placed in Plexiglas restrainers for 6-hours. Rats in the CON and ALC groups were handled accordingly to control for additional handling as a result of restraint. Immediately after the 6-hour restraint period, all rats received 2 g/kg of either ethanol (20% v/v) or saline via gastric gavage. Additionally on day-1, all rats were tail-nicked and blood was drawn to measure levels of corticosterone and blood alcohol content 30-40 minutes after the gavage procedure. The 30-40 minute time window was chosen as a means of assessing peak BAC and CORT levels. The stress and alcohol treatments continued and on day-7, rats were sacrificed 30-40 minutes after gavage. At this time, blood and brain samples were taken for further analysis (western blots and spine density).

Restraint and Drug Administration

The following procedures were taken from Aim 2, for further detail please refer back. Rats were restrained, not immobilized, for 6hr/day/7days (10:00-16:00) in a Plexiglas restrainer measuring 21.5cm long x 6.3cm internal diameter (Harvard Apparatus). Pure ethanol (200 proof, Sigma-Aldrich) was diluted in saline (0.9% NaCl, Fisher Scientific) to produce a concentration of 20% v/v ethanol. All rats were gavaged with a dose of 2 g/kg of alcohol or saline in a counterbalanced fashion at the same time every day (~16:15). Blood alcohol content was analyzed using 5µl of sera in an Analox GM7 micro-stat machine.

Western Blots and Corticosterone Assay

Western blots and corticosterone procedures were identical to those described in Aim 2; however, the day of sample collection differd. In Aim 4, blood was collected on day-1 and day-7 and brains were collected on day-7 for neurotransmitter receptor expression. For specific detail, refer to Aim 2.

Statistical Analysis

Blood alcohol content was analyzed using a 2x2 ANOVA to test for differences between groups, between days, and any group by day interaction. Corticosterone levels and body weights were analyzed using repeated-measures ANOVA to test within subject differences of day and between subject differences of group. Post-hoc ANOVAs were run to test any significant main effects. Protein expression levels were tested using one-way ANOVAs and significant results were tested with a post-hoc LSD.

Results

Blood alcohol content was measured and metabolism was not altered by treatment on either day-1 or day-7. A 2-way repeated-measures ANOVA found no significant main effects of Group [$F_{(1,26)}$=1.10, p=0.30], Day [$F_{(1,26)}$=0.26, p=0.61], or Group by Day interaction [$F_{(1,26)}$=0.18, p=0.68] (Figure 17), suggesting no stress-induced change in metabolism.

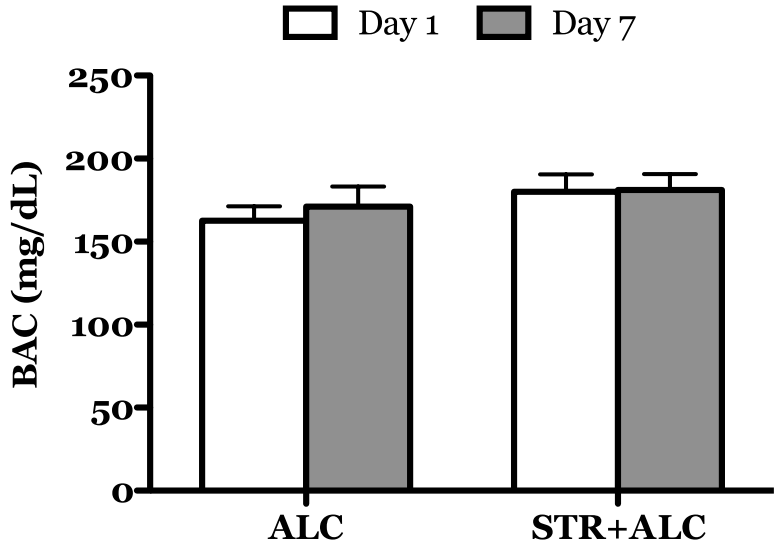

Figure 17. Blood alcohol content (means±SEM) as measured in tail-nick samples 30-40 minutes after alcohol was gavaged on day-1 and day-7. No significant differences were found.

The stress response to restraint and alcohol intake was measured via corticosterone (CORT) levels on day-1 and day-7. Repeated-measures 2-way ANOVA showed significant within subjects' main effects of CORT [$F_{(1,27)}$=15.31, p=0.001] and a CORT by Day interaction [$F_{(3,27)}$=17.03, p=0.0001]. Additionally, between subjects analysis also showed an effect of Group [$F_{(3,27)}$=10.23, p=0.0001] (Figure 18). On day-1, the STR+ALC group had a higher CORT level (158 ng/ml) compared to the other groups ($p<0.05$). Also on day-1, the ALC group had a greater CORT level than the STR group ($p<0.05$), but was statistically the same as the CON group ($p>0.05$). On day-7, the STR and STR+ALC group had lower CORT levels than the CON group ($p<0.005$). Within groups, only the STR+ALC group showed a difference in CORT levels between day-1 (158.9 ng/ml) and day-7 (19.9 ng/ml) ($p<0.002$), all other groups had equal CORT levels between days (Figure 18).

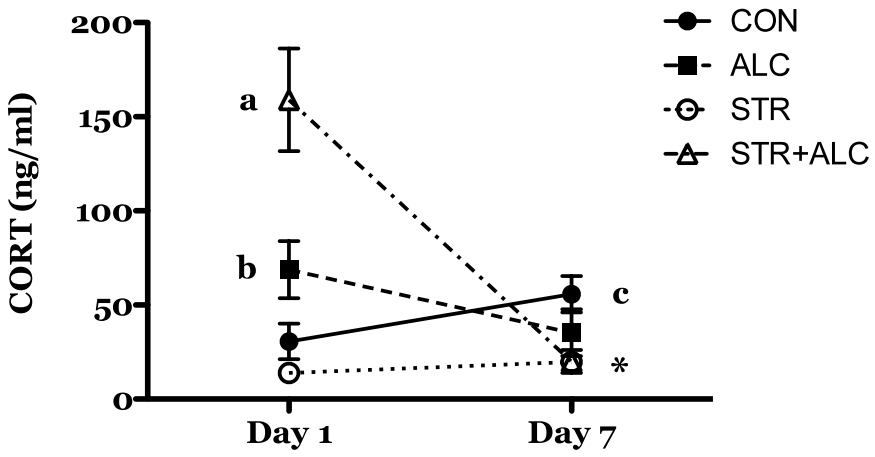

Figure 18. Levels of corticosterone measured 30-40 minutes after stress/alcohol treatment from tail-nick samples on day-1 and day-7 (means±SEM). |a| indicates the STR+ALC group had the highest level of CORT on day-1 compared to all other groups ($p<0.05$). |b| indicates the ALC group had higher CORT on day-1 than the STR group ($p<0.05$). |c| indicates the CON group had a significantly greater level of CORT on day-7 compared to the STR and STR+ALC groups. Asterisk |*| shows only the STR+ALC group had a significant within group difference in CORT levels from day-1 to day-7.

Body weights were taken over the 7-day treatment and used to determine alcohol volume and assess stress reactivity. As expected, repeated-measures ANOVA showed within subject main effects of Day [$F_{(6,162)}=52.75$, $p=0.0001$] and a Day by Group interaction [$F_{(18,162)}=6.26$, $p=0.0001$]. Between subjects' analysis showed a significant main effect of Group [$F_{(3,27)}=21.49$, $p=0.0001$]. There was no difference between groups on day-1, but from day-2 to day-4 the CON and ALC groups outweighed the STR and STR+ALC groups ($p<0.05$). From day-5 to day-7 the CON group weighed more than all other groups ($p<0.05$), but the ALC group was still greater than the STR and STR+ALC groups ($p<0.05$) (Figure 19).

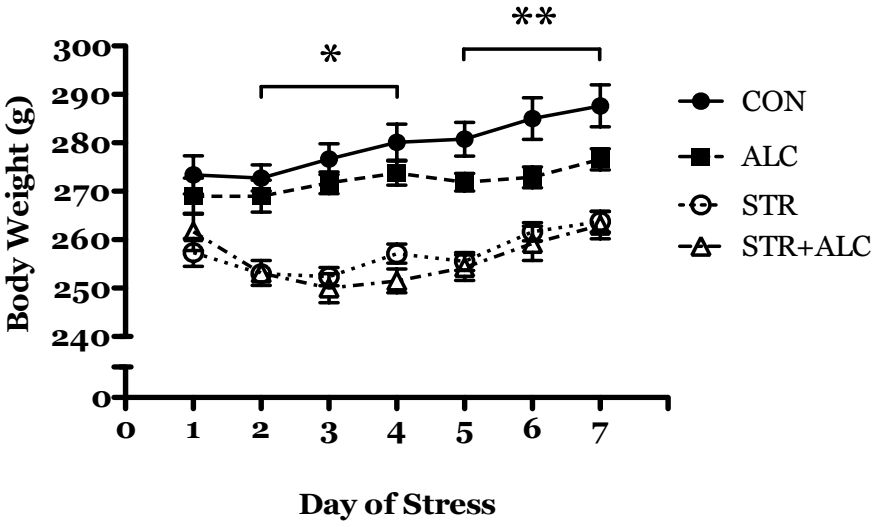

Figure 19. Body weight over the 7 days of treatment (means±SEM). No difference was found on day-1 between groups. Asterisk |*| significant difference between the CON/ALC groups and the STR/STR+ALC groups over day range. Double Asterisk |**| significant difference between CON, ALC, and STR/STR+ALC groups over day range ($p<0.05$).

On the final day of treatment (day-7), hippocampal tissue was collected and levels of GABAα4 and GluN2B protein expression were measured using western blot techniques. An ANOVA showed a significant difference in levels of GABAα4 between groups [$F_{(3,25)}=3.82$, $p=0.024$]. The STR+ALC group had the highest level of GABAα4 compared to the other groups ($p<0.02$). For GluN2B, a significant difference was also found between groups [$F_{(3,27)}=3.62$, $p=0.027$]. Here, the two groups treated with alcohol (ALC and STR+ALC) had greater expression of the glutamate receptor protein compared to the CON and STR groups ($p<0.03$) (Figure 20).

