Spring 2015

Chronic Stress during Adolescence Alters Alcohol-Induced Conditioned Place Preference in Mice Selectively Bred for High Alcohol Preference but no Low Alcohol Preference

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Graduate School Form 30
Updated 1/15/2015

PURDUE UNIVERSITY
GRADUATE SCHOOL
Thesis/Dissertation Acceptance

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By Kristen R. Breit

Entitled

Chronic Stress During Adolescence Alters Alcohol-Induced Conditioned Place Preference in Mice Selectively Bred for High Alcohol Preference but not Low Alcohol Preference

For the degree of Doctor of Philosophy

Is approved by the final examining committee:

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CHRONIC STRESS DURING ADOLESCENCE ALTERS ALCOHOL-INDUCED CONDITIONED PLACE PREFERENCE IN MICE SELECTIVELY BRED FOR HIGH ALCOHOL PREFERENCE BUT NOT LOW ALCOHOL PREFERENCE

A Dissertation

Submitted to the Faculty

of

Purdue University

by

Kristen R. Breit

In Partial Fulfillment of the

Requirements for the Degree

of

Doctor of Philosophy

May 2015

Purdue University

West Lafayette, Indiana

ACKNOWLEDGEMENTS

In reality, I would like to thank everyone that has crossed my path for bringing me to this point in my life, because I am sure you all influenced my life choices in some way or another. I realize that doing so would make my acknowledgements section longer than the body of this work. So, I will mention a few specifics, and I hope anyone not mentioned by name accepts my humble attempt to include everyone that has enabled me to pursue this degree.

I would like to thank my Ph.D. advisor, Dr. Julia Chester, for bringing me into her laboratory space and encouraging me to complete the challenging project I wanted to pursue. Thank you, Julia, for not telling me it was too large or too complicated, and for helping me figure out what it all meant in the end when it did turn out to be long and complicated. Thank you for the lengthy conversations, both in person and via email, and for always supporting all of my decisions after I joined your lab. I am so glad I finished my graduate career with you!

I would like to thank the members of the Chester lab for all of their help, both professional and social. Matt, because of you, I am now also a CPP master (at least I hope you think so!). Molly, thank you for all of the breeding work you did for me. I will never be able to repay you for that monstrosity. Marcus, thanks for being awesome and always working around my scheduling and breeding demands. Aaron, thank you for taking all of the blood samples…I legitimately could not have finished this project

without your help. You are much better at handling those animals and taking blood samples than I could ever hope to be.

Many thanks to my committee members, as well. You always provided surprising and challenging questions, which encouraged me to think critically about my work. Susie, Ed, and Steve, I appreciate your input more than you probably know. Thank you for listening to all of the presentations and reading all of the papers in the past three years. Your valuable comments will impact how I interpret my future scientific work.

It would not be right to miss an opportunity to acknowledge my M.A. advisor, Dr. Jennifer Thomas. Although I completed my Ph.D. at a different university and with a different advisor, Jen provided me with a firm base in the research process. I had never touched a laboratory rodent before you graciously accepted me into your lab. The two years I spent in the Thomas lab evoked a love for science and the scientific community within me that has followed me through my last three years at Purdue. So, thank you Jen, and thanks to the rest of the Thomas lab as well (Lia, Joe, and Kyle, this means you!).

Thank you to my friends back home in California for supporting my decision to move from a place with earthquakes to a place with tornados. You guys have always stayed in touch with text messages, phone calls, Skype calls, and countless Facebook posts and Snapchats. Sometimes it feels like I never left you all (although it does when there's snow on the ground). I'm sure I will forget a few, but thank you Betsey, Lindsey, Marcus, Adam, Julie, Michael, Justin, Stephanie, Arthur, Kelsey, Daryl, and everybody else back home in California. West coast, best coast!

Many, many thanks to my wonderful and encouraging boyfriend, Aaron. This has been a rough journey for me, and it must have been rough for you, as well. Thank

you for never giving me a hard time when I want to work late, sleep early, or just take some time to myself. Most importantly, thank you for being so supportive of my choices, and being willing to do whatever makes me happy. I am eternally grateful for your presence in my life, and so happy that I get to celebrate this accomplishment with you.

Lastly, to my family, Mom, Dad, and Jentry. I cannot express how lucky I am to have a family that fully supported my nomad lifestyle as a graduate student. You have always put my best interest as your priority, even though I know you hated that I moved away to Indiana. I appreciate the daily text messages and phone calls, and the occasional traditional Breit-style gifts in the mail. I am so happy to have you all as my family, and I love you to the moon and back! (And thank you, Brewski, for never having a vendetta against my laptop.)

I would like to dedicate this work to my grandfather, Joe Breit, and my grandmother, Leonore Boulden. I hope this accomplishment makes you proud. I miss you both.

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ABSTRACT

Breit, Kristen R. Ph.D., Purdue University, May 2015. Chronic Stress During Adolescence Alters Alcohol-Induced Conditioned Place Preference in Mice Selectively Bred for High Alcohol Preference but not Low Alcohol Preference. Major Professor: Julia A. Chester.

Chronic stress exposure during adolescence is associated with more long-lasting negative consequences than exposure during adulthood. Adolescent chronic stress exposure has long-lasting effects on physiology and behavior, including an increased risk of developing an alcohol use disorder (AUD) later in life. This relationship is particularly true in individuals with a familial history of AUDs. Recent research has shown that chronic stress in adolescent mice increased voluntary alcohol consumption in adulthood, but did not do so in adult mice. However, little is known about the mechanism of the relationship between adolescent chronic stress and increased alcohol consumption in adulthood. Evidence suggests that chronic stress exposure during adolescence has long-term effects on the developing brain, including areas important for sensitivity to the rewarding effects of alcohol. The over-arching aim of the current study was to explore the effects of adolescent chronic stress on sensitivity to the motivational effects of alcohol in adulthood. Three stress treatment groups were used, including subjects exposed to stress during adolescence, subjects exposed to stress during adulthood, and subjects not exposed to stress. Within each stress treatment group, high-alcohol preferring (HAP2) and low-alcohol preferring (LAP2) mice were represented, to mimic differences in familial AUD history. Thirty days after stress exposure, all subjects began a conditioned place preference (CPP) paradigm, a behavioral task that measures the sensitivity to alcohol's rewarding effects. Since re-exposure to a stressor has been associated with an increased risk in relapse and other drug-seeking behaviors, half of the subjects in each stress treatment group were re-exposed to the original stressor (RS) before the CPP posttest. Overall, LAP2 mice showed greater CPP than HAP2 mice, which supports more recent literature

suggesting that an inverse relationship between alcohol consumption and CPP expression may exist. In contrast to what was hypothesized, adolescent stress exposure decreased CPP expression in the HAP2 subjects during the first portion of testing. This finding may support an inverse relationship between alcohol consumption and CPP expression, when interpreted such that subjects exposed to stress during adolescence may drink more during adulthood because they are less sensitive to the rewarding effects of alcohol. In LAP2 subjects, there were no differences in CPP expression between the stress treatment groups, supporting past research suggesting that HAP2 mice are more sensitive to alterations in drug-related behaviors following stress exposure. RS did not produce alterations in CPP in either line. Overall, the findings of the current study suggest that one explanation for why individuals exposed to stress during adolescence may increase alcohol consumption during adulthood might be because more alcohol is required in order to reach the desired perceived rewarding effects of the drug, especially in those with a familial history of AUDs.

INTRODUCTION

The term alcohol use disorder (AUD) is used to encompass the spectrum of alcohol abuse, alcohol dependence, and alcoholism (Boschloo, van den Brink, Penninx, Wall, & Hasin, 2012). Approximately 18 million individuals in the United States suffer from AUDs, and at least 100,000 deaths per year are related to alcohol use (Li, Hewitt, & Grant, 2004), costing the nation 235 billion dollars annually (Gunzerath, Hewitt, Li, & Warren, 2011). Understanding what makes an individual more susceptible to developing specific alcohol-related behaviors is vital to the prediction and treatment of AUDs. The literature on the development of AUD characteristics is multi-faceted, such that a variety of genetic and environmental influences may interact and influence the likelihood that an individual could develop an AUD throughout his or her lifetime. One important environmental factor that can influence one's likeliness of developing an AUD is stress exposure, which has been associated with an increased risk of AUD development (Enoch, 2011). However, there are a variety of ways in which stress exposure may alter alcohol-related behaviors, and the mechanism of this relationship may differ depending on specific characteristics of the stressor. By better understanding what features of stress exposures influence specific characteristics of alcohol's effects, we will be better able to understand the complicated relationship between stress exposure and alcohol-related behaviors, and better predict and treat individuals with AUDs in at-risk populations.

Stress Exposure

The Hypothalamic Pituitary Adrenal Axis

Stress is a complex physiological response to a stimulus that can have both immediate and long-term consequences on behavior. A stressor is a stimulus that evokes a stress response. When an individual is in the presence of a stressor, the hypothalamic pituitary adrenal (HPA) axis, or stress axis, is activated. Corticotrophin-releasing hormone (CRH) is released by the hypothalamus, signaling the pituitary to

discharge adrenocorticotrophin (ACTH), resulting in the release of glucocorticoids and norepinephrine (NE) from the adrenal glands into the body (Elenkov, Webster, Torpy, & Chrousos, 1999). The glucocorticoid in humans is cortisol, and in rodents it is corticosterone (CORT). When glucocorticoids are released into the body, they signal the hypothalamus and pituitary gland to stop releasing CRH and ACTH; this is known as an HPA negative feedback loop (Kudielka & Kirschbaum, 2005). In this way, the HPA axis self-regulates responses to stress exposure. However, an alteration in the function of the HPA axis can lead to maladaptive responses to stress. Glucocorticoid exposure is associated with alterations in emotion and cognition in the face of an immediate stressor, such as an increase in alertness. Excessive glucocorticoid exposure has been associated with long-term and sometimes maladaptive alterations in the HPA axis, such as an inefficient or overactive HPA negative feedback loop, which can lead to excessive or blunted glucocorticoid release in the face of a stressor. These types of alterations may lead to increases in anxiety-related behaviors and alterations in drug-related behaviors (Kudielka & Kirschbaum, 2005). The current study will focus on both immediate and long-term changes in CORT levels following stress exposure, and how these may be related to alcohol-related behaviors.

The Stress Vulnerability Hypothesis

Importantly, stress is a natural response that promotes survival, and individuals can be either vulnerable to the negative consequences of stress or resilient after stress exposure (Charney & Manji, 2004). The vulnerability hypothesis states that long-lasting consequences of stress may not result from stress exposure alone, but from a pre-existing level of vulnerability an individual has for the development of anxiety-related behaviors that interacts with other genetic influences or environmental factors, like stress exposure (Charney & Manji, 2004). Stress is not inherently negative; stress can be viewed as either positive or negative, depending on the type of stressor and the resulting individual consequences (Lupien, McEwen, Gunnar, & Heim, 2009). However, repeated exposure to stressors may produce maladaptive effects, as repeated stress exposure is associated with an increased susceptibility to developing psychopathology and drug addiction. In general, stressors such as maltreatment or other taxing life events, including divorce, violence, death, or illness, have been associated with harmful emotional and cognitive consequences compared to minute daily stressors (Enoch, 2011). Unfortunately, exposure to both maltreatment

and other stressful life events is fairly common worldwide in both child and adult populations (Enoch, 2011).

Specific characteristics of stress exposure may predict whether positive or negative consequences may result, including stress exposure length, the age at which stress exposure occurs (see review by McCormick, Mathews, Thomas, & Waters, 2010), severity of the stressor, predictability of the stressor, re-exposure to the stressor, and differences in the individual that may make them more or less likely to develop maladaptive physical and behavioral stress-related alterations (Lupien et al., 2009). Different types of stressors can occur simultaneously; in fact, individuals are typically exposed to multiple stressors at a time (Dong et al., 2004). Thus, the characteristics of any exposure to a stressor play an important role in possible resulting behaviors, and can interact at multiple levels (Enoch, 2011).

Sex differences. Importantly, a variety of studies in clinical and animal research suggest that male and female individuals may have different responses to stress, and that inherent levels of resilience may differ between the sexes.

Clinical research. Research in clinical populations suggests that men and women may have different vulnerabilities for anxiety-related and substance use disorders. Varying neural processes and brain region activation between the sexes in response to both stress and alcohol exposure have been identified. Seo et al. (2011) demonstrated that men show greater brain activation in the face of stress, while women display greater activity when shown an alcohol-related cue. Increased stress in human populations can lead to higher drinking incidences, and self-reports indicate that men and women differ in the lengths between stress exposure and drinking onset depending on the characteristics of the stressor (Ayer, Harder, Rose, & Helzer, 2011). These types of differences have been observed as early as adolescence (Burk et al., 2011), suggesting that the effects of specific stressors vary at this developmental stage, and thus could have different long-term effects on drinking behaviors. Importantly, sex can be a mediating factor, such that women generally show higher rates of resilience than men in clinical populations (Schilling, Aseltine, & Gore, 2007). Thus, when discussing stress-related research, it is important to clarify the sex of the population being discussed.

Animal research. Similarly, animal research suggests that differences in responses to stressors exist between male and female rodents. Male and female rats

exposed to severe, sporadic stressors during adolescence both showed an increase in anxiety-related behaviors during adulthood, although the alterations manifested in different specific behaviors (Pohl et al., 2007). Past research has shown that male mice generally show greater startle amplitude than female mice (Barrenha & Chester, 2007). Animal research has also identified sex-specific effects of stress on drinking behaviors. Prenatal maternal separation has been shown to increase adulthood voluntary drinking in male mice (Cruz, Quadros, S. Planeta, & Miczek, 2008) and male rats (Roman & Nylander, 2005), but not their female counterparts. Furthermore, after chronic stress, male mice show an increase in voluntary alcohol consumption, but females do not show this effect (Chester et al., 2006). These data mimic the clinical data, and suggest that male rodents may be generally more susceptible to long-term effects of early-life stress than females. In contrast, there have been animal studies that have reported increased long-term effects of stress on drug-related behaviors specifically in females (McCormick, Robarts, Gleason, & Kelsey, 2004; McCormick, Smith, & Mathews, 2008). It is possible that the array of discrepancy in this type of literature could be due to varying characteristics of stress exposure among study designs and the species of rodent used in research.

As stated, the resulting behavioral alterations from stress exposure can vary widely, and the choices for each study design should be made with specific research questions in mind. Due to these discrepancies, the current coverage of background literature will primarily focus on animal studies performed in male rodents, and will specify the sex of the rodents used in each study. In addition, the sex of human subjects in the clinical background literature will also be specified.

Chronicity of a Stressor

Much of the resulting consequences from stress exposure have to do with the stress exposure length, such as whether the stressor is acute or chronic (Enoch, 2011). The chronicity of a stressor refers to whether the stress exposure has a short-term or long-term timeline, such as whether exposure to a stressor happens in a single incident (acute) or repeatedly (chronic).

Clinical research. Clinical research suggests that chronic stressors have greater and longer-lasting effects on our long-term memory that acute stress. When male and female clinical subjects were asked to self-report their biggest source of stress, chronic stressors were mentioned more often than acute stressors (Mattlin,

Wethington, & Kessler, 1990; McGonagle & Kessler, 1990). Acute stress can actually enhance short-term memory, working morale, and focus, suggesting that acute stress may heighten cognitive functioning; however, chronic stress may have detrimental effects on cognitive functioning. In humans, chronic stress is more associated with long-lasting psychological adjustments than acute stress (Avison & Turner, 1988), and is associated with the development of depressive- or anxiety-related disorders (McGonagle & Kessler, 1990) and substance use disorders, even simultaneously (Brady & Sinha, 2005) in both sexes.

Animal research. Similarly, research in rodents suggests that acute exposure to a nonthreatening stressor, such as noise and light, heightens exploratory activity (Katz, Roth, & Carroll, 1981), increases arousal (Keeney et al., 2006), and enhances immune function (Dhabhar & McEwen, 1997) in male rodents. However, animal research has also shown that a chronic battery of threatening stressors, including foot shock and forced swim tests, are associated with long-term depressive-related symptoms (Katz et al., 1981), immune function suppression (Dhabhar & McEwen, 1997), and detrimental alterations in HPA responses to stress (Keeney et al., 2006) in male rodents. This suggests that acute stress may have beneficial effects in animals, but chronic stress in animal models could lead to long-term changes in physiological, emotional, psychological, and cognitive responses.

At some point between acute and chronic timelines, a “switch” occurs in which the stress becomes maladaptive. Chronic stress has shown both long-term physiological changes in the HPA axis in male mice (Keeney et al., 2006) and behavioral alterations indicative of depressive and anxiety-related behaviors in male rats (Katz et al., 1981). Other animal research in male rats has shown that while acute stress exposure depletes the levels of NE in the brain and body, repeated exposure to stress can increase the overall levels of serotonin (5HT) and NE, which are both stress-related neurotransmitters, over time (Adell, Garcia-Marquez, Armario, & Gelpi, 1988). Additionally, research by Isgor et al. (2004) showed that chronic stress exposure in adult rats resulted in reduced brain volume, specifically inhibited growth in the CA1 pyramidal cell layer of the hippocampus and in the dentate gyrus. These animals also displayed impaired working memory, down-regulated CORT receptors, and deficits in HPA negative feedback (Isgor, Kabbaj, Akil, & Watson, 2004). Translating the relevant animal and clinical research together, these results suggest

that chronic stress is more likely to result in behavioral and physiological changes, and should be more of a focus in stress-related research than acute stressors when long-lasting consequences are the focus.

Stress Exposure During Early-Life

The age at which stress exposure occurs can also greatly affect the types of consequences that result; these findings have been demonstrated in both clinical and animal research (Enoch, 2011; Lupien et al., 2009).

Clinical research. Young children and adolescents in clinical populations are more susceptible to the negative effects of stress than adults, in general, and the long-lasting behavioral consequences resulting from stress exposure are less easily reversed (Lupien et al., 2009). Early life stress is associated with greater cognitive and emotional deficits in adulthood, including learning impairments, increased sensitivity to drug use, and anxiety-related disorders in both males and females (Lupien et al., 2009). Clinical research further suggests that the specific developmental time period at which stress exposure occurs is important. Interestingly, one study found that stress exposure before adolescence was more likely to lead to the development of major depression, whereas stress exposure during adolescence was more likely to lead to the development of post-traumatic stress disorder (PTSD) in females (Maercker, Michael, Fehm, Becker, & Margraf, 2004). Importantly, even though the long-term effects of stress exposure in early-life age groups may result in similar psychological, emotional, and cognitive characteristics in clinical populations, they may have different impacts on specific brain regions, depending on the developmental period of the individual during the stress exposure.

Animal research. Findings from animal research suggest that the hippocampus may be more vulnerable to CORT exposure during prenatal and early postnatal stress exposure, whereas CORT release during adolescent stress exposure may have greater effects on the frontal cortex. This difference in affected areas between the prenatal and adolescent periods may be due to the prefrontal cortex's development during puberty (Lupien et al., 2009). This rationale correlates to both clinical and animal anatomical research showing that pre-adolescent trauma is correlated with a reduced hippocampal volume, but similar trauma experienced during adolescence leads to reduced prefrontal cortex volume (Teicher, Tomoda, & Andersen, 2006) and reduced neuronal integrity of the frontal cortex (R. Cohen et al.,

2006) in male rodents. Naturally, it is presumptuous to assume that stress exposure, such as trauma, to an animal would have identical effects than it would to a human participant. In this way, we are limited in the conclusions we can make from research using rodents in terms of clinical translation. However, using animal research as a tool to create a more-controlled environment than is available via human subjects helps us better understand aspects of the relationship between stressors, the age of stress exposure, and possible resulting psychopathology.

Past research has been more focused on the effects of pre-natal and early post-natal stress rather than stress exposure that occurs specifically during adolescence (Lupien et al., 2009); thus, there is a gap in the literature. Prior work in animal research has shown that male (Pohl, Olmstead, Wynne-Edwards, Harkness, & Menard, 2007; Tsoory, Cohen, & Richter-Levin, 2007; Vidal et al., 2007) and female (Pohl et al., 2007) adolescent rodents show enhanced anxiety-related symptoms during adulthood, and adolescent male mice show greater fear conditioning compared to adult subjects (Hefner & Holmes, 2007), suggesting that they may be more susceptible to developing stress-related anxiety behaviors later in life. Furthermore, stress exposure during adolescence has additionally been suggested to have longer-lasting implications on long-term memory, emotional behaviors, and sensitivity to a variety of drugs in male and female rodents than stress exposure during adulthood (see review by McCormick et. al, 2010). However, the mechanisms of this relationship are still not well understood. There is a great need for research that focuses specifically on the sensitivity of stress exposure during adolescence in both human and animal populations to close this gap in the literature. The current study sought to examine the long-term effects of stress exposure during adolescence in male mice, specifically, on drug-related behaviors during adulthood.

Stress Exposure During the Adolescent Period

Stress exposure during the adolescent period has the potential to cause greater neural, hormonal, and morphological changes to brain systems than stress that occurs later in life, including changes in stress circuitry (Enoch, 2011). Adolescence is characterized by a number of “sensitive periods” in which individuals are more vulnerable to a variety of external stimuli while undergoing maturation and neurological, biological, and neurochemical changes (Witt, 1994). Thus, the adolescent brain is generally thought to be more sensitive to the effects of stress

exposure than the adult brain(Enoch, 2011; McCormick et al., 2010). Stress reactivity during adolescence is different than that during adulthood, and this has been demonstrated in both clinical and animal research (Spear, 2000).

Clinical research. Clinical research has shown that male and female adolescent individuals have higher basal and stress-induced cortisol levels than adult humans (Gunnar, Wewerka, Frenn, Long, & Griggs, 2009). This difference in basal glucocorticoid levels suggests two main possibilities. First, it suggests that adolescent individuals' HPA axes may be more active than adults', thus resulting in more cortisol release at a basal level. Secondly, it is possible that the negative feedback loops of the HPA axes in adolescents may not function at a mature level, thus, the HPA axis does not have the same capacity to inhibit its own glucocorticoid release during stress exposure. Given the lower level of circulating cortisol,, stress exposure during adolescence could presumably lead to even further cortisol release above the levels of an adult exposed to stress.

Animal research. The clinical importance of adolescent stress exposure is echoed through research using rodents. Generally, both male and female adolescent rodents show a prolonged HPA response to stressors compared to adult rodents, including a delayed rise and normalization of CORT levels in the presence of a stressor (Vazquez & Akil, 1993), and males show a potentiated CORT response to repeated chronic stress exposure (Romeo et al., 2006). In contrast, CORT release in male adult rodents typically habituates to chronic stress exposure over time (Girotti et al., 2006). Animal research has shown that stress exposure during adolescence in males is associated with long-lasting biological consequences, such as changes in DNA methylation and chronic hypersecretion of CORT (Murgatroyd et al., 2009) due to the early-life exposure to CORT. Such biological consequences could be correlated with later behavioral changes, including reduced stress-coping ability (Murgatroyd et al., 2009), reduced exploratory behaviors, and reduced avoidance learning (Tsoory & Richter-Levin, 2006). These findings complement those of the clinical research, suggesting that the negative feedback loop of the HPA axes in adolescents may not be as mature as the axes of adults, and thus glucocorticoid levels in adolescents may be higher at basal levels and also in the face of a stressor. These findings further suggest that excessive glucocorticoid exposure is more likely to happen when stress occurs during adolescence than during adulthood, since the negative feedback in the

HPA axis could be under-developed and unable to regulate responses to stress exposure.

Although clinical and animal CORT and brain development research are not directly comparable due to the species difference (Lupien et al., 2009), clinical research does align with animal research in this area, showing that male and female adolescent individuals have higher basal and stress-induced glucocorticoid levels than adult humans (Gunnar et al., 2009). Excessive glucocorticoid exposure during adolescence can lead to an altered stress response in adulthood, caused by potentiation and incubation effects, where symptoms are not present until adulthood when synaptic organization is complete (Lupien et al., 2009). It is important to mention that although the timelines are different between humans and rodents, adolescence can be observed in both human and animal models. In humans, adolescence is defined as the period between childhood and adulthood, in which psychological, social, and reproductive development occur. In rodents, adolescence occurs at the end of puberty and at the point of sexual maturation during the peri-pubertal period, and generally takes place between postnatal days (PD) 20 and 45 (Witt, 1994), with the late-adolescent period extending up to PD 59 (Lupien et al., 2009). Importantly, research on the long-term effects of chronic stress exposure during adolescence in rodents is sparse compared to that of research on stress exposure during the prenatal and early postnatal periods (Lupien et al., 2009), but is needed to understand the specific vulnerabilities present during this time.

The Severity of a Stressor

The severity of a stressor may also influence the behavioral consequences of stress exposure. Stress is a broad term; a wide variety of environmental influences may induce stress, and the level of stress perceived from any influence may vary between individuals. In both clinical and animal populations, the severity, or degree of impact, of a stressor can differentially be associated with positive and negative consequences of stress exposure.

Clinical research. In clinical populations, a higher severity of a stressor is associated with greater risk for externalizing disorders in adulthood (Hicks, South, Dirago, Iacono, & McGue, 2009), and specifically poorer outcomes in anxiety-related and substance use disorders during adulthood (Enoch, 2011) in both males and females. Similarly, a higher cumulative number of stressors are associated with more

severe psychopathology in both sexes (Schmid et al., 2010). Importantly, these associations have been observed either when stressors are experienced with others (such as family members or friends) or when they are experienced alone (Goodman-Brown, Edelstein, Goodman, Jones, & Gordon, 2003). These findings suggest that if an individual interprets a stressor to be more impactful on his or her life, then the behavioral results following stress exposure are more likely to be altered in a negative manner.

Animal research. Although stressors may not be directly comparable between clinical and animal research (Schmidt et al., 2007), a variety of techniques have been used in rodent models to mimic chronic stress exposure in humans. Foot shock is commonly used due to its many benefits compared to other stress paradigms, including restraint stress, social stress, and the forced swim test. Foot shock administration has been established as both a physical and psychological stressor for male rodents (Matsuzawa & Suzuki, 2002). Research suggests that, although exact brain regions are still unknown, foot shock stress causes an interaction between corticotrophin-releasing factor CRF and 5HT, and the effects can be mitigated by SSRIs in male and female rodents (Le & Shaham, 2002), making foot shock a more well-understood stress paradigm as far as brain mechanisms that influence responses to this type of stressor. Importantly, the effects of foot shock stress in male rodents have been shown to alter drug-related behaviors, but do not generalize to non-drug reinforcers, such as sucrose (Le et al., 1998) or food pellets (Ahmed & Koob, 1997),

The use of foot shock as a stress paradigm has successfully elicited both chronic stress effects (Song, Wang, Zhao, Zhai, & Lu, 2007) and acute re-exposure effects (Matsuzawa, Suzuki, & Misawa, 1998) on conditioned place preference in male adolescent subjects, as well as long-term chronic stress effects on voluntary alcohol consumption in both males and females (Chester, Barrenha, Hughes, & Keuneke, 2008). Importantly, foot shock has not been shown to cause an increase to pain sensitivity in either male adult or adolescent mice thus far (Hefner & Holmes, 2007). However, not all strains or lines of rodents have been tested, so it is important to monitor physical responses to foot shocks when using foot shock as a chronic stress paradigm.

An important advantage of using foot shock as a stressor is that physical responses to the shocks, termed tactile startle, can be recorded for every shock

exposure in grams of force measurements (g/F). G/F measurements can be analyzed to detect sensitization or habituation to the foot shocks and compare the physical responses to stress exposure between subgroups. Thus, foot shock is an effective, well-established, viable, and advantageous method to model chronic stress in adolescent and adult mice and elicits both immediate and long-term effects in alcohol-related behavior.

Predictability of a Stressor

Clinical research. Clinical research suggests that the predictability of stress exposure provides a sense of control to an individual, and that the unpredictability of stress is more associated with the development of anxiety- and depressive-related disorders (Grillon et al., 2008). Because there is little ability to control whether individuals experience a predictable or unpredictable stressor, it is difficult to study this topic in a clinical population. Interestingly, one study found that male and female clinical participants reported paying more attention to unpredictable stressors and reported more severe symptoms following the unpredictable stressors than those in the predictable stressor group (Matthews, Scheier, Brunson, & Carducci, 1980). However, animal research provides more information about the influence of predictability in a stress exposure paradigm.

Animal research. Previous literature using rodent models suggests that the predictability of a stressor may influence resulting physical and behavioral alterations (De Boer, Van Der Gugten, & Slangen, 1989; Mormede, Dantzer, Michaud, Kelley, & Le Moal, 1988). In general, stress exposure that is unpredictable is more likely to lead to lead to an increase in anxiety-related symptoms. For example, one study by Pohl et al. (2007) showed that severe, sporadic stress showed a greater increase in anxiety-related behaviors in male and female rats than a chronic, mild stressor. Furthermore, past research by Tsuda et al. (1989) found that unpredictability of a stressor was associated to greater NE turnover in areas included in the HPA axis and other stress-related brain areas, including the hypothalamus, amygdala, midbrain, cerebral cortex, thalamus and locus coeruleus. Importantly, male subjects in the predictable stress exposure group only showed NE turnover in the hypothalamus, amygdala, midbrain, and cerebral cortex, and showed less overall NE turnover in these areas compared to the unpredictable stress group (Tsuda et al., 1989). These findings suggest that both predictable and unpredictable foot shocks are associated with increases in

stress-related neurotransmitters, but unpredictable foot shocks elicit a greater impact on the brain, overall.

Re-Exposure to a Stressor

Re-exposure to a stressor after prior chronic stress exposure can also lead to physiological and cognitive changes, as seen in both animal and clinical research.

Clinical research. In clinical populations, re-exposure to a stressor has important implications for relapses in drug-seeking behavior (Koob & Le Moal, 2002; Stewart, 2000). More recent work in clinical populations suggest that this relationship may be related to a stress-induced craving for the drug, motivating individuals with a prior drug addiction to relapse (Breese et al., 2005). This is particularly true in individuals who have been abstinent for a shorter amount of time and for those with AUDs compared to other drugs of abuse (Breese et al., 2005). Similar to other stress-related research, more evidence is available via animal research.

Animal research. Chronic stress has been shown to raise 5HT and NE levels over time in rodents due to adaptations of the body in neurotransmitter synthesis and metabolism in response to chronic stress exposure. In rats re-exposed to a stressor after previous chronic stress, the stress re-exposure significantly decreased both 5HT and NE levels in male subjects (Adell et al., 1988). This suggests that the chronically-stressed subjects could have a sensitized response to acute stress.