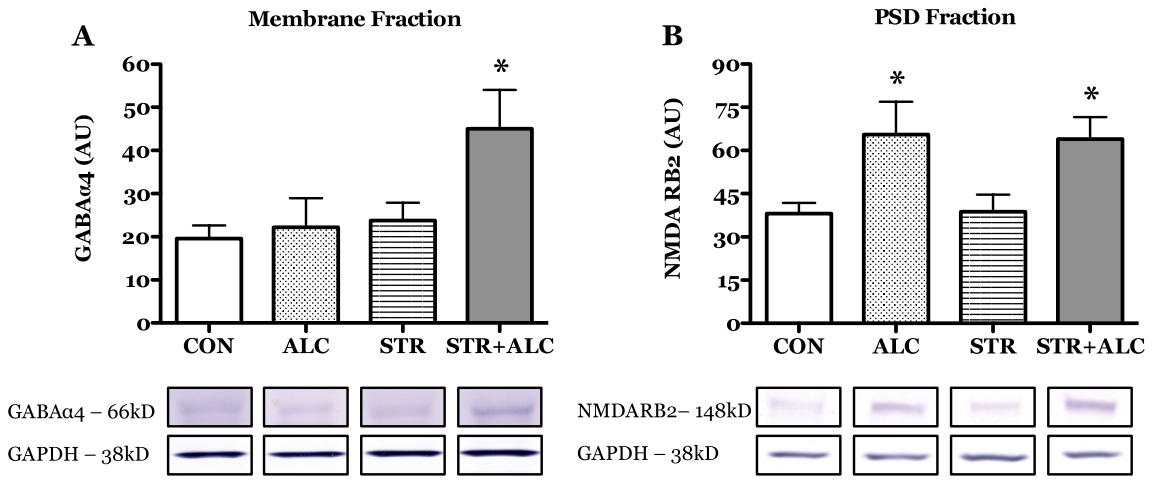

Figure 20. Level of neurotransmitter receptor protein expression in the hippocampus of samples taken 30-40 minutes after final stress/alcohol treatment (AU: Arbitrary Units - mean±SEM). (A) GABAα4 analysis shows a significant difference, the STR+ALC group had higher levels compared to other groups (Asterisk |*| $p<0.05$). (B) GluN2B analysis shows a significant difference, ALC and STR+ALC were greater than CON and STR groups (Asterisk |*| $p<0.05$).

Discussion

The physiological and neurological effects of stress, alcohol, and the combination were tested after 7-days of treatment in male rats. No change was found in blood alcohol content (i.e. metabolism) between rats in stressed and non-stressed groups. The corticosterone response was not altered by stress compared to control on day-1. However, the combination treatment elevated the level of CORT 10-fold, but this elevation subsided by day-7. As an indirect measure of stress effect, body weights were analyzed to test for changes induced by treatment. Both stress groups gained weight at a lower rate than controls starting on day-2. By day-5 and persisting to day-7, all groups were lower in body weight compared to controls. Treatment had an effect on neurotransmitter levels in hippocampal tissue. GABAα4 receptor expression was elevated in the combination group, while all other groups were equal. GluN2B receptor expression was elevated in rats treated with alcohol and the combination group. These results should be contrasted with

those obtained when subjects were sacrificed after 3-days of behavioral testing immediately after the forced swim task (Aim 2). The results obtained in Aim 4 indicate that the treatment with stress or alcohol had effects on physiology and neurochemistry, which can normalize after 3-days of behavioral testing. More importantly, Aim 4 provides a baseline from which animals start as they are cognitively tested.

Stress can affect the response to alcohol consumption and alcohol can affect the response to stress exposure. Spencer and McEwen (1990) showed the CORT response to chronic alcohol intake adapts over time much like the CORT response to a chronic stressor. Following 6-hours of restraint stress or not and a 2 g/kg alcohol dose and/or saline, the ALC and STR+ALC groups elicited a higher CORT response than the CON and STR groups on day-1. Only the STR+ALC group showed an adaptive effect between day-1 and day-7. The effect may be explained by duration of treatment only being 7-days. Other studies find that the changes in CORT occur over longer periods of time (i.e. 21-days) (Spencer and McEwen, 1990; Dhabhar et al., 1997). Corticosterone levels have been shown to return to normal following 3- to 6-hours of restraint stress (Galea et al., 1997). We also showed that CORT was not elevated in the stress group after 6-hours of restraint stress, but an additive effect was present in the combination group, suggesting an interaction that resulted in an enhancement of the CORT response. Trudeau et al. (1990) found that alcohol increases the CORT response to a 60-minute restraint session in rats, which supports our findings. Others have found that rats exposed to a stressor after alcohol injection display lower peak blood alcohol concentrations, suggesting that stress may increase alcohol metabolism (Mezey et al., 1979; Ryabinin et al., 1995). We did not find any change in blood alcohol concentration between the stress and non-stress groups. However, unlike studies that have found a difference, we administered alcohol after the stressor. Perhaps the increase in alcohol metabolism was present only when animals were exposed to a stressor after consumption, reported in humans as a “sobering” effect (Breslin et al., 1994). The stress-induced increase in alcohol metabolism may explain the stress-induced increase in alcohol

consumption, as a lower BAC may drive an individual to drink more to attain a regular blood alcohol concentration obtained when not stressed (Sommer et al. 2008).

The body weights recorded in our experiment suggest that stress had a physiological effect, however, after 5-days of treatment the alcohol group also showed a difference from the control group, but still greater weight than the stress groups. The results were slightly different than those found in Aim 2, in which only stress altered weight gain. The only difference between studies was the males in Aim 2 had an extra week of habituation before treatment began, which may have influenced the effects of alcohol on weight gain. It was hypothesized that in Aim 2 neurotransmitter levels normalized following 3-days of behavioral testing with no stress or alcohol treatment. In the present experiment, tissue was collected on the final day of treatment to determine GABAα4 and GluN2B receptor expression levels before the rats experienced behavior and possible recovery. We found that GABAα4 was elevated in the STR+ALC group. The alpha-4 subunit was chosen due to its high association with anxiolytic effects caused by alcohol consumption or intake of benzodiazepines (Gulinello et al. 2001). The upregulation of alpha-4 subunit helps to explain the difference in anxiety found between the STR and STR+ALC groups. GluN2B was elevated in the groups exposed to alcohol, showing a non-specific action of alcohol on upregulation. The N2B receptor is associated with the withdrawal symptoms and in compensation for constant NMDA antagonism, by alcohol, the N2B receptor is upregulated (Follesa and Ticku, 1995; Hu et al., 1996). However, the change in receptor expression has been shown to be transient and others have found an upregulation of GluN2B immediately after 5- or 6-days of alcohol treatment and a return to control levels 48-hours post-treatment (Kalluri et al., 1998; Narita et al., 2000). The results found in Aim 4 help in part to clarify the lack of effect on neural measures in Aim 2 and comparisons between all Aims will be discussed in the next section. In conclusion, Aim 4 shows that the physiological and neurological effects of stress and alcohol are time-dependent and may alter behavior.

General Discussion

Overall we found that one-week of restraint stress and/or alcohol was sufficient to change various aspects of behavior and physiology. The effects were dependent not only on treatment, but also on gender. Aim 1 showed, as expected, alcohol consumption increases following a stressor. However, unexpectedly, the availability of voluntary alcohol after a stressor blocked the memory impairments on the object placement task seen in the alcohol and stress alone groups (Gomez et al., 2012). The former result supports others who show a stress-induced increase in alcohol consumption (Bowers et al., 1997; Sillaber et al., 2002; Farook et al., 2008). However, to our knowledge, no one has shown an alleviation of stress- or alcohol-induced memory impairments when treatments are combined. Additionally, the increase in stress-induced consumption is mainly reported in male subjects (Pohorecky, 1991). For instance, restraint for 2-hours/day increases intake in male but not female rats (Chester et al., 2006). The increase of consumption has been attributed to activation/deactivation of the hypothalamic-pituitary-adrenal axis. Alcohol before a stress tends to augment the stress response (Patterson-Buchendahl et al., 2005), but alcohol after a stressor tends to blunt the stress response (Shirao et al., 1988).

In Aim 1, it was believed that the availability of alcohol after the stressor influenced stress hormone levels and subsequent memory on the OP task. The OP task was chosen due to its low stress, no training requirement, and non-reinforcement qualities (Ennaceur et al., 1997). Studies have shown impairment in OP performance following chronic stress (Bowman et al., 2006; Eiland and McEwen, 2012). The effects were replicated in Aim 2 using the Y-maze, another hippocampal-dependent spatial memory task that is impaired by stress or alcohol (Conrad et al., 1996; White and Best, 2000). A slight difference was seen between voluntary consumption and forced intake on the effects of body weight. Voluntary consumption led to an alleviation of the stress-induced weight reduction present in the stress groups. Forced intake did not block the reduction in weight gain. This difference may be attributed to the differences in

duration and dose of alcohol consumed. Rats in Aim 1 were only restrained for 1-hour per day and drank an average of 0.5 g/kg. Rats in Aim 2 were restrained for 6-hours per day and were given 2.0 g/kg. Despite the differing weight gain results found between Aims, it was clear that additive effects were present in male rats given alcohol during times of stress.

As a way of controlling for the variance of individual alcohol consumption Aims 2, 3, and 4 administered a controlled oral dose via gastric gavage. By controlling for alcohol intake, we were able to ensure that all subjects received the same amount of alcohol and that behavioral effects reflected the actions of alcohol on the stressor. Gavage treatment also ensured that treatments were the same in both sexes, which was critical for assessing sex differences. Generally, male rats exposed to both stress and alcohol showed effects on the object recognition, Y-Maze, elevated plus maze, and forced swim task equal to the control group. In contrast, female rats exposed to the combination treatment showed detrimental effects on the aforementioned tasks. The sex-dependent effects on anxiety and depression are of particular interest as they relate to aspects of substance abuse and dependence.

Comorbidity exists between anxiety disorders and alcohol dependence, with a greater rate found in women (Conway et al., 2006). Our study found that alcohol blocks the stress-induced increase in anxiety in male rats. This finding provided validity for male individual's inclination to use alcohol as a way of reducing anxiety. Increased anxiety during alcohol intake generally occurs only during the withdrawal phase (Menzaghi et al., 1994; Rasmussen et al., 2001). We found the levels of anxiety in the ALC group, as evaluated on the EPM, was equal to CON, thus it can be assumed that no withdrawal from alcohol was present. Treatments did not affect performance on the elevated plus maze in female rats, a result that is consistent with previous studies (Mitra et al., 2005; Chadda and Devaud, 2005; McCormick et al., 2008; Bowman et al., 2009). On the other hand, the elevated plus maze, under the current conditions, may have been inadequate for testing anxiety in female rats. Others have found stress-induced increases in anxiety using the open field task by measuring latency to enter the field and center

crossings (Beck and Luine, 2002; Walf and Frye, 2005). Under the current paradigm follow-up studies should use other anxiety sensitive measures when testing female subjects in order to further validate or repudiate the current results.

Distinct from the effects on anxiety, the forced swim task also showed opposing effects of treatments in male and female rats. Depression, much like anxiety, is highly comorbid with chronic alcohol consumption (Grant and Harford, 1995). Treatment with alcohol has shown mixed results on depression tasks. Pre-treatment (24-hours pre-test) with alcohol, either voluntary or intra-gastric, elicits an anti-depressant like effect on the FST (Ciccocioppo et al., 1999). However, after 2-weeks of ethanol vapor exposure, rats show an increase in depression levels on the FST (Walker et al., 2010). In other depression models, such as the tail suspension task, rats exposed to alcohol register increased levels of depressive-like behaviors (Boyce-Rustay et al., 2006). With respects to chronic stress, previous studies found that chronic stress increases depressive like behaviors on the FST (Molina et al., 1994). In our study, male rats displayed depressive-like behaviors after chronic exposure to alcohol or stress alone and the effect was blocked when treatments were combined. Female rats showed an increase in depressive behaviors associated with alcohol intake, suggesting non-selective alcohol-induced effect. Women are more susceptible to depressive disorders and those depressed are twice as likely to abuse alcohol (Dixit and Crum, 2000). It may be possible that a lower dose of alcohol is needed to produce equivalent effects on the swim task given the elevated ACTH and CORT release to the same alcohol dose seen in females (Rivier, 1993). Agreement for our results can be found. When compared to depressed women, only depressed men report a reduction in depression after drinking alcohol (Berger and Adesso, 1991). Similar to anxiety, a male individual may drink alcohol during times of stress as a way of reducing the negative associations of depressive-like feelings (Levenson et al., 1980; Kushner et al., 1994).