Furthermore, research in our laboratory demonstrated that continuous intermittent re-exposure to a stressor before limited access drinking gradually increased voluntary alcohol intake in male high-alcohol preferring mice (Chester, de Paula Barrenha, DeMaria, & Finegan, 2006). Such results could reflect the idea of reinforcing self-medication; however, more work is needed to understand the mechanism by which stress increases voluntary alcohol consumption, such as if sensitivity to the rewarding effects of alcohol is increased with stress re-exposure. There is some evidence from animal research that suggests that re-exposure to a stressful situation can further increase sensitivity to the rewarding effects of alcohol. This effect has been studied by giving male and female rodents stress re-exposure before expression of conditioned reward-related behavior (Sinha, 2001). Stress re-exposure has become an important area of research for drug-related relapse, and has been suggested to be one of the more effective research designs for re-instating drug-seeking behaviors in rodents (Stewart, 2000). Research in male rats suggests that

CORT and CRF may modulate this response, with more evidence indicating that CRF plays a more prominent role in this relationship due to its actions on and near the hypothalamus (Lê et al., 2000).

Individual Differences

Of course, individual differences also play a role in the relationship between stress exposure and resulting consequences. It is important to note that even chronic, severe stress does not guarantee a poor outcome in adulthood. Mediating factors can occur and end in resilience in the individual instead of psychopathology (Uhart & Wand, 2009). Important factors include peer relationships (Fergusson, Woodward, & Horwood, 1999), familial history of substance use (Jaffee, Caspi, Moffitt, Polo-Tomas, & Taylor, 2007), and parenting styles (DuMont, Widom, & Czaja, 2007). Furthermore, the role of genetic and environmental interactions is important. Relevant to the current study, resilience has been shown to protect individuals from high levels of alcohol-seeking behavior and AUD development (Enoch, 2011). Even though resilience can take place, stress-induced psychopathology is an important topic that requires more research due to the wide prevalence of chronic stress and resulting anxiety-related and substance use disorders.

Adolescent Stress Exposure and Alcohol Use

Clinical research has linked adolescent stress exposure to an increased risk for lifetime AUD development in both male and female individuals (Anda et al., 2006). This is a multi-tiered relationship, and specific characteristics of this relationship have been illustrated through clinical and animal research.

Clinical Research

Stress exposure can alter alcohol consumption, and stress exposure during adolescence has been associated with changes in several alcohol-related behaviors. Clinical research has linked adolescent stress to an increased risk for early-life binge drinking (Labouvie, 1986; Pilowsky, Keyes, & Hasin, 2009) and developing a lifetime AUD (Anda et al., 2006). Importantly, stress exposure during adolescence can lead to both immediate (Kabbaj, Isgor, Watson, & Akil, 2002) and long-term (McCormick et al., 2004) increases in drug use, including alcohol use. The differences in cortisol levels during adolescence compared to levels during adulthood may play a role in the mechanisms of alcohol dependence, especially if alcohol is consumed during adolescence. Alcohol influences the HPA axis, and since the HPA axis is still

developing during adolescence, alcohol exposure during this time may lead to a heightened risk of alcohol dependence (Prendergast & Little, 2007). Interestingly, one study found that male and female adolescents in a clinical population with alterations in the corticotrophin-releasing hormone receptor 1 (CRHR1; a stress-related neurotransmitter receptor) exhibited higher rates of alcohol-drinking throughout their lifetime when exposed to negative stressors (Blomeyer et al., 2008). This finding is important because it suggests that alterations in the HPA axis and its related receptors may influence the relationship between adolescent stress exposure and the development of an AUD.

One characteristic of AUDs is a maladaptive increase in alcohol-seeking behaviors above other behaviors. An important influence on a person's individual drug-seeking behaviors is the individual's level of sensitivity to the perceived rewarding effects of the drug (Stephens et al., 2010). Alcohol is known to interact with several areas of the brain that have suggested involvement in the regulation of the reinforcing aspects of drugs, such as the ventral tegmental area (VTA) and the nucleus accumbens (NAc) (Stephens et al., 2010) as well as with neurotransmitters involved in the stress response, including dopamine (DA) (Brady & Sinha, 2005) and 5HT (Enoch, 2011). Importantly, stress exposure during adolescence has been associated with long-term changes in the mesolimbic DA pathway in the brain, which is one brain region associated with the perceived rewarding effects of alcohol (Brady & Sinha, 2005; Enoch, 2011). DA neurons near the basal ganglia are susceptible to early life stress and are necessary for incentive behaviors (Enoch, 2011), such as the incentive towards drug-seeking behaviors. These are important findings, as alcohol consumption is associated with an increased presence of DA in the NAc and other areas associated with the rewarding effects of drugs. It is possible that an altered DA pathway could alter an individual's sensitivity to the positive rewarding effects of alcohol when it is consumed. Thus, stress exposure during adolescence may increase the perceived rewarding effects of alcohol, in both immediate and long-term timelines.

Animal Research

Results from animal research examining the effects of stress exposure and alcohol consumption throughout the lifetime are complicated, and results vary depending on the specific design of the study and its subjects (see review by Becker, Lopez, & Doremus-Fitzwater, 2011). For example, past research has found that

exposure to alcohol during adolescence followed by stress during adulthood did not increase adult alcohol consumption in male mice (Tambour, Brown, & Crabbe, 2008), but does increase adult alcohol consumption in female mice (Tambour et al., 2008) and female rats (Füllgrabe, Vengeliene, & Spanagel, 2007). In addition, alcohol-drinking initiation during adolescence did not predict drinking behaviors or relapse behaviors in male Wistar rats; although, subjects who began drinking during adolescence did reflect a sensitized response to acute stress in terms of alcohol consumption (Siegmund, Vengeliene, Singer, & Spanagel, 2005). Chronic stress exposure during adolescence has been shown to increase subsequent voluntary alcohol consumption during adulthood in high-alcohol preferring (HAP2) male mice using foot shock stress (Chester et al., 2008) and in male and female C57BL/6J mice using social stress (Lopez, Doremus-Fitzwater, & Becker, 2011). A study by Advani et al. (2007) showed that social isolation during adolescence (post-weaning) increased alcohol intake and preference during adulthood, as well as an increase in 5HT receptor function in the dorsal raphe nucleus in both male and female mice (Advani et al., 2007). This research suggests that stress exposure during adolescence may lead to long-term alterations in both drug-related behavior and neurotransmitters important for stress- and drug-seeking behaviors.

Importantly, self-administration studies in animal research provide limited information regarding the motivational properties behind increased consumption, and conclusions about motivational changes in drug consumption can be strengthened by converging or diverging information from other behavioral models. Thus, animal research utilizing other behavioral paradigms that better measure the motivational influences behind drug consumption is needed to better understand the relationship between adolescent stress exposure and adult drug consumption, and to relate the findings to clinical populations.

Research in rodents suggests that psychological stress could play an important role in the perceived rewarding effects of alcohol, and that 5HT and DA may be involved in this relationship (Matsuzawa & Suzuki, 2002). Other research shows that early life stress alters the DAergic systems of the brain associated with the rewarding effects of drugs in female rodents, and supports the hypothesis of DA involvement in this relationship (Matthews & Robbins, 2003). Although increased magnitude and duration of DA in the DA reward-related pathways and increased 5HT

release are not the only indicators of the perceived rewarding effects of a drug, several animal studies have shown that early life stress, including during adolescence, does seem to increase the sensitivity to the rewarding effects of drugs (Enoch, 2011). Changes in the DA pathway or 5HT levels resulting from stress exposure during adolescence could be possible explanations for the drug-related behavioral changes observed following such stress exposure, but this relationship is still not well understood.

Genetically Influenced Predisposition to Drinking Behaviors

The association between chronic stress exposure during adolescence and AUDs is especially pronounced in individuals with a familial history of AUDs (Dube et al., 2001). Clinical research in at-risk children has found that there are neural, cognitive, and electrophysiological differences between children with a family history of alcoholism and those with no family history (Witt, 1994). Having a family history of AUDs is a risk factor for developing anxiety-related and substance use disorders after chronic stress exposure (Jaffee et al., 2007). For example, blunted HPA axis responses to stress have been observed in clinical populations of male and female individuals with a history of AUDs, regardless of whether or not the individual suffers from an AUD him- or herself, and blunted CORT responses in the face of a stressor can alter an individual's behavioral responses to stress (Dai, Thavundayil, & Gianoulakis, 2005; Dai, Thavundayil, Santella, & Gianoulakis, 2007). Past research suggests that specific genetic influences may serve as risk factors for developing stress-related psychopathology, and these genetic influences can interact with environmental mediating factors (Moffitt, Caspi, & Rutter, 2005), including the characteristics of stress exposures throughout the lifetime. Thus, it is important to examine these different familial predispositions toward alcohol drinking and how they may be differentially affected by environmental manipulations.

Selectively-Bred Lines

Much research has been done to mimic familial histories of AUDs in animal models, such as using rodents selectively bred to either prefer or not prefer alcohol. In our laboratory, we use the high-alcohol preferring (HAP) and low-alcohol preferring (LAP) selectively bred mouse lines. The HAP and LAP mouse lines were generated from the out-bred stock Hs/Ibg, and were selectively-bred over 10 generations based on their inherent alcohol drinking behaviors, as demonstrated by a 30-day two-bottle

choice drinking paradigm (Grahame, Li, & Lumeng, 1999). The extremely high- and extremely-low drinking mice were selected to originally generate the HAP1 and LAP1 (first replicate) lines, where, the HAP1 mice consumed over 12 g/kg of 10% alcohol and the LAP1 mice consumed less than 2 g/kg of 10% alcohol (Grahame et al., 1999). Since the first selective breeding process, second and third replicate lines have been generated, as well as a line of c-HAP mice that are cross-bred between the HAP2 and HAP3 lines. Over the selective breeding process, these lines reflect genetically-influenced drinking behaviors. In general, the HAP line serves as a model of inherited propensity (family history positive) toward AUD development, as HAP mice will voluntarily drink significantly more alcohol than LAP mice (Grahame et al., 1999).

Using these selectively-bred lines simultaneously allows for an establishment of different propensities in alcohol drinking behaviors and responses to alcohol consumption (Crabbe, 1989). Furthermore, directly comparing data between these selectively bred lines allows researchers to show that behaviors in response to a substance can vary based on genetic influences and environmental manipulations, such as stress exposure. Comparing data between the HAP and LAP lines of mice has allowed for examination of the various effects of stress exposure in subjects with different drinking behaviors. HAP2 mice show greater baseline startle responses than LAP2s, particularly in male subjects (Chester & Barrenha, 2007). Male and female HAP2 mice also show greater fear-potentiated startle (FPS) overall than LAP2 mice (Barrenha & Chester, 2007), and exhibit lower CORT levels after foot shock and fear conditioning than LAP2 mice (Chester, Kirchhoff, & Barrenha, 2013). Increases in anxiety-related behaviors and blunted CORT responses to stress exposure have been identified as characteristics of AUD individuals, reinforcing the use of HAP mice to mimic AUD familial history. These selectively-bred lines are relevant in examining the relationship between adolescent stress exposure and characteristics of AUD development.

Importantly, in male HAP2 mice, chronic adolescent stress has been shown to increase voluntary alcohol consumption during adulthood (Chester et al., 2008). However, as mentioned, self-administration models provide limited insight into the nature of the motivation behind voluntary drinking behaviors, which can include both positive and negative motivational effects. Increasing levels of intoxication can hinder the assessment of sensitivity to the rewarding effects of alcohol due to effects on

motor behavior, and alcohol drinking can be influenced by taste factors that could confound interpretation of results. Thus, additional research using other behavioral paradigms that are more sensitive to the motivational properties that underlie alcohol consumption and using the HAP and LAP lines is needed to better understand the established relationship between adolescent stress exposure and adult AUD development and apply it towards a clinical population.

The Conditioned Place Preference Paradigm (CPP)

Paradigms such as place conditioning allow for the assessment of sensitivity to either the rewarding or aversive effects of drugs in rodents without relying on oral consumption of alcohol, because animals are tested in a drug-free state. They also allow for the assessment of learning and memory mechanisms involved in alcohol's motivational effects, which are thought to play a critical role in the maintenance of reward-related behaviors (Cunningham, Fidler, & Hill, 2000). The conditioned place preference (CPP) behavioral paradigm effectively measures the role of learning and memory involved in the perceived rewarding effects of alcohol. This measurement is important in understanding the "appetitive" processes in drug addiction and the role in drug relapse in humans (Cunningham et al., 2000). A variety of drugs induce CPP in rodents, and several neuroanatomical pathways have been shown to mediate CPP in rodents, including the VTA, NaC, medial prefrontal cortex, ventral pallidum, amygdala, and the pedunculopontine tegmental nucleus (Tzschentke, 1998). This suggests that the CPP paradigm effectively evokes preference for a drug-related context based on the associated perceived rewarding effects of a drug, and that the task is capable of measuring subjects' sensitivity to the rewarding effects of a drug. The CPP paradigm has been widely used to show differences in sensitivity to the perceived rewarding effects of alcohol between subjects in different stress conditions, age groups, sexes, and drinking propensities (see review by Tzschentke, 2007). Although it is difficult to directly translate preference data from rodent studies to a clinical application (Spanagel, 2003), the data from CPP research provides valuable information regarding the motivational properties involved in alcohol-seeking behaviors, and how these may be altered by environmental variables.

Stress Exposure and CPP

Previous research examining the effects of stress exposure on CPP has been performed in recent years (see review by Tzschentke, 2007). In general, results

suggest that exposure to chronic stress results in increased CPP when CPP immediately follows stress exposure (Bahi, 2013). Studies have also examined the direct effects of CORT administration on alcohol-induced CPP. For example, previous research suggests that direct manipulation of CORT levels in male mice does not alter the acquisition or expression of CPP (Chester & Cunningham, 1998). A similar study by Brooks et al. (2004) showed that CORT administration while using the traditional alternate-day CPP paradigm decreased CPP expression in male mice, but CORT administration using the rapid-approach CPP paradigm increased CPP expression at lower doses (Brooks, Hennebry, Croft, Thomas, & Little, 2004). These results likely vary due to the differences between the study designs and specific strains of rodents used.

Relevant to the current study, Song et al. (2007) used CPP to examine different alcohol doses between male and female adult and adolescent subjects in stress and no-stress conditions. Interestingly, chronic stress in adolescent subjects leads to a significant increase in CPP at the 2.0 g/kg dose, while acute stress did not show this effect. Neither stress exposure affected adult subjects' CPP at the 1.0 g/kg dose (Song et al., 2007). One limitation of this study is that the adolescent and adult groups were not compared between stress conditions at the same dose of alcohol, so information regarding the effects of chronic versus acute stress in the adult subjects at the equivalent dose of the adolescent subjects is unavailable. Furthermore, this study did not provide information as to how long lasting these effects are, such as if the effects of chronic stress on CPP in the adolescent subjects would have persisted into adulthood. The current study sought to fill this gap in the literature by examining if chronic stress exposure during adolescence would increase sensitivity to the rewarding effects of alcohol during adulthood.

Stress Re-Exposure and CPP

The effects of stress re-exposure in animal research on alcohol-induced CPP are less prevalent, but the available results are promising. In one study, a history of chronic stress exposure increased alcohol-induced CPP in male rats compared to those without stress exposure history, and those who were re-exposed to the original stressor once again directly before CPP testing showed a greater enhancement of alcohol-induced CPP (Matsuzawa et al., 1998). However, these subjects were all of adult age during chronic stress exposure, and thus information investigating if similar

or more pronounced results are obtainable when chronic stress occurs during adolescence and the CPP paradigm takes place during adulthood is not currently available. The current study used repeated intermittent stress re-exposure to examine if re-exposure would further increase sensitivity to the rewarding effects of alcohol following adolescent stress exposure.

Hypotheses

The current study sought to fill important gaps in the literature regarding the relationship between adolescent stress exposure and increased risk for AUD development in adulthood. Overall, this research examined if chronic stress exposure during adolescence would increase sensitivity to the rewarding effects of alcohol during adulthood, and how these effects may depend on stress re-exposure and propensity for high or low alcohol drinking in a male mouse model. Three main hypotheses were developed to address this research question.

Hypothesis 1

The overall prediction for Hypothesis 1 was that chronic stress exposure during adolescence would significantly increase sensitivity to the rewarding effects of alcohol during adulthood, as measured by CPP. To evaluate this overall hypothesis effectively, two sub-hypotheses and planned comparisons were used.

To address the first sub-hypothesis, CPP expression during adulthood was compared between a group exposed to chronic stress during adolescence and a group exposed to chronic stress during adulthood. This approach addressed the sub-hypothesis that the age of stress exposure during adolescence would increase adult CPP more than stress exposure during adulthood, based on previous findings by Song et al. (2007) and research examining voluntary alcohol consumption in our own laboratory (Chester et al., 2008).

To address the second sub-hypothesis, CPP expression during adulthood was directly compared between the group exposed to chronic stress during adolescence and another group not exposed to stress, but matched in age. This addressed the sub-hypothesis that stress exposure during adolescence would increase adult CPP more than a lack of stress exposure during adolescence, based on previous work by Song et al. (2007).

Hypothesis 2

We hypothesized that re-exposure to the original stressor would enhance sensitivity to the rewarding effects of alcohol, particularly in subjects exposed to chronic stress during adolescence. To evaluate this hypothesis, half the subjects in each stress treatment group were re-exposed to the stressor before CPP Posttest 1 and CPP Posttest 2, and the other half were not re-exposed to stress before either Posttest. This allowed for direct comparison between the re-exposed and non re-exposed subjects at each level of stress treatment (adolescent stress exposure, adult stress exposure, and no stress exposure). This hypothesis was based on research showing that chronically-stressed subjects show an increased sensitivity to acute stress (Adell et al., 1988) and that re-exposure to a stressor increases CPP expression (Matsuzawa et al., 1998). Importantly, the current study used two Posttests (1 and 2) 24 hours apart. Using the two consecutive CPP Posttests allowed us to measure if the second re-exposure to the stressor before Posttest 2 would further increase the sensitivity to the rewarding effects of alcohol compared to the results of Posttest 1. This hypothesis was based on previous research in our laboratory, which showed that intermittent re-exposure to a stressor increased voluntary alcohol consumption in male mice (Chester et al., 2006).

Hypothesis 3

We expected that stress-related alterations in sensitivity to the rewarding effects of alcohol would be evident in the HAP line of mice, but not in the LAP line of mice. To evaluate this hypothesis, equal representation of HAP2 and LAP2 mice were used within each stress treatment and stress re-exposure groups. This hypothesis was based on extensive research between the HAP and LAP lines showing that HAP2 mice show greater fear-conditioning behaviors (Barrenha & Chester, 2007) and alterations in CORT levels (Chester et al., 2013), indicative of differences in behavioral and physiological stress-related changes between the two drinking propensities lines.

Rationale

The current study sought to answer an important gap in the research literature regarding a possible mechanism for the relationship between adolescent stress exposure and increased alcohol consumption during adulthood. This relationship has been established through both clinical and animal research, and importantly has been observed in our laboratory. A prior study by Chester et al. (2008) used a 10-day foot

shock design to mimic a chronic stress paradigm, and the current study utilized the same paradigm. This paradigm elicited 15 foot shocks (0.2 mA) over a 30 min period, with one foot shock presented every 2 min (fixed schedule). While it is true that variable stress exposure is generally more effective at eliciting anxiety-related behaviors (Pohl et al., 2007), it was important that the stress paradigm used in the current study was replicated as closely as possible to that used in the prior study to allow for a direct comparison between the results. A shock amplitude of 0.2 mA was selected for use in the prior study because this amplitude was within a range deemed safe for adolescent mice to avoid pain sensitization, and the same amplitude was used in the current study design. Using this chronic foot shock paradigm, the previous study successfully showed that adolescent stress exposure significantly increased voluntary alcohol consumption during adulthood, whereas adult stress exposure had no effect on later alcohol consumption (Chester et al., 2008). Limiting the amount of extraneous variables in the stress paradigm used between the prior and current study better enabled us to understand the relationship between voluntary alcohol consumption and sensitivity to the rewarding effects of alcohol following adolescent stress exposure. Thus, the current study sought to mimic the prior study's stress paradigm as much as possible, and chose to use the same stress paradigm as the Chester et al. (2008) study.

In addition, the previous study by Chester et al. (2008) used male HAP2 mice, suggesting that the described chronic foot shock stress paradigm is indeed effective in the HAP2 line of mice for eliciting drug-related behavioral changes during adulthood. LAP2 mice were not used in the prior study, but use of both the HAP2 and LAP2 mice in the current study provided an opportunity to investigate how environmental manipulations, such as stress exposure, could interact with genetically-influenced factors, such as drinking propensity. Behavioral differences between the HAP and LAP lines have been studied repeatedly since the first replicate line (Barrenha & Chester, 2007; Chester et al., 2013; Grahame, Chester, Rodd-Henricks, Li, & Lumeng, 2001; Grahame et al., 1999), but the possible difference in the HAP2 and LAP2 lines in typical alcohol-induced CPP expression or how stress exposure may influence CPP expression has not been examined in depth between the lines. Prior research showed that the HAP1 and LAP1 lines differed in CPP expression only at the 4.0 g/kg dose, but not at the 0, 1.5, or 3 g/kg dose (Grahame et al., 1999). The current study used a

2.0 g/kg dose of alcohol, which was well within the range used in prior research that showed no difference between the lines. Thus, the current study provided valuable information regarding differences in sensitivity to the rewarding effects of alcohol in conditions where subjects are given a 2.0 g/kg dose and exposed to stress within the study design.

Furthermore, this study focused on male mice due to prior research suggesting that male rodents show greater and more long-lasting behavioral alterations following stress exposure (Barrenha & Chester, 2007; Chester et al., 2006; Cruz et al., 2008; Roman & Nylander, 2005). Importantly, sex differences in the sensitivity to the rewarding effects of alcohol have not been found consistently across recent research, although isolated studies have identified differences in CPP between male and female rodents. In a study by Roger-Sanchez et al. (2012), both early and late adolescent female mice showed significant alcohol-induced CPP, whereas in males, only early adolescent subjects showed CPP (Roger-Sanchez, Aguilar, Rodriguez-Arias, Aragon, & Minarro, 2012). Another recent study by Torres et al. (2013) showed that a moderate dose of ethanol produced CPP in adult and adolescent rats, but not in males of either age group. Furthermore, female rats that were ovariextomized (OVX) show no CPP (Torres et al., 2013), suggesting that ovarian hormones may mediate levels of sensitivity to the rewarding effects of alcohol. However, Song et al. (2007) found no evidence of sex differences when examining the effects of stress on CPP in adult and adolescent mice. Other studies have similarly found no evidence of sex differences in CPP (Bechtholt, Smith, Raber, & Cunningham, 2004). Thus far, sex differences have not been found in CPP using mice specifically bred for high- or low-taste aversion (Phillips et al., 2005). Since the current study wanted to primarily focus on possible alterations on CPP resulting from stress exposure at different ages, and the evidence of sex differences during CPP has thus been inconsistent, the current study included only male mice.

The use of stress re-exposure in the current study is also based on research in our laboratory showing that intermittent re-exposure increased alcohol consumption (Chester et al., 2006), and similar results have been reflected in CPP research (Matsuzawa et al., 1998). In one particular study, a history of chronic stress exposure increased alcohol-induced CPP in mice compared to those without stress exposure history, and those who were re-exposed to the original stressor once again directly

before CPP testing showed a greater enhancement of alcohol-induced CPP (Matsuzawa et al., 1998). However, these subjects were all of adult age during chronic stress exposure. This study sought to investigate if similar or more pronounced results were obtainable when chronic stress occurred during adolescence and the CPP paradigm took place during adulthood.

As previously noted, the literature investigating the effects of stress exposure on alcohol-induced CPP is complicated. The discrepancies among recent studies are likely due to a wide variety in the characteristics of stress exposure, the specific rodent lines and sexes used, and the exact CPP paradigm used. The current study sought to answer a very specific research question regarding how adolescent stress exposure could alter sensitivity to the rewarding effects of alcohol during adulthood. Importantly, the current study used a longitudinal design that has not been investigated using a CPP model before, and the results of this study provided important information regarding the relationship among the long-term effects of adolescent stress exposure, stress re-exposure, different drinking propensities, and how such variables could interact to alter alcohol-related behaviors and ultimately the likelihood of AUD development.

METHODS

Subjects

Subjects were male HAP2 and LAP2 mice (the second replicate of the HAP and LAP lines) from generations 44a and 44b, generated at Purdue University. The current study utilized a separate breeding colony from the rest of the laboratory space due to the constraints of timing the adolescent period and to allow for counterbalancing between breeding pairs. Harem pairings of male breeders (A-E) and female breeders (A1-A5, B1-B5, C1-C5, D1-D5, and E1-E5) were used for both HAP2 and LAP2 mice in generations 44a and 44b. On alternating breeding periods (odd-numbered female breeders in breeding cohort 1, even-numbered female breeders in breeding cohort 2), 1 male breeders and their 2 (breeding cohort 2) or 3 (breeding cohort 1) female partners were placed in a cage for 2 weeks. At the end of 2 weeks, female breeders were separated and placed in individual cages while pregnant. When pups were born (PD 1), the day was noted. Weanings took place between PD 21-23, at which time pups were slated for use in the Adolescent Stress, Adult Stress, or Control groups. Whenever possible, weaned litters were split into 3 cages for use in the 3 stress treatment groups, to allow for counterbalancing between the breeding pairs and parity status of the female breeder.

Approximately 16 subjects were run per stress group, re-exposure group, line, and CPP conditioning subgroup, based on previous research in our laboratory for adequate power needed to detect changes in CPP. All mice were housed in clear polycarbonate cages (11.5 X 7.5 X 5 in) with ad libitum access to food and water throughout the experiment. All behavioral experiments began at 0700.

General Design

There were 3 Stress Treatment groups present in the study design. Two of the groups received chronic stress exposure (CSE). Half of the mice in these groups received stress exposure during adolescence (Adolescent Stress) and half received stress exposure during adulthood (Adult Stress). The third group did not receive any

stress exposure and served as a control group (Stress Control). There was a 30-day interim between stress exposure and the start of conditioned place preference (CPP) conditioning trials to allow the Adolescent Stress and Stress Control subjects to reach adulthood before the start of conditioning. Half the subjects in each Stress Treatment group were re-exposed to the original stressor (RS) immediately before the first CPP posttest (Adolescent Stress-RS, Adult Stress-RS, Stress Control-RS). A second CPP posttest took place the day immediately following the first posttest (see Fig. 1). To account for possible litter effects, subjects in each group were counterbalanced between breeding pairs and parity status of the dam. All subjects were bred from breeding pairs specific to this study. Each subgroup was equally represented within HAP2/LAP2 mice. Due to the magnitude of this study design, several cohorts of subjects were run to reach the appropriate number of subjects per group. Within each cohort, subgroup representation was balanced to the best of our ability to account for possible litter effects and environmental variations over time.

Chronic Stress Exposure (CSE) Procedure

For 10 consecutive days, Adolescent Stress and Adult Stress subjects received 15 foot shocks (0.2 mA) during a 30-min time period (Chester et al., 2008). Control subjects were placed in the foot shock chambers for 30 min, with no foot shocks given, to ensure that all subjects were experientially matched and to avoid novelty effects when any Control subjects were later exposed to the chambers during the RS phase. Grams of force (g/F) measurements were recorded for each of the 15 foot shocks across the 10 days of CSE to be compared between subgroups. The ability to record g/F measurements allowed us to ensure that the CSE was, in fact, inducing a tactile response to stress. Subjects were weighed on Days 1-10 of CSE to monitor changes in body weight as a function of stress treatments. All subjects were handled normally during routine animal husbandry.

On Days 1 and 10 of CSE, all subjects had blood samples taken to measure CORT changes across CSE. Collecting blood samples on these days allowed us to see if CORT levels increased across CSE, whether the CORT levels differed between the Stress Treatment groups, and allowed us to compare differences in CORT levels between the HAP2 and LAP2 lines.

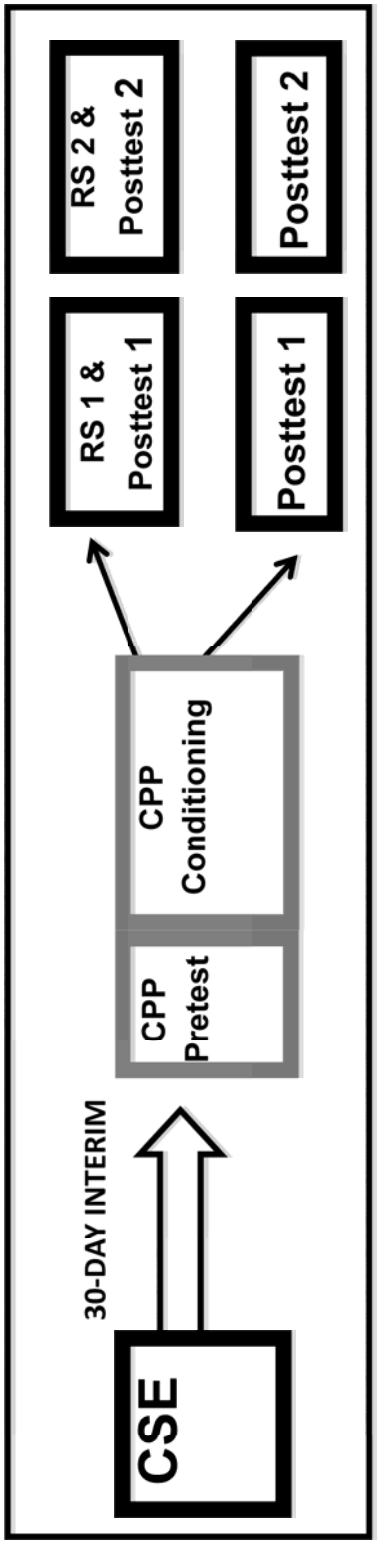

Figure 1. Methods flowchart for the behavioral paradigms in the current study.

Alcohol–Induced CPP Procedure

The CPP procedure included 3 phases: pretest, conditioning, and posttests. The CPP paradigm proposed in this study was based on extensive research on experimental variables in CPP apparati (Cunningham, Gremel, & Groblewski, 2006). In this model, tactile cues are used to distinguish the two distinct floor types (GRID and HOLE), which have been found to produce robust CPP. This paradigm uses a 2-compartment chamber, with no neutral chamber separating the two separate tactile cues, so that the total time spent on the two floors is equal across groups. In unbiased CPP procedures, subjects are assigned to conditioning stimuli (i.e., tactile floor stimuli) without regard to initial floor preference. An advantage of using unbiased versus biased place conditioning procedures is that the data can be more easily understood and interpreted. For example, in a biased procedure, it is difficult to interpret whether the unconditioned stimulus is enhancing the unlearned motivation response to the conditioned stimulus or if the unconditioned stimulus is motivating in itself (Cunningham et al., 2006). Using an unbiased CPP procedure is important in regards to measurement (Cunningham, Ferree, & Howard, 2003) and increasing the likelihood of producing CPP in subjects (Cunningham et al., 2006). When an unbiased procedure is used, such as the one in this proposed study, and subjects in different groups are properly counterbalanced into subgroups and floor order, results from data analysis can be understood more easily by ensuring that any possible floor assignment effects are dispersed evenly throughout groups. Thus, any differences in CPP can be attributed to group or line differences rather than possible floor assignment effects, such as conditioned stimulus (CS) +/- pairing or floor exposure order (Cunningham et al., 2003).

During the pretest, subjects were placed in the middle of 2 distinct floor types (GRID and HOLE). Subjects were allowed to roam freely for 60 min to measure baseline preference. Four conditioning trials took place for each alcohol (+) and saline (-) pairing, with a total of 8 conditioning trials. There was a 2-day break between the first 4 and last 4 conditioning trials. The CPP paradigm is a differential conditioning procedure; all subjects received equal exposure to the conditioning stimuli and drug treatments. Floor pairings and exposure order were assigned with no regard to any initial preference so that the paradigm remained unbiased.

On alternating days during conditioning, subjects in the GRID+ (G+) subgroup received an IP injection of alcohol at a dose of 2 g/kg (Powers, Barrenha, Mlinac, Barker, & Chester, 2010) and were immediately placed on a GRID floor in the apparatus for 5 min. Conversely, the GRID- (G-) subgroup was injected with saline and placed on the GRID floor for 5 min. During the intervening days, subjects in each subgroup received the opposite injection and were placed on the opposite floor from the previous trial. Throughout the entire CPP procedure, apparatus enclosure, alcohol floor pairing, and floor placement order were counterbalanced within groups.

Each subject was tested on 2 drug-free posttests (CPP Posttest 1 and CPP Posttest 2). During each drug-free posttest, subjects had free access to both the GRID and HOLE floors for 60 min, to measure alcohol-induced CPP. No injections were given during either posttest in order to ensure a drug-free testing environment and to avoid any cue-induced behaviors, since each floor was previously associated with either an alcohol (+) or saline (-) injection cue. CPP Posttest 2 took place 24 hours after CPP Posttest 1.

A Note About CPP in the HAP/LAP Lines and CPP

Both HAP1 and LAP1 mice produce equivalent CPP at the 0, 1.5, and 3 g/kg doses. LAP1 mice showed greater CPP expression only at the 4.0 g/kg dose (Grahame et al., 2001). This prior research suggests that both lines are similarly sensitive to the rewarding effects of alcohol at the 2.0 g/kg dose, since it is bracketed by the lower doses in the previous research. The current study used HAP2 and LAP2 mice, and thus far HAP2 and LAP2 have not been tested for alcohol-induced CPP. Thus, the Control groups in the proposed study will serve an important role in illustrating any differences in CPP between lines at the 2.0 g/kg dose regardless of age of stress exposure. Importantly, HAP1 and LAP1 and HAP2 and LAP2 mice have been shown to have similar alcohol metabolism, BAC dose response curves, (Grahame et al., 1999), and BAC elimination (Chester & Barrenha, 2007) when alcohol is administered based on body weight, such as in the current study. Previous research has found no difference in BAC levels two hours post-alcohol injection between lines (Chester & Barrenha, 2007), and since trials were five minutes long, BAC levels were not expected to differ between lines during conditioning. Thus, it is assumed that any differences in CPP seen between the lines in the proposed study were not solely attributable to metabolic differences.

Re-Exposure to the Stressor (RS) Procedure

Half of the subjects in each group (Adolescent Stress, Adult Stress, Stress Control) were re-exposed to the original stressor (RS), receiving 15 foot shocks (0.2 mA) in 30 minutes, immediately before each CPP posttest (Adolescent Stress-RS, Adult Stress-RS, Stress Control-RS). The remaining non-RS subjects (Adolescent Stress-noRS, Adult Stress-noRS, Control Stress-noRS) were exposed to the chambers before each posttest, but no foot shocks were given. The RS phase prior to the first CPP posttest was the first foot shock exposure for the Stress Control-RS group, which allowed us to measure acute effects of foot shock stress on CPP (see Table 1). After the RS phase, all subjects were immediately tested for CPP. A blood sample was taken after each CPP posttest to measure CORT levels during CPP Posttest 1 and CPP Posttest 2 and to compare levels between Stress Treatment, RS, and Line subgroups.

The purpose of CPP Posttest 2 was to explore whether alterations in sensitivity to the rewarding effects of alcohol may have served as a mechanism for previous results in which repeated intermittent re-exposure to a stressor before limited access drinking increased voluntary alcohol consumption over time (Chester et al., 2006). It was predicted that CPP would be greater in the RS groups due to the repeated re-exposures to the foot shocks. CPP Posttests 1 and 2 were conducted identically in regards to RS to explore this possibility.

Corticosterone Samples

Blood samples were taken on Days 1 and 10 of CSE and after CPP posttests 1 and 2. Blood samples for CORT analysis were obtained using the submandibular collection technique. A small sterile lancet (5 mm; Goldman) was used to puncture the skin at the vascular bundle behind the jawbone. 10-15 microliters of blood was collected in a 75 mm capillary tube. The samples were placed on dry ice until the samples were centrifuged and plasma extraction occurred (no more than 5 min took place between collection and extraction). Plasma samples were kept frozen in a -80 freezer until CORT analysis was performed.

CORT analysis was run according to the “Small Volumes Protocol” from Assay Designs, using an enzyme immunoassay kit from the same company. Resulting CORT densities were read by a microplate reader at a 405 nm wavelength. All

Table 1

Chronic Stress Exposure and Stress Re-exposure Behavioral Assignments for Subjects Exposed to Stress During Adolescence, Stress During Adulthood, and No Stress

Stress Treatment Group:	CSE	RS Subgroup:	CPP Posttest #1	CPP Posttest #2
Adolescent Stress:	Foot shocks	Adolescent-RS	RS	RS
		Adolescent-noRS	no RS	no RS
Adult Stress:	Foot shocks	Adult-RS	RS	RS
		Adult-noRS	no RS	no RS
Control:	Placed in bins	Control-RS	Acute Stress	Acute Stress
		Control-noRS	Control CPP	Control CPP

samples were run in duplicate and correlation values between each duplicate were analyzed.

Statistical Analyses

Data was analyzed using analysis of variance (ANOVA) in the Statistical Package for Social Sciences (SPSS). The significance level was set at $p < 0.05$. Bonferroni-corrected t-tests were used where appropriate.

Grams of force (g/F) per kg measurements were used as dependent variables for CSE data.

The CPP posttest data was analyzed in several ways to facilitate our interpretation of evidence for conditioned changes in behavior. The pretest provided valuable information for any baseline differences in floor preference. This was also important to assess baseline floor preference given that mice were exposed to a grid-like floor (for foot shock) during the chronic stress procedures. Since the paradigm was implemented as an unbiased design, any significant difference between the raw time scores on the grid floor in the G+ and G- subgroups indicated a baseline preference. If baseline differences between floor types (GRID or HOLE) were present, the GRID difference scores (time spent on the GRID floor during the posttest minus time spent on the GRID floor during the posttest) was analyzed instead. Using the GRID difference score is an advantageous way of interpreting CPP data, because it allows the researcher to account for any initial grid or hole floor preference in the analysis (Cunningham et al., 2003). The GRID difference score reduces variation in initial preference, as it is a within-subject dependent measure that can facilitate the detection of group differences. Alternatively, the raw time spent on the floor paired with alcohol (CS+) versus the floor paired with saline (CS-) could also be used. Past research in our lab has found that even when baseline floor preferences are present, counterbalancing floor assignments allows equal dispersion between the CS+ and CS- subgroups, such that equal preference to alcohol-paired floors and saline-paired floors is present at baseline (unpublished pilot data). The current study initially used the GRID difference score to interpret the data, but also performed analyses using the within-subjects CS+ versus CS- approach.

Since the paradigm was unbiased, any significant difference between the raw time scores on the GRID floor or GRID difference scores between the Conditioning

Subgroups (G+, G-) indicated CPP. Importantly, any interactions with Conditioning Subgroup (G+, G-) indicated differences in CPP magnitude.

Activity rates during the conditioning trials and posttest were also analyzed as dependent variables. This is also an important variable to correlate with CPP, as activity rates have been correlated with CPP expressions in previous research (Cunningham, 2014). Correlations between activity levels during the CPP conditioning trials and CPP posttest data were calculated using Pearson's product moment correlation.

CORT levels at each of the 4 time points (CSE Day 1, CSE Day 10, CPP Posttest 1, and CPP Posttest 2) were used in several analyses. At each time point, differences between Stress Treatment groups and Line were analyzed. For the CPP Posttest time points, differences between RS subgroups were also assessed. In addition, changes in CORT between CSE Days 1 and 10 and CPP Posttests 1 and 2 provided important within-subject information regarding change over time. Correlations between CORT levels during CSE and CPP posttest data were calculated using Pearson's product moment correlation. Area under the curve (AUC) for CORT levels during the 2 CSE time points (Day 1 and Day 10) and during the 2 CPP time points (Posttest 1 and Posttest 2) were also calculated.

For each analysis, a full ANOVA including all relevant factors were performed initially for each paradigm, and follow-up planned comparisons addressing specific research questions were also conducted in order to maximize our ability to detect small or moderate sized effects or interactions that require greater statistical power to detect in a multi-factorial ANOVA. For the CSE analyses, Stress Treatment (Adolescent Stress, Adult Stress, Stress Control) and Line (HAP2, LAP2) were used as independent variables. CPP analyses additionally included RS subgroups (RS, noRS) and conditioning subgroup (GRID+, GRID-) as independent variables. Any interactions between the conditioning subgroups and the other independent variable(s) suggested that the independent variable(s) altered the expression of CPP (Cunningham et al., 2006). Planned comparisons for the CPP data included direct comparisons of the Adolescent Stress and Adult Stress groups and the Adolescent Stress and Stress Control groups, separately. These planned comparisons were designed to directly test the hypotheses regarding age of CSE (Adolescent Stress vs. Adult Stress subjects) and between CSE vs. no CSE in animals of the same age

(Adolescent Stress vs. Stress Control subjects). Since this design was complicated, it was possible that these smaller, direct effects were not detectable in an overall ANOVA, and thus addressing them more specifically was beneficial to the research question at hand. All CPP posttest analyses were run for each posttest individually (Posttest 1, Posttest 2). In addition, a within-subjects repeated measures ANOVA between Posttest 1 and Posttest 2 were run to address the hypothesis about repeated stress re-exposure on CPP expression.

RESULTS

Subjects

A grand total of 419 mice were run over the course of the current study. A goal of 16 mice per subgroup was desired, but due to the complicated design of the study, we anticipated subjects would need to be dropped over the course of the study for a variety of reasons. In total, eleven subjects needed to be excluded from data analyses. One subject was dropped when it received alcohol on a CS- day during CPP. Eight subjects were humanely euthanized due to fighting wounds. Two subjects died during the course of the CPP paradigm. A total of 408 mice (210 HAP2, 198 LAP2) were used for final data analyses. Overall, the numbers across groups were not altered significantly once the described subjects were eliminated.

In total, there were 138 subjects in the Adolescent Stress group, which began CSE between PD 22-34 (*M* = 28) and CPP between PD 62-76 (*M* = 70). There were131 subjects in the Adult Stress group, which began CSE between PD 63-162 (*M* = 95) and began CPP between PD 104-202 (*M* = 137). The age range of the Adult Stress group was larger than desired due to the timing limitations of counterbalancing the breeding pairs. There were 11 subjects aged between PD 136-162 when CSE began, which greatly influenced the age range. However, when these subjects are removed from the data sets, the results for both CSE and CPP are not altered, suggesting that the older subjects in the Adult Stress group did not largely influence the results of the current study. Lastly, there were 139 subjects in the Stress Control group, which were placed in the bins between PD 22-34(*M* = 28) and began CPP between PD 62-76 (*M* = 70) (see Table 2). The final number of subjects in each Line, Stress Treatment, RS, and Conditioning Subgroup are listed in Table 3.

Chronic Stress Exposure

The equipment used to emit foot shocks during CSE also records the amount of force exerted by the subjects for each shock, known as the grams of force (g/F). The g/F per kg data provides information about group difference in startle responses

Table 2

Mean Ages of Subjects in the Adolescent Stress, Control, and Adult Stress Groups at the Start of the Chronic Stress Exposure and Conditioned Place Preference Paradigms

	CSE Day 1			CPP Prettest		
	Adolescent Stress	Control	Adult Stress	Adolescent Stress	Control	Adult Stress
Age (PD):	28	28	95	70	70	137

Table 3

Numbers of Subjects With Behavioral Data Represented in Each Line, Stress Treatment, Stress Re-exposure, and Conditioning Subgroup

	Adolescent Stress				Adult Stress				Control			
	RS		noRS		RS		noRS		RS		noRS	
	G+	G-	G+	G-	G+	G-	G+	G-	G+	G-	G+	G-
HAP2:	18	18	17	17	17	18	17	17	19	18	17	17
LAP2:	17	17	17	17	16	15	15	16	17	17	17	17

in relation to body weight, as well as information about sensitization or habituation to the foot shocks over time.

To examine group differences in shock responses over the 10 days of CSE, g/F per kg data was analyzed using a 2 (Stress Treatment: Adolescent Stress, Adult Stress) x 2 (Line: HAP2, LAP2) repeated measures ANOVA on CSE Days 1 and 10. Since there was no g/F data for the Stress Control subjects, only the Adolescent Stress and Adult Stress subjects were represented in this analysis.

Within-subjects, a main effect of CSE Day was present (F[1, 265] = 46.09, p < 0.001). Between groups, there was an interaction between Stress Treatment and Line (F[1, 265] = 18.04, p < 0.001), where HAP2 mice showed greater g/F responses than LAP2 mice, particularly the HAP2 Adult Stress subjects (see Fig. 2).

To better interpret the differences in g/F responses between Stress Treatment groups, all data were analyzed separately for HAP2 and LAP2 mice. A follow-up repeated measures ANOVA was run on the 2 Stress Treatment groups within each Line, and Bonferroni-corrected adjustments were made (p < 0.025). In HAP2 subjects, there was a within-subjects main effect of CSE Day (F[1, 137] = 26.82, p < 0.001), due to the fact that both the HAP2 Adult Stress and Adolescent Stress groups habituated to the foot shocks overall. The HAP2 Adult Stress subjects showed overall greater g/F responses than the HAP2 Adolescent Stress subjects (F[1, 137] = 28.08, p < 0.001), as evident by a between-subjects effect of Stress Treatment in the HAP2 subjects. In contrast, LAP2 subjects showed a main effect of habituation over the 10 days of foot shocks (F[1, 128] = 20.19, p < 0.001), but there was no between-subjects effect of Stress Treatment group, suggesting that the LAP2 Adolescent Stress and LAP2 Adult Stress subjects did not differ in g/F per kg tactile responses. In general, all subjects habituated to the foot shocks over the course of the 10 days.

In addition to looking at the change in g/F responses over the 10 days of CSE, CSE Days 1-10 were analyzed individually to examine possible group differences on each day. A 2 (Stress Treatment) x 2 (Line) univariate ANOVA was performed for the g/F per kg responses on Days 1-10 of CSE, separately. On every Day of CSE (1-10), there was a significant interaction between Stress Treatment and Line (all ps < 0.001). Follow-up analyses used univariate ANOVAs to examine differences between the 2 Lines within each Stress Treatment group on each Day of CSE (1-10; Bonferroni-corrected: p < 0.025). In the Adolescent Stress subjects, there was no difference in

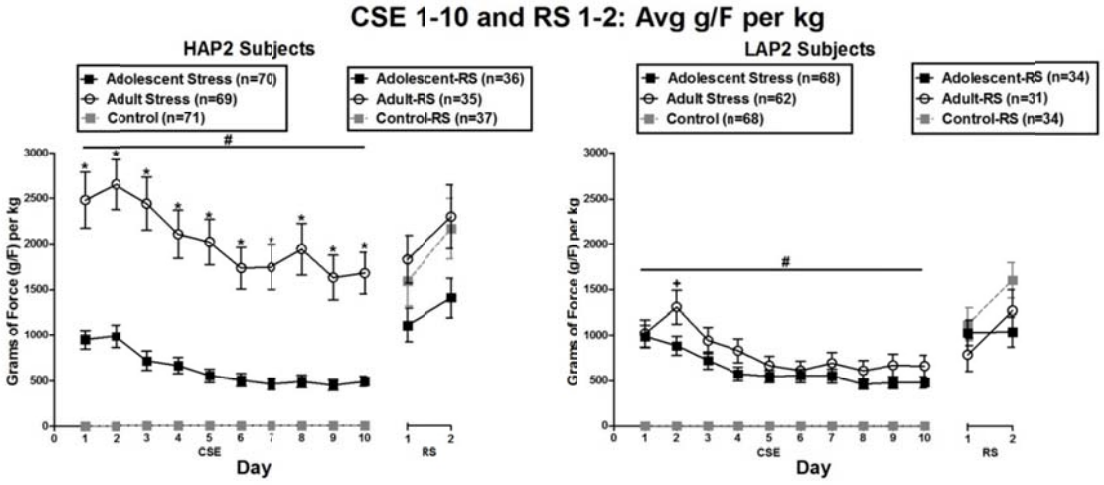

Figure 2. Mean (±SEM) grams of force per kilogram on CSE Days 1-10 and RS 1-2 in HAP2 mice and LAP2 mice exposed to adolescent stress and adult stress. Stress control mice are showed at 0 g/F. *$p < 0.001$, Adult Stress > Adolescent Stress. +$p < 0.05$, Adult Stress > Adolescent Stress. #$p < 0.05$, CSE Day 1 > CSE Day 10.

g/F per kg responses between the HAP2 and LAP2 subjects on any day of CSE (1-10). However, in the Adult Stress subjects, the HAP2 Adult Stress subjects showed greater g/F per kg responses than the LAP2 Adult Stress subjects on every day of CSE (1-10; all *p*s < 0.01). Importantly, these differences were seen in spite of the habituation in g/F responses indicated by the previous repeated-measures analyses. This suggests that the HAP2 Adult Stress subjects showed significantly greater responses to the foot shocks than the LAP2 Adult Stress subjects, whereas this Line difference was not observed between the HAP2 and LAP2 Adolescent Stress groups.

Re-Exposure to the Stressor

G/F per kg data collected during re-exposure to the stressor were also analyzed before CPP Posttest 1 (RS 1) and CPP Posttest 2 (RS 2). Since there was no g/F data for the non Re-exposed (noRS) subjects, only the Adolescent-RS, Adult-RS, and Control-RS subjects are represented in these analyses.

To see if g/F per kg responses changed from the last day of CSE (CSE 10) to RS 1, a 3 (Stress Treatment: Adolescent Stress, Adult Stress, Stress Control) x 2 (Line: HAP2, LAP2) repeated measures ANOVA was performed on CSE 10 and RS 1. There was a within-subjects interaction between Day and Stress Treatment group (F[2, 201] = 16.60, p < 0.001). Additionally, there was a Line by Stress Treatment interaction between groups (F[2, 201] = 4.87, p < 0.01) (see Fig. 2). To better investigate these interactions, follow-up analyses used a repeated measures ANOVA on the 2 lines for CSE 10 and RS 1 within each Stress Treatment group (Bonferroni corrected: p < 0.017). In the Adolescent Stress subjects, there was an overall effect of Day (F[1, 68] = 24.66, p < 0.001), such that g/F per kg responses increased between CSE 10 and the RS 1. A similar effect was seen in the Stress Control subjects (F[1, 69] = 65.44, p < 0.001).There was no within-subjects change in g/F responses between CSE 10 and RS 1 in the Adult Stress subjects. However, the HAP2 Adult Stress subjects showed greater overall g/F responses than the LAP2 Adult Stress subjects, as indicated by a between-subjects effect of Line (F[1,64] = 9.91, p <0.01), mimicking the Line difference in g/F responses during CSE. Importantly, this Line difference was not seen in the Adolescent Stress or Stress Control subjects in the follow-up analyses.

To see if g/F per kg responses changed with repeated re-exposure to the stressor, a 3 (Stress Treatment) x 2 (Line) repeated-measures ANOVA was used

between the g/F per kg responses on RS 1 and RS 2. A main effect of Day was present (F[1,201] = 27.37, p < 0.001), and an interaction between Day and Stress Treatment group was trending (F[2, 201] = 2.60, p = 0.08). In addition, a main effects of Line was significant between-subjects (F[1, 201] = 10.79, p < 0.01) and a main effect of Stress Treatment was also trending (F[2, 201] = 2.71, p = 0.07). Overall, the LAP2 subjects showed lower g/F per kg responses than the HAP2 subjects (see Fig. 2). To further interpret these interactions, follow-up analyses used a repeated measures ANOVA on the 2 lines for RS 1 and RS 2 within each Stress Treatment group (Bonferroni-corrected: p < 0.017). In the Adolescent Stress subjects, there were no within-subject effects of Day or Line, suggesting that g/F responses did not change between RS 1 and RS 2 and that there were no differences between Lines. However, both the Adult Stress and Stress Control groups showed a main effect of Day overall (F[1, 64] = 11.38, p < 0.01; F[1, 69] = 16.67, p < 0.001), where g/F responses increased between RS 1 and RS 2 in each Stress Treatment group. In addition, HAP2 Adult Stress subjects showed greater overall g/F responses than their LAP2 counterparts (F[1, 64] = 8.48, p < 0.01), again mimicking the Line difference present in the Adult Stress subjects during CSE 1-10 and between CSE 10 and RS 1. The Stress Control subjects showed no overall difference between the HAP2 and LAP2 subjects.

In addition to investigating the change in g/F responses over time, the g/F responses on RS 1 and RS 2 were analyzed individually to examine possible group differences on each day. A 3 (Stress Treatment) x 2 (Line) univariate ANOVA was performed on RS 1 and RS 2, separately. On RS 1, an interaction between Line and Stress Treatment group was trending (F[2, 206] = 2.55, p = 0.08), and a main effect of Line was present (F[1,206] = 9.33, p < 0.001), where HAP2 subjects generally showed greater g/F per kg responses than the LAP2 subjects .

On RS 2, there were main effects of Line (F[1, 206] = 9.35, p < 0.01) and Stress Treatment group (F[2, 206] = 3.78, p < 0.05), but no interactions occurred between the two variables, in contrast to the interaction seen during RS 1. Overall, HAP2 subjects showed greater g/F responses than LAP2 subjects. The Stress Control subjects showed greater g/F per kg responses than the Adolescent Stress subjects (p < 0.05), overall, while the other groups did not differ from one another. This suggests that repeated re-exposure to the stressor actually may have decreased g/F responses in the Adolescent Stress subjects compared to the Control subjects.

Conditioned Place Preference

Pretest

To begin interpretation of the CPP data, the average time on the GRID floor during the pretest was analyzed using a 3 (Stress Treatment: Adolescent Stress, Adult Stress, Stress Control) x 2 (Line: HAP2, LAP2) x 2 (Conditioning Subgroup: G+, G-) ANOVA. The pretest analysis served to investigate any initial preferences between the conditioning subgroups toward the GRID or HOLE floor. Main effects of Line (F[1, 407] = 131.01, $p < 0.001$) and Stress Treatment group (F[2, 407] = 28.57, $p < 0.001$) were seen during the pretest. Overall, LAP2 subjects spent significantly more time on the GRID floor than HAP2 subjects during the pretest (see Fig. 3). Adult Stress subjects spent more time on the GRID floor than both Adolescent Stress subjects and Stress Control subjects during the pretest, with significant differences between each group (all ps < 0.05). Importantly, these effects did not interact with Conditioning Subgroup assignments, nor was there a main effect of conditioning subgroup in the analysis. This suggests that the initial preference toward the GRID floor was not different between those with G+ or G- assignments, and was more of a global effect across subjects.

To ensure that the initial GRID floor preference was counterbalanced between the alcohol-paired (CS+) and saline-paired (CS-) floor assignments across groups, average time on the CS+ and CS- floors during the pretest were analyzed using a 3 (Stress Treatment) x 2 (Line) repeated-measures design. Importantly, there were no main effects of Line or Stress Treatment group on the average time spent on the CS+ versus CS- floors during the pretest (see Fig. 4), which suggests that the initial GRID preference was not specific to subjects who had the GRID floor assigned as their CS+ or CS- cue. This further supports the interpretation that the initial GRID floor preference was a global effect across subjects in the study.

The average activity level during the pretest was analyzed using a 3 (Stress Treatment) x 2 (Line) univariate ANOVA on the average activity across the 60 min of the pretest. Main effects of Line (F[1, 407] = 312.11, $p < 0.001$) and Stress Treatment (F[2, 407] = 17.52, $p < 0.001$) were observed (see Fig. 5). Overall, HAP2 subjects showed greater activity levels than LAP2 subjects. Additionally, the Adolescent Stress ($p < 0.001$) and Stress Control subjects ($p < 0.001$) generally showed greater activity

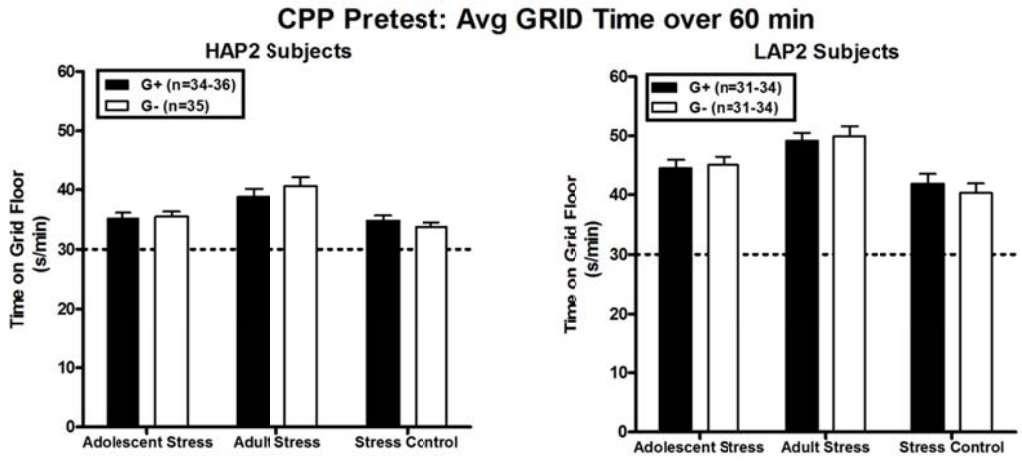

Figure 3. Mean (±SEM) raw time (sec/min) spent on the GRID floor in G+ and G- conditioning subgroups during the pretest for HAP2 and LAP2 mice exposed to adolescent stress, adult stress, and no stress.

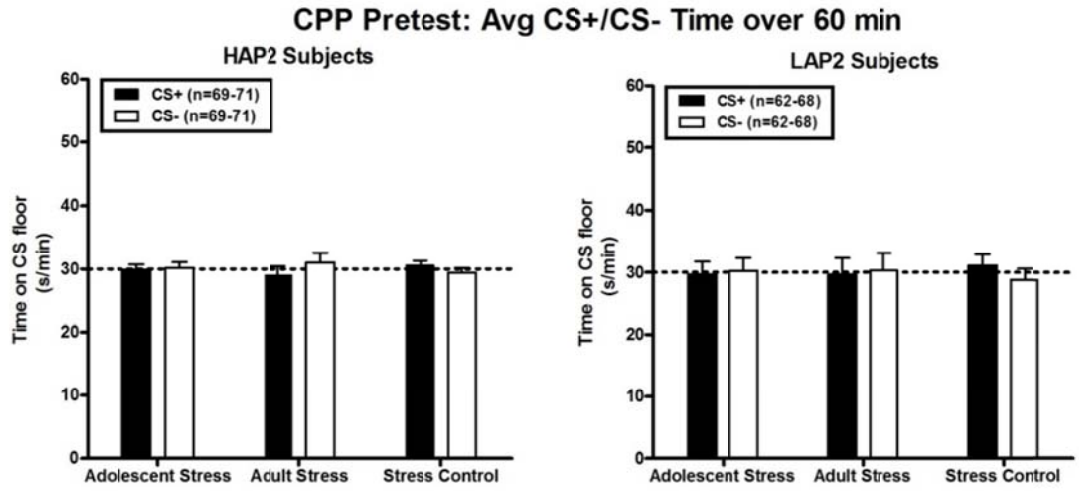

Figure 4. Mean (±SEM) raw time (sec/min) spent on the CS+ and CS- floor during the pretest for HAP2 and LAP2 mice exposed to adolescent stress, adult stress, and no stress.

levels than the Adult Stress subjects (*p*s < 0.001), while the two groups did not differ from one another.

Since the pretest was 60 min long, it was possible that activity levels changed over the course of the pretest. A 3 (Stress Treatment) x 2 (Line) repeated measures analysis on minutes 1-60 of the pretest indicated a 3-way interaction between Minute, Line, and Stress Treatment on activity levels within-subjects (F[118, 23,718] = 1.23, p = 0.05), as well main effects of Line (F[1, 402] = 312.11, p < 0.001) and Stress Treatment (F[2, 402] = 17.52, p < 0.001) between groups (see Fig. 6). To obtain a better interpretation of general activity level changes, a 3 (Stress Treatment) x 2 (Line) repeated measures analysis was performed specific on Min 1 and Min 60 of the pretest. In this analysis, there was a within-subject interaction with Minute and Line (F[1, 402] = 171.28, p < 0.001) and a between-group main effect of Line (F[1, 402] = 21.63, p < 0.001). Both HAP2 (F[1, 207] = 378.92, p < 0.001) and LAP2 (F[1, 195] = 1449.64, p < 0.001) subjects showed a general decrease in activity between Min 1 and Min 60, although LAP2 subjects were less active overall and decreased in activity more drastically than HAP2 subjects over the 60 min of the pretest.