An aspect of substance abuse and dependence often overlooked is the effects on cognitive function. Environmental context has been shown to influence reinstatement of

alcohol-seeking behavior (Burattini et al., 2006; Zironi et al., 2006). Of course this effect is dependent on the animal remembering the context. For memory function, a sexually dimorphic effect exists with stress causing impairments in males but not females (Beck and Luine, 2002; Shors, 2001; Conrad et al., 2003; Dalla et al., 2005). The ability of alcohol to impair memory does not show sexual dimorphisms (Ryabinin et al., 2002; Ranney and Petro, 2009). However, we report a sexual dimorphism when stress and alcohol were combined. When exposed to chronic stress followed by alcohol ingestion, males display intact working and spatial memory, while females were impaired. The effects seen on memory are important in that drugs of addition activate structures highly integrated with limbic areas associated with memory and emotion, which are essential for survival (Swanson, 2000). There is great plasticity in neocortical structures compared to more diencephalic areas, and the vast communication between the two allows for autonomic responses to drugs to readily alter behavior (Wang and McGinty, 1996).

The GABAergic and glutamatergic responses to stress and alcohol play fundamental roles in behavioral outcome. Neuronal dendritic atrophy and impaired hippocampal dependent memory are associated with chronic stress, but the effects of stress can be blocked with a competitive $NMDA_R$ antagonist (Magariños and McEwen, 1995; Luine et al., 1994). Additionally, chronic stress or alcohol has been shown to suppress hippocampal LTP (Pavlides et al., 2002; Roberto et al., 2002). However, the alcohol-induced suppression of LTP can be reversed with administration of a 5α-reductase inhibitor, which suppresses the alcohol-induced increase of GABAα4 (Talani et al., 2011). In Aim 4, we found an upregulation of the GABAα4 receptor in rats treated with the combination of stress and alcohol. We also found an upregulation of GluN2B receptors in rats exposed to alcohol, regardless of stress. In comparison to Aim 2, rats in Aim 4 were not exposed to behavioral testing, thus, neurotransmitter receptor expression may not have had sufficient time to recover. Our data support others who found changes in receptor levels on the final day of treatment and a rebound to normal 48-hours post-treatment

(Kalluri et al., 1998; Narita et al., 2000). Rats in Aim 4 were used as a basis for understanding the neuronal composition of animals before testing behavioral effects. The increase in GABAα4 may have been associated with the alcohol-mediated reduction of anxiety on the elevated plus maze. Although increases in GABAα4 have been associated with increases in anxiety in males and females (Gulinello et al. 2002), this only occurred during withdrawal after 21-days of treatment. Shorter duration and lower dose of alcohol treatment (≤ 7-days, 3 g/kg) are generally associated with increases in Cl^- influx mediated $GABA_A$ receptor function, which often leads to decreases in anxiety (review Grobin et al. 1998). Stress and alcohol-induced increases in glutamatergic function have been associated with impairment on spatial memory tasks (Cui et al., 2009; Hicklin et al., 2011). However, the increase was caused by treatment lasting >30-days. Results in Aim 4 show a possible mechanism for the impairment of memory seen in the alcohol alone group in Aim 2. Despite the increase in GluN2B receptor expression in the STR+ALC group, the interactive effect was not reflected in memory function.

Spine density in the prefrontal cortex and hippocampus was analyzed in both male and female rats following behavioral tests. Much like neurotransmitter receptor levels, no changes in spine density were seen between groups. It is possible that the 3-days of behavioral testing allowed sufficient time for spine density to normalize as with neurotransmitter expression. However, we did find a correlation between Y-maze performance and CA1 apical spine density in female rats, with more spines correlating with greater preference for the novel arm. McLaughlin et al. (2009) found similar results; female rats treated with stress and estrogen had higher levels of CA1 spine densities and performed better on the Morris water maze. Future experiments will analyze spine density immediately after the final stress and alcohol treatment. Much like receptor protein expression, it is expected that spine density may be different between groups if measured at an earlier time point.

In conclusion, the results presented show a clear interaction between chronic restraint stress and alcohol consumption. The data provide information relevant to positive aspects

involved in reduction of anxiety and depression for male rats. In addition, these results provide support for the self-medication hypothesis and elevated use of alcohol during times of stress. With respects to female subjects, alcohol consumption after a stressor results in negative outcomes, which, along with physiological differences, substantiates lower rates of female alcohol abusers. The associations between physical or neurological changes and behavioral outcome are transient and need to be studied at various time frames to fully understand the interaction between stress and alcohol. Finally, we provide empirical support for sex specific examination in the development of substance abuse or dependence during times of stress and rationale for the establishment of sex specific prevention methods and treatments for chronic stress and alcohol abuse.

References

Abel, T., and Lattal, K.M. (2001). Molecular mechanisms of memory acquisition, consolidation and retrieval. Curr Opin Neurobiol *11*, 180-187.

Adinoff, B., Ruether, K., Krebaum, S., Iranmanesh, A., and Williams, M.J. (2003). Increased salivary cortisol concentrations during chronic alcohol intoxication in a naturalistic clinical sample of men. Alcohol Clin Exp Res *27*, 1420-1427.

Aggleton, J.P., Hunt, P.R., and Rawlins, J.N. (1986). The effects of hippocampal lesions upon spatial and non-spatial tests of working memory. Behav Brain Res *19*, 133-146.

Alkadhi, K.A., Alzoubi, K.H., Srivareerat, M., and Tran, T.T. (2011). Chronic psychosocial stress exacerbates impairment of synaptic plasticity in beta-amyloid rat model of Alzheimer's disease: prevention by nicotine. Curr Alzheimer Res *8*, 718-731.

Allan, C.A. (1995). Alcohol problems and anxiety disorders--a critical review. Alcohol Alcohol *30*, 145-151.

Anderson, C.R. (1976). Coping behaviors as intervening mechanisms in the inverted-U stress-performance relationship. J Appl Psychol *61*, 30-34.

Aneshensel, C.S., and Huba, G.J. (1983). Depression, alcohol use, and smoking over one year: a four-wave longitudinal causal model. J Abnorm Psychol *92*, 134-150.

Aronsson, G., and Rissler, A. (1998). Psychophysiological stress reactions in female and male urban bus drivers. J Occup Health Psychol *3*, 122-129.

Baker, K.B., and Kim, J.J. (2002). Effects of stress and hippocampal NMDA receptor antagonism on recognition memory in rats. Learn Memory *9*, 58-65.

Baran, S.E., Campbell, A.M., Kleen, J.K., Foltz, C.H., Wright, R.L., Diamond, D.M., and Conrad, C.D. (2005). Combination of high fat diet and chronic stress retracts hippocampal dendrites. Neuroreport *16*, 39-43.

Bates, M.E., Bowden, S.C., and Barry, D. (2002). Neurocognitive impairment associated with alcohol use disorders: implications for treatment. Exp Clin Psychopharmacol *10*, 193-212.

Beck, K.D., and Luine, V.N. (1999). Food deprivation modulates chronic stress effects on object recognition in male rats: role of monoamines and amino acids. Brain Res *830*, 56-71.

Beck, K.D., and Luine, V.N. (2002). Sex differences in behavioral and neurochemical profiles after chronic stress: role of housing conditions. Physiol Behav *75*, 661-673.

Becker, H.C., Lopez, M.F., and Doremus-Fitzwater, T.L. (2011). Effects of stress on alcohol drinking: a review of animal studies. Psychopharmacology (Berl) *218*, 131-156.

Belda, X., Fuentes, S., Nadal, R., and Armario, A. (2008). A single exposure to immobilization causes long-lasting pituitary-adrenal and behavioral sensitization to mild stressors. Horm Behav *54*, 654-661.

Bellani, R., Luecken, L.J., and Conrad, C.D. (2006). Peripubertal anxiety profile can predict predisposition to spatial memory impairments following chronic stress. Behav Brain Res *166*, 263-270.

Berger, B.D., and Adesso, V.J. (1991). Gender differences in using alcohol to cope with depression. Addict Behav *16*, 315-327.

Berman, R.F., and Hannigan, J.H. (2000). Effects of prenatal alcohol exposure on the hippocampus: spatial behavior, electrophysiology, and neuroanatomy. Hippocampus *10*, 94-110.

Berman, R.F., Hannigan, J.H., Sperry, M.A., and Zajac, C.S. (1996). Prenatal alcohol exposure and the effects of environmental enrichment on hippocampal dendritic spine density. Alcohol *13*, 209-216.

Berner, P., Lesch, O.M., and Walter, H. (1986). Alcohol and depression. Psychopathology *19 Suppl 2*, 177-183.

Berry, R.B., and Matthews, D.B. (2004). Acute ethanol administration selectively impairs spatial memory in C57BL/6J mice. Alcohol *32*, 9-18.

Bisagno, V., Grillo, C.A., Piroli, G.G., Giraldo, P., McEwen, B., and Luine, V.N. (2004). Chronic stress alters amphetamine effects on behavior and synaptophysin levels in female rats. Pharmacol Biochem Behav *78*, 541-550.

Boulouard, M., Lelong, V., Daoust, M., and Naassila, M. (2002). Chronic ethanol consumption induces tolerance to the spatial memory impairing effects of acute ethanol administration in rats. Behav Brain Res *136*, 239-246.

Bowers, W.J., Sabongui, A.G., and Amit, Z. (1997). The role of ethanol availability on stress-induced increases in ethanol consumption. Alcohol *14*, 551-556.

Bowman, R.E., Beck, K.D., and Luine, V.N. (2003). Chronic stress effects on memory: sex differences in performance and monoaminergic activity. Horm Behav *43*, 48-59.

Bowman, R.E., Ferguson, D., and Luine, V.N. (2002). Effects of chronic restraint stress and estradiol on open field activity, spatial memory, and monoaminergic neurotransmitters in ovariectomized rats. Neuroscience *113*, 401-410.

Bowman, R.E., Maclusky, N.J., Diaz, S.E., Zrull, M.C., and Luine, V.N. (2006). Aged rats: sex differences and responses to chronic stress. Brain Res *1126*, 156-166.

Bowman, R.E., Micik, R., Gautreaux, C., Fernandez, L., and Luine, V.N. (2009). Sex-dependent changes in anxiety, memory, and monoamines following one week of stress. Physiol Behav *97*, 21-29.

Boyce-Rustay, J.M., Cameron, H.A., and Holmes, A. (2007). Chronic swim stress alters sensitivity to acute behavioral effects of ethanol in mice. Physiol Behav *91*, 77-86.

Boyce-Rustay, J.M., Janos, A.L., and Holmes, A. (2008). Effects of chronic swim stress on EtOH-related behaviors in C57BL/6J, DBA/2J and BALB/cByJ mice. Behav Brain Res *186*, 133-137.

Boyce-Rustay, J.M., Wiedholz, L.M., Millstein, R.A., Carroll, J., Murphy, D.L., Daws, L.C., and Holmes, A. (2006). Ethanol-related behaviors in serotonin transporter knockout mice. Alcohol Clin Exp Res *30*, 1957-1965.

Breslin, F.C., Hayward, M., and Baum, A. (1994). Effect of stress on perceived intoxication and the blood alcohol curve in men and women. Health Psychol *13*, 479-487.

Brick, J., and Pohorecky, L.A. (1982). Ethanol-stress interaction: biochemical findings. Psychopharmacology (Berl) *77*, 81-84.

Broadbent, N.J., Squire, L.R., and Clark, R.E. (2004). Spatial memory, recognition memory, and the hippocampus. Proc Natl Acad Sci U S A *101*, 14515-14520.

Burattini, C., Gill, T.M., Aicardi, G., and Janak, P.H. (2006). The ethanol self-administration context as a reinstatement cue: acute effects of naltrexone. Neuroscience *139*, 877-887.

Cagetti, E., Pinna, G., Guidotti, A., Baicy, K., and Olsen, R.W. (2004). Chronic intermittent ethanol (CIE) administration in rats decreases levels of neurosteroids in hippocampus, accompanied by altered behavioral responses to neurosteroids and memory function. Neuropharmacology *46*, 570-579.

Calvo, N., Martijena, I.D., Molina, V.A., and Volosin, M. (1998). Metyrapone pretreatment prevents the behavioral and neurochemical sequelae induced by stress. Brain Res *800*, 227-235.

Carpenter-Hyland, E.P., and Chandler, L.J. (2006). Homeostatic plasticity during alcohol exposure promotes enlargement of dendritic spines. Eur J Neurosci *24*, 3496-3506.

Caspi, A., Sugden, K., Moffitt, T.E., Taylor, A., Craig, I.W., Harrington, H., McClay, J., Mill, J., Martin, J., Braithwaite, A.*, et al.* (2003). Influence of life stress on depression: moderation by a polymorphism in the 5-HTT gene. Science *301*, 386-389.

Chadda, R., and Devaud, L.L. (2005). Differential effects of mild repeated restraint stress on behaviors and GABA(A) receptors in male and female rats. Pharmacol Biochem Behav *81*, 854-863.

Chester, J.A., Blose, A.M., Zweifel, M., and Froehlich, J.C. (2004). Effects of stress on alcohol consumption in rats selectively bred for high or low alcohol drinking. Alcohol Clin Exp Res *28*, 385-393.

Chester, J.A., de Paula Barrenha, G., DeMaria, A., and Finegan, A. (2006). Different effects of stress on alcohol drinking behaviour in male and female mice selectively bred for high alcohol preference. Alcohol Alcohol *41*, 44-53.

Ciccocioppo, R., Panocka, I., Froldi, R., Colombo, G., Gessa, G.L., and Massi, M. (1999). Antidepressant-like effect of ethanol revealed in the forced swimming test in Sardinian alcohol-preferring rats. Psychopharmacology (Berl) *144*, 151-157.

Conrad, C.D. (2006). What is the functional significance of chronic stress-induced CA3 dendritic retraction within the hippocampus? Behav Cogn Neurosci Rev *5*, 41-60.

Conrad, C.D., Galea, L.A., Kuroda, Y., and McEwen, B.S. (1996). Chronic stress impairs rat spatial memory on the Y maze, and this effect is blocked by tianeptine pretreatment. Behav Neurosci *110*, 1321-1334.

Conrad, C.D., Grote, K.A., Hobbs, R.J., and Ferayorni, A. (2003). Sex differences in spatial and non-spatial Y-maze performance after chronic stress. Neurobiol Learn Mem *79*, 32-40.

Conrad, C.D., Jackson, J.L., Wieczorek, L., Baran, S.E., Harman, J.S., Wright, R.L., and Korol, D.L. (2004). Acute stress impairs spatial memory in male but not female rats: influence of estrous cycle. Pharmacol Biochem Behav *78*, 569-579.

Conrad, C.D., LeDoux, J.E., Magarinos, A.M., and McEwen, B.S. (1999). Repeated restraint stress facilitates fear conditioning independently of causing hippocampal CA3 dendritic atrophy. Behav Neurosci *113*, 902-913.

Conrad, C.D., McLaughlin, K.J., Harman, J.S., Foltz, C., Wieczorek, L., Lightner, E., and Wright, R.L. (2007). Chronic glucocorticoids increase hippocampal vulnerability to neurotoxicity under conditions that produce CA3 dendritic retraction but fail to impair spatial recognition memory. J Neurosci *27*, 8278-8285.

Conrad, C.D., McLaughlin, K.J., Huynh, T.N., El-Ashmawy, M., and Sparks, M. (2012). Chronic stress and a cyclic regimen of estradiol administration separately facilitate spatial memory: relationship with hippocampal CA1 spine density and dendritic complexity. Behav Neurosci *126*, 142-156.

Conway, K.P., Compton, W., Stinson, F.S., and Grant, B.F. (2006). Lifetime comorbidity of DSM-IV mood and anxiety disorders and specific drug use disorders: results from the National Epidemiologic Survey on Alcohol and Related Conditions. J Clin Psychiatry *67*, 247-257.

Cooper, M.L., Russell, M., Skinner, J.B., Frone, M.R., and Mudar, P. (1992). Stress and alcohol use: moderating effects of gender, coping, and alcohol expectancies. J Abnorm Psychol *101*, 139-152.

Crabbe, J.C., Harris, R.A., and Koob, G.F. (2011). Preclinical studies of alcohol binge drinking. Ann N Y Acad Sci *1216*, 24-40.

Cryan, J.F., and Lucki, I. (2000). Antidepressant-like behavioral effects mediated by 5-Hydroxytryptamine(2C) receptors. J Pharmacol Exp Ther *295*, 1120-1126.

Cryan, J.F., Markou, A., and Lucki, I. (2002). Assessing antidepressant activity in rodents: recent developments and future needs. Trends Pharmacol Sci *23*, 238-245.

Cui, B., Wu, M.Q., She, X.J., and Liu, H.T. (2009). [Effects of noise exposure on event-related potential P300 and mechanism in hippocampus of rats]. Zhongguo Ying Yong Sheng Li Xue Za Zhi *25*, 404-407.

Cullinan, W.E., Ziegler, D.R., and Herman, J.P. (2008). Functional role of local GABAergic influences on the HPA axis. Brain Struct Funct *213*, 63-72.

Dalla, C., Antoniou, K., Drossopoulou, G., Xagoraris, M., Kokras, N., Sfikakis, A., and Papadopoulou-Daifoti, Z. (2005). Chronic mild stress impact: are females more vulnerable? Neuroscience *135*, 703-714.

Dawson, G.R., and Tricklebank, M.D. (1995). Use of the elevated plus maze in the search for novel anxiolytic agents. Trends Pharmacol Sci *16*, 33-36.

Deroche, V., Piazza, P.V., Casolini, P., Maccari, S., Le Moal, M., and Simon, H. (1992). Stress-induced sensitization to amphetamine and morphine psychomotor effects depend on stress-induced corticosterone secretion. Brain Res *598*, 343-348.

Devenport, L., Stidham, J., and Hale, R. (1989). Ethanol and spatial localization. Behav Neurosci *103*, 1259-1266.

Dhabhar, F.S., McEwen, B.S., and Spencer, R.L. (1997). Adaptation to prolonged or repeated stress--comparison between rat strains showing intrinsic differences in reactivity to acute stress. Neuroendocrinology *65*, 360-368.

Dixit, A.R., and Crum, R.M. (2000). Prospective study of depression and the risk of heavy alcohol use in women. Am J Psychiatry *157*, 751-758.

Drapier, D., Bentue-Ferrer, D., Laviolle, B., Millet, B., Allain, H., Bourin, M., and Reymann, J.M. (2007). Effects of acute fluoxetine, paroxetine and desipramine on rats tested on the elevated plus-maze. Behav Brain Res *176*, 202-209.

Durand, D., and Carlen, P.L. (1984). Impairment of long-term potentiation in rat hippocampus following chronic ethanol treatment. Brain Res *308*, 325-332.

Eilam-Stock, T., Serrano, P., Frankfurt, M., and Luine, V. (2012). Bisphenol-A impairs memory and reduces dendritic spine density in adult male rats. Behav Neurosci *126*, 175-185.

Eiland, L., and McEwen, B.S. (2012). Early life stress followed by subsequent adult chronic stress potentiates anxiety and blunts hippocampal structural remodeling. Hippocampus *22*, 82-91.

Engelmann, M., Ebner, K., Landgraf, R., and Wotjak, C.T. (2006). Effects of Morris water maze testing on the neuroendocrine stress response and intrahypothalamic release of vasopressin and oxytocin in the rat. Horm Behav *50*, 496-501.

Ennaceur, A., and Aggleton, J.P. (1994). Spontaneous recognition of object configurations in rats: effects of fornix lesions. Exp Brain Res *100*, 85-92.

Ennaceur, A., Neave, N., and Aggleton, J.P. (1997). Spontaneous object recognition and object location memory in rats: the effects of lesions in the cingulate cortices, the medial prefrontal cortex, the cingulum bundle and the fornix. Exp Brain Res *113*, 509-519.

Farook, J.M., Lewis, B., Littleton, J.M., and Barron, S. (2009). Topiramate attenuates the stress-induced increase in alcohol consumption and preference in male C57BL/6J mice. Physiol Behav *96*, 189-193.

Follesa, P., and Ticku, M.K. (1995). Chronic ethanol treatment differentially regulates NMDA receptor subunit mRNA expression in rat brain. Brain Res Mol Brain Res *29*, 99-106.

Frezza, M., di Padova, C., Pozzato, G., Terpin, M., Baraona, E., and Lieber, C.S. (1990). High blood alcohol levels in women. The role of decreased gastric alcohol dehydrogenase activity and first-pass metabolism. N Engl J Med *322*, 95-99.

Galea, L.A., McEwen, B.S., Tanapat, P., Deak, T., Spencer, R.L., and Dhabhar, F.S. (1997). Sex differences in dendritic atrophy of CA3 pyramidal neurons in response to chronic restraint stress. Neuroscience *81*, 689-697.

Galea, S., Ahern, J., Resnick, H., Kilpatrick, D., Bucuvalas, M., Gold, J., and Vlahov, D. (2002). Psychological sequelae of the September 11 terrorist attacks in New York City. N Engl J Med *346*, 982-987.

Girotti, M., Weinberg, M.S., and Spencer, R.L. (2007). Differential responses of hypothalamus-pituitary-adrenal axis immediate early genes to corticosterone and circadian drive. Endocrinology *148*, 2542-2552.

Givens, B.S., and Breese, G.R. (1990). Site-specific enhancement of gamma-aminobutyric acid-mediated inhibition of neural activity by ethanol in the rat medial septal area. J Pharmacol Exp Ther *254*, 528-538.

Gomez, J.L., Lewis, M.J., and Luine, V.N. (2012). The interaction of chronic restraint stress and voluntary alcohol intake: Effects on spatial memory in male rats. Alcohol *46*, 499-504.

Gould, E., Woolley, C.S., Frankfurt, M., and McEwen, B.S. (1990). Gonadal steroids regulate dendritic spine density in hippocampal pyramidal cells in adulthood. J Neurosci *10*, 1286-1291.