Conditioning Trials

CS+ trials. To investigate differences in activity levels during the alcohol-paired conditioning trials, activity levels during Trials 1 and 4 of the CS+ trials were analyzed using a 3 (Stress Treatment) x 2 (Line) design repeated measures ANOVA. During the CS+ trials, there was an interaction between Trial and Line (F[1, 402] = 3.73, p < 0.05). Additionally, there were main effects of Line (F[1, 402] = 31.54, p < 0.001) and Stress Treatment (F[2, 402] = 10.68, p < 0.001). Overall, LAP2 subjects showed greater activity during the CS+ trials than HAP2 subjects during the CS+ trials (see Fig. 7). In addition, Adult Stress subjects showed less activity than the Adolescent Stress (p < 0.001) and Stress Control subjects (p < 0.001), in general. To interpret the interactions, a follow-up analysis used a repeated measures ANOVA on the 3 Stress Treatment groups for CS+ Trials 1 and 4, within each Line (Bonferroni-corrected: p < 0.025). Both the HAP2 (F[1, 207] = 41.43, p < 0.001) and LAP2 subjects (F[1, 195] = 12.82, p < 0.001) showed an increase in activity levels over the course of the CS+ conditioning trials, suggesting an overall sensitization to alcohol's locomotor effects. In the HAP2 mice, there was a main effect of Stress Treatment between groups (F[2, 207] = 8.71, p < 0.001), where the Adult Stress subjects showed

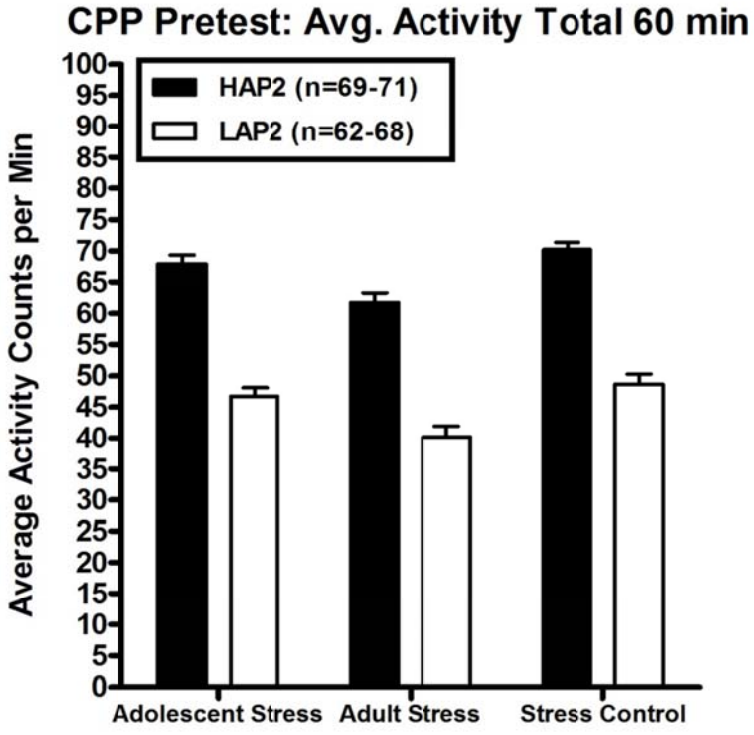

Figure 5. Mean (±SEM) activity counts per minute during the pretest for HAP2 and LAP2 mice exposed to adolescent stress, adult stress, and no stress.

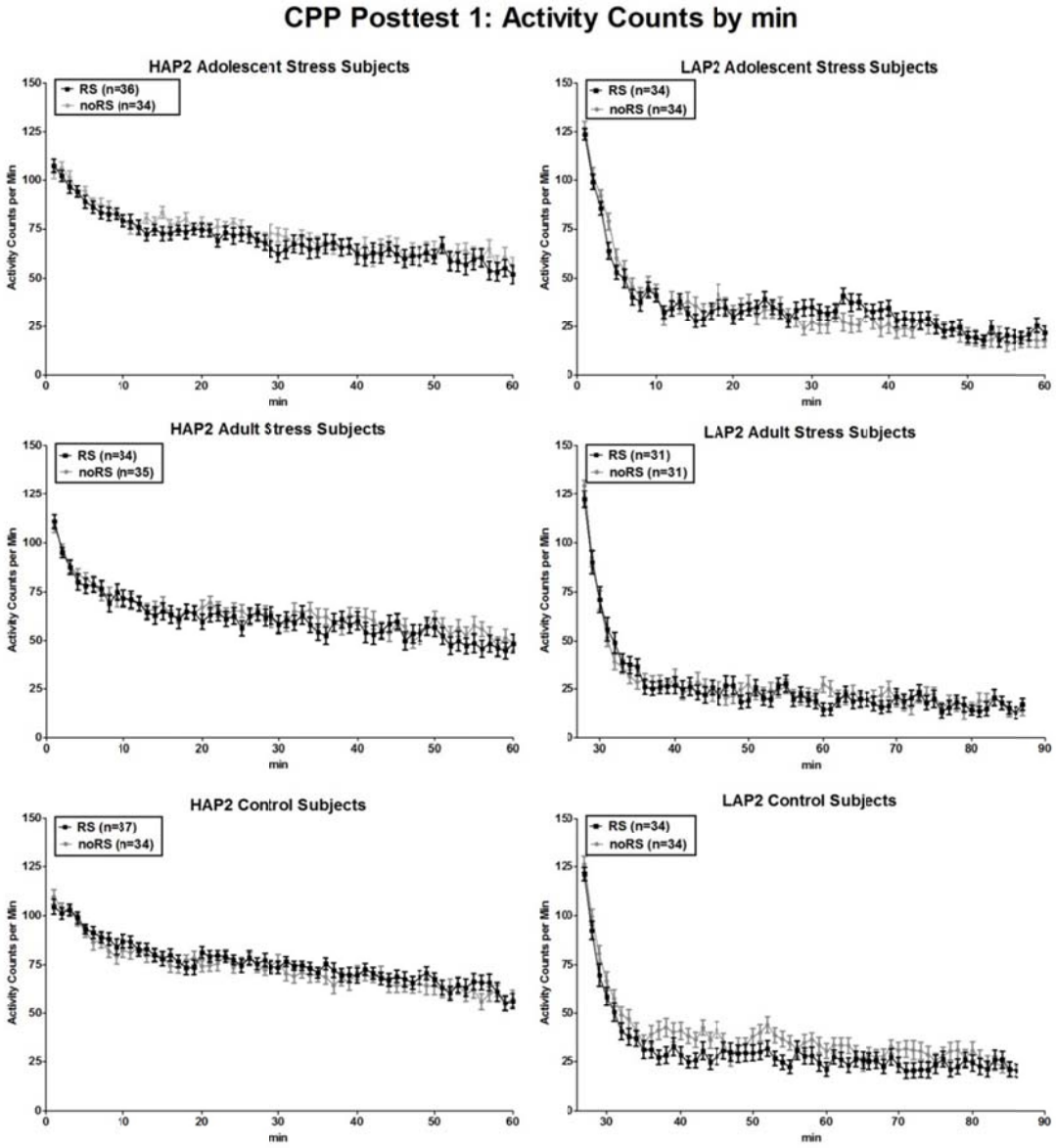

Figure 6. (±SEM) Raw activity counts during the pretest for all HAP2 and LAP2 mice, separated by stress treatment and stress re-exposure subgroups.

lower activity than the Adolescent Stress ($p < 0.025$) and Stress Control subjects ($p < 0.001$). A main effect of Stress Treatment was near significance in the LAP2 subjects ($F[2, 195] = 3.55$, $p = 0.03$), but did not meet Bonferroni-corrected criteria.

CS- trials. To investigate differences in activity levels during the saline-paired conditioning trials, activity levels during Trials 1 and 4 of the CS- trials were analyzed using a 3 (Stress Treatment) x 2 (Line) design repeated measures ANOVA. During the CS- trials, there was an interaction between Trial and Line ($F[1, 402] = 58.85$, $p < 0.001$) within-subjects. Between groups, there was a trend towards an interaction between Line and Stress Treatment ($F[2, 402] = 2.64$, $p = 0.07$) and a main effect of Stress Treatment ($F[2, 402] = 3.19$, $p < 0.05$). Overall, Adult Stress subjects showed less activity than the Adolescent Stress subjects ($p < 0.05$), and trended towards significance compared to the Stress Controls ($p = 0.08$; see Fig. 7). To follow-up the near-significant interaction, a repeated measures ANOVA on the 3 Stress Treatment groups for CS- Trials 1 and 4 was performed (Bonferroni-corrected: $p < 0.025$). The HAP2 subjects showed an overall habituation in activity across the CS- trials ($F[1, 207] = 62.69$, $p < 0.001$), and a main effect of Stress Treatment between groups ($F[2, 207] = 6.74$, $p < 0.01$). HAP2 Adult Stress subjects showed less activity than Adolescent Stress ($p < 0.025$) and Stress Control subjects ($p < 0.01$). LAP2 subjects also showed a habituation across CS- trials ($F[1, 195] = 267.18$, $p < 0.001$), with no differences between Stress Treatment groups.

Posttest 1

CPP Posttest data can be analyzed in several ways (see review by Cunningham et al., 2003). Posttest data for this study was initially analyzed using raw time on the GRID floor, GRID difference scores (time on the GRID floor during the pretest minus time on the GRID floor during the posttest) and raw time on the CS+ and CS- floors, separately. Importantly, the use of these three different dependent variables yielded the same overall interactions and main effects in the data.

Due to the initial GRID floor bias present during the pretest, data was most effectively shown using the GRID difference score. The difference score removed the initial bias of the GRID floor from the Posttest data interpretation and allowed for the clearest interpretation of the data.

In addition to using different dependent variables to interpret CPP during the posttests, it was also important to investigate a portion of time from the posttest that

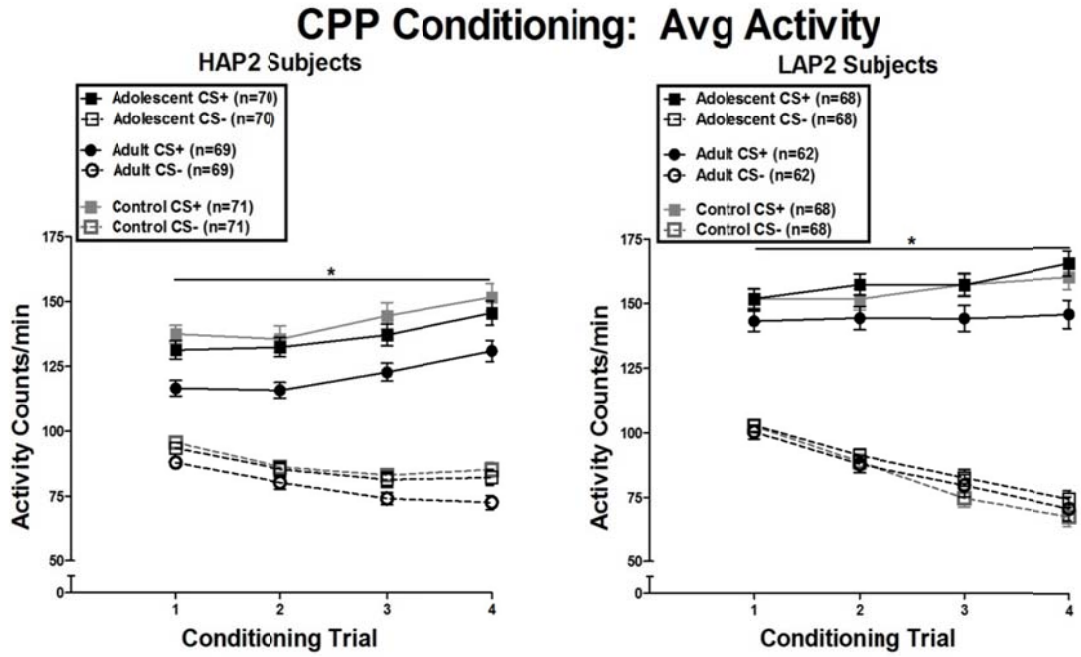

Figure 7. Mean (±SEM) activity counts during CS+ and CS- conditioning trials 1-4 in HAP2 and LAP2 mice exposed to adolescent stress, adult stress, and no stress. *p < 0.05 CS+ Trial 4 > CS+ Trial 1 and CS- Trial 1 > CS- Trial 4.

would allow for the most accurate interpretation of the data. Since each Posttest was 60 min long, and both activity levels and CPP magnitude can vary over time, analyses of the minute by minute change of the Posttests were performed. To look at the change in time spent on the GRID floor over the course of the Posttest, a 3 (Stress Treatment: Adolescent Stress, Adult Stress, Stress Control) x 2 (Line: HAP2, LAP2) x 2 (RS: RS, noRS) x 2 (Conditioning Subgroup: G+, G-) repeated measures ANOVA was performed on the GRID floor time for min 1-60 of Posttest 1. There was a significant 3-way interaction between Minute, Line, and Conditioning Subgroup (F[59, 22656] = 2.30, $p < 0.001$), and a trend towards significance in a 5-way interaction between Minute, Line, Stress Treatment, RS, and Conditioning Subgroup (F[118, 22656] = 1.19, $p = 0.078$). Importantly, using the repeated-measures ANOVA model to investigate change in CPP magnitude in terms of time spent on the CS+ floor as the dependent variable yielded the same 3-way interaction of Minute, Line, and Conditioning Subgroup (F[59, 22656] = 2.52, $p < 0.001$), as well as a 3-way interaction between Minute, Line, and Stress Treatment (F[118, 22656] = 1.24, $p < 0.05$). These interactions suggest that CPP magnitude likely changed over the course of the 60 min Posttest, and that only using an average of the 60 min to run CPP Posttest analyses would lead to misinterpretation of the data (Cunningham et al., 2006).

We decided to split the Posttest 1 and Posttest 2 data into 3 separate time bins in order to investigate how CPP expression had changed over the 60 min. Visual comparisons of time spent on the GRID floor on minutes 1-60 between the GRID+ and GRID- subjects in each Stress Treatment and Line subgroup suggested that the greatest CPP magnitude was seen within the first 20 min of the CPP Posttests, and then continued to decline over the remaining 40 min (see Fig. 8). Thus, we decided to split the 60 min Posttest into 3 separate 20 min time bins. For the analyses, there were 3 separate GRID difference score analyses for each Posttest, where the dependent variables were the average GRID difference scores over the first 20 min, over the second 20 min, and over the third 20 min of the 60 min Posttests.

GRID difference score during the first 20 min of posttest 1. A 3 (Stress Treatment: Adolescent Stress, Adult Stress, Stress Control) x 2 (Line: HAP2, LAP2) x 2 (RS: RS, noRS) x 2 (Conditioning Subgroup: G+, G-) univariate ANOVA on the GRID difference score during the first 20 min of Posttest 1 was used to interpret the first 20 min of the Posttest 1 data. Because we used the GRID difference score

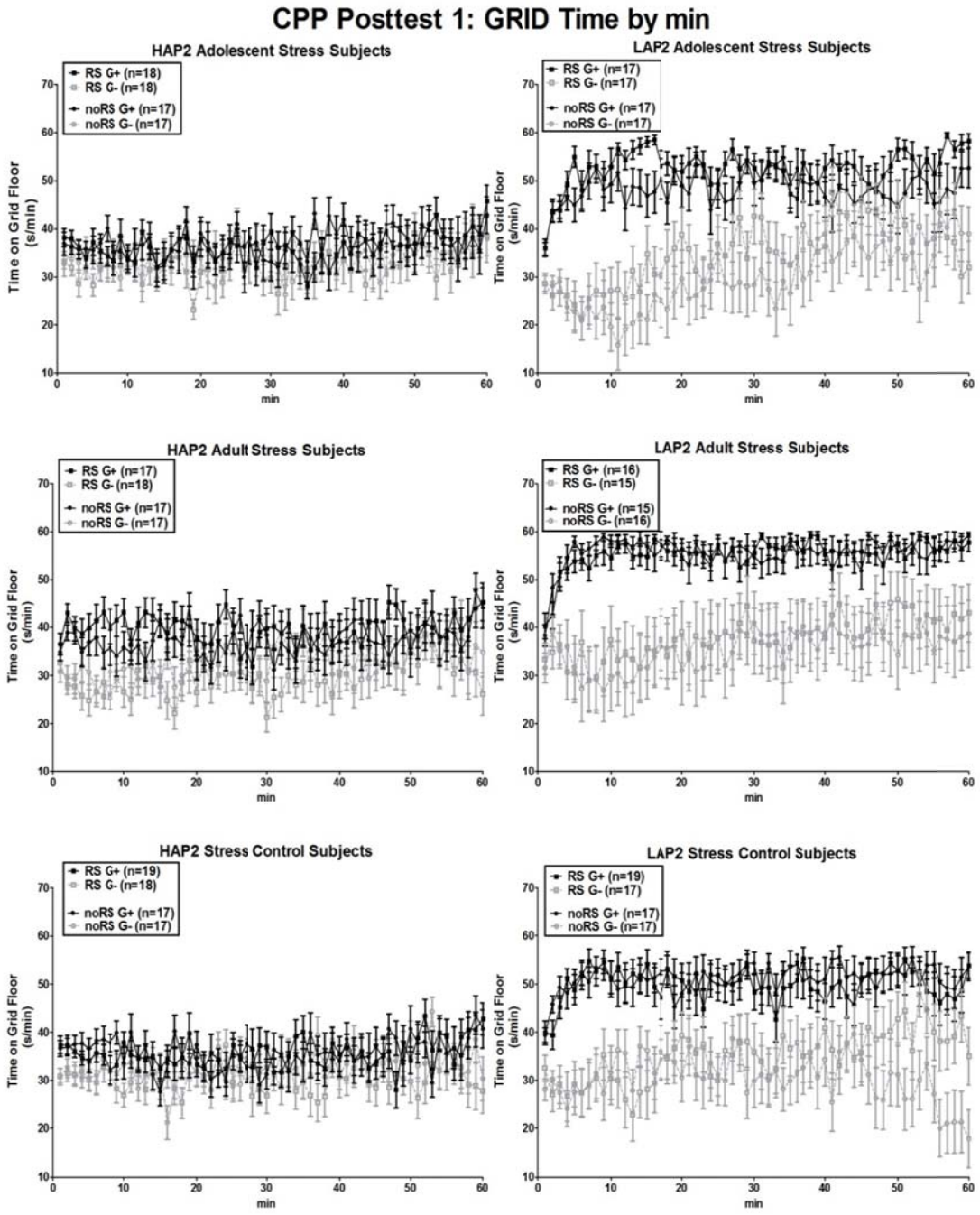

Figure 8. (±SEM) Raw time spent on the GRID floor during the pretest for G+ and G- conditioning subgroups for all HAP2 and LAP2 mice, separated by stress treatment and stress re-exposure subgroups.

analysis, a main effect or interaction with Conditioning Subgroup indicated significant CPP, as it suggested the subjects that had the GRID floor paired with alcohol (G+) spent significantly more time on the GRID floor than those that had the GRID floor paired with saline (G-).

An interaction between Line and Conditioning Subgroup was present (F[1, 407] = 36.70, $p < 0.001$), indicating that LAP2 subjects showed greater overall CPP than HAP2 subjects (see Fig. 9).

To better understand this data, a follow-up analysis used a 3 (Stress Treatment) x 2 (RS) x 2 (Conditioning Subgroup) univariate ANOVA on the GRID difference score during the first 20 min of Posttest 1 within each Line (Bonferroni-corrected: $p < 0.025$). In the HAP2 subjects, there was an interaction between Stress Treatment and Conditioning Subgroup (F[2, 209] = 5.28, $p < 0.01$). Thus, a second follow-up analysis used a 2 (RS) x 2 (Conditioning Subgroup) univariate ANOVA on the GRID difference score during the first 20 min of Posttest 1 within each Stress Treatment group in the HAP2 subjects (Bonferroni-corrected: $p < 008$). The HAP2 Adolescent Stress subjects showed no main effect of Conditioning Subgroup (F[1, 69] = 3.47, $p = 0.07$), suggesting that these subjects did not show alcohol-induced CPP. However, both the HAP2 Adult Stress (F[1, 68] = 19.26, $p < 0.001$) and HAP2 Stress Control subjects (F[1, 70] = 11.67, $p < 0.008$) did show significant CPP.

In the LAP2 subjects, there was a main effect of Conditioning Subgroup (F[1,197] = 99.05, $p < 0.001$), suggesting an overall presence of CPP in the LAP2 mice. However, there were no interactions between Stress Treatment and Conditioning Subgroup in the LAP2 subjects, suggesting that there were no differences in CPP magnitude between the Stress Treatment groups in LAP2 subjects.

Importantly, no interactions between RS and Conditioning Subgroup were seen during CPP Posttest 1. This suggests that stress re-exposure did not alter CPP magnitude in any Line or Stress Treatment subgroup.

Planned comparisons. In addition to the overall ANOVA, the specified planned comparisons between the Adolescent Stress and Adult Stress subjects and the Adolescent Stress and Stress Control subjects were performed.

Adolescent stress vs. adult stress. A 2 (Stress Treatment: Adolescent Stress, Adult Stress) x 2 (Line) x 2 (RS) x 2 (Conditioning Subgroup) univariate ANOVA on the GRID difference score during the first 20 min of Posttest 1 was

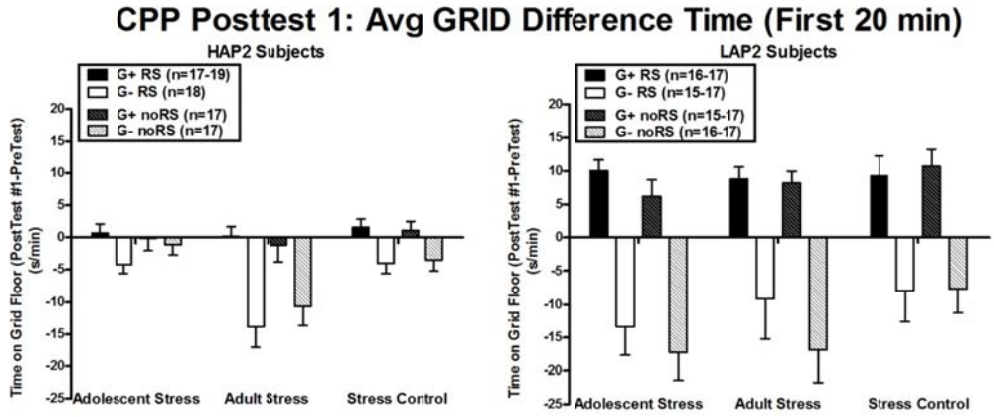

Figure 9. Average (±SEM) GRID difference time during the first 20 minutes of Posttest 1 for G+ and G- conditioning subgroups (separated by stress re-exposure subgroup) for all HAP2 and LAP2 mice exposed to adolescent stress, adult stress, and no stress.

performed to analyze the planned comparison regarding age of stress exposure (Adolescent Stress vs. Adult Stress). There were significant interactions between Line and Conditioning Subgroup (F[1, 268] = 25.22, $p < 0.001$) and Line and Stress Treatment (F[1, 268] = 4.76, $p < 0.05$). There were also trending interactions between Line, Stress Treatment, and Conditioning Subgroup (F[1, 268] = 3.18, $p = 0.076$) and Line and RS (F[1, 268] = 2.85, $p = 0.09$). To better understand the interactions in this planned comparison, a follow-up analysis used a 2 (Stress Treatment) x 2 (RS) x 2 (Conditioning Subgroup) univariate ANOVA on the GRID difference score during the first 20 min of Posttest 1 within each Line (Bonferroni-corrected: $p < 0.025$). In the HAP2 subjects, there was an interaction between Stress Treatment and Conditioning Subgroup (F[1, 138] = 8.00, $p < 0.01$). A second follow-up analysis used a 2 (RS) x 2 (Conditioning Subgroup) univariate ANOVA within each Stress Treatment group in the HAP2 subjects (Bonferroni-corrected: $p < 0.0125$). The HAP2 Adult Stress subjects showed significant CPP (F[1, 68] = 19.23, $p < 0.001$) while the HAP2 Adolescent Stress subjects did not (F[1, 69] = 3.47, $p = 0.07$). In the LAP2 subjects, an overall main effect of Conditioning Subgroup was seen (F[1, 129] = 73.38, $p < 0.001$), indicating CPP, but no other interactions were present. There were no interactions of RS and Conditioning Subgroup. Thus, the results from this planned comparison showed similar results to that of the overall ANOVA.

Adolescent stress vs. stress control. A 2 (Stress Treatment: Adolescent Stress, Stress Control) x 2 (Line) x 2 (RS) x 2 (Conditioning Subgroup) univariate ANOVA on the GRID difference score during the first 20 min of Posttest 1 was performed to analyze the planned comparison regarding the effects of stress exposure versus no stress exposure in subjects of the same age (Adolescent Stress vs. Stress Control). There was an interaction between Line and Subgroup (F[1, 276] = 39.58, $p < 0.001$), indicating that LAP2 subjects showed greater overall CPP than HAP2 subjects. However, there were no interactions between Stress Treatment and Conditioning Subgroup or RS and Conditioning Subgroup in this planned comparison.

Activity levels during the first 20 min of posttest 1. Since activity levels could be related to CPP expression, a 3 (Stress Treatment) x 2 (Line) x 2 (RS) x 2 (Conditioning Subgroup) univariate ANOVA on the average activity levels during the first 20 min of Posttest 1 was performed to examine possible group differences in activity levels. There was a main effect of Line (F[1, 407] = 542.65, $p < 0.001$), in

which HAP2 subjects showed greater activity during the first 20 min of Posttest 1 than LAP2 subjects (see Fig. 10). In addition, a main effect of Stress Treatment was present (F[2, 407] = 18.48, $p < 0.001$), such that Adolescent Stress and Stress Control subjects showed greater activity levels than the Adult Stress subjects overall ($p < 0.001$), but did not differ from each other. These results suggest that increased activity levels in the Adolescent Stress and Stress Control subjects were not due to differences in stress history, but may reflect a difference in age compared to the Adult Stress groups.

Correlations between activity levels and time spent on the GRID floor during the first 20 min of the Posttest were analyzed. Activity levels were significantly negatively related to time spent on the GRID floor (r = -0.299, $p < 0.001$), suggesting that lower activity levels could have contributed to greater CPP expression.

To better understand the relationship between activity levels and CPP expression, two individual follow-up analyses examined the data within each Line and within each Stress Treatment group, separately, since there were main effects of Line and Stress Treatment in the activity level analyses. Activity levels were only significantly correlated with time spent on the GRID floor in LAP2 subjects (r = -0.250, $p < 0.001$), and not HAP2 subjects (r = -0.03, p = 0.67). Activity levels were significantly negatively correlated with time spent on the GRID floor in the Adult Stress (r = -0.36, $p < 0.001$) and Stress Control subjects (r = -0.35, $p < 0.001$), but not in the Adolescent Stress subjects (r = 0.15, p = 0.09). This suggests that activity levels may have contributed to CPP expression in specific subgroups during the first 20 min of Posttest 1, but did not have an overall effect on the subjects in the study and their CPP expression.

GRID difference score during the second 20 min of posttest 1. A 3 (Stress Treatment: Adolescent Stress, Adult Stress, Stress Control) x 2 (Line: HAP2, LAP2) x 2 (RS: RS, noRS) x 2 (Conditioning Subgroup: G+, G-) univariate ANOVA on the GRID difference score during the second 20 min of Posttest 1 was used to interpret the second 20 min of the Posttest 1 data. An interaction between Line and Conditioning Subgroup was present (F[1, 407] = 27.36, $p < 0.001$), indicating that LAP2 subjects showed greater overall CPP than HAP2 subjects (see Fig. 11). In addition, a 3-way interaction between Line, RS, and Conditioning Subgroup was trending towards significance (F[2, 407] = 3.63, p = 0.057).

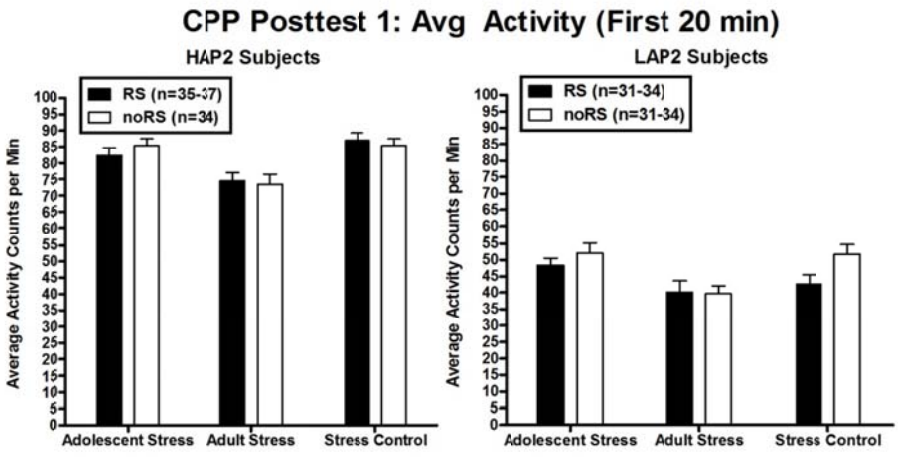

Figure 10. Average (±SEM) activity levels during the first 20 minutes of Posttest 1 for stress re-exposed and non stress re-exposed HAP2 and LAP2 mice exposed to adolescent stress, adult stress, or no stress.

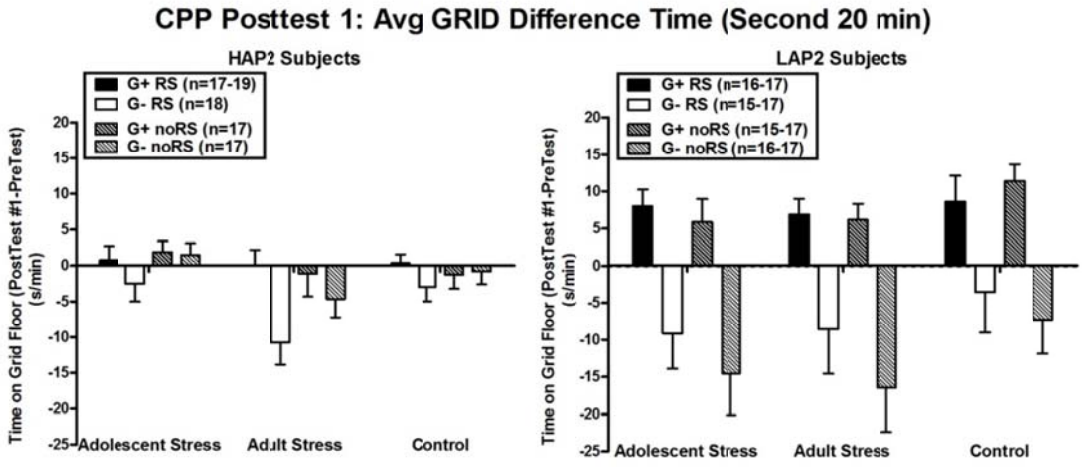

Figure 11. Average (±SEM) GRID difference time during the second 20 minutes of Posttest 1 for G+ and G- conditioning subgroups (separated by stress re-exposure subgroup) for all HAP2 and LAP2 mice exposed to adolescent stress, adult stress, and no stress.

A follow-up analysis used a 3 (Stress Treatment) x 2 (RS) x 2 (Conditioning Subgroup) univariate ANOVA on the GRID difference score during the first 20 min of Posttest 1 within each Line (Bonferroni-corrected: $p < 0.025$). In the HAP2 subjects, there was a main effect of Conditioning Subgroup ($F[2, 209] = 7.65$, $p < 0.01$), suggesting that HAP2 subjects overall expressed CPP. Similarly, in the LAP2 subjects, there was a main effect of Conditioning Subgroup ($F[1,197] = 51.77$, $p<0.001$), suggesting an overall presence of CPP in the LAP2 mice. There were no interactions between Stress Treatment or RS and Conditioning Subgroup in either Line, suggesting that there were no differences in CPP magnitude between the Stress Treatment or RS subgroups within each Line by the second 20 min of Posttest 1.

Planned comparisons. In addition to the overall ANOVA, the specified planned comparisons between the Adolescent Stress and Adult Stress subjects and the Adolescent Stress and Stress Control subjects were performed.

Adolescent stress vs. adult stress. A 2 (Stress Treatment: Adolescent Stress, Adult Stress) x 2 (Line) x 2 (RS) x 2 (Conditioning Subgroup) univariate ANOVA on the GRID difference score during the second 20 min of Posttest 1 was performed to analyze the planned comparison regarding age of stress exposure (Adolescent Stress vs. Adult Stress). There was a significant interaction between Line and Conditioning Subgroup ($F[1, 268] = 17.59$, $p < 0.001$). There were no interactions of Stress Treatment or RS and Conditioning Subgroup. Thus, the results from this planned comparison showed similar results to that of the overall ANOVA.

Adolescent stress vs. stress control. A 2 (Stress Treatment: Adolescent Stress, Stress Control) x 2 (Line) x 2 (RS) x 2 (Conditioning Subgroup) univariate ANOVA on the GRID difference score during the second 20 min of Posttest 1 was performed to analyze the planned comparison regarding the effects of stress exposure versus no stress exposure in subjects of the same age (Adolescent Stress vs. Stress Control). There was an interaction between Line and Subgroup ($F[1, 276] = 23.61$, $p < 0.001$), indicating that LAP2 subjects showed greater overall CPP than HAP2 subjects. However, there were no interactions between Stress Treatment and Conditioning Subgroup or RS and Conditioning Subgroup in this planned comparison.

Activity levels during the second 20 min of posttest 1. A 3 (Stress Treatment) x 2 (Line) x 2 (RS) x 2 (Conditioning Subgroup) univariate ANOVA on the average activity levels during the second 20 min of Posttest 1 was performed to

examine possible group differences in activity levels. There was a main effect of Line (F[1, 407] = 608.98, p < 0.001), in which HAP2 subjects showed greater activity during the second 20 min of Posttest 1 than LAP2 subjects (see Fig. 12). In addition, there was a main effect of Stress Treatment (F[1, 407] = 622.90, p < 0.001), in which the Adolescent Stress (p < 0.001) and Control (p < 0.001) subjects showed greater activity than the Adult Stress subjects. No effects of RS subgroup were observed.

Correlations between activity levels and time spent on the GRID floor during the second 20 min of the Posttest were analyzed. Activity levels were significantly negatively related to time spent on the GRID floor (r = -0.32, p < 0.001), suggesting that lower activity levels could have contributed to greater CPP expression.

To better understand the relationship between activity levels and CPP expression, a follow-up analysis examined the activity data within each Line and Stress Treatment group, separately. Activity levels were only significantly correlated with time spent on the GRID floor in LAP2 subjects (r = -0.21, p < 0.01), and not HAP2 subjects (r = 0.005, p = 0.94). Activity levels were significantly correlated with time spent on the GRID floor in the Adolescent Stress (r = -.28, p < 0.01), Adult Stress (r = -0.31, p < 0.001), and Control subjects (r = -0.37, p < 0.001). This suggests that activity levels may have contributed to CPP expression in specific subgroups during the second 20 min of Posttest 1, particularly in the LAP2 subjects.

GRID difference score during the third 20 min of posttest 1. A 3 (Stress Treatment: Adolescent Stress, Adult Stress, Stress Control) x 2 (Line: HAP2, LAP2) x 2 (RS: RS, noRS) x 2 (Conditioning Subgroup: G+, G-) univariate ANOVA on the GRID difference score during the third 20 min of Posttest 1 was used to interpret the third 20 min of the Posttest 1 data. An interaction between Line and Conditioning Subgroup was present (F[1, 407] = 12.09, p < 0.01), indicating that LAP2 subjects showed greater overall CPP than HAP2 subjects (see Fig. 13). In addition, a 3-way interaction between Line, RS, and Conditioning Subgroup was trending towards significance (F[2, 407] = 2.82, p = 0.094).

A follow-up analysis used a 3 (Stress Treatment) x 2 (RS) x 2 (Conditioning Subgroup) univariate ANOVA on the GRID difference score during the first 20 min of Posttest 1 within each Line (Bonferroni-corrected: p < 0.025). In the HAP2 subjects, there was a main effect of Conditioning Subgroup (F[2, 209] = 14.28, p < 0.001), suggesting that HAP2 subjects overall expressed CPP. Similarly, in the LAP2

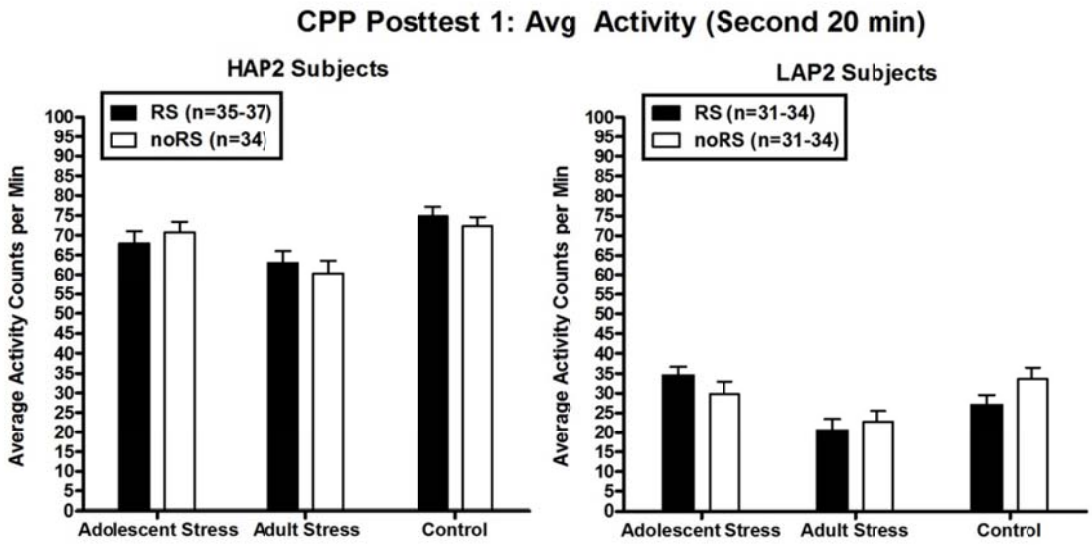

Figure 12. Average (±SEM) activity levels during the second 20 minutes of Posttest 1 for stress re-exposed and non stress re-exposed HAP2 and LAP2 mice exposed to adolescent stress, adult stress, or no stress.

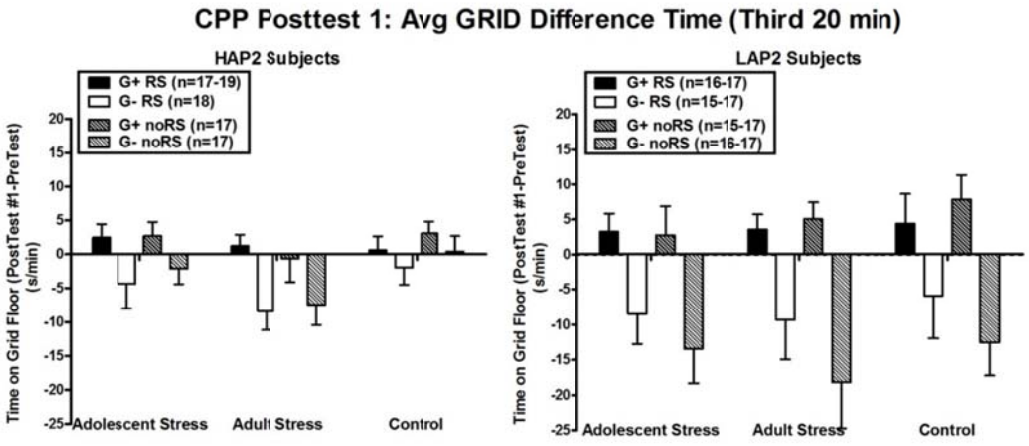

Figure 13. Average (±SEM) GRID difference time during the third 20 minutes of Posttest 1 for G+ and G- conditioning subgroups (separated by stress re-exposure subgroup) for all HAP2 and LAP2 mice exposed to adolescent stress, adult stress, and no stress.

subjects, there was a main effect of Conditioning Subgroup (F[1, 197] = 37.11, p < 0.001), suggesting an overall presence of CPP in the LAP2 mice. There were no interactions between Stress Treatment or RS and Conditioning Subgroup in either Line, suggesting that there were no differences in CPP magnitude between the Stress Treatment or RS subgroups within each Line during the third 20 min of Posttest 1.

Planned comparisons. In addition to the overall ANOVA, the specified planned comparisons between the Adolescent Stress and Adult Stress subjects and the Adolescent Stress and Stress Control subjects were performed.

Adolescent stress vs. adult stress. A 2 (Stress Treatment: Adolescent Stress, Adult Stress) x 2 (Line) x 2 (RS) x 2 (Conditioning Subgroup) univariate ANOVA on the GRID difference score during the third 20 min of Posttest 1 was performed to analyze the planned comparison regarding age of stress exposure (Adolescent Stress vs. Adult Stress). There was a significant interaction between Line and Conditioning Subgroup (F[1, 268] = 6.33, p < 0.05). There were no interactions of Stress Treatment or RS and Conditioning Subgroup. Thus, the results from this planned comparison showed similar results to that of the overall ANOVA.

Adolescent stress vs. stress control. A 2 (Stress Treatment: Adolescent Stress, Stress Control) x 2 (Line) x 2 (RS) x 2 (Conditioning Subgroup) univariate ANOVA on the GRID difference score during the third 20 min of Posttest 1 was performed to analyze the planned comparison regarding the effects of stress exposure versus no stress exposure in subjects of the same age (Adolescent Stress vs. Stress Control). There was an interaction between Line and Subgroup (F[1, 276] = 8.66, p < 0.01), indicating that LAP2 subjects showed greater overall CPP than HAP2 subjects. However, there were no interactions between Stress Treatment and Conditioning Subgroup or RS and Conditioning Subgroup in this planned comparison.

Activity levels during the third 20 min of posttest 1. Since activity levels could be related to CPP expression, a 3 (Stress Treatment) x 2 (Line) x 2 (RS) x 2 (Conditioning Subgroup) univariate ANOVA on the average activity levels during the third 20 min of Posttest 1 was performed to examine possible group differences in activity levels. There was a main effect of Line (F[1, 407] = 482.495, p < 0.001), in which HAP2 subjects showed greater activity during the second 20 min of Posttest 1 than LAP2 subjects (see Fig. 14). There was also a main effect of Stress treatment (F[1, 407] = 10.28, p < 0.001), in which the Adolescent Stress (p < 0.05) and Control

($p < 0.001$) subjects showed greater activity than the Adult Stress subjects. No further effects of RS subgroup were observed.

Correlations between activity levels and time spent on the GRID floor during the third 20 min of the Posttest were analyzed. Activity levels were significantly negatively related to time spent on the GRID floor ($r = -0.33$, $p < 0.001$), suggesting that lower activity levels could have contributed to greater CPP expression.

To better understand the relationship between activity levels and CPP expression, follow-up analyses correlation examined the activity data within each Line and Stress Treatment group, separately. Activity levels were only significantly correlated with time spent on the GRID floor in LAP2 subjects ($r = -0.24$, $p < 0.01$), and not HAP2 subjects ($r = -.116$, $p = 0.09$). Activity levels were significantly correlated with time spent on the GRID floor in the Adolescent Stress ($r = -.34$, $p < 0.001$), Adult Stress ($r = -0.35$, $p < 0.001$), and Control subjects ($r = -0.28$, $p < 0.01$). This suggests that activity levels may have contributed to CPP expression in specific subgroups during this third 20 min of Posttest 1, particularly in LAP2 subjects.

Posttest 2

GRID difference score during the first 20 min of posttest 2. A 3 (Stress Treatment) x 2 (Line) x 2 (RS) x 2 (Conditioning Subgroup) univariate ANOVA was used to analyze the GRID difference scores for the first 20 min of Posttest 2. Similar to Posttest 1, there was an interaction between Line and Conditioning Subgroup ($F[1, 407] = 18.565$, $p < 0.001$), in which LAP2 subjects showed greater overall CPP than HAP2 subjects (see Fig. 15). No interactions between Stress Treatment and Conditioning Subgroup or RS and Conditioning Subgroup were observed during the first 20 min of Posttest 1.

Planned comparisons. The same planned comparisons performed for CPP Posttest 1 were also performed for Posttest 2.

Adolescent stress vs. adult stress. A 2 (Stress Treatment: Adolescent Stress, Adult Stress) x 2 (Line) x 2 (RS) x 2 (Conditioning Subgroup) univariate ANOVA on the GRID difference score during the first 20 min of Posttest 2 was performed to analyze the planned comparison regarding age of stress exposure (Adolescent Stress vs. Adult Stress). There was an overall interaction between Line and Conditioning Subgroup ($F[1, 268] = 12.62$, $p < 0.001$), suggesting greater CPP in LAP2 subjects than HAP2 subjects. No other interactions between Stress Treatment

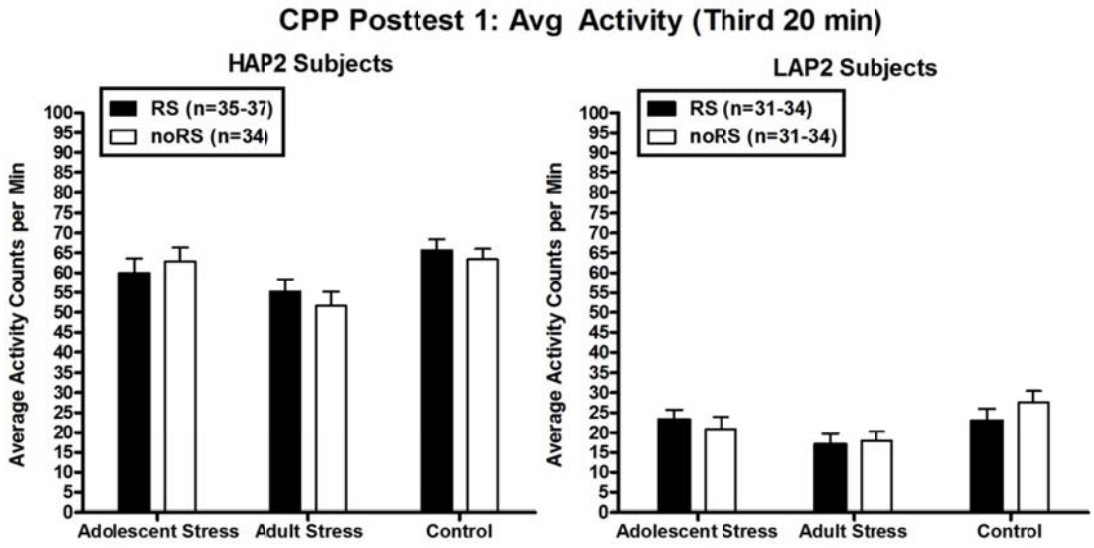

Figure 14. Average (±SEM) activity levels during the third 20 minutes of Posttest 1 for stress re-exposed and non stress re-exposed HAP2 and LAP2 mice exposed to adolescent stress, adult stress, or no stress.

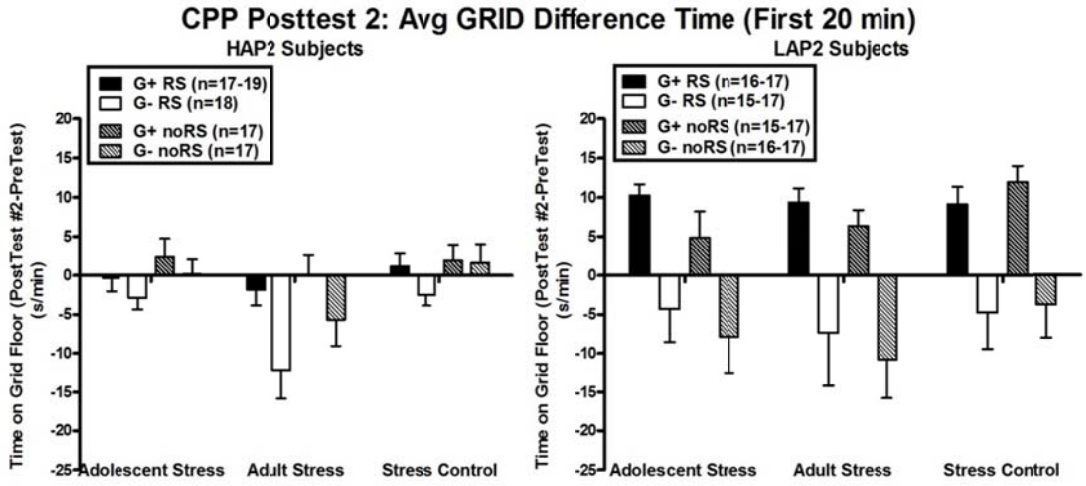

Figure 15. Average (±SEM) GRID difference time during the first 20 minutes of Posttest 2 for G+ and G- conditioning subgroups (separated by stress re-exposure subgroup) for all HAP2 and LAP2 mice exposed to adolescent stress, adult stress, and no stress.

and Conditioning Subgroup or RS and Conditioning Subgroup were observed, similar to the results of the overall ANOVA.

Adolescent stress vs. stress control. A 2 (Stress Treatment: Adolescent Stress, Stress Control) x 2 (Line) x 2 (RS) x 2 (Conditioning Subgroup) univariate ANOVA on the GRID difference score during the first 20 min of Posttest 2 was performed to analyze the planned comparison regarding the effects of stress exposure versus no stress exposure in subjects of the same age (Adolescent Stress vs. Stress Control). An interaction between Line and Conditioning Subgroup was observed (F[1, 276] = 21.03, $p < 0.001$), where LAP2 subjects showed greater CPP than HAP2 subjects. No other interactions between Stress Treatment and Conditioning Subgroup or RS and Conditioning Subgroup were observed, similar to the results of the overall ANOVA.

Activity levels during the first 20 min of posttest 2. A 3 (Stress Treatment) x 2 (Line) x 2 (RS) x 2 (Conditioning Subgroup) univariate ANOVA was performed on the activity levels during the first 20 min of Posttest 2. There were main effects of Line (F[1, 407] = 341.87, $p < 0.001$) and Stress Treatment (F[2, 407] = 19.15, $p < 0.001$) in the activity levels. In general, HAP2 subjects showed greater activity during the first 20 min of Posttest 2 than LAP2 subjects (see Fig. 16). Adolescent Stress and Stress Control subjects showed greater activity than Adult Stress subjects overall (ps < 0.001), but the two groups did not differ from one another. Similar to Posttest 1, these results reflect a possible age difference in activity levels during Posttest 2.

Average activity levels during the first 20 min of Posttest 2 were significantly and negatively correlated with time spent on the GRID floor (r = -0.38, $p < 0.001$). Similar to Posttest 1, this suggests that activity levels could have been related to CPP expression during Posttest 2.

To mimic the differences in activity level analyses during Posttest 2, two individual follow-up analyses examined the data within each Line and within each Stress Treatment group, separately, since there were main effects of Line and Stress Treatment in the activity level analyses. In the HAP2 subjects, activity was not significantly negatively correlated with GRID time (r = -0.15, p = 0.026). However, in LAP2 subjects, activity was significantly negatively correlated with GRID time (r = -0.33, $p < 0.001$). This suggests that activity-related changes in CPP expression were more present in the LAP2 subjects than the HAP2 subjects. Activity levels were

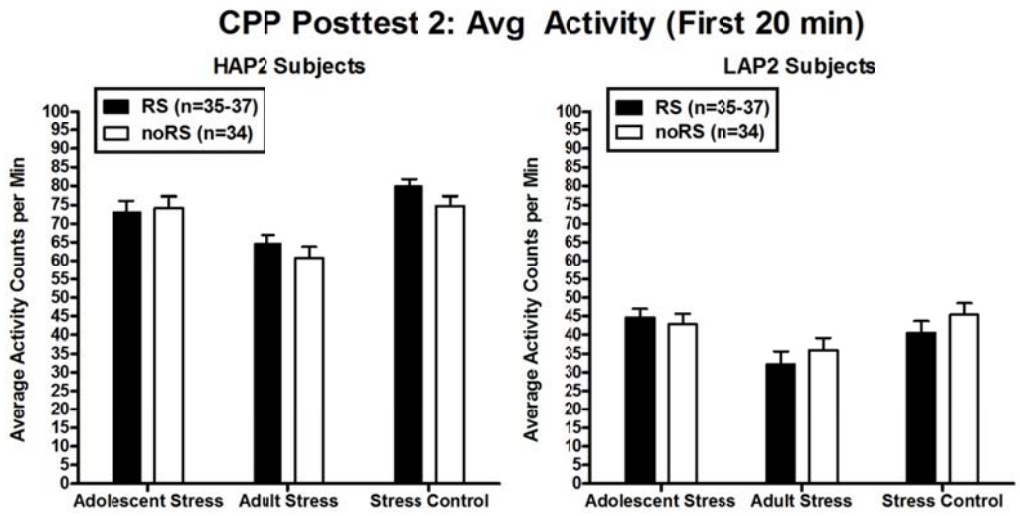

Figure 16. Average (±SEM) activity levels during the first 20 minutes of Posttest 2 for stress re-exposed and non stress re-exposed HAP2 and LAP2 mice exposed to adolescent stress, adult stress, or no stress.

significantly and negatively correlated with time spent on the GRID floor in the Adolescent Stress (r = -0.39, p < 0.001), Adult Stress (r = -0.33, p < 0.001), and Stress Control subjects (r = -0.43, p < 0.001), suggesting that activity levels may have had a more universal influence across Stress Treatment groups, particularly in the LAP2 subjects.

GRID difference score during the second 20 min of posttest 2. A 3 (Stress Treatment: Adolescent Stress, Adult Stress, Stress Control) x 2 (Line: HAP2, LAP2) x 2 (RS: RS, noRS) x 2 (Conditioning Subgroup: G+, G-) univariate ANOVA on the GRID difference score during the second 20 min of Posttest 2 was used to interpret the second 20 min of the Posttest 2 data. An interaction between Line and Conditioning Subgroup was present (F[1, 407] = 24.52, p < 0.001), indicating that LAP2 subjects showed greater overall CPP than HAP2 subjects (see Fig. 17). No further interactions between Stress Treatment or RS and Conditioning Subgroup were observed during the second 20 min of Posttest 2.

Planned comparisons. In addition to the overall ANOVA, the specified planned comparisons between the Adolescent Stress and Adult Stress subjects and the Adolescent Stress and Stress Control subjects were performed.

Adolescent stress vs. adult stress. A 2 (Stress Treatment: Adolescent Stress, Adult Stress) x 2 (Line) x 2 (RS) x 2 (Conditioning Subgroup) univariate ANOVA on the GRID difference score during the second 20 min of Posttest 2 was performed to analyze the planned comparison regarding age of stress exposure (Adolescent Stress vs. Adult Stress). There was a significant interaction between Line and Conditioning Subgroup (F[1, 268] = 14.72, p < 0.001). There were no interactions of Stress Treatment or RS and Conditioning Subgroup. Thus, the results from this planned comparison showed similar results to that of the overall ANOVA.

Adolescent stress vs. stress control. A 2 (Stress Treatment: Adolescent Stress, Stress Control) x 2 (Line) x 2 (RS) x 2 (Conditioning Subgroup) univariate ANOVA on the GRID difference score during the second 20 min of Posttest 2 was performed to analyze the planned comparison regarding the effects of stress exposure versus no stress exposure in subjects of the same age (Adolescent Stress vs. Stress Control). There was an interaction between Line and Subgroup (F[1, 276] = 14.76, p < 0.001), indicating that LAP2 subjects showed greater overall CPP than HAP2

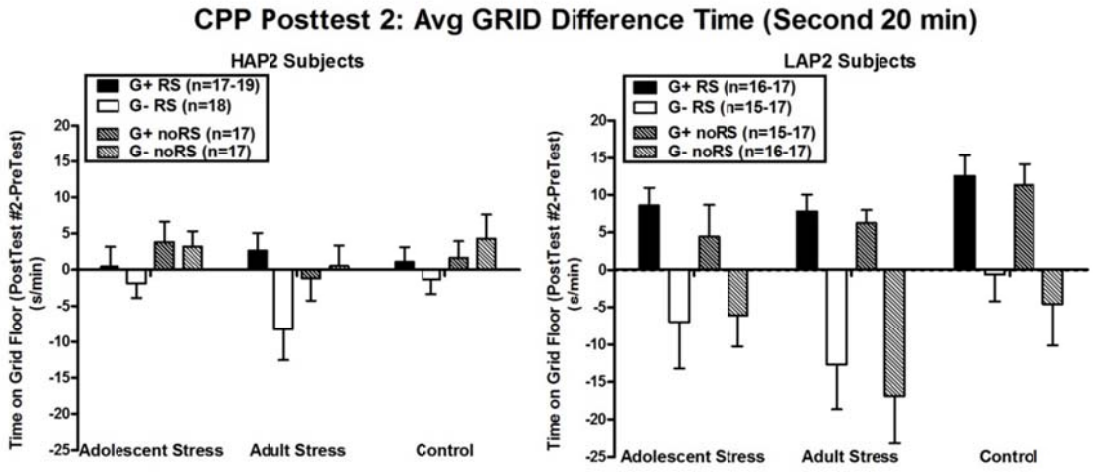

Figure 17. Average (±SEM) GRID difference time during the second 20 minutes of Posttest 2 for G+ and G- conditioning subgroups (separated by stress re-exposure subgroup) for all HAP2 and LAP2 mice exposed to adolescent stress, adult stress, and no stress.

subjects. However, there were no interactions between Stress Treatment and Conditioning Subgroup or RS and Conditioning Subgroup in this planned comparison.

Activity levels during the second 20 min of posttest 2. Since activity levels could be related to CPP expression, a 3 (Stress Treatment) x 2 (Line) x 2 (RS) x 2 (Conditioning Subgroup) univariate ANOVA on the average activity levels during the second 20 min of Posttest 2 was performed to examine possible group differences in activity levels. There was a main effect of Line (F[1, 407] = 408.42, $p < 0.001$), in which HAP2 subjects showed greater activity during the second 20 min of Posttest 2 than LAP2 subjects (see Fig. 18). In addition, there was a main effect of Stress Treatment (F[1, 407] = 14.21, $p < 0.001$), in which the Adolescent Stress ($p < 0.01$) and Control ($p <0.001$) showed greater activity than the Adult Stress subjects. No further effects of RS subgroup were observed.

Correlations between activity levels and time spent on the GRID floor during the second 20 min of Posttest 2 were analyzed. Activity levels were significantly negatively related to time spent on the GRID floor (r = -0.32, $p < 0.001$), suggesting that lower activity levels could have contributed to greater CPP expression.

To better understand the relationship between activity levels and CPP expression, a follow-up analysis examined the activity data within each Line and Stress Treatment group, separately. Activity levels were significantly correlated with time spent on the GRID floor in HAP2 subjects (r = -0.15, $p < 0.05$) and LAP2 subjects (r = -.23, $p < 0.01$). In addition, activity levels were significantly correlated with time spent on the GRID floor in the Adolescent Stress (r = -0.36, $p < 0.001$), Adult Stress (r = -0.22, $p < 0.05$), and Control subjects (r = -0.38, $p < 0.001$). This suggests that activity levels may have contributed to CPP expression during the second 20 min of Posttest 2 in a more universal manner.

GRID difference score during the third 20 min of posttest 2. A 3 (Stress Treatment: Adolescent Stress, Adult Stress, Stress Control) x 2 (Line: HAP2, LAP2) x 2 (RS: RS, noRS) x 2 (Conditioning Subgroup: G+, G-) univariate ANOVA on the GRID difference score during the third 20 min of Posttest 2 was used to interpret the third 20 min of the Posttest 2 data. An interaction between Line and Conditioning Subgroup was present (F[1, 407] = 13.95, $p < 0.001$), indicating that LAP2 subjects showed greater overall CPP than HAP2 subjects (see Fig. 19). No further interactions

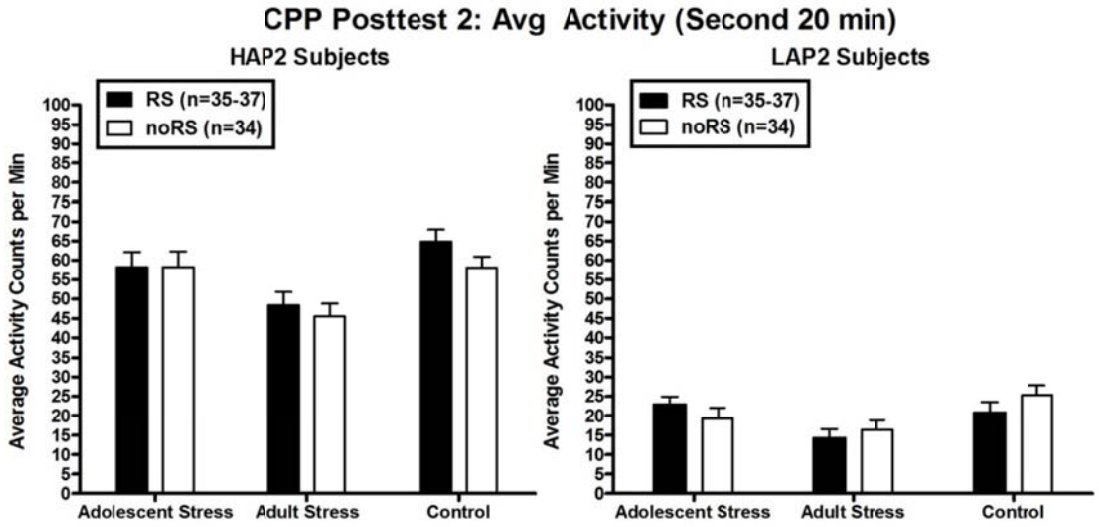

Figure 18. Average (±SEM) activity levels during the second 20 minutes of Posttest 2 for stress re-exposed and non stress re-exposed HAP2 and LAP2 mice exposed to adolescent stress, adult stress, or no stress.

between Stress Treatment or RS and Conditioning Subgroup were present during the third 20 min of Posttest 2.