Grant, B.F., and Harford, T.C. (1995). Comorbidity between DSM-IV alcohol use disorders and major depression: results of a national survey. Drug Alcohol Depend *39*, 197-206.

Grant, K.A., Valverius, P., Hudspith, M., and Tabakoff, B. (1990). Ethanol withdrawal seizures and the NMDA receptor complex. Eur J Pharmacol *176*, 289-296.

Griffiths, K.L., Mackey, M.G., and Adamson, B.J. (2011). Behavioral and psychophysiological responses to job demands and association with musculoskeletal symptoms in computer work. J Occup Rehabil *21*, 482-492.

Grobin, A.C., Matthews, D.B., Devaud, L.L., and Morrow, A.L. (1998). The role of GABA(A) receptors in the acute and chronic effects of ethanol. Psychopharmacology (Berl) *139*, 2-19.

Gulinello, M., Gong, Q.H., Li, X., and Smith, S.S. (2001). Short-term exposure to a neuroactive steroid increases alpha4 GABA(A) receptor subunit levels in association with increased anxiety in the female rat. Brain Res *910*, 55-66.

Gulinello, M., Gong, Q.H., and Smith, S.S. (2002). Progesterone withdrawal increases the alpha4 subunit of the GABA(A) receptor in male rats in association with anxiety and altered pharmacology - a comparison with female rats. Neuropharmacology *43*, 701-714.

Haney, M., Maccari, S., Le Moal, M., Simon, H., and Piazza, P.V. (1995). Social stress increases the acquisition of cocaine self-administration in male and female rats. Brain Res *698*, 46-52.

Hansson, A.C., Cippitelli, A., Sommer, W.H., Fedeli, A., Bjork, K., Soverchia, L., Terasmaa, A., Massi, M., Heilig, M., and Ciccocioppo, R. (2006). Variation at the rat Crhr1 locus and sensitivity to relapse into alcohol seeking induced by environmental stress. Proc Natl Acad Sci U S A *103*, 15236-15241.

Harbuz, M.S., and Lightman, S.L. (1992). Stress and the hypothalamo-pituitary-adrenal axis: acute, chronic and immunological activation. J Endocrinol *134*, 327-339.

Hellemans, K.G., Verma, P., Yoon, E., Yu, W.K., Young, A.H., and Weinberg, J. (2010). Prenatal alcohol exposure and chronic mild stress differentially alter depressive- and anxiety-like behaviors in male and female offspring. Alcohol Clin Exp Res *34*, 633-645.

Henry, C., Kabbaj, M., Simon, H., Le Moal, M., and Maccari, S. (1994). Prenatal stress increases the hypothalamo-pituitary-adrenal axis response in young and adult rats. J Neuroendocrinol *6*, 341-345.

Hernandez-Avila, C.A., Rounsaville, B.J., and Kranzler, H.R. (2004). Opioid-, cannabis- and alcohol-dependent women show more rapid progression to substance abuse treatment. Drug Alcohol Depend *74*, 265-272.

Hicklin, T.R., Wu, P.H., Radcliffe, R.A., Freund, R.K., Goebel-Goody, S.M., Correa, P.R., Proctor, W.R., Lombroso, P.J., and Browning, M.D. (2011). Alcohol inhibition of the NMDA receptor function, long-term potentiation, and fear learning requires striatal-enriched protein tyrosine phosphatase. Proc Natl Acad Sci U S A *108*, 6650-6655.

Hoffmann, S.E., and Matthews, D.B. (2001). Ethanol-induced impairments in spatial working memory are not due to deficits in learning. Alcohol Clin Exp Res *25*, 856-861.

Hommer, D., Momenan, R., Rawlings, R., Ragan, P., Williams, W., Rio, D., and Eckardt, M. (1996). Decreased corpus callosum size among alcoholic women. Arch Neurol *53*, 359-363.

Hu, X.J., Follesa, P., and Ticku, M.K. (1996). Chronic ethanol treatment produces a selective upregulation of the NMDA receptor subunit gene expression in mammalian cultured cortical neurons. Brain Res Mol Brain Res *36*, 211-218.

Hutchinson, K.M., McLaughlin, K.J., Wright, R.L., Bryce Ortiz, J., Anouti, D.P., Mika, A., Diamond, D.M., and Conrad, C.D. (2012). Environmental enrichment protects against the effects of chronic stress on cognitive and morphological measures of hippocampal integrity. Neurobiol Learn Mem *97*, 250-260.

Huynh, T.N., Krigbaum, A.M., Hanna, J.J., and Conrad, C.D. (2011). Sex differences and phase of light cycle modify chronic stress effects on anxiety and depressive-like behavior. Behav Brain Res *222*, 212-222.

Ising, H., and Ising, M. (2002). Chronic Cortisol Increases in the First Half of the Night Caused by Road Traffic Noise. Noise Health *4*, 13-21.

Izidio, G.S., and Ramos, A. (2007). Positive association between ethanol consumption and anxiety-related behaviors in two selected rat lines. Alcohol *41*, 517-524.

Jezova, D., Jurankova, E., Mosnarova, A., Kriska, M., and Skultetyova, I. (1996). Neuroendocrine response during stress with relation to gender differences. Acta Neurobiol Exp (Wars) *56*, 779-785.

Kalia, M. (2002). Assessing the economic impact of stress--the modern day hidden epidemic. Metabolism *51*, 49-53.

Kalluri, H.S., Mehta, A.K., and Ticku, M.K. (1998). Up-regulation of NMDA receptor subunits in rat brain following chronic ethanol treatment. Brain Res Mol Brain Res *58*, 221-224.

Kant, G.J., Lenox, R.H., Bunnell, B.N., Mougey, E.H., Pennington, L.L., and Meyerhoff, J.L. (1983). Comparison of stress response in male and female rats: pituitary cyclic AMP and plasma prolactin, growth hormone and corticosterone. Psychoneuroendocrinology *8*, 421-428.

Kessler, R.C. (2003). Epidemiology of women and depression. J Affect Disord *74*, 5-13.

Khantzian, E.J. (1997). The self-medication hypothesis of substance use disorders: a reconsideration and recent applications. Harv Rev Psychiatry *4*, 231-244.

Khisti, R.T., Boyd, K.N., Kumar, S., and Morrow, A.L. (2005). Systemic ethanol administration elevates deoxycorticosterone levels and chronic ethanol exposure attenuates this response. Brain Res *1049*, 104-111.

Kitraki, E., Kremmyda, O., Youlatos, D., Alexis, M., and Kittas, C. (2004). Spatial performance and corticosteroid receptor status in the 21-day restraint stress paradigm. Ann N Y Acad Sci *1018*, 323-327.

Kleen, J.K., Sitomer, M.T., Killeen, P.R., and Conrad, C.D. (2006). Chronic stress impairs spatial memory and motivation for reward without disrupting motor ability and motivation to explore. Behav Neurosci *120*, 842-851.

Kliethermes, C.L., Cronise, K., and Crabbe, J.C. (2004). Anxiety-like behavior in mice in two apparatuses during withdrawal from chronic ethanol vapor inhalation. Alcohol Clin Exp Res *28*, 1012-1019.

Koob, G.F. (1992). Drugs of abuse: anatomy, pharmacology and function of reward pathways. Trends Pharmacol Sci *13*, 177-184.

Koob, G.F., and Le Moal, M. (2008). Addiction and the brain antireward system. Annu Rev Psychol *59*, 29-53.

Kuczewski, N., Langlois, A., Fiorentino, H., Bonnet, S., Marissal, T., Diabira, D., Ferrand, N., Porcher, C., and Gaiarsa, J.L. (2008). Spontaneous glutamatergic activity induces a BDNF-dependent potentiation of GABAergic synapses in the newborn rat hippocampus. J Physiol *586*, 5119-5128.

Kushner, M.G., Sher, K.J., and Beitman, B.D. (1990). The relation between alcohol problems and the anxiety disorders. Am J Psychiatry *147*, 685-695.

Kushner, M.G., Sher, K.J., Wood, M.D., and Wood, P.K. (1994). Anxiety and drinking behavior: moderating effects of tension-reduction alcohol outcome expectancies. Alcohol Clin Exp Res *18*, 852-860.

Le, A.D., Harding, S., Juzytsch, W., Funk, D., and Shaham, Y. (2005). Role of alpha-2 adrenoceptors in stress-induced reinstatement of alcohol seeking and alcohol self-administration in rats. Psychopharmacology (Berl) *179*, 366-373.

Le, A.D., Poulos, C.X., Harding, S., Watchus, J., Juzytsch, W., and Shaham, Y. (1999). Effects of naltrexone and fluoxetine on alcohol self-administration and reinstatement of alcohol seeking induced by priming injections of alcohol and exposure to stress. Neuropsychopharmacology *21*, 435-444.

Lee, S., Kim, C.K., and Rivier, C. (1999). Nitric oxide stimulates ACTH secretion and the transcription of the genes encoding for NGFI-B, corticotropin-releasing factor, corticotropin-releasing factor receptor type 1, and vasopressin in the hypothalamus of the intact rat. J Neurosci *19*, 7640-7647.

Lee, S., and Rivier, C. (1997). An initial, three-day-long treatment with alcohol induces a long-lasting phenomenon of selective tolerance in the activity of the rat hypothalamic-pituitary-adrenal axis. J Neurosci *17*, 8856-8866.

Leggio, M.G., Mandolesi, L., Federico, F., Spirito, F., Ricci, B., Gelfo, F., and Petrosini, L. (2005). Environmental enrichment promotes improved spatial abilities and enhanced dendritic growth in the rat. Behav Brain Res *163*, 78-90.

Leuner, B., and Shors, T.J. (2004). New spines, new memories. Mol Neurobiol *29*, 117-130.

Levenson, R.W., Sher, K.J., Grossman, L.M., Newman, J., and Newlin, D.B. (1980). Alcohol and stress response dampening: pharmacological effects, expectancy, and tension reduction. J Abnorm Psychol *89*, 528-538.

Lewis, M.J. (1996). Alcohol reinforcement and neuropharmacological therapeutics. Alcohol Alcohol *1*, 17-25.

Li, C.S., Luo, X., Yan, P., Bergquist, K., and Sinha, R. (2009). Altered impulse control in alcohol dependence: neural measures of stop signal performance. Alcohol Clin Exp Res *33*, 740-750.

Lister, R.G. (1987). The use of a plus-maze to measure anxiety in the mouse. Psychopharmacology (Berl) *92*, 180-185.

Liu, X., and Weiss, F. (2003). Stimulus conditioned to foot-shock stress reinstates alcohol-seeking behavior in an animal model of relapse. Psychopharmacology (Berl) *168*, 184-191.

Lonstein, J.S. (2005). Reduced anxiety in postpartum rats requires recent physical interactions with pups, but is independent of suckling and peripheral sources of hormones. Horm Behav *47*, 241-255.

Luine, V. (2002). Sex differences in chronic stress effects on memory in rats. Stress *5*, 205-216.

Luine, V., Villegas, M., Martinez, C., and McEwen, B.S. (1994). Repeated stress causes reversible impairments of spatial memory performance. Brain Res *639*, 167-170.