Planned comparisons. In addition to the overall ANOVA, the specified planned comparisons between the Adolescent Stress and Adult Stress subjects and the Adolescent Stress and Stress Control subjects were performed.

Adolescent stress vs. adult stress. A 2 (Stress Treatment: Adolescent Stress, Adult Stress) x 2 (Line) x 2 (RS) x 2 (Conditioning Subgroup) univariate ANOVA on the GRID difference score during the third 20 min of Posttest 2 was performed to analyze the planned comparison regarding age of stress exposure (Adolescent Stress vs. Adult Stress). There was a significant interaction between Line and Conditioning Subgroup ($F[1, 268] = 7.29$, $p < 0.01$). There were no interactions of Stress Treatment or RS and Conditioning Subgroup. Thus, the results from this planned comparison showed similar results to that of the overall ANOVA.

Adolescent stress vs. stress control. A 2 (Stress Treatment: Adolescent Stress, Stress Control) x 2 (Line) x 2 (RS) x 2 (Conditioning Subgroup) univariate ANOVA on the GRID difference score during the third 20 min of Posttest 2 was performed to analyze the planned comparison regarding the effects of stress exposure versus no stress exposure in subjects of the same age (Adolescent Stress vs. Stress Control). There was an interaction between Line and Subgroup ($F[1, 276] = 9.29$, $p < 0.01$), indicating that LAP2 subjects showed greater overall CPP than HAP2 subjects. However, there were no interactions between Stress Treatment and Conditioning Subgroup or RS and Conditioning Subgroup in this planned comparison.

Activity levels during the third 20 min of posttest 2. Since activity levels could be related to CPP expression, a 3 (Stress Treatment) x 2 (Line) x 2 (RS) x 2 (Conditioning Subgroup) univariate ANOVA on the average activity levels during the second 20 min of Posttest 2 was performed to examine possible group differences in activity levels. There was a main effect of Line ($F[1, 407] = 282.16$, $p < 0.001$), in which HAP2 subjects showed greater activity during the third 20 min of Posttest 2 than LAP2 subjects (see Fig. 20). In addition, there was a main effect of Stress Treatment ($F[1, 407] = 9.95$, $p < 0.001$), in which the Adolescent Stress ($p < 0.05$) and Control ($p < 0.001$) showed greater activity than the Adult Stress subjects. No further effects of RS subgroup were observed.

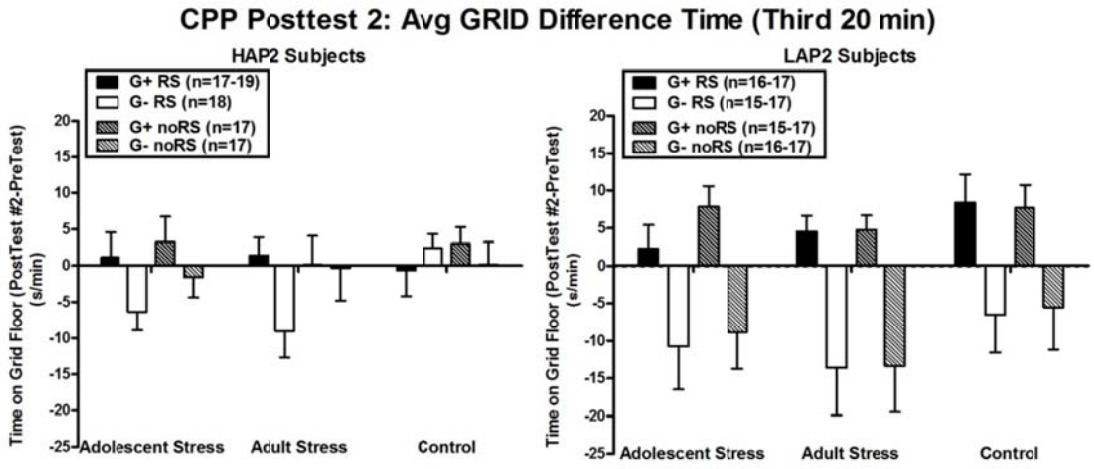

Figure 19. Average (±SEM) GRID difference time during the third 20 minutes of Posttest 2 for G+ and G- conditioning subgroups (separated by stress re-exposure subgroup) for all HAP2 and LAP2 mice exposed to adolescent stress, adult stress, and no stress.

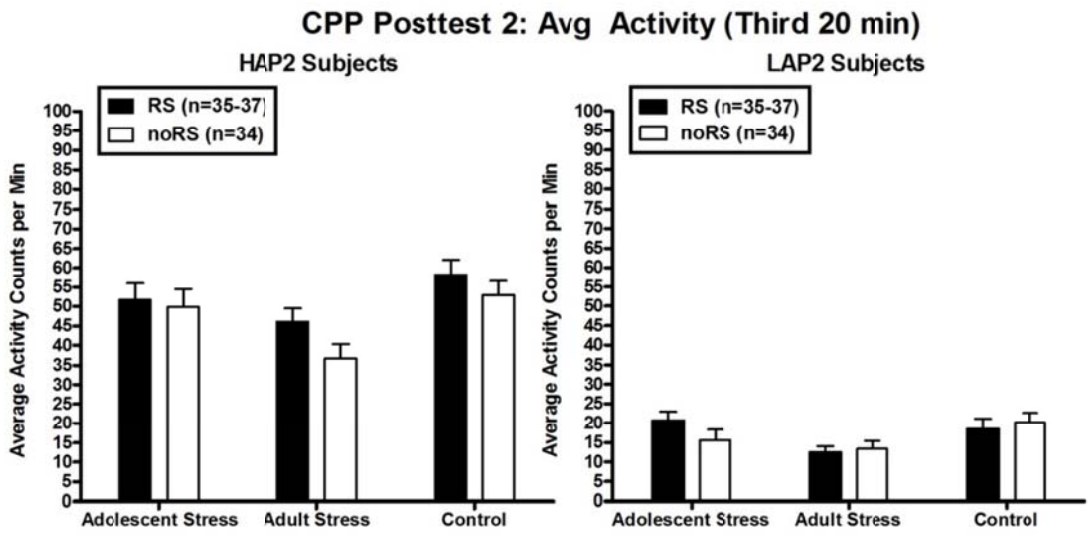

Figure 20. Average (±SEM) activity levels during the third 20 minutes of Posttest 2 for stress re-exposed and non stress re-exposed HAP2 and LAP2 mice exposed to adolescent stress, adult stress, or no stress.

Correlations between activity levels and time spent on the GRID floor during the second 20 min of Posttest 2 were analyzed. Activity levels were significantly negatively related to time spent on the GRID floor (r = -0.33, p < 0.001), suggesting that lower activity levels could have contributed to greater CPP expression.

To better understand the relationship between activity levels and CPP expression, a follow-up analysis examined the activity data within each Line and Stress Treatment group, separately. Activity levels were significantly correlated with time spent on the GRID floor in HAP2 subjects (r = -0.21, p < 0.01) and LAP2 subjects (r = -.20, p < 0.01). In addition, activity levels were significantly correlated with time spent on the GRID floor in the Adolescent Stress (r = -0.37, p < 0.001), Adult Stress (r = -0.27, p < 0.01), and Control subjects (r = -0.33, p < 0.001). This suggests that activity levels may have contributed to CPP expression during this time bin of Posttest 2.

Change in CPP Within Posttests 1 and 2

To analyze possible changes in CPP over the course of the two CPP posttests, a 3 (Stress Treatment: Adolescent Stress, Adult Stress, Stress Control) x 2 (Line: HAP2, LAP2) x 2 (RS: RS, noRS) x 2 (Conditioning Subgroup: GRID+, GRID-) repeated measures ANOVA on the GRID difference score during the first 20 min of CPP Posttests 1 and 2 was used. There was a 4-way interaction between Posttest, Line, Stress Treatment, and Conditioning Subgroup (F[2, 558] = 3.39, p < 0.05) within-subjects. Additionally, there was an interaction of Line and Conditioning Subgroup (F[1, 558] = 41.02, p < 0.001) between groups. To better understand these interactions, a follow-up analysis used a 3 (Stress Treatment) x 2 (RS) x 2 (Conditioning Subgroup) repeated measures ANOVA on the GRID difference during the first 20 min of CPP Posttests 1 and 2 was used within each Line (Bonferroni-corrected: p < 0.025). In the HAP2 subjects, there was an interaction between Posttest and Conditioning Subgroup (F[1, 285] = 15.30, p < 0.001), such that CPP generally decreased between Posttest 1 and 2. There was a trend towards a 3-way interaction between Posttest, Stress Treatment, and Conditioning Subgroup (F[2, 285] = 3.03, p = 0.05); however, this effect did not reach Bonferroni criteria. Similarly, in the LAP2 subjects, there was an interaction between Posttest and Conditioning Subgroup (F[1, 273] = 26.07, p < 0.001), indicating that CPP generally decreased between Posttests 1 and 2.

Planned comparisons. In the same manner as the univariate ANOVAs, planned comparisons were used to compare the change in CPP across Posttests 1 and 2 between the Adolescent Stress versus Adult Stress and the Adolescent Stress versus Stress Control subjects, separately.

Adolescent stress vs. adult stress. A 2 (Stress Treatment: Adolescent Stress, Adult Stress) x 2 (Line) x 2 (RS) x 2 (Conditioning Subgroup) repeated-measures ANOVA on the GRID difference scores of Posttest 1 and Posttest 2 was performed to analyze the planned comparison regarding age of stress exposure (Adolescent Stress vs. Adult Stress). There was a 3-way interaction between Posttest, Line, and Conditioning Subgroup (F[1, 253] = 5.18, $p < 0.05$), and a near-significant 4-way interaction between Posttest, Line, Stress Treatment, and Conditioning Subgroup (F[1, 253] = 3.64, $p = 0.058$) within-subjects. A between-group interaction of Line and Conditioning Subgroup was also present (F[1, 253] = 18.14, $p < 0.001$). To better understand this interaction, a follow-up analysis used a 2 (Stress Treatment) x 2 (RS) x 2 (Conditioning Subgroup) repeated measures ANOVA was used within each Line (Bonferroni-corrected: $p < 0.025$). In the HAP2 subjects, there was a trend towards an interaction between Posttest and Conditioning Subgroup within-subjects, but this effect did not reach Bonferroni criteria (F[1, 131] = 3.48, $p = 0.06$). A between-groups interaction of Stress Treatment and Conditioning Subgroup was present in the HAP2 subjects (F[1, 131] = 5.32, $p < 0.025$), indicating that the overall CPP expression was greater in HAP2 Adult Stress subjects than the HAP2 Adolescent Stress subjects. However, the between-groups interaction in this analysis did not indicate that the magnitude of change in CPP differed between the two groups. In the LAP2 subjects, there was an interaction between Posttest and Conditioning Subgroup (F[1, 122] = 14.21, $p < 0.001$), but no interactions between Stress Treatment and Conditioning Subgroup or RS and Conditioning Subgroup were observed.

Adolescent stress vs. stress control. A 2 (Stress Treatment: Adolescent Stress, Stress Control) x 2 (Line) x 2 (RS) x 2 (Conditioning Subgroup) repeated-measures ANOVA on the GRID difference score during the first 20 min of Posttest 2 was used to analyze the planned comparison regarding the effects of stress exposure versus no stress exposure in subjects of the same age (Adolescent Stress vs. Stress Control). Within subjects, there was a significant interaction between Posttest and Conditioning Subgroup (F[1, 261] = 12.05, $p < 0.01$). In addition, there were

near-significant interactions between Posttest, Line, Stress Treatment, and Conditioning Subgroup (F[1, 261] = 3.81, p = 0.52) and Posttest, Line, and Conditioning Subgroup (F[1, 261] = 3.64, p = 0.058). Between groups, there was an interaction between Line and Conditioning Subgroup (F[1, 261] = 33.74, p < 0.001). To better understand these interactions, a follow-up analysis used a 2 (Stress Treatment) x 2 (RS) x 2 (Conditioning Subgroup) repeated measures ANOVA was used within each Line (Bonferroni-corrected: p < 0.025). In the HAP2 subjects, there were no significant follow-up interactions with Conditioning Subgroup, although an interaction between Posttest and Conditioning Subgroup within-subjects was trending towards significance (F[1, 133] = 2.88, p = 0.09). In the LAP2 subjects, there was an interaction between Posttest and Conditioning Subgroup (F[1, 128] = 8.95, p < 0.01), where CPP magnitude generally decreased from Posttest 1 to Posttest 2.

Corticosterone Samples

The blood samples from 129 subjects were analyzed for CORT concentrations at all 4 time points (CSE 1, CSE 10, Posttest 1, Posttest 2), with a goal of samples from 10 subjects for each Line, Stress Treatment, and RS subgroup represented (with 5 samples from G+ subjects and 5 from G- subjects). Samples were counterbalanced among cohorts as much as possible, and duplicate representation from a cage within a subgroup was avoided. The 4 time point samples from 2 subjects had to be dropped because at least one time point was an outlier, based on Dixon's Extreme Test. The total number of subjects' samples used for CORT analyses at the 4 time points are depicted in Table 4.

Days 1 and 10 of CSE

To analyze changes in CORT over CSE Day 1 and CSE Day 10, a 3 (Stress Treatment: Adolescent Stress, Adult Stress, Stress Control) x 2 (Line: HAP2, LAP2) repeated measures ANOVA was performed. There was a within-subject interaction of Day and Stress Treatment (F[2, 121] = 3.13, p < 0.05). To follow-up this interaction, a repeated-measures ANOVA on the 2 Lines for CSE Day 1 and CSE Day 10 CORT levels was used within each Stress Treatment group (Bonferroni-corrected, p < 0.017). The Adolescent Stress, Adult Stress, and Stress Control subjects all showed an overall decrease in CORT levels between Day 1 and Day 10 of CSE (ps < 0.01), however the decrease in the Adolescent Stress subjects was more drastic, indicating a steeper slope (see Fig. 21).

Table 4

Numbers of Subjects With Corticosterone Data Represented in Each Line, Stress Treatment, Stress Re-exposure, and Conditioning Subgroup

	Adolescent Stress				Adult Stress				Control			
	RS		noRS		RS		noRS		RS		noRS	
	G+	G-	G+	G-	G+	G-	G+	G-	G+	G-	G+	G-
HAP2:	5	6	6	6	5	5	5	5	5	5	5	5
LAP2:	6	5	6	6	5	5	5	5	6	5	5	5

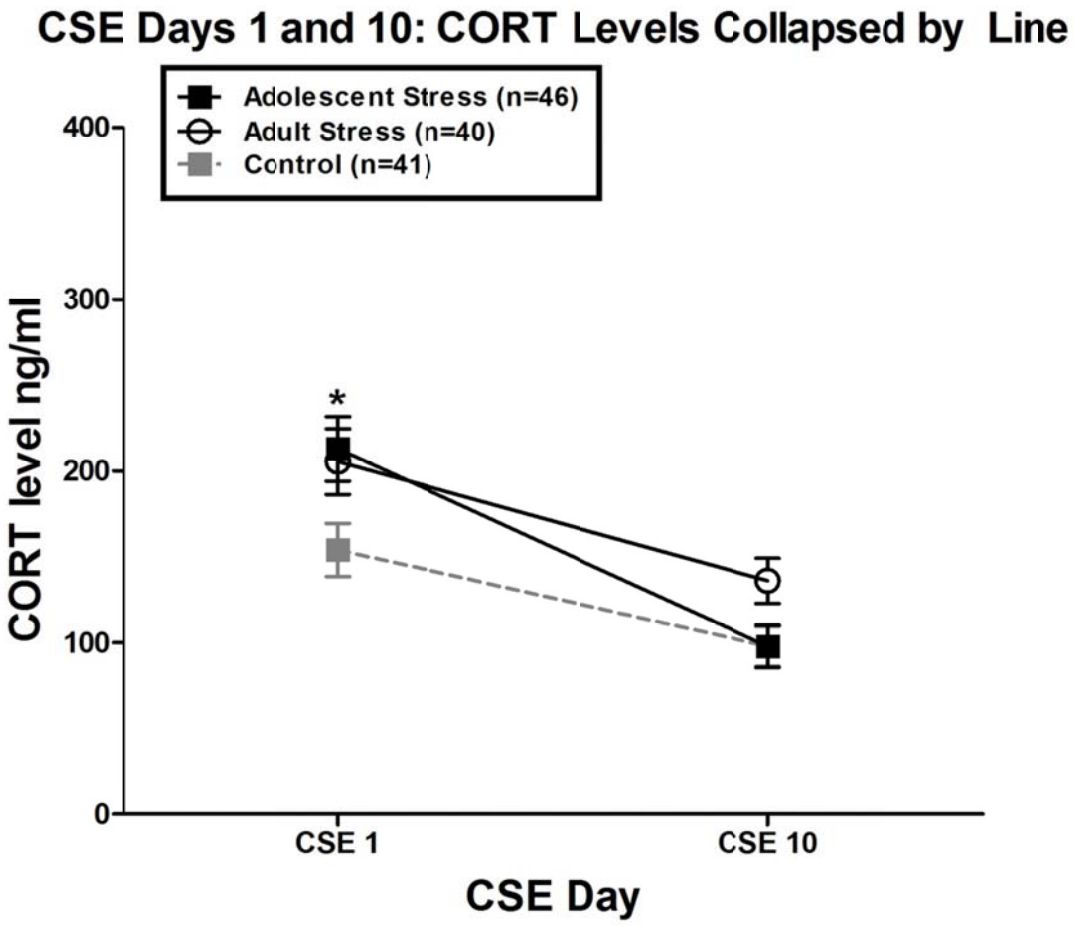

Figure 21. Mean (±SEM) CORT levels (ng/ml) on CSE Day 1 and CSE Day 10 for mice exposed to adolescent stress, adult stress, or no stress (collapsed across HAP2 and LAP2 subjects). *$p < 0.05$, Adolescent Stress > Stress Control.

In addition to investigating the change in CORT level changes over time, CORT levels on CSE Day 1 and CSE Day 10 were analyzed individually to examine possible group differences on each day. A 3 (Stress Treatment) x 2 (Line) univariate ANOVA was performed on CSE Day 1 and CSE Day 10, separately. On CSE 1, there was a near-significant main effect of Stress Treatment on CORT levels, such that the Adolescent Stress subjects generally showed greater CORT levels than the Stress Control subjects ($p = 0.053$), while no other significant differences between groups were seen. On CSE 10, there was a near-significant effect of Stress Treatment ($F[2, 126] = 3.04$, $p = 0.051$). However, on Day 10, the Adult Stress subjects generally showed higher CORT levels than the Adolescent Stress ($p = 0.07$) and Stress Control subjects ($p = 0.09$), while the latter groups did not differ.

Correlation with g/F. Because the Stress Control subjects did not have g/F data, only the Adolescent Stress and Adult Stress data were represented in this analysis. G/F data was significantly and positively correlated with CORT values on CSE 1 ($r = 0.25$, $p < 0.05$). To better mimic the group differences seen in the CORT analysis on CSE 1, data was separated by Stress Treatment group to see if specific groups showed correlation between CORT and g/F exerted during CSE 1. Importantly, CORT values were not correlated with g/F in the Adolescent Stress subjects, but there was a significant correlation present in the Adult Stress subjects on CSE 1.

On CSE 10, CORT values were not significantly correlated with g/F exerted.

Posttest 1 and Posttest 2

To analyze possible changes in CORT levels from Posttest 1 and Posttest 2, a 3 (Stress Treatment: Adolescent Stress, Adult Stress, Stress Control) x 2 (Line: HAP2, LAP2) x 2 (RS, noRS) repeated measures ANOVA was performed. There was a within-subjects interaction between Day and Stress Treatment ($F[2, 115] = 4.69$, $p < 0.05$) and Day and Line ($F[1, 115] = 5.244$, $p < 0.05$). Additionally, there was a near-significant 3-way interaction between Day, Line, and Stress Treatment ($F[2, 115] = 2.82$, $p = 0.06$). To further investigate these interactions, a follow-up analysis used a 3 (Stress Treatment) x 2 (RS) repeated measures ANOVA was used within each Line (Bonferroni-corrected: $p < 0.025$). In the HAP2 subjects, there was an overall effect of Day, where CORT levels decreased between the two Posttests ($F[1, 57] = 17.63$, $p < 0.001$; see Fig. 22). There was a near-significant interaction between Day and Stress Treatment, but this interaction did not reach Bonferroni criteria ($F[2, 57] = 2.696$, $p =$

0.07). In the LAP2 subjects, there was a significant interaction between Day and Stress Treatment (F[2, 58] = 4.19, p < 0.025). To better understand these interactions, a second follow-up analysis used a repeated measures ANOVA on the 2 RS subgroups (RS, noRS) within each Line and Stress Treatment subgroup (Bonferroni-corrected: p < 0.004). In the HAP2 Adolescent Stress and HAP2 Stress Control subjects, no effects of Day or RS were observed. However, in the HAP2 Adult Stress subjects, CORT levels overall decreased between the two Posttests (F[1, 18] = 24.94, p < 0.001). Both the LAP2 Adult Stress and Stress Control subjects showed a decrease in CORT over the Posttests (ps < 0.004). In contrast, the LAP2 Adolescent Stress subjects showed no effect of Day, but did show a main effect of RS (F[1, 21] = 13.25, p < 0.004), where the LAP2 Adolescent-RS subjects increased CORT levels over the Posttests 1 and 2, whereas the noRS subjects showed decreased CORT levels.

In addition to investigating the change in CORT level changes between Posttest 1 and Posttest 2, CORT levels on Posttest 1 and Posttest 2 were analyzed individually to examine possible group differences on each day. Separate univariate ANOVAs were performed for CORT levels on Posttest 1 and Posttest 2, using a 3 (Stress Treatment) x 2 (Line) x 2 (RS) design. On Posttest 1, there was an interaction between Line and Stress treatment (F[2, 126] = 5.65, p < 0.01). There was also a main effect of RS (F[1, 126] = 4.59, p < 0.05) where the RS subjects generally showed lower CORT levels than noRS subjects. To follow-up the interaction, a 3 (Stress Treatment) x 2 (RS) univariate ANOVA on CORT levels during Posttest 1 was used within each Line (Bonferroni-corrected: p < 0.025). In the HAP2 subjects, there was a trend towards a main effect of RS where RS subjects showed lower CORT than the noRS subjects, but this effect did not meet Bonferroni criteria (p = 0.07). In the LAP2 subjects, there was a main effect of Stress Treatment (F[2, 63] = 8.12, p < 0.01), where the LAP2 Adult Stress subjects showed greater CORT levels than the Adolescent Stress (p < 0.01) and Stress Control subjects (p < 0.025), while the latter groups did not differ.

During Posttest 2, there was an interaction between Line and Stress Treatment in the CORT levels (F[2, 126] = 4.62, p < 0.05). To follow-up this interaction, a 3 (Stress Treatment) x 2 (RS) univariate ANOVA on CORT levels during Posttest 2 was used within each Line (Bonferroni-corrected: p < 0.025). In the HAP2 subjects, there

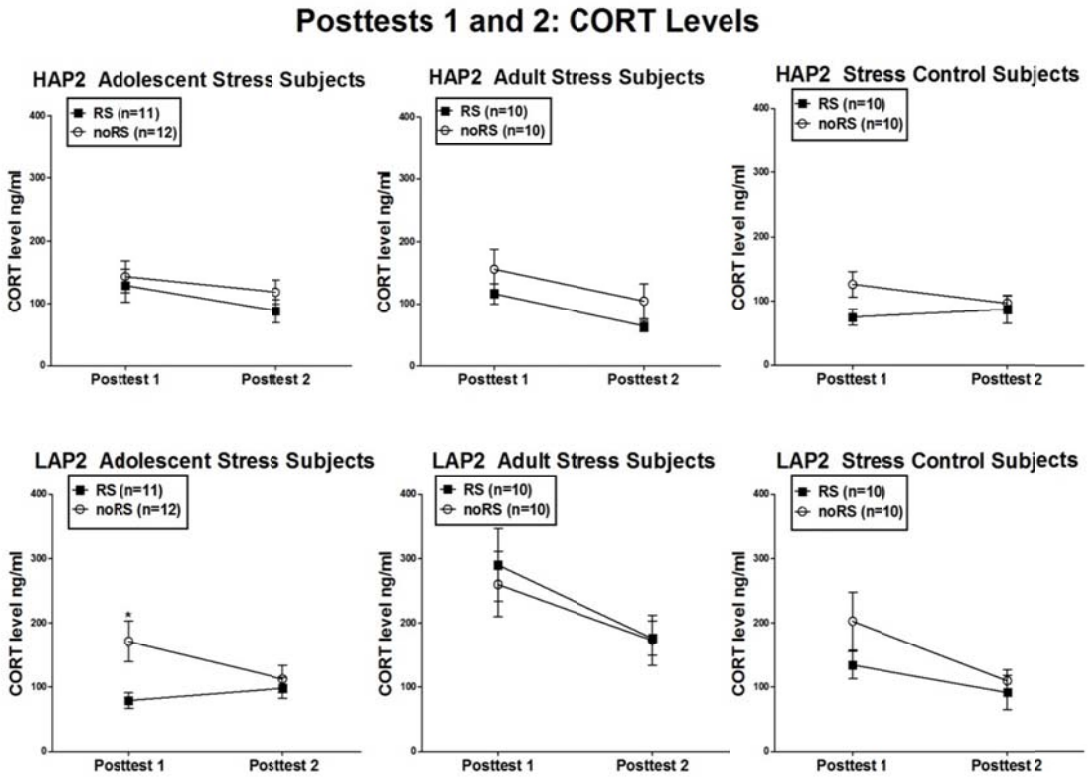

Figure 22. Mean (±SEM) CORT levels (ng/ml) on Posttest 1 and Posttest 2 for all HAP2 and LAP2 subjects, separated by stress treatment and stress re-exposure subgroups. *$p < 0.05$, noRS > RS.

was a trend towards a main effect of RS (p = 0.09), where RS subjects showed lower CORT than the noRS subjects, but this effect did not meet Bonferroni criteria. In the LAP2 subjects, there was a significant main effect of Stress Treatment (F[2, 63] = 5.13, p < 0.025), where the Adult Stress subjects again showed higher CORT levels than the Adolescent Stress (p < 0.025) and Stress Control subjects (p < 0.025), but the latter two groups did not differ.

Correlation with CPP expression. Correlations between time spent on the GRID floor during the Posttests and CORT levels after the Posttests were analyzed. Time spent on the GRID floor was not significantly correlated with CORT levels during Posttest 1 or Posttest 2.

Area Under the Curve (AUC)

AUC for CSE days 1 and 10. The area under the curve (AUC) across the 2 time points for CORT levels during CSE was analyzed using a 3 (Stress Treatment) x 2 (Line) univariate ANOVA on CSE CORT AUC. There was a near-significant main effect of Stress Treatment (F[1, 126) = 3.07, p = 0.05), where Adult Stress subjects showed a higher AUC than the Control subjects (p < 0.05; see Fig. 23). There were no differences between the HAP2 and LAP2 lines.

AUC for CPP posttests 1 and 2. The area under the curve (AUC) across the 2 time points for CORT levels during CPP Posttests 1 and 2 was analyzed using a 3 (Stress Treatment) x 2 (Line) x 2 (RS) univariate ANOVA on CPP CORT AUC. There was an interaction between Line and Stress Treatment (F[2, 126] = 5.88, p < 0.001), and a main effect of and RS (F[1, 126) = 3.99, p < 0.05), where RS subjects generally showed a lower AUC than noRS subjects. To better understand the interaction, a 3 (Stress Treatment) x 2 (RS) univariate ANOVA on CORT AUC was used within each Line (Bonferroni-corrected: p < 0.025). In the HAP2 subjects, there was a trend towards a main effect of RS in the CORT AUCs (F[1, 126) = 3.77, p = 0.06); however, this did not meet Bonferroni criteria. In the LAP2 subjects, a main effect of Stress Treatment was evident (F[2, 63] = 7.90, p < 0.01). LAP2 Adult Stress subjects showed a higher CORT AUC over the course of the study than the LAP2 Adolescent Stress (p < 0.01) and LAP2 Stress Control subjects (p < 0.01), and the latter two groups did not differ (see Fig. 24).

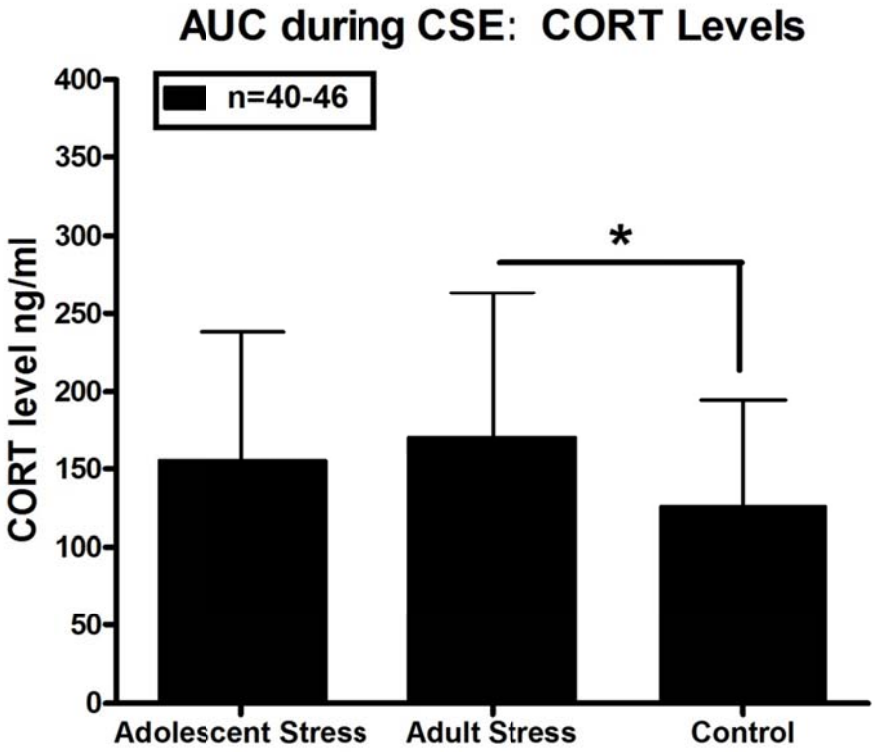

Figure 23. Area under the curve (±SEM) of corticosterone levels (ng/ml) for CSE Day 1 and CSE Day 10 for mice exposed to adolescent stress, adult stress, and no stress, collapsed by line.

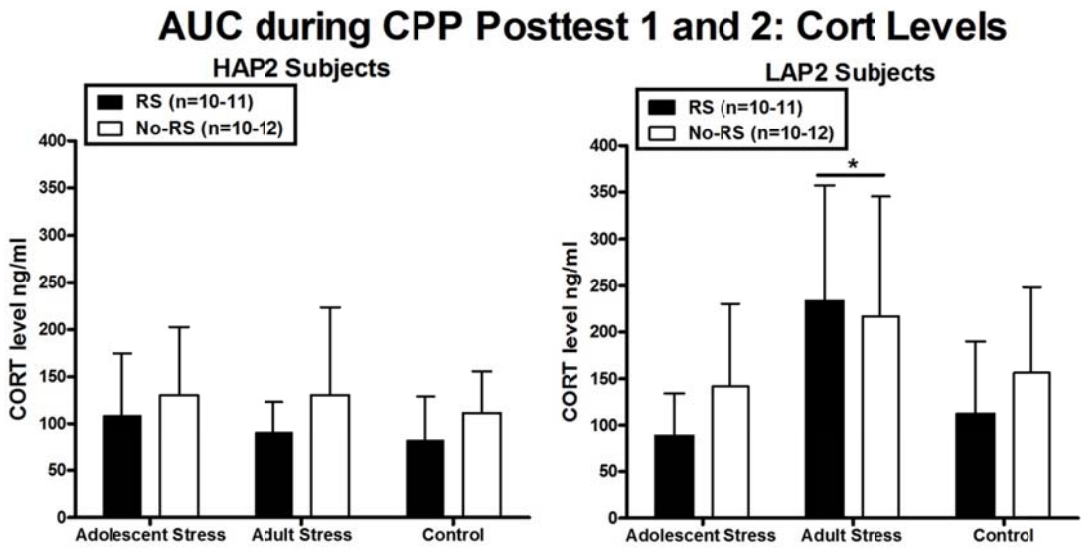

Figure 24. Area under the curve (±SEM) of corticosterone levels (ng/ml) for CPP Posttests 1 and 2 for stress re-exposed and stress non re-exposed HAP2 and LAP2 mice exposed to adolescent stress, adult stress, and no stress.

DISCUSSION

The overall purpose of this study was to determine if adolescent chronic stress exposure increases sensitivity to the rewarding effects of alcohol during adulthood. Revisiting each hypothesis helps clarify the overall answer to this research question.

Hypotheses Revisited

The first hypothesis was that subjects exposed to adolescent chronic stress would show greater CPP during adulthood than subjects exposed to stress during adulthood and to those not exposed to stress. The results of this study do not support this hypothesis. In fact, specifically in the HAP2 mice, the subjects exposed to adolescent stress were the only subjects that failed to show significant CPP specifically during the first 20 min of Posttest 1. During the remaining time of Posttest 1 and all of Posttest 2, there were no differences in CPP magnitude between the different stress treatment groups. In the LAP2 subjects, there were no differences in CPP expression between the stress treatment groups in either Posttest 1 or 2. These findings were true both in the overall ANOVAs and in the planned comparisons. These results suggest that adolescent stress exposure may potentially decrease CPP expression during adulthood, specifically in those bred for high-alcohol drinking behaviors, and that this effect extinguishes with time. Conversely, stress exposure does not appear to have any long-term effects on CPP in LAP2 subjects.

The second hypothesis predicted that subjects who were re-exposed to the original stressor directly before the CPP posttest would show increased CPP compared to those not re-exposed to the stressor before the posttest. This hypothesis was also not supported. Re-exposure to the stressor produced no alterations in CPP expression during either Posttest 1 or Posttest 2. The second hypothesis also predicted that CPP would increase between Posttests 1 and 2 in subjects re-exposed to the stressor, because intermittent re-exposure to stress has been shown to linearly increase voluntary alcohol consumption (Chester et al., 2006). However, this effect

was not observed. CPP decreased in all subjects between the two posttests, regardless of whether or not subjects were re-exposed to stress before each posttest.

The third hypothesis stated that HAP2 subjects would show the predicted effects in hypotheses 1 and 2 to a greater degree than LAP2 subjects. Overall, LAP2 subjects showed a greater magnitude of CPP; however, LAP2 subjects showed no alterations in CPP based on differential stress treatment, whereas differences in CPP expression between stress treatment groups in HAP2 subjects were seen during the first 20 min of Posttest 1. Importantly, even though adolescent stress exposure actually decreased CPP compared to adult stress exposure and no stress exposure in the HAP2 subjects, adolescent stress exposure did significantly alter CPP in the HAP2 mice compared to the other stress treatment groups. These findings are important, and suggest that the HAP2 subjects were more sensitive to the effects of stress exposure during adolescence than LAP2 subjects, even though the direction of the observed effect was opposite than expected. Effects of stress re-exposure did not differ between the HAP2 and LAP2 lines, nor did the effects of intermittent re-exposure across CPP testing. In fact, overall CPP decreased between Posttests 1 and 2, even in the subjects re-exposed to stress before each test. Thus, this hypothesis was supported in regards to the prediction that differential effects of stress treatment on CPP would be seen in the HAP2 subjects, and not LAP2 subjects. However, the remaining and majority of aspects of this hypothesis were not supported.

Tactile Responses During Chronic Stress Exposure and Re-Exposure

Tactile startle responses have been used in animal research to measure a subjects' response to a stimuli, typically an aversive stimuli. Startle responses are suggested to reflect the emotional response to an environmental stimulus (Brown, Kalish, & Farber, 1951; Geyer & Swerdlow, 2001). When an animal is exposed to a stimulus, the innate startle response begins at the animal's head, and then travels down the body as flexor contractions occur. Thus, the g/F recorded during a startle response reflects the net force of the animal's response to the stimuli being administered (Szabo & Hazafi, 1965).

Importantly, the g/F per kg data in the current study suggested that all subjects might have habituated to the foot shocks over the 10 days of CSE. The overall habituation in g/F per kg responses observed in the current study was unexpected, and suggests that the chronic foot shock paradigm utilized in this study design may

not have been as severe as paradigms used in other stress-related research in rodents. Although we cannot determine for certain whether all subjects in the current study experienced chronic stress based on g/F per kg tactile responses alone, we also cannot rule out the possibility that the chronic stress paradigm we sought to deliver may not have actually evoked a chronic stress response. It is possible that the foot shocks were only perceived as stressful during the first day of CSE, and the paradigm may have mimicked more of an acute stress exposure rather than a chronic stress exposure. This possibility should be considered throughout the discussion of this study's data interpretation.

Other portions of the results from the CSE portion results of this study were as expected. In the current study, there were no differences in g/F exerted between the lines in the Adolescent Stress group. However, the HAP2 Adult Stress subjects showed significantly greater startle responses than the LAP2 Adult Stress subjects. Importantly, this difference was not due to differences in body weight, because the g/F responses were calculated in relation to subjects' body weights (g/F per kg). The g/F per kg results in the current data suggest that the HAP2 Adult Stress subjects were more responsive to the foot shock exposure than the LAP2 Adult Stress subjects, since the net responses in g/F per kg were statistically significant between the two groups. These results support past research that the HAP2 line is generally more responsive and sensitive to the effects of stress exposure on anxiety-related behaviors. Past research in our laboratory has shown that adult HAP2 subjects are more sensitive to fear conditioning and show greater startle response amplitude than adult LAP2 subjects (Barrenha & Chester, 2007). In the current study, there was no difference between the adolescent HAP2 and LAP2 subjects' startle responses. This is consistent with past literature comparing startle responses between adolescent alcohol-preferring (P) and alcohol non-preferring (NP) rats (Bell et al., 2003) and a variety of other adolescent rat strains (Blaszczyk, 1996). Since the adolescent subjects were smaller than the adult subjects (approximately 15 grams vs. 30 grams), the possibility that using g/F as a measure of tactile response amplitude was not sensitive enough to detect differences between the adolescent groups cannot be ruled out. However, line differences are typically not seen in adolescent subjects across the literature, particularly in rats, which are presumably larger than the mice in the current study (Bell et al., 2003; Blaszczyk, 1996). Thus, a different rationale may better

explain why a line difference was present in the adult subjects but not the adolescent subjects.

As mentioned previously, a proposed reason for why stress exposure during adolescence may not manifest into altered behaviors until adulthood could be due to potentiation and incubation effects resulting from excessive glucocorticoid exposure during adolescence (Lupien et al., 2009). Many neural, hormonal, morphological, changes occur during adolescence, including those involved in stress circuitry (Enoch, 2011), and the effects of excessive glucocorticoid exposure may not be observed until later in life, when synaptic organization is complete (Lupien et al., 2009). This rationale proposes an explanation for why past research has shown no differences between rodent strains and lines in startle responses during adolescence, but clear differences can be seen during adulthood (Barrenha & Chester, 2007; Bell et al., 2003; Blaszczyk, 1996).

However, the Adolescent Stress subjects in the current study did not show a greater g/F per kg response during RS 1. In fact, the HAP2 Adolescent Stress subjects showed a lower g/F per kg response than the HAP2 Adult Stress subjects and Stress Control subjects. The LAP2 subjects showed no differences between the three Stress Treatment groups. This is contradictory to past research suggesting that rats with a chronic stress history show a sensitized neuronal response in 5HT and NE levels to acute stress (Adell et al., 1988). Of course, behavioral responses have been shown to differ from physiological responses to acute stress following prior chronic stress exposure in both clinical and animal research (Miller, Chen, & Zhou, 2007). There is very little data available regarding raw tactile startle responses in subjects exposed to stress during adolescence and later re-exposed to stress during adulthood. Unpublished data in our laboratory suggests that chronic stress history increases startle responses during a fear-potentiated startle (FPS) task when FPS directly follows stress exposure (unpublished data). However, these unpublished results did not use an interim period between stress exposure and FPS conditioning, nor did the design use adolescent subjects. A follow-up study to the current study has been planned to investigate changes in adult tactile startle amplitude and fear conditioning in subjects exposed to chronic stress during adolescence to help fill this gap in the literature.

CORT Concentrations

CORT During CSE

Little was known about what differences in CORT levels would emerge between groups in the current study, because CORT levels in the HAP2 and LAP2 lines have not yet been investigated in the adolescent population. In the current study, no differences between the HAP2 and LAP2 lines were observed during CSE Days 1 or 10, which was unexpected given past research in our laboratory showing differences in CORT levels between the HAP2 and LAP2 lines. Previously, we have observed lower CORT responses in HAP2 mice following stress exposure, which could be due to either enhanced negative feedback or a blunted response (Chester et al., 2013).

However, there were overall differences between the stress treatment groups during CSE. On the first day of CSE, the Adolescent Stress and Adult Stress subjects showed greater CORT levels than the Stress Control subjects, with the Adolescent Stress group showing the highest CORT levels. This finding was important, as it suggested that the foot shocks did elicit a physiological stress response in the HPA axis in comparison to the stress control. Since the stress control groups were placed in the bins, even though no shocks were administered, it was important to ensure that the stress control groups showed a different physiological response following CSE than the Adolescent Stress and Adult Stress groups. The CORT levels on Day 1 of CSE provided valuable information about the effects of acute stress exposure (since this was the first instance of exposure) on CORT levels, and the results from the current study suggested that the foot shocks elicited a rise in CORT levels in the Adolescent Stress and Adult Stress subjects compared to the Stress Control subjects, regardless of whether the subjects were HAP2 or LAP2 mice. Previous literature suggests that CORT levels generally rise during acute stress exposure, and suggests that a physiological HPA response was elicited (Mizoguchi et al., 2001; Ottenweller et al., 1992; Shanks, Griffiths, Zalcman, Zacharko, & Anisman, 1990). The difference in CORT levels on Day 1 of CSE suggest that the Stress Control group did, in fact, serve as a valid control, which was vital for later comparisons between stress treatment groups for the CPP data.

Importantly, the Adolescent Stress subjects showed the highest CORT levels, even though they were not statistically different from those of the Adult Stress

subjects. Previous research suggests that acute stress exposure during adolescence may result in excessive glucocorticoid release due to a prolonged HPA response to stressors compared to adult rodents (Vazquez & Akil, 1993), and that adolescent rodents undergoing stress show greater CORT levels than adult rodents in the same conditions (Laviola, Adriani, Morley-Fletcher, & Terranova, 2002). However, similar results were not seen in the current study. There are many discrepancies in rodent research examining CORT responses to acute and chronic stress (Miller et al., 2007), particularly between different strains of mice (Shanks et al., 1990). The HAP2 and LAP2 mice are bred from an out-bred stock strain that includes 8 different strains of mice, and only some of these strains have been shown to increase CORT levels in response to acute and chronic stress (Shanks et al., 1990). Furthermore, the CORT levels of adolescent mice within the HAP2 and LAP2 mice have not been well characterized, so little is known about what differences could have been expected. Based on the results of the current study, acute foot shock exposure did increase CORT levels overall when compared to controls, but there was no differentiation between the adolescent and adult subjects on Day 1 of CSE.

Interestingly, on Day 10 of CSE, the Adult Stress group showed a higher CORT response than both the Adolescent Stress and Stress Control groups. These results are contradictory to some of the literature, which suggests that a potentiated CORT response to repeated chronic stress exposure usually occurs in adolescents (Laviola et al., 2002; Romeo et al., 2006). . Over the course of the 10 days, the Adolescent Stress group's CORT levels decreased at a faster rate than the Adult Stress and Stress Control groups. Even though the Adolescent Stress subjects showed the highest CORT levels on Day 1 of CSE, their levels eventually returned to a level similar to those of the Stress Control group. Furthermore, the CORT levels of the Adolescent Stress group were lower than the levels of the Adult Stress group by CSE Day 10. In contrast, the Adult Stress and Stress Control groups' CORT levels decreased over the time course, although the change was less drastic than that seen in the Adolescent Stress group. Considering the habituation in g/F per kg responses during the 10 days of CSE, it is possible that the repeated foot shock paradigm used in the current study did not, in fact, mimic a chronic stressor. Based on the differences in CORT levels on Day 1, there is evidence that the foot shocks on Day 1 served as an acute stressor on that day. However, there is little evidence that the repeated foot

shock paradigm served as an efficient chronic stressor. It cannot be ruled out that the CORT levels in the Adolescent and Adult Stress subjects declined over the 10 days of CSE because the foot shock paradigm was not perceived as a chronic stressor.

Conversely, other research suggests that chronic stress exposure may disrupt the HPA axis in a way that enhances negative feedback and down-regulates CORT expression (Mizoguchi, Ishige, Aburada, & Tabira, 2003) or CORT receptors in the prefrontal cortex and hippocampus (Mizoguchi et al., 2001), and this has also been seen in adolescent subjects (Schmidt et al., 2007). Thus, an alternative explanation for the faster decline in CORT levels over the 10 days of CSE and the lower CORT levels in the Adolescent Stress subjects compared to the Adult Stress subjects on Day 10 of CSE may have been related to a disruption in the negative feedback of the HPA axis, such that CORT levels were down-regulated in these subjects more than in the adult subjects due to enhanced negative feedback. The results of the current study may also support the interpretation that CORT levels in the Adolescent Stress may have been down regulated more than those of the Adult Stress subjects over the course of CSE, possibly due to an enhanced negative feedback loop.

Blunted glucocorticoid responses have been associated with familiar characteristics of AUDs (Adinoff, Junghanns, Kiefer, & Krishnan-Sarin, 2005; Sorocco, Lovallo, Vincent, & Collins, 2006) and PTSD (de Kloet et al., 2006) in the literature. Importantly, blunted levels of cortisol in response to a stressor (Yehuda, McFarlane, & Shalev, 1998) and enhanced negative feedback in the HPA axis (Yehuda, 2001) have been suggested to serve as mechanisms by which individuals with PTSD show altered HPA functionality. Past research in our laboratory has shown that HAP2 mice show blunted CORT responses in response to a stressor compared to LAP2 mice (Chester et al., 2013). This was an important finding, as the HAP2 line of mice has been used to mimic genetically influenced disposition toward high-alcohol drinking and PTSD-symptom development (Chester et al., 2013). Additional research in rats has also shown that PTSD-related symptoms are associated with blunted CORT responses to a stressor (H. Cohen et al., 2006; King, Abend, & Edwards, 2001; Zoladz, Fleshner, & Diamond, 2012). Furthermore, CORT administration has been shown to decrease anxiety-related behaviors in rats (Cohen, Matar, Buskila, Kaplan, & Zohar, 2008; H. Cohen et al., 2006), similar to clinical data showing that cortisol administration decreased the likelihood of PTSD development following a traumatic event (Schelling

et al., 2001) and decreased symptoms in individuals diagnosed with PTSD (Aerni et al., 2004). Thus, strains or lines of rodents that show consistent alterations in CORT responses may serve as models of AUD or PTSD susceptibility.

To explore whether an altered negative feedback loop in the HPA axis was responsible for the faster decline in CORT levels during CSE in the Adolescent Stress subjects, it would be valuable to replicate this study design and use a synthetic glucocorticoid, such as dexamethasone (DEX). DEX mimics glucocorticoids and acts on the hypothalamus, and due to the negative feedback loop in the HPA axis, inhibits further release of ACTH and glucocorticoids. When the feedback loop is nonresponsive or down regulated, DEX does not suppress glucocorticoid release. DEX administration has been widely used during chronic stress paradigms in both clinical (de Kloet et al., 2006; Dinan, 1994; Kudielka & Kirschbaum, 2005; Miller et al., 2007; Raison & Miller, 2003; Yehuda, Boisoneau, Lowy, Giller, & Jr, 1995) and animal (Mizoguchi et al., 2003; Mizoguchi et al., 2001; Yehuda, Giller, Southwick, Lowy, & Mason, 1991) research to test feedback sensitivity of the HPA axis. Importantly, the proposed follow-up study using DEX would further help us understand if the decreased CORT levels over the 10 days of CSE, particularly in the Adolescent Stress subjects, can be attributed to an ineffective chronic stress paradigm or enhanced negative feedback following chronic stress exposure.

CORT During CPP

In contrast to the CORT levels during CSE, the CORT levels during Posttests 1 and 2 did differ between the HAP2 and LAP2 subjects, on each day and looking at the change in CORT levels between the two Posttests. The HAP2 mice showed no significant differences between the stress treatment groups. However, the LAP2 Adult Stress subjects showed generally higher CORT levels than the LAP2 Adolescent Stress subjects and the LAP2 Stress Control subjects. In addition, the LAP2 Adolescent Stress subjects showed differences in CORT levels depending on whether or not the subjects were re-exposed to the stressor before the Posttest. This was the only significant effect of RS seen in the current study. The LAP2 Adolescent Stress subjects who were re-exposed to the stressor showed lower CORT levels on Posttest 1 and increased CORT levels during Posttest 2, where the opposite results were seen in the LAP2 Adolescent Stress subjects who were not re-exposed to stress.

These were complicated results to interpret, based on the current available literature. In general, acute stress following chronic stress tends to produce a potentiated CORT response (Laviola et al., 2002; Miller et al., 2007); however, the only subgroup that showed a higher CORT response during Posttest 1 was the LAP2 Adult Stress group, and the Adult Stress subjects (when collapsed across the lines) had previously shown higher CORT levels during CSE Day 10, compared to the Adolescent Stress and Stress Control subjects. Furthermore, there were no differences between the RS and noRS subjects in the LAP2 Adult Stress group, which suggests that the higher level of CORT did not differ based on acute stress re-exposure. The results seen in the LAP2 Adolescent Stress subjects seem to contradict the literature; based on previous research, we would expect the LAP2 Adolescent-RS subjects to show a potentiated CORT response compared to the LAP2 Adolescent-noRS subjects (Laviola et al., 2002; Miller et al., 2007), but the opposite was seen. In line with the previous discussion, it is possible that the LAP2 Adolescent-RS subjects were showing a blunted CORT response to stress re-exposure, possibly due to down-regulated CORT levels resulting from enhanced negative feedback in the HPA axis resulting from excessive CORT exposure during adolescence (Mizoguchi et al., 2003). This could explain why this effect was seen in the LAP2 Adolescent Stress subjects, and not in the LAP2 Adult Stress or LAP2 Stress Control groups.

It was interesting that the effects of RS on CORT levels were only seen in the LAP2 Adolescent Stress subjects, and not in the HAP2 counterpart subjects. Since HAP2 mice are generally more sensitive to developing anxiety-related behaviors (Barrenha & Chester, 2007), one would predict that the HAP2 mice would show greater changes in CORT than the LAP2 mice. However, given that prior research suggests that the HAP2 mice generally show blunted CORT responses to a stressor compared to LAP2 mice (Chester et al., 2013), it is possible that differences in CORT levels between the stress treatment groups or RS subgroups could not be detected.

Conditioned Place Preference

Initial GRID Floor Preference

There were several unexpected but important effects that emerged from this study that need to be addressed. First, there was an initial preference for the GRID floor during the CPP pretest, particularly in the LAP2 subjects. A moderate preference for the GRID floor in CPP studies using the GRID and HOLE floor types has been

seen before in our laboratory (unpublished data), but the extent of the initial preference in LAP2 subjects was not seen until this study. A review on CPP by Cunningham (2014) examined CPP expression in a 15 inbred mouse strains, including the 8 strains used in the original out-bred stock of the HAP and LAP lines. Past research shows that some of the strains have historically shown an initial GRID preference during the pretest, but not all strains have. Since the HAP and LAP lines likely have differing genetically-influenced histories from the out-bred stock, it is possible that one or more of the strains examined in Cunningham's (2014) review is more represented in the LAP2 mice than the HAP2 mice familial history, and this could help explain the difference in the magnitude of initial GRID preference between the lines in the current study.

There was an initial concern before the study began that subjects exposed to the stress paradigm might avoid the GRID floor during the pretest due to its similar texture to the grid-type floor in the foot shock bin, even though the grid-type foot shock bins and CPP GRID floors are not similar in grid width or placement. However, the data do not suggest that adolescent stress or adult stress subjects were the only subjects showing an initial GRID floor preference or aversion. In other words, the initial preference to the GRID floor does not appear to be stress treatment group specific, but rather an overall phenomenon that is particularly present in the LAP2 subjects.

Due to the initial GRID floor preference seen in the current study, the raw time on the GRID floor was not suitable for use as the dependent variable for the CPP analyses (Cunningham et al., 2003). Instead, the GRID difference score was a more optimal dependent variable for the analyses, because it accounted for the initial GRID floor preference in the data. Importantly, even though the LAP2 subjects initially preferred the GRID floor more than the HAP2 subjects, the LAP2 subjects still showed significant CPP. Even further, the LAP2 subjects showed significantly greater CPP than the HAP2 subjects overall, which suggests that the initial GRID floor preference in the LAP2 subjects did not inhibit the expression of alcohol-induced CPP or produce a ceiling effect in the data. Thus, the initial GRID floor preference was not a major concern in overall data interpretation. Additionally, we also analyzed the data using the raw time on the GRID floor and raw time on the CS+ (alcohol-paired) floor to see if similar results were observed when different dependent variables were used (data not

shown). Importantly, no matter which dependent variables were used, the results of the study remained the same.

Change in CPP Over Time

Selecting what portion of time during the CPP posttest to use for data analysis was also an important implication of this study. The default time period to show in CPP data is the average time spent on the floor of interest (GRID or CS+) over the total 60 min of the test. However, the reason that the posttests are 60 min in length is to ensure that the height of CPP expression is captured during the posttest, as it may wax and wane over time (Cunningham et al., 2003). In specific study designs, CPP may begin at a high level and then substantially decrease over the 60 min period, or vice versa (Cunningham, 2014; Cunningham, Dickinson, & Okorn, 1995; Cunningham et al., 2006; Cunningham, Henderson, & Bormann, 1998). For example, Cunningham et al. (1995; 1998) used naloxone to examine alterations in CPP expression, but effects were only seen during the first 30 min of the Posttest. Using analyses that averaged over the full 60 min of the Posttest led to conclusions of null results, because the effect during the first 30 min could not be detected (Cunningham et al., 1995; Cunningham et al., 1998). Thus, even though a Posttest may contain 60 min of data, CPP expression is likely to reduce over time, and identifying the temporal period in which differences in CPP expression can be identified is vital to accurate interpretation of the data (Cunningham et al., 2006).

The current study was especially sensitive to changes in CPP over the course of the 60 min due to the nature of the design. In general, activity levels have been shown to alter the magnitude of CPP expression (Cunningham, 2014). During the pretest, there were initial differences between activity in the HAP2 and LAP2 mice, such that the HAP2 mice showed greater activity than the LAP2 subjects. This is consistent with previous literature showing that HAP1 mice showed greater activity during a CPP Posttest compared to LAP1 mice (Grahame et al., 2001). Furthermore, even though the Adolescent Stress and Stress Control subjects were adults during the CPP paradigm, they were still younger than the subjects who underwent CSE during adulthood (Adult Stress subjects). Younger rodents typically show higher activity levels than older subjects (Tzschentke, 2007). Lastly, foot shock exposure has not directly been shown to increase locomotor activity levels during CPP in some studies (Sanchez, Bailie, Wu, Li, & Sorg, 2003), but the majority of the studies that have used

foot shock as a stress re-exposure paradigm before CPP did not report overall locomotor activity levels (Matsuzawa et al., 1998; Song et al., 2007; Wang, Luo, Ge, Fu, & Han, 2002). Thus, it could not be ruled out that activity levels and/or CPP expression could alter CPP expression over the course of the CPP posttests.

We wanted to ensure that the data adequately represented the portion of the posttest in which CPP magnitude differences were most clear. Thus, preliminary analyses were performed to see if CPP expression did, in fact, significantly change over the 60 min period. CPP expression significantly interacted with genotype and the conditioning subgroup of the subjects, and there was a near-significant interaction between CPP expression, stress treatment, and stress re-exposure subgroups of the subjects. These findings strongly suggested that CPP expression changed over time, depending on the specific subgroup assignment of the animal (24 subgroups total). The CPP posttest data was examined minute-by-minute, and visual representation indicated that the greatest CPP was seen in the first 20 min of the posttests across groups, after which CPP decreased over time during the last 40 min of the posttests. Thus, using data from only the first 20 min of the posttests allowed for a more clear interpretation of the CPP results; using the data from the total 60 min provided an inaccurate representation of the results due to the change in CPP expression over time.

CPP Magnitude Difference Between the HAP and LAP Lines

Another important effect that emerged from this study was the clear difference in CPP magnitude between the HAP2 and LAP2 lines. The LAP2 subjects showed significantly greater CPP than the HAP2 subjects in every analysis of the CPP data. Recent research directly comparing CPP between the HAP2 and LAP2 lines has not been performed in a study of this size, and thus the magnitude of difference in CPP between the lines was not initially expected. Past research has shown that LAP1 mice showed greater CPP than HAP1 mice at a 4.0 g/kg dose, but not at a 1.5 or 3.0 g/kg dose (2.0 g/kg was the dose used in the current study; Grahame et al., 2001). The current study examined the effects of stress exposure, and thus is not directly comparable to the Grahame et al. (2001) HAP1/LAP1 comparison study. However, the stress control groups of the current study provide important information about the difference in CPP magnitude between the HAP2 and LAP2 lines. The LAP2 stress control-noRS subjects showed significantly greater CPP than the HAP2 stress

control-noRS subjects, suggesting that the observed line difference was not dependent on prior stress exposure history or stress re-exposure. Rather, the difference in CPP between the HAP2 and LAP2 lines was a general overall effect.

Furthermore, the Grahame et al. (2001) study was performed in the first replicate of the HAP and LAP lines, while the current study used the second replicate of the lines. This is an important differentiation, as differences between replicate lines and even generations within replicate lines may occur within behavioral paradigms, even though the mice are bred for specific behaviors (Bice et al., 2006; Crabbe, Phillips, Kosobud, & Belknap, 1990). Not only may interactions between genotype and specific laboratory locations occur (Crabbe, Wahlsten, & Dudek, 1999), but other phenotypes related to drinking behaviors may differ between replicates and generations. This could help explain why LAP1 subjects showed greater CPP than HAP1 subjects only at a high dose of alcohol in past research (Grahame et al., 2001), while the current study showed the same effect at a lower dose. It would be greatly beneficial to repeat the current study with the third replicate line (HAP3 and LAP3) to see if similar results are found.

The fact that LAP2 subjects showed significantly greater CPP than the HAP2 subjects at the 2.0 g/kg dose in the current study is an important finding for researchers using these selectively-bred lines. Previous research has shown that HAP and LAP mice in the first and second replicates have similar alcohol metabolism rates, BAC response curves (Grahame et al., 1999), and BAC elimination (Chester & Barrenha, 2007) when alcohol is administered according to body weight. This information suggests that the difference in CPP magnitude between the HAP2 and LAP2 lines in the current study should not be attributed to metabolic differences, but reflects a genetically-influenced difference in the sensitivity to the rewarding effects of alcohol between these lines, which may occur at a lower dose than previously recorded (Grahame et al., 2001). Importantly, this is a new finding for the HAP2 and LAP2 lines, and it is complementary to the prior research in the HAP1 and LAP1 lines.

Inverse Relationship Between Alcohol Drinking and CPP

The current study hypothesized that the HAP2 mice would show greater CPP following stress exposure, specifically adolescent stress exposure, because HAP2 mice are more sensitive to the effects of stress on alcohol-related behaviors (Chester et al., 2006; Chester et al., 2008). Unfortunately, this hypothesis did not take into

account that LAP2 mice would show greater overall CPP than HAP2 mice, because a difference of this magnitude between the lines in CPP expression was not expected. The relationship between CPP and drinking behaviors is not well understood (see review by Green & Grahame, 2008), due in part to a lack of research directly comparing drinking phenotypes and different inbred mouse strains in CPP study designs. However, a more recent literature review assessing the inverse relationship between voluntary drinking and sensitivity to the rewarding effects of alcohol has been published (Cunningham, 2014), and may help explain one rationale as to why the difference in CPP magnitude between the LAP2 and HAP2 lines in this study should have been expected.