Luine, V.N., Beck, K.D., Bowman, R.E., Frankfurt, M., and Maclusky, N.J. (2007). Chronic stress and neural function: accounting for sex and age. J Neuroendocrinol *19*, 743-751.

Luine, V.N., Jacome, L.F., and Maclusky, N.J. (2003). Rapid enhancement of visual and place memory by estrogens in rats. Endocrinology *144*, 2836-2844.

Luine, V.N., Spencer, R.L., and McEwen, B.S. (1993). Effects of chronic corticosterone ingestion on spatial memory performance and hippocampal serotonergic function. Brain Res *616*, 65-70.

Lynch, W.J., Kushner, M.G., Rawleigh, J.M., Fiszdon, J., and Carroll, M.E. (1999). The effects of restraint stress on voluntary ethanol consumption in rats. Exp Clin Psychopharmacol *7*, 318-323.

Macbeth, A.H., and Luine, V.N. (2010). Changes in anxiety and cognition due to reproductive experience: a review of data from rodent and human mothers. Neurosci Biobehav *34*, 452-467.

Madden, J.S. (1993). Alcohol and depression. Br J Hosp Med *50*, 261-264.

Magarinos, A.M., Deslandes, A., and McEwen, B.S. (1999). Effects of antidepressants and benzodiazepine treatments on the dendritic structure of CA3 pyramidal neurons after chronic stress. Eur J Pharmacol *371*, 113-122.

Magarinos, A.M., and McEwen, B.S. (1995). Stress-induced atrophy of apical dendrites of hippocampal CA3c neurons: involvement of glucocorticoid secretion and excitatory amino acid receptors. Neuroscience *69*, 89-98.

Magarinos, A.M., McEwen, B.S., Flugge, G., and Fuchs, E. (1996). Chronic psychosocial stress causes apical dendritic atrophy of hippocampal CA3 pyramidal neurons in subordinate tree shrews. J Neurosci *16*, 3534-3540.

Maier, D.M., and Pohorecky, L.A. (1986). The effect of ethanol and sex on radial arm maze performance in rats. Pharmacol Biochem Behav *25*, 703-709.

Mancinelli, R., Binetti, R., and Ceccanti, M. (2007). Woman, alcohol and environment: Emerging risks for health. Neurosci Biobehav Rev *31*, 246-253.

Mancinelli, R., Ceccanti, M., Guiducci, M.S., Sasso, G.F., Sebastiani, G., Attilia, M.L., and Allen, J.P. (2003). Simultaneous liquid chromatographic assessment of thiamine, thiamine monophosphate and thiamine diphosphate in human erythrocytes: a study on alcoholics. J Chromatogr B Analyt Technol Biomed Life Sci *789*, 355-363.

Marin, M.T., Cruz, F.C., and Planeta, C.S. (2007). Chronic restraint or variable stresses differently affect the behavior, corticosterone secretion and body weight in rats. Physiol Behav *90*, 29-35.

Marks-Kaufman, R., and Lewis, M.J. (1984). Early housing experience modifies morphine self-administration and physical dependence in adult rats. Addict Behav *9*, 235-243.

Martin, K.P., and Wellman, C.L. (2011). NMDA receptor blockade alters stress-induced dendritic remodeling in medial prefrontal cortex. Cereb Cortex *21*, 2366-2373.

Martinetti, M.P., Andrzejewski, M.E., Hineline, P.N., and Lewis, M.J. (2000). Ethanol consumption and the matching law: a choice analysis using a limited-access paradigm. Exp Clin Psychopharmacol *8*, 395-403.

Matthews, D.B., Ilgen, M., White, A.M., and Best, P.J. (1999). Acute ethanol administration impairs spatial performance while facilitating nonspatial performance in rats. Neurobiol Learn Mem *72*, 169-179.

Matthews, D.B., and Morrow, A.L. (2000). Effects of acute and chronic ethanol exposure on spatial cognitive processing and hippocampal function in the rat. Hippocampus *10*, 122-130.

Matthews, D.B., Morrow, A.L., O'Buckley, T., Flanigan, T.J., Berry, R.B., Cook, M.N., Mittleman, G., Goldowitz, D., Tokunaga, S., and Silvers, J.M. (2008). Acute mild footshock alters ethanol drinking and plasma corticosterone levels in C57BL/6J male mice, but not DBA/2J or A/J male mice. Alcohol *42*, 469-476.

Matthews, D.B., Morrow, A.L., Tokunaga, S., and McDaniel, J.R. (2002). Acute ethanol administration and acute allopregnanolone administration impair spatial memory in the Morris water task. Alcohol Clin Exp Res *26*, 1747-1751.

Mattucci-Schiavone, L., and Ferko, A.P. (1987). Effect of muscimol on ethanol-induced central nervous system depression. Pharmacol Biochem Behav *27*, 745-748.

McCormick, C.M., Smith, C., and Mathews, I.Z. (2008). Effects of chronic social stress in adolescence on anxiety and neuroendocrine response to mild stress in male and female rats. Behav Brain Res *187*, 228-238.

McEwen, B.S. (2008). Central effects of stress hormones in health and disease: Understanding the protective and damaging effects of stress and stress mediators. Eur J Pharmacol *583*, 174-185.

McEwen, B.S., Magarinos, A.M., and Reagan, L.P. (2002). Structural plasticity and tianeptine: cellular and molecular targets. Eur Psychiatry *17 Suppl 3*, 318-330.

McLaughlin, K.J., Baran, S.E., and Conrad, C.D. (2009). Chronic stress- and sex-specific neuromorphological and functional changes in limbic structures. Mol Neurobiol *40*, 166-182.

McLaughlin, K.J., Baran, S.E., Wright, R.L., and Conrad, C.D. (2005). Chronic stress enhances spatial memory in ovariectomized female rats despite CA3 dendritic retraction: possible involvement of CA1 neurons. Neuroscience *135*, 1045-1054.

McLaughlin, K.J., Bimonte-Nelson, H., Neisewander, J.L., and Conrad, C.D. (2008). Assessment of estradiol influence on spatial tasks and hippocampal CA1 spines: evidence that the duration of hormone deprivation after ovariectomy compromises 17beta-estradiol effectiveness in altering CA1 spines. Horm Behav *54*, 386-395.

McLaughlin, K.J., Gomez, J.L., Baran, S.E., and Conrad, C.D. (2007). The effects of chronic stress on hippocampal morphology and function: an evaluation of chronic restraint paradigms. Brain Res *1161*, 56-64.

McLaughlin, K.J., Wilson, J.O., Harman, J., Wright, R.L., Wieczorek, L., Gomez, J., Korol, D.L., and Conrad, C.D. (2010). Chronic 17beta-estradiol or cholesterol prevents stress-induced hippocampal CA3 dendritic retraction in ovariectomized female rats: possible correspondence between CA1 spine properties and spatial acquisition. Hippocampus *20*, 768-786.

McLay, R.N., Freeman, S.M., and Zadina, J.E. (1998). Chronic corticosterone impairs memory performance in the Barnes maze. Physiol Behav *63*, 933-937.

Menzaghi, F., Rassnick, S., Heinrichs, S., Baldwin, H., Pich, E.M., Weiss, F., and Koob, G.F. (1994). The role of corticotropin-releasing factor in the anxiogenic effects of ethanol withdrawal. Ann N Y Acad Sci *739*, 176-184.

Mezey, E., Potter, J.J., and Kvetnansky, R. (1979). Effect of stress by repeated immobilization on hepatic alcohol dehydrogenase activity and ethanol metabolism. Biochem Pharmacol *28*, 657-663.

Mitra, R., Vyas, A., Chatterjee, G., and Chattarji, S. (2005). Chronic-stress induced modulation of different states of anxiety-like behavior in female rats. Neurosci Lett *383*, 278-283.

Molina, V.A., Heyser, C.J., and Spear, L.P. (1994). Chronic variable stress or chronic morphine facilitates immobility in a forced swim test: reversal by naloxone. Psychopharmacology (Berl) *114*, 433-440.

Morris, R.G., Garrud, P., Rawlins, J.N., and O'Keefe, J. (1982). Place navigation impaired in rats with hippocampal lesions. Nature *297*, 681-683.

Morrow, A.L., Biggio, G., Serra, M., Becker, H.C., Lopez, M.F., Porcu, P., Alward, S.E., and O'Buckley, T.K. (2009). The role of neuroactive steroids in ethanol/stress interactions: proceedings of symposium VII at the Volterra conference on alcohol and stress, May 2008. Alcohol *43*, 521-530.

Murakami, S., Imbe, H., Morikawa, Y., Kubo, C., and Senba, E. (2005). Chronic stress, as well as acute stress, reduces BDNF mRNA expression in the rat hippocampus but less robustly. Neurosci Res *53*, 129-139.

Narita, M., Soma, M., Mizoguchi, H., Tseng, L.F., and Suzuki, T. (2000). Implications of the NR2B subunit-containing NMDA receptor localized in mouse limbic forebrain in ethanol dependence. Eur J Pharmacol *401*, 191-195.

Nisenbaum, L.K., Zigmond, M.J., Sved, A.F., and Abercrombie, E.D. (1991). Prior exposure to chronic stress results in enhanced synthesis and release of hippocampal norepinephrine in response to a novel stressor. J Neurosci *11*, 1478-1484.

Olivier, J.D., Van Der Hart, M.G., Van Swelm, R.P., Dederen, P.J., Homberg, J.R., Cremers, T., Deen, P.M., Cuppen, E., Cools, A.R., and Ellenbroek, B.A. (2008). A study in male and female 5-HT transporter knockout rats: an animal model for anxiety and depression disorders. Neuroscience *152*, 573-584.

Orchinik, M., Weiland, N.G., and McEwen, B.S. (1995). Chronic exposure to stress levels of corticosterone alters GABAA receptor subunit mRNA levels in rat hippocampus. Brain Res Mol Brain Res *34*, 29-37.

Padovan, C.M., Del Bel, E.A., and Guimaraes, F.S. (2000). Behavioral effects in the elevated plus maze of an NMDA antagonist injected into the dorsal hippocampus: influence of restraint stress. Pharmacol Biochem Behav *67*, 325-330.

Patterson-Buckendahl, P., Kubovcakova, L., Krizanova, O., Pohorecky, L.A., and Kvetnansky, R. (2005). Ethanol consumption increases rat stress hormones and adrenomedullary gene expression. Alcohol *37*, 157-166.

Pavlides, C., Nivon, L.G., and McEwen, B.S. (2002). Effects of chronic stress on hippocampal long-term potentiation. Hippocampus *12*, 245-257.

Paxinos, G., and Watson, C. (2005). The rat brain in stereotaxic coordinates, 5th edn (Amsterdam ; Boston, Elsevier Academic Press).

Pellow, S., Chopin, P., File, S.E., and Briley, M. (1985). Validation of open:closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. J Neurosci Methods *14*, 149-167.

Pfefferbaum, A., and Sullivan, E.V. (2005). Disruption of brain white matter microstructure by excessive intracellular and extracellular fluid in alcoholism: evidence from diffusion tensor imaging. Neuropsychopharmacology *30*, 423-432.

Piazza, P.V., Deminiere, J.M., le Moal, M., and Simon, H. (1990). Stress- and pharmacologically-induced behavioral sensitization increases vulnerability to acquisition of amphetamine self-administration. Brain Res *514*, 22-26.