The review by Cunningham et al. (2014) suggests that the literature comparing alcohol drinking and CPP expression is complicated, hence why there has been such discrepancy in the literature thus far. Cunningham's review examined 15 inbred mouse strains (8 represented in the HAP/LAP lines) and a variety of alcohol-related behaviors, including blood ethanol concentrations, ethanol withdrawal severity, voluntary alcohol consumption, conditioned taste aversion (CTA), and locomotor activity, and assessed how these behaviors related to CPP expression. Importantly, there was a wide range of magnitude in CPP expression across the strains at both 2.0 and 4.0 g/kg doses, suggesting that genetic influences may alter alcohol-induced CPP in a general manner (Cunningham, 2014).

An important finding of this review suggested that there is a significant and negative relationship between alcohol intake and CPP at the 2 g/kg dose (Cunningham, 2014), such that mouse strains that drink more alcohol voluntarily tend to show lower CPP expression. The LAP lines of mice are selectively-bred for low alcohol preference, and this finding helps explain why the LAP1 mice in the Grahame et al. (2001) and the LAP2 mice in the current study showed greater CPP than their HAP counterparts. An explanation for this inverse relationship has been proposed, suggesting that rodents who drink more voluntarily may be drinking more because they are less sensitive to the rewarding effects of the drug, and thus require greater amounts of alcohol to reach their desired rewarding state (Cunningham, 2014).

The literature has also proposed a notion that LAP2 mice may be more sensitive to the aversive effects of alcohol, and that this may lead to greater alcohol-induced CPP. Some literature suggests that alcohol consumption is more initially

aversive to rodents selectively bred for low-drinking, and thus the rewarding effects of alcohol are not initially experienced, in contrast to using injections during a CPP paradigm and avoiding an aversive taste cute (Cunningham, Gremel, & Groblewski, 2009). It is important to keep in mind that the HAP2 and LAP2 lines were bred over 20 generations for their drinking behaviors, not for their sensitivity to the rewarding effects of alcohol. As discussed previously, voluntary alcohol consumption is influenced by many factors, including taste factors. The CPP paradigm uses injections as a route of administration, essentially bypassing any confounds of taste-related behaviors. It is possible that one of the reasons why the LAP2 mice may drink less alcohol voluntarily is because the taste of alcohol is aversive to them. LAP1 and LAP2 mice show greater conditioned taste aversion (CTA) than HAP1 and HAP2 mice (Chester, Lumeng, Li, & Grahame, 2003). This suggests that LAP2 mice may have a greater sensitivity to the aversive effects of alcohol, although the magnitude of CTA has been proposed to reflect a general sensitivity to either rewarding or aversive effects of a drug, applicable to the current results. Importantly, drinking propensity and CTA expression are negatively related (Cunningham, 2014).

Thus, LAP2 mice may show enhanced CPP expression compared to the HAP2 mice because they are more sensitive to the rewarding effects of alcohol, but a positive relationship between drinking behaviors and CPP expression is not reflected due to extraneous taste influences. In other words, an inverse relationship between drinking propensity and sensitivity to the rewarding effects of alcohol may exist, but this relationship may also be influenced by a variety of other factors selectively represented between the HAP2 and LAP2 lines, besides just drinking propensity. However, one limitation of this explanation is that it fails to explain the increase in preference to the alcohol-paired floor, particularly in studies that use a difference score to calculate CPP (Cunningham, 2014), such as the current study.

In addition, the literature suggests a positive relationship between CPP expression and chronic ethanol withdrawal severity, such that strains that show severe ethanol withdrawal also show a high CPP expression (Cunningham, 2014). For example, past research has shown that withdrawal-seizure prone (WSP) mice and high-alcohol withdrawal (HAW) mice show greater CPP than withdrawal-seizure resistant (WSR) mice or low-alcohol withdrawal (LAW) mice (Chester, Risinger, & Cunningham, 1998; Crabbe, Phillips, Cunningham, & Belknap, 1992). The LAP2 mice

in our laboratory have historically shown more severe chronic ethanol withdrawal than the HAP2 mice (Chester & Barrenha, 2007). Overall, the difference in CPP magnitude in the current study between the LAP2 and HAP2 lines is in agreement with previous literature, and should have been expected.

Alternative Explanations

It is important to ensure that the difference in CPP magnitude between the HAP2 and LAP2 lines seen in the current study was not due to other differences in aspects of the CPP paradigm, including differences in activity levels or learning and memory. There were significant differences in activity between the HAP2 and LAP2 subjects, such that LAP2 mice showed lower activity levels than the HAP2 mice. Previous research has shown that lower activity levels have been associated with higher CPP expression (Cunningham, 2014). This association stems from the concept that less active mice may choose a specific CS floor and remain there, whereas more active mice may initially choose a CS floor but continue to move around the apparatus after a certain period of time. However, this relationship has primarily been established in CPP paradigms that use a posttest in which a drug has been administered (Gremel & Cunningham, 2007), whereas the current study used a drug-free posttest, and less so for CPP paradigms using different lines or strains of rodents. In the current study, activity levels were significantly correlated with time spent on the GRID floor, but the Pearson correlation coefficients were relatively low, at approximately $r = 0.3$ or 0.4. This suggests that activity levels can only account for approximately 15% of the variance of the data between the lines. Furthermore, in both the HAP2 and LAP2 subjects, activity levels were highest during the first 20 min of the posttests (data not shown), the same time at which CPP expression was highest. If higher activity levels were associated with lower CPP expression, then we would expect that CPP expression would have been highest at the end of the 60 min session; in fact, the opposite was true. The relationship between locomotor activity and CPP expression in the current study suggests that the differences in activity levels between the lines may have contributed to a portion of the difference in CPP magnitude, but cannot explain the difference in CPP magnitude between the HAP2 and LAP2 lines entirely.

Furthermore, the differences in CPP magnitude in the current study do not appear to be due to line differences in learning or memory mechanisms that support the development and expression of classically conditioned behavior. Both HAP2 and

LAP2 mice show evidence of learning in other behavioral paradigms, such as FPS and CTA (Barrenha & Chester, 2007; Chester et al., 2003). LAP2 mice have shown decreased FPS and increased CTA expression compared to HAP2 mice, and in the current study, LAP2 mice showed increased CPP compared to HAP2 mice. Taken together, these results suggest that both lines are capable of learning classical conditioning paradigms, and suggest that differences between the two lines in CPP expression are not specifically due to differences in learning mechanisms, because the differences are not always in the same direction. Similarly, meta-analyses in past research comparing CPP expression between different strains of rodents do not suggest that differences in CPP are due to differences in learning or memory mechanisms, but rather should be attributed to differences in genetically- or environmentally-influenced behaviors (see review by Cunningham et al., 2014). However, it is important to note that since the LAP2 mice show enhanced CPP and CTA expression compared to HAP2 mice, it is possible that line differences in learning behavioral paradigms that use alcohol as a cue, specifically, may exist. Further research is needed to explore this possibility.

In the current study, both the HAP2 and LAP2 subjects showed overall CPP, though the magnitude greatly differed between the lines. Importantly, there were no differences in activity levels during the CPP conditioning trials, suggesting that one line did not sensitize to the alcohol-paired conditioning trails more than the other. It is true that behavioral paradigms can be inherently stressful and may alter motivational behaviors (McCormick et al., 2010), leading to differences in performance of the task. It is therefore possible that the decreased CPP expression in the HAP2 subjects, particularly the HAP2 subjects exposed to stress during adolescence, could stem from an increase in anxiety-related behaviors during the CPP posttest. It would be beneficial to see if the HAP2 and LAP2 lines also differ in a separate study using the same chronic stress paradigm, but using a different classical conditioning paradigm as the outcome. One example would be to replace the CPP paradigm in the current study with an FPS paradigm, to see if HAP2 subjects exposed to stress during adolescence are sensitized to fear conditioning during adulthood. A follow-up study has been planned to investigate this possibility.

Alterations in CPP Due to Adolescent Stress Exposure

The supported rationale that alcohol-drinking behaviors and sensitivity to the rewarding effects of alcohol are inversely related may additionally provide an explanation for why the HAP2 adolescent stress subjects failed to show CPP compared to the HAP2 Adult Stress and HAP2 Stress Control subjects, the opposite of what was hypothesized.

The literature suggests that stress exposure during adolescence increases voluntary alcohol consumption both directly following stress exposure (Becker et al., 2011; Croft, Brooks, Cole, & Little, 2005; Hilakivi-Clarke & Lister, 1992; Kudryavtseva, Madorskaya, & Bakshtanovskaya, 1991; Little et al., 1999; Siegmund et al., 2005; Sperling, Gomes, Sypek, Carey, & McLaughlin, 2010; Vengeliene et al., 2003) and later during adulthood (Chester et al., 2008). In general, adolescents will voluntarily consume less alcohol than adult rodents (Siegmund et al., 2005). While acute stress exposure in adolescent and adult rodents typically decreases or has no effect on alcohol consumption (Becker et al., 2011; Croft et al., 2005), chronic or intermittent stress exposure increases consumption (Becker et al., 2011; Croft et al., 2005; Hilakivi-Clarke & Lister, 1992; Kudryavtseva et al., 1991; Little et al., 1999; Siegmund et al., 2005; Sperling et al., 2010; Vengeliene et al., 2003), especially in adolescent rodents (Becker et al., 2011; Siegmund et al., 2005). Chronic social stress has been shown to increase alcohol drinking in adult rodents, and severely wounded subjects showed significant alcohol preference following social stress (Hilakivi-Clarke & Lister, 1992). Importantly, this effect was not due to differences in aggression levels. Foot shock exposure has also been shown to increase voluntary alcohol consumption immediately following stress exposure (Becker et al., 2011; Siegmund et al., 2005; Sperling et al., 2010; Vengeliene et al., 2003), particularly in adolescent subjects, whereas forced swim stress does not increase drinking in either age group (Siegmund et al., 2005).

Previous research in our laboratory showed that chronic stress exposure during adolescence significantly increased voluntary alcohol consumption in HAP2 mice during adulthood, whereas stress exposure during adulthood did not increase later consumption (Chester et al., 2008). LAP2 mice were not tested in the Chester et al. (2008) study, so it is unknown if adolescent stress exposure would alter voluntary alcohol consumption in LAP2 mice. The current study revolved around the hypothesis

that the relationship between adolescent stress and increased consumption in HAP2 mice may exist due to an increase in the rewarding or reinforcing effects of alcohol exposure. However, an alternate explanation provides an applicable explanation of why the results were in the opposite direction of the hypothesis: perhaps the reason why HAP2 subjects exposed to stress during adolescence consumed more alcohol during adulthood in the Chester et al. (2008) study was because these subjects were less sensitive to the rewarding effects of alcohol following adolescent stress exposure. This rationale is better supported by the literature (Cunningham, 2014) than the original hypothesis and rationale, and suggests that voluntary alcohol consumption and CPP expression may also be inversely related when stress exposure is involved in the relationship.

Additionally, another possible explanation for the decreased CPP seen in the HAP2 adolescent subjects during the first 20 min of Posttest 1 might stem from previous research suggesting that adolescent stress exposure may increase the threshold for the rewarding effects of a drug. Research by Mathews et al. (2008) exposed adolescent rats to a social stress paradigm and tested amphetamine-induced CPP during adulthood, and found modest, dose-specific changes in CPP expression. For example, the subjects exposed to stress during adolescence showed a decrease in CPP at the 0.5 mg/kg dose but an increase in CPP at the 1.0 mg/kg dose in female subjects compared to the stress control subjects (Mathews, Mills, & McCormick, 2008). These findings suggest that stress exposure during adolescence may shift the dose-response curve to sensitivity to the rewarding effects of amphetamine, thus increasing the threshold for the rewarding effects of the drug. The current study is not directly comparable to the Mathews et al. (2008) study due to the fact that all-male selectively-bred lines and a different drug were used, but it is possible that a similar shift in threshold could explain the decreased alcohol-induced CPP expression seen specifically in the HAP2 adolescent stress subjects. To explore this possibility, a follow-up study using several different doses of alcohol should be used, as the current study only used a 2.0 g/kg dose.

Importantly, the explanation of the results in the current study may require a combination of this rationale. For example, the inverse relationship between voluntary drinking and CPP may initially help us understand why the HAP2 Adolescent Stress subjects showed decreased CPP during the first 20 min of Posttest 1 compared to all

other subgroups, but the mechanism by which this inverse relationship was seen specifically in the HAP2 mice and not LAP2 mice could be due to an altered threshold to the rewarding effects of alcohol resulting from adolescent stress exposure in subjects prone to developing anxiety-related behaviors (Barrenha & Chester, 2007). Combining this rationale may help explain why the HAP2 Adolescent Stress subjects showed decreased CPP, specifically, and not the LAP2 Adolescent Stress subjects. Replicating the current study using other several doses of alcohol during CPP as well as other behavioral paradigms used to measure subjects' sensitivity to the rewarding effects of alcohol, such as a 2-bottle choice or CTA paradigm, would benefit the interpretation of these data (Lederle et al., 2011; Lynch, Nicholson, Dance, Morgan, & Foley, 2010; Sanchis‐Segura & Spanagel, 2006).

We must also consider the possibility that the chronic stress paradigm used in the current study may not have been severe enough to evoke clear behavioral changes between the stress treatment groups. The HAP2 Adolescent Stress subjects showed an alteration in CPP compared to the HAP2 Adult Stress and HAP2 Control subjects, but this effect was only observed during the first 20 min of Posttest 1. Throughout the remaining duration of Posttest 1 and all of Posttest 2, there were no differences between the Stress Treatment groups in either the HAP2 or LAP2 mice. Importantly, there were no significant differences specifically between the HAP2 Adolescent Stress and HAP2 Control subjects during CPP. It is possible that a different stress exposure paradigm could have elicited clearer or more long-lasting results between the stress treatment groups. In addition, the results of the current study could be partially due to the effects of age differences in CPP expression (comparing the Adolescent Stress and Control subjects' CPP magnitude to that of the Adult Stress subjects in the HAP2 mice). All mice were adults by the time the CPP paradigm took place, but the Adult Stress subjects (M =137) were approximately 67 days older than the Adolescent Stress and Stress Control subjects (M =70) during CPP.

The hypothesis that adolescent chronic stress exposure would increase CPP during adulthood was also based upon previous research showing that adolescent stress increases CPP expression (Song et al., 2007). It is not surprising that the current study did not mimic these results. The Song et al. (2007) study found that chronic stress increased CPP in adolescent subjects, and that stress exposure had no

effect on CPP in adult subjects. However, the Song et al. (2007) study compared CPP between the adolescent group at a 2 g/kg dose and the adult group at a 1 g/kg dose (Song et al., 2007), so the differential effects of the age of stress exposure on CPP were not directly comparable between the two age groups. The current study conditioned all subjects with a 2 g/kg dose of alcohol so that the groups may be directly compared and to allow for a more clear interpretation of the data. In addition, the Song et al. (2007) design began the CPP paradigm directly following stress exposure, focusing on a more immediate effect of stress exposure on sensitivity to the rewarding effects of alcohol than long-term effects. The current study used a longitudinal design with a 3-day interim between stress exposure and CPP, and stress differs in its immediate and long-term effects on drug-related behaviors (Becker et al., 2011).

The second hypothesis of the current study was based on previous research by Matsuzawa et al. (1998), which showed that groups exposed to stress showed greater CPP than those not exposed to stress, and that re-exposure before the CPP posttest further increased CPP expression. The current study did not mimic these results, but there are key differences between the current study and the previous study. The research by Matsuzawa et al. (1998) exposed subjects to foot shock during the CPP conditioning trials, so that the stress exposure and exposure to the alcohol-paired floors occurred on the same day. This design allowed for the fear stimulus to be simultaneously conditioned to alcohol, whereas the current study focused more on the long-term effects of exposure to stress on CPP expression, separately, without combining stress-related cues and the CPP stimuli simultaneously.

Additionally, similar to the Song et al. (2007) study, the study by Matsuzawa et al. (1998) tested a more immediate effect of stress exposure on CPP expression, whereas the current study was focused on the long-term effects of stress exposure on CPP expression. This reinforces the differences in the immediate and long-term effects of stress, and how they may differentially alter the sensitivity to the rewarding effects of a drug (see review by (Becker et al., 2011). Furthermore, re-exposure immediately before the CPP posttest increased CPP in the Matsuzawa et al. (1998) study, but not in the current study. This is not surprising, given that the stress exposure paradigm was more recently concluded when subjects were re-exposed to stress in the Matsuzawa et al. (1998) study, whereas a 30-day interim period took

place in the current study between stress and stress re-exposure. Thus, the re-exposure stimulus was likely more salient to subjects with a stress exposure history in the Matsuzawa et al (1998) study, and may have had a more potent effect on CPP expression compared to re-exposed subjects in the current study. Lastly, the Matsuzawa et al. (1998) study used rats, and mice and rats often show differences in CPP expression, particularly when stress exposure is used as a variable (Blanchard, McKittrick, & Blanchard, 2001).

Specific Characteristics of the Stressor

It is important to note that the effects of stress exposure on drug-related behaviors can be highly variable between stressor types, laboratory practices, and rodent species (see review by McCormick et al., 2010). In general, adolescent stress exposure tends to alter the sensitivity to the various effects of drugs (McCormick, 2009); importantly, the type of stressor used can variably alter the sensitivity to the rewarding effects of drugs, specifically. For example, research by Burke et al. (2011) found that social stress exposure during adolescence significantly increased amphetamine-induced CPP during adulthood, but using foot shock stress as a stressor during adolescence had no effects on CPP expression (Burke, Watt, & Forster, 2011). Thus, it is possible that the use of a different stressor in the current study could have produced different results.

The current study sought to use the same chronic stress paradigm that had previously been shown to increase voluntary alcohol consumption in our laboratory (Chester et al., 2008) to see if alterations in sensitivity to the rewarding effects of alcohol could explain the relationship between adolescent stress and adult alcohol consumption. Furthermore, the use of foot shock as a stressor was important to the design of this study because it is a well-established physical and psychological stressor (Matsuzawa & Suzuki, 2002), and the brain mechanisms that influence foot shock stress are more well understood than other stressor types (Le & Shaham, 2002). Despite the rationale for using the repeated foot shock paradigm in the current study to mimic chronic stress exposure, we cannot rule out the possibility that the stressor in the current study may not have effectively mimicked chronic stress in any of the subjects. Alterations in CPP were observed in the HAP2 mice exposed to stress during adolescence; however, these results did not persist throughout the entirety of

the posttests, and a more severe stress paradigm may have evoked clearer differences in CPP magnitude between the stress treatment groups.

Future Directions

Several follow-up studies to the current study have been proposed throughout this discussion. One planned follow-up study to the current study has been planned to examine changes in adult tactile startle amplitude and fear conditioning in subjects exposed to chronic stress during adolescence. This follow-up study will use the same chronic stress exposure paradigm as the current study, followed by an interim period to allow subjects to mature into adulthood. Following the interim period, subjects will undergo an FPS conditioning and testing paradigm. The data from this follow-up study will provide important information as to whether adolescent stress exposure increases anxiety-related behaviors during adulthood more than adult stress exposure or no stress exposure, and how these may differ by drinking propensity.

In addition, other future directions should be taken based on the results of the current study. Future research should examine whether adolescent stress exposure alters the threshold to the rewarding effects of drugs. This could be investigated using several different doses of alcohol during a CPP paradigm in a study designed similar to the current study. Investigating multiple doses of alcohol instead of one, like the current study, will provide information about whether the threshold for sensitivity to the rewarding effects of alcohol may have been altered in the HAP2 Adolescent Stress subjects, or if sensitivity to the rewarding effects of alcohol were abolished regardless of alcohol dosage.

Furthermore, future research that replicates the current study but also implements DEX treatment during chronic adolescent stress exposure would provide information regarding if the subjects exposed to adolescent stress experienced enhanced negative feedback in the HPA axis during chronic stress exposure. A separate future study could also replicate the current study but use an alternate behavioral paradigm that assesses sensitivity to the rewarding effects of alcohol, such as a 2-bottle choice paradigm or CTA. A replication of the current study using female subjects would also be beneficial, especially if the future study examined the influence of estrous stage and how hormone fluctuations may alter the effects of stress exposure on CPP expression.

The current study should additionally be replicated using the HAP2 and LAP3 lines, to see if similar results are observed. This would be especially valuable due to the differences in CPP magnitude observed between the HAP1/HAP2 and LAP1/LAP2 replicate lines at differing doses. By replicating the current study using the HAP3 and LAP3 lines, we may better understand the genetic correlation of the effects observed in the current study.

Lastly, it would be greatly beneficial to replicate the current study with a different or more severe stress paradigm to mimic chronic stress. For example, using a chronic social stressor may evoke more long-lasting results, based on more recent research. Future research using a different stress paradigm would also benefit from using a more unpredictable stressor. Completing this future research would help us understand whether the stress paradigm used in the current study was, in fact, an effective chronic stressor.

Conclusions

In summary, the current study sought to answer if chronic stress exposure during adolescence increased sensitivity to the rewarding effects of alcohol during adulthood, and how the effects may vary based on genetic propensity toward high or low alcohol preference. It was hypothesized that adolescent stress would increase CPP expression during adulthood, based on previous research suggesting that adolescent stress increases voluntary alcohol consumption during adulthood (Chester et al. 2008). Previous research also suggests that adolescent stress may alter brain-related pathways associated with the reward effects of drugs (Brady & Sinha, 2005; Enoch, 2011), and that adolescent stress exposure increases CPP expression (Song et al., 2007). This hypothesis was not supported. In the current study, adolescent stress exposure actually decreased CPP in HAP2 mice during the first 20 min of the Posttest. A more recent literature review shows that an inverse relationship exists between alcohol consumption and CPP expression (Cunningham et al., 2014), and suggests that specific subjects may require higher alcohol consumption because they are less sensitive to the rewarding effects of the drug. Furthermore, research by Mathews et al. (2008) suggested that adolescent stress exposure results in an altered threshold to the rewarding effects of drugs during adulthood; this rationale may also apply to the current study.

The current study also hypothesized that stress re-exposure would further increase CPP, based on previous work showing that stress re-exposure further increases CPP expression (Matsuzawa et al., 1998). This hypothesis was also not supported. In the current study, stress re-exposure before the CPP posttests resulted in no alterations in CPP expression. Furthermore, intermittent re-exposure to the stressor did not increase CPP expression between the two CPP posttests. In fact, CPP expression decreased between the posttests, overall.

Lastly, it was hypothesized that adolescent stress exposure and stress re-exposure would lead to alterations in CPP particularly in the HAP2 subjects, and less so in the LAP2 subjects. This hypothesis did not take into account the significant difference in CPP magnitude between the lines; LAP2 mice showed a greater magnitude of CPP expression than HAP2 mice overall, which supports recent research suggesting that there may be an inverse relationship between voluntary drinking behaviors and sensitivity to the rewarding effects of alcohol (Cunningham, 2014). Due to the inherent difference in CPP magnitude between the lines, this hypothesis was partially supported. Adolescent stress exposure did significantly alter CPP expression in the HAP2 subjects during adulthood, specifically during the first 20 minutes of the Posttest, although the results in the current study were in the opposite direction than expected. Stress re-exposure did not alter CPP expression in either the HAP2 or LAP2 subjects.

Importantly, analyses of CORT levels during stress exposure and the CPP posttests helps provide a mechanistic rationale for why alterations in CPP were seen in the subjects exposed to stress during adolescence. Both HAP2 and LAP2 subjects exposed to adolescent stress showed a more rapid decline in CORT levels across stress exposure compared to subjects exposed to stress during adulthood and subjects not exposed to stress. The literature suggests that this difference may be due to enhanced negative feedback in the HPA axis of the subjects exposed to stress during adolescence, as excessive CORT exposure during this developmental time period may disrupt the negative feedback loop of the immature HPA axis (Kudielka & Kirschbaum, 2005). However, these results could also be due to an ineffective chronic stressor. Overall, long-term alterations in CORT levels were not observed in the current study.

Overall, these results suggest that chronic stress exposure during adolescence may decrease alcohol-induced CPP expression during adulthood, particularly in rodents bred for high-drinking propensity. These data provide some support for an inverse relationship between genetically influenced alcohol consumption and CPP expression, and suggest that this relationship may also extend into the stress literature. The current study suggests that an inverse relationship between drinking and sensitivity to the rewarding effects of alcohol may help explain why adolescent stress exposure is associated with increased alcohol consumption during adulthood; individuals exposed to stress during adolescence may increase alcohol consumption during adulthood because more alcohol is needed by these individuals in order to reach the desired perceived rewarding effects of the drug.

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Yehuda, R., Giller, E. L., Southwick, S. M., Lowy, M. T., & Mason, J. W. (1991). Hypothalamic-pituitary-adrenal dysfunction in posttraumatic stress disorder. *Biological Psychiatry, 30*(10), 1031-1048. doi: http://dx.doi.org/10.1016/0006-3223(91)90123-4

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Zoladz, P. R., Fleshner, M., & Diamond, D. M. (2012). Psychosocial animal model of ptsd produces a long-lasting traumatic memory, an increase in general anxiety and ptsd-like glucocorticoid abnormalities. *Psychoneuroendocrinology, 37*(9), 1531-1545. doi: http://dx.doi.org/10.1016/j.psyneuen.2012.02.007

VITA

VITA

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EDUCATION

PhD, Psychology, Concentration in Behavioral Neuroscience May 2015
Purdue University (anticipated)
Frederick N. Andrews Fellowship Recipient for Psychology, 2012
Advisor: Dr. Julia A. Chester
Dissertation Title: "Chronic Stress During Adolescence Alters Alcohol-Induced Conditioned Place Preference in Mice Selectively Bred for High Alcohol Preference but not Low Alcohol Preference"

MA, Psychology, Concentration in Behavioral Neuroscience December 2012
San Diego State University
Advisor: Dr. Jennifer D. Thomas
Thesis Title: "The Effects of Choline on Stress Regulation in Rats Exposed to Alcohol during Development"

BA (with honors), Psychology May 2010
Chapman University
Graduated Magna Cum Laude
Provost's List 2006; Chancellor's List 2007; Provost's List 2008; Chancellor's List 2009

RESEARCH EXPERIENCE

Chester Lab, Purdue University August 2012 – Present
Research focus: Stress and Alcoholism
Mentor: Dr. Julia Chester
Graduate Fellow
Dissertation Research
Dissertation Title: "The Effects of Chronic Adolescent Stress Exposure on the Sensitivity to the Rewarding Effects of Alcohol during Adulthood"
Skills: Animal handling and care; injections (i.p.), ELISA corticosterone assay, SPSS statistical software use, manuscript preparation, grant application submission (F31)
Behavioral Tasks: pre-pulse inhibition, fear-potentiated startle, conditioned place preference, handling-induced convulsions

Center for Behavioral Teratology, Thomas Lab, SDSU August 2010 – July 2012

Research focus: Fetal Alcohol Spectrum Disorder Deficit Intervention

Mentor: Dr. Jennifer Thomas

Graduate Student Research Assistant, Student Mentor

Master's Thesis

Breit, K. The effects of choline on stress regulation in rats exposed to alcohol during development (*Currently Writing for Publication with Dr. Thomas for ACER*). San Diego State University, San Diego, CA.

Also Printed by Montezuma Publishing for the SDSU Library

Skills: Animal handling and care; alcohol intubations, drug injection (s.c. and i.p.), instracardial perfusion, collection of brain and organ tissue, SPSS statistical software use, manuscript preparation

Behavioral Tasks: working memory Morris water maze, elevated plus maze, stress platform, open field activity, odor and object recognition

Study of Aging Experience (SAGE) Lab at Chapman University September 2009 – May 2010

Research focus: Social Smoking and Delay Discounting

Mentor: Dr. Ruby Brougham

Undergraduate Research Assistant

This study examined the effects of message-framing and memory of anti-smoking messages towards social and light smokers.

Skills: survey administration, APRIL aging software, writing manuscripts

Independent Undergraduate Senior Thesis August 2009 – May 2010

Research Title: Child Eyewitness Age and Testimony Accuracy: Who Do We Believe?

Chapman University

Mentor: Dr. Steven Schandler

Previous research suggests a downward shift in accuracy in child eyewitnesses around the age of 5 years due to older children's awareness of socioemotional and environmental pressures and their ability to speculate. This review paper hypothesized that if an eyewitness is a young child (5 years or younger), then the eyewitness testimony presented in court will be more accurate than if the eyewitness is an older child. Results were various, but the hypothesis was overall refuted. Although increased accuracy can be predicted in older children, individual differences in the child and the event need to be considered.

RESEARCH AWARDS

APA Dissertation Research Award November 2014

Awarded by the American Psychological Association (APA)

Recipient of funding for dissertation research that reflects excellence in scientific psychology.

Basic Psychological Research Grant February 2013

Awarded by the American Psychological Association of Graduate Students (APAGS)

Recipient of a competitive scholarship award intended to support current dissertation research.

RESEARCH PRESENTATIONS

Breit, K. R. & Chester, J. A. (2014). The effects of adolescent chronic stress on alcohol-related reward in adulthood in mice selectively bred for high and low alcohol preference. Poster presented at the Research Society for Alcoholism Annual Conference in Bellevue, WA.

Chronic stress exposure during adolescence has long-lasting effects on physiology and behavior and is associated with an increased risk of developing an alcohol use disorder (AUD) later in life. Evidence suggests that chronic stress exposure during

adolescence has long-term effects on the developing brain reward systems, which could affect later-life sensitivity to the rewarding effects of alcohol. Male and female HAP2 and LAP2 mice were assigned to 3 groups. One group received chronic stress exposure (CSE) during the adolescent period, one group received CSE during adulthood, and one group served as a stress control group. Thirty days after CSE, all subjects were exposed to CPP conditioning procedures. In each subgroup, half of the subjects were re-exposed to stress (RS) before the CPP posttests. LAP2 mice showed significantly greater alcohol-induced CPP than HAP2 mice. The data also suggested that stress exposure during adolescence may increase CPP in females more than males and that RS may further increase CPP in females. We are currently conducting replications of this experiment. Supported by an APAGS Basic Psychological Science Grant

Breit, K., Sullivan, M., Ostberg, K., Issler, E., Thomas, J. (2012). The effects of choline on stress regulation in rats exposed to alcohol during development. Poster presented at the Student Research Symposium at San Diego State University, San Diego, CA