Pietraszek, M.H., Urano, T., Sumioshi, K., Serizawa, K., Takahashi, S., Takada, Y., and Takada, A. (1991). Alcohol-induced depression: involvement of serotonin. Alcohol Alcohol *26*, 155-159.

Pitman, D.L., Ottenweller, J.E., and Natelson, B.H. (1988). Plasma corticosterone levels during repeated presentation of two intensities of restraint stress: chronic stress and habituation. Physiol Behav *43*, 47-55.

Ploj, K., Roman, E., and Nylander, I. (2003). Long-term effects of maternal separation on ethanol intake and brain opioid and dopamine receptors in male Wistar rats. Neuroscience *121*, 787-799.

Pohorecky, L.A. (1991). Stress and alcohol interaction: an update of human research. Alcohol Clin Exp Res *15*, 438-459.

Pohorecky, L.A. (2008). Psychosocial stress and chronic ethanol ingestion in male rats: effects on elevated plus maze behavior and ultrasonic vocalizations. Physiol Behav *94*, 432-447.

Porsolt, R.D., Le Pichon, M., and Jalfre, M. (1977). Depression: a new animal model sensitive to antidepressant treatments. Nature *266*, 730-732.

Porsolt, R.D., Le Pichon, M., and Jalfre, M. (1977). Depression: a new animal model sensitive to antidepressant treatments. Nature *266*, 730-732.

Porter, R.S., Kaplan, J.L., Homeier, B.P., and Albert, R.K. (2009). The Merck manual home health handbook, [3rd edn (Whitehouse Station, NJ, Merck Research Laboratories).

Prince, C.R., and Anisman, H. (1984). Acute and chronic stress effects on performance in a forced-swim task. Behav Neural Biol *42*, 99-119.

Pucilowski, O., Overstreet, D.H., Rezvani, A.H., and Janowsky, D.S. (1993). Chronic mild stress-induced anhedonia: greater effect in a genetic rat model of depression. Physiol Behav *54*, 1215-1220.

Radley, J.J., Rocher, A.B., Miller, M., Janssen, W.G., Liston, C., Hof, P.R., McEwen, B.S., and Morrison, J.H. (2006). Repeated stress induces dendritic spine loss in the rat medial prefrontal cortex. Cereb Cortex *16*, 313-320.

Ranney, A., and Petro, M.S. (2009). Resveratrol protects spatial learning in middle-aged C57BL/6 mice from effects of ethanol. Behav Pharmacol *20*, 330-336.

Rasmussen, D.D., Mitton, D.R., Green, J., and Puchalski, S. (2001). Chronic daily ethanol and withdrawal: 2. Behavioral changes during prolonged abstinence. Alcohol Clin Exp Res *25*, 999-1005.

Rassnick, S., Heinrichs, S.C., Britton, K.T., and Koob, G.F. (1993). Microinjection of a corticotropin-releasing factor antagonist into the central nucleus of the amygdala reverses anxiogenic-like effects of ethanol withdrawal. Brain Res *605*, 25-32.

Richardson, H.N., Lee, S.Y., O'Dell, L.E., Koob, G.F., and Rivier, C.L. (2008). Alcohol self-administration acutely stimulates the hypothalamic-pituitary-adrenal axis, but alcohol dependence leads to a dampened neuroendocrine state. Eur J Neurosci *28*, 1641-1653.

Rivier, C. (1993). Female rats release more corticosterone than males in response to alcohol: influence of circulating sex steroids and possible consequences for blood alcohol levels. Alcohol Clin Exp Res *17*, 854-859.

Roberto, M., Nelson, T.E., Ur, C.L., and Gruol, D.L. (2002). Long-term potentiation in the rat hippocampus is reversibly depressed by chronic intermittent ethanol exposure. J Neurophysiol *87*, 2385-2397.

Rockman, G.E., and Glavin, G.B. (1984). Ethanol-stress interaction: differences among ethanol-preferring rats' responses to restraint. Alcohol *1*, 293-295.

Roman, E., Ploj, K., and Nylander, I. (2004). Maternal separation has no effect on voluntary ethanol intake in female Wistar rats. Alcohol *33*, 31-39.

Roohi, N., and Hayee, S. (2010). Work stress related physiological responses in professional bus drivers. Acta Physiol Hung *97*, 408-416.

Roozendaal, B., Williams, C.L., and McGaugh, J.L. (1999). Glucocorticoid receptor activation in the rat nucleus of the solitary tract facilitates memory consolidation: involvement of the basolateral amygdala. Eur J Neurosci *11*, 1317-1323.

Rosenbaum, R.S., Winocur, G., Grady, C.L., Ziegler, M., and Moscovitch, M. (2007). Memory for familiar environments learned in the remote past: fMRI studies of healthy people and an amnesic person with extensive bilateral hippocampal lesions. Hippocampus *17*, 1241-1251.

Roske, I., Baeger, I., Frenzel, R., and Oehme, P. (1994). Does a relationship exist between the quality of stress and the motivation to ingest alcohol? Alcohol *11*, 113-124.

Russo, S.J., Wilkinson, M.B., Mazei-Robison, M.S., Dietz, D.M., Maze, I., Krishnan, V., Renthal, W., Graham, A., Birnbaum, S.G., Green, T.A., *et al.* (2009). Nuclear factor kappa B signaling regulates neuronal morphology and cocaine reward. J Neurosci *29*, 3529-3537.

Ryabinin, A.E., Melia, K.R., Cole, M., Bloom, F.E., and Wilson, M.C. (1995). Alcohol selectively attenuates stress-induced c-fos expression in rat hippocampus. J Neurosci *15*, 721-730.

Ryabinin, A.E., Miller, M.N., and Durrant, S. (2002). Effects of acute alcohol administration on object recognition learning in C57BL/6J mice. Pharmacol Biochem Behav *71*, 307-312.

Ryabinin, A.E., Wang, Y.M., Freeman, P., and Risinger, F.O. (1999). Selective effects of alcohol drinking on restraint-induced expression of immediate early genes in mouse brain. Alcohol Clin Exp Res *23*, 1272-1280.

Sacharczuk, M., Juszczak, G., Swiergiel, A.H., Jaszczak, K., Lipkowski, A.W., and Sadowski, B. (2009). Alcohol reverses depressive and pronociceptive effects of chronic stress in mice with enhanced activity of the opioid system. Acta Neurobiol Exp (Wars) *69*, 459-468.

Sacktor, T.C., Osten, P., Valsamis, H., Jiang, X., Naik, M.U., and Sublette, E. (1993). Persistent activation of the zeta isoform of protein kinase C in the maintenance of long-term potentiation. Proc Natl Acad Sci U S A *90*, 8342-8346.

Santucci, A.C., Cortes, C., Bettica, A., and Cortes, F. (2008). Chronic ethanol consumption in rats produces residual increases in anxiety 4 months after withdrawal. Behav Brain Res *188*, 24-31.

Sapolsky, R.M. (1997). McEwen-Induced Modulation of Endocrine History: A Partial Review. Stress *2*, 1-12.

Sapolsky, R.M., Krey, L.C., and McEwen, B.S. (1983). The adrenocortical stress-response in the aged male rat: impairment of recovery from stress. Exp Gerontol *18*, 55-64.

Sartorius, N., Ustun, T.B., Lecrubier, Y., and Wittchen, H.U. (1996). Depression comorbid with anxiety: results from the WHO study on psychological disorders in primary health care. Br J Psychiatry Suppl, 38-43.

Savage, L.M., Candon, P.M., and Hohmann, H.L. (2000). Alcohol-induced brain pathology and behavioral dysfunction: using an animal model to examine sex differences. Alcohol Clin Exp Res *24*, 465-475.

Schaaf, M.J., de Jong, J., de Kloet, E.R., and Vreugdenhil, E. (1998). Downregulation of BDNF mRNA and protein in the rat hippocampus by corticosterone. Brain Res *813*, 112-120.

Schneider, A.M., Simson, P.E., Spiller, K., Adelstein, J., Vacharat, A., Short, K.R., and Kirby, L.G. (2009). Stress-dependent enhancement and impairment of retention by naloxone: evidence for an endogenous opioid-based modulatory system protective of memory. Behav Brain Res *205*, 290-293.

Schuckit, M.A., Gold, E., and Risch, C. (1987). Plasma cortisol levels following ethanol in sons of alcoholics and controls. Arch Gen Psychiatry *44*, 942-945.

Schuckit, M.A., Risch, S.C., and Gold, E.O. (1988). Alcohol consumption, ACTH level, and family history of alcoholism. Am J Psychiatry *145*, 1391-1395.

Schummers, J., and Browning, M.D. (2001). Evidence for a role for GABA(A) and NMDA receptors in ethanol inhibition of long-term potentiation. Brain Res Mol Brain Res *94*, 9-14.

Scullion, G.A., Kendall, D.A., Sunter, D., Marsden, C.A., and Pardon, M.C. (2009). Central noradrenergic depletion by DSP-4 prevents stress-induced memory impairments in the object recognition task. Neuroscience *164*, 415-423.

Seeley, J.R. (1960). Death by liver cirrhosis and the price of beverage alcohol. Can Med Assoc J *83*, 1361-1366.

Selye, H. (1956). What is stress? Metabolism *5*, 525-530.

Shirao, I., Tsuda, A., Ida, Y., Tsujimaru, S., Satoh, H., Oguchi, M., Tanaka, M., and Inanaga, K. (1988). Effect of acute ethanol administration on noradrenaline metabolism in brain regions of stressed and nonstressed rats. Pharmacol Biochem Behav *30*, 769-773.

Shors, T.J. (2001). Acute stress rapidly and persistently enhances memory formation in the male rat. Neurobiol Learn Mem *75*, 10-29.

Shors, T.J., Lewczyk, C., Pacynski, M., Mathew, P.R., and Pickett, J. (1998). Stages of estrous mediate the stress-induced impairment of associative learning in the female rat. Neuroreport *9*, 419-423.

Sillaber, I., Rammes, G., Zimmermann, S., Mahal, B., Zieglgansberger, W., Wurst, W., Holsboer, F., and Spanagel, R. (2002). Enhanced and delayed stress-induced alcohol drinking in mice lacking functional CRH1 receptors. Science *296*, 931-933.

Sinha, R. (2001). How does stress increase risk of drug abuse and relapse? Psychopharmacology (Berl) *158*, 343-359.

Sinha, R. (2008). Chronic stress, drug use, and vulnerability to addiction. Ann N Y Acad Sci *1141*, 105-130.

Sircar, R., Basak, A.K., and Sircar, D. (2009). Repeated ethanol exposure affects the acquisition of spatial memory in adolescent female rats. Behav Brain Res *202*, 225-231.

Sircar, R., and Sircar, D. (2005). Adolescent rats exposed to repeated ethanol treatment show lingering behavioral impairments. Alcohol Clin Exp Res *29*, 1402-1410.

Sommer, W.H., Rimondini, R., Hansson, A.C., Hipskind, P.A., Gehlert, D.R., Barr, C.S., and Heilig, M.A. (2008). Upregulation of voluntary alcohol intake, behavioral sensitivity to stress, and amygdala crhr1 expression following a history of dependence. Biol Psychiatry *63*, 139-145.

Spencer, R.L., and McEwen, B.S. (1990). Adaptation of the hypothalamic-pituitary-adrenal axis to chronic ethanol stress. Neuroendocrinology *52*, 481-489.

Spencer, R.L., and McEwen, B.S. (1997). Impaired adaptation of the hypothalamic-pituitary-adrenal axis to chronic ethanol stress in aged rats. Neuroendocrinology *65*, 353-359.

Spinetta, M.J., Woodlee, M.T., Feinberg, L.M., Stroud, C., Schallert, K., Cormack, L.K., and Schallert, T. (2008). Alcohol-induced retrograde memory impairment in rats: prevention by caffeine. Psychopharmacology (Berl) *201*, 361-371.

Sprague, J.E., and Maickel, R.P. (1994). Effects of stress and ebiratide (Hoe-427) on free-choice ethanol consumption: comparison of Lewis and Sprague-Dawley rats. Life Sci *55*, 873-878.

Squire, L.R. (1992). Memory and the hippocampus: a synthesis from findings with rats, monkeys, and humans. Psychol Rev *99*, 195-231.

Steigerwald, E.S., and Miller, M.W. (1997). Performance by adult rats in sensory-mediated radial arm maze tasks is not impaired and may be transiently enhanced by chronic exposure to ethanol. Alcohol Clin Exp Res *21*, 1553-1559.

Swanson, L.W. (2000). Cerebral hemisphere regulation of motivated behavior. Brain Res *886*, 113-164.

Talani, G., Biggio, G., and Sanna, E. (2011). Enhanced Sensitivity to Ethanol-Induced Inhibition of LTP in CA1 Pyramidal Neurons of Socially Isolated C57BL/6J Mice: Role of Neurosteroids. Front Endocrinol (Lausanne) *2*, 56.

Thatcher-Britton, K., and Koob, G.F. (1986). Alcohol reverses the proconflict effect of corticotropin-releasing factor. Regul Pept *16*, 315-320.

Thayer, J.F., Hall, M., Sollers, J.J., 3rd, and Fischer, J.E. (2006). Alcohol use, urinary cortisol, and heart rate variability in apparently healthy men: Evidence for impaired inhibitory control of the HPA axis in heavy drinkers. Int J Psychophysiol *59*, 244-250.

Tokunaga, S., McDaniel, J.R., Morrow, A.L., and Matthews, D.B. (2003). Effect of acute ethanol administration and acute allopregnanolone administration on spontaneous hippocampal pyramidal cell neural activity. Brain Res *967*, 273-280.

Torres, J.M., and Ortega, E. (2004). Alcohol intoxication increases allopregnanolone levels in male adolescent humans. Psychopharmacology (Berl) *172*, 352-355.

Tretter, V., Revilla-Sanchez, R., Houston, C., Terunuma, M., Havekes, R., Florian, C., Jurd, R., Vithlani, M., Michels, G., Couve, A., *et al.* (2009). Deficits in spatial memory correlate with modified {gamma}-aminobutyric acid type A receptor tyrosine phosphorylation in the hippocampus. Proc Natl Acad Sci U S A *106*, 20039-20044.

Trevisan, L., Fitzgerald, L.W., Brose, N., Gasic, G.P., Heinemann, S.F., Duman, R.S., and Nestler, E.J. (1994). Chronic ingestion of ethanol up-regulates NMDAR1 receptor subunit immunoreactivity in rat hippocampus. J Neurochem *62*, 1635-1638.

Trudeau, L.E., Aragon, C.M., and Amit, Z. (1990). Effects of ethanol on locomotor depression and corticosterone release induced by restraint-stress: support for a stress-ethanol interaction. Pharmacol Biochem Behav *36*, 273-278.

Trudeau, L.E., Aragon, C.M., and Amit, Z. (1991). Involvement of endogenous opioid mechanisms in the interaction between stress and ethanol. Psychopharmacology (Berl) *103*, 425-429.

Tsien, J.Z., Huerta, P.T., and Tonegawa, S. (1996). The essential role of hippocampal CA1 NMDA receptor-dependent synaptic plasticity in spatial memory. Cell *87*, 1327-1338.

Ukai, W., Ishii, T., Hashimoto, E., Tateno, M., Yoshinaga, T., Ono, T., Watanabe, K., Watanabe, I., Shirasaka, T., and Saito, T. (2009). The common aspects of pathophysiolgy of alcoholism and depression. Nihon Arukoru Yakubutsu Igakkai Zasshi *44*, 704-711.

Valdez, G.R., Roberts, A.J., Chan, K., Davis, H., Brennan, M., Zorrilla, E.P., and Koob, G.F. (2002). Increased ethanol self-administration and anxiety-like behavior during acute ethanol withdrawal and protracted abstinence: regulation by corticotropin-releasing factor. Alcohol Clin Exp Res *26*, 1494-1501.

Valdez, G.R., Zorrilla, E.P., Roberts, A.J., and Koob, G.F. (2003). Antagonism of corticotropin-releasing factor attenuates the enhanced responsiveness to stress observed during protracted ethanol abstinence. Alcohol *29*, 55-60.

Vallee, M., Mayo, W., Dellu, F., Le Moal, M., Simon, H., and Maccari, S. (1997). Prenatal stress induces high anxiety and postnatal handling induces low anxiety in adult offspring: correlation with stress-induced corticosterone secretion. J Neurosci *17*, 2626-2636.

Venzala, E., Garcia-Garcia, A.L., Elizalde, N., and Tordera, R.M. (2012). Social vs. environmental stress models of depression from a behavioural and neurochemical approach. Eur Neuropsychopharmacol.

Verkuyl, J.M., Karst, H., and Joels, M. (2005). GABAergic transmission in the rat paraventricular nucleus of the hypothalamus is suppressed by corticosterone and stress. Eur J Neurosci *21*, 113-121.

Vlahov, D., Galea, S., Resnick, H., Ahern, J., Boscarino, J.A., Bucuvalas, M., Gold, J., and Kilpatrick, D. (2002). Increased use of cigarettes, alcohol, and marijuana among Manhattan, New York, residents after the September 11th terrorist attacks. Am J Epidemiol *155*, 988-996.

Vyas, A., Mitra, R., Shankaranarayana Rao, B.S., and Chattarji, S. (2002). Chronic stress induces contrasting patterns of dendritic remodeling in hippocampal and amygdaloid neurons. J Neurosci *22*, 6810-6818.

Walf, A.A., and Frye, C.A. (2005). Antianxiety and antidepressive behavior produced by physiological estradiol regimen may be modulated by hypothalamic-pituitary-adrenal axis activity. Neuropsychopharmacology *30*, 1288-1301.

Walker, B.M., Drimmer, D.A., Walker, J.L., Liu, T., Mathe, A.A., and Ehlers, C.L. (2010). Effects of prolonged ethanol vapor exposure on forced swim behavior, and neuropeptide Y and corticotropin-releasing factor levels in rat brains. Alcohol *44*, 487-493.

Walker, D.W., Barnes, D.E., Zornetzer, S.F., Hunter, B.E., and Kubanis, P. (1980). Neuronal loss in hippocampus induced by prolonged ethanol consumption in rats. Science *209*, 711-713.

Wang, J.Q., and McGinty, J.F. (1996). Acute methamphetamine-induced zif/268, preprodynorphin, and preproenkephalin mRNA expression in rat striatum depends on activation of NMDA and kainate/AMPA receptors. Brain Res Bull *39*, 349-357.

Weinberger, A.H., Maciejewski, P.K., McKee, S.A., Reutenauer, E.L., and Mazure, C.M. (2009). Gender differences in associations between lifetime alcohol, depression, panic disorder, and posttraumatic stress disorder and tobacco withdrawal. Am J Addict *18*, 140-147.

White, A.M., and Best, P.J. (2000). Effects of ethanol on hippocampal place-cell and interneuron activity. Brain Res *876*, 154-165.

Whitlock, J.R., Heynen, A.J., Shuler, M.G., and Bear, M.F. (2006). Learning induces long-term potentiation in the hippocampus. Science *313*, 1093-1097.

Williams, T.J., Torres-Reveron, A., Chapleau, J.D., and Milner, T.A. (2011). Hormonal regulation of delta opioid receptor immunoreactivity in interneurons and pyramidal cells in the rat hippocampus. Neurobiol Learn Mem *95*, 206-220.

Wilson, M.A., Burghardt, P.R., Ford, K.A., Wilkinson, M.B., and Primeaux, S.D. (2004). Anxiolytic effects of diazepam and ethanol in two behavioral models: comparison of males and females. Pharmacol Biochem Behav *78*, 445-458.

Windle, R.J., Wood, S.A., Shanks, N., Lightman, S.L., and Ingram, C.D. (1998). Ultradian rhythm of basal corticosterone release in the female rat: dynamic interaction with the response to acute stress. Endocrinology *139*, 443-450.

Wright, R.L., and Conrad, C.D. (2005). Chronic stress leaves novelty-seeking behavior intact while impairing spatial recognition memory in the Y-maze. Stress *8*, 151-154.

Wright, R.L., and Conrad, C.D. (2008). Enriched environment prevents chronic stress-induced spatial learning and memory deficits. Behav Brain Res *187*, 41-47.

Wright, R.L., Lightner, E.N., Harman, J.S., Meijer, O.C., and Conrad, C.D. (2006). Attenuating corticosterone levels on the day of memory assessment prevents chronic stress-induced impairments in spatial memory. Eur J Neurosci *24*, 595-605.

Yaroslavsky, I., and Tejani-Butt, S.M. (2010). Voluntary alcohol consumption alters stress-induced changes in dopamine-2 receptor binding in Wistar-Kyoto rat brain. Pharmacol Biochem Behav *94*, 471-476.

Ylikahri, R.H., Huttunen, M.O., and Harkonen, M. (1980). Hormonal changes during alcohol intoxication and withdrawal. Pharmacol Biochem Behav *13 Suppl 1*, 131-137.

Zarrindast, M.R., Babapoor-Farrokhran, S., and Rezayof, A. (2008). Involvement of opioidergic system of the ventral hippocampus, the nucleus accumbens or the central amygdala in anxiety-related behavior. Life Sci *82*, 1175-1181.

Zhang, J.M., Tonelli, L., Regenold, W.T., and McCarthy, M.M. (2010). Effects of neonatal flutamide treatment on hippocampal neurogenesis and synaptogenesis correlate with depression-like behaviors in preadolescent male rats. Neuroscience *169*, 544-554.

Zhang, Z., Morse, A.C., Koob, G.F., and Schulteis, G. (2007). Dose- and time-dependent expression of anxiety-like behavior in the elevated plus-maze during withdrawal from acute and repeated intermittent ethanol intoxication in rats. Alcohol Clin Exp Res *31*, 1811-1819.

Zimmerberg, B., Martinez, A.R., Skudder, C.M., Killien, E.Y., Robinson, S.A., and Brunelli, S.A. (2010). Effects of gestational allopregnanolone administration in rats bred for high affective behavior. Physiol Behav *99*, 212-217.

Zironi, I., Burattini, C., Aicardi, G., and Janak, P.H. (2006). Context is a trigger for relapse to alcohol. Behav Brain Res *167*, 150-155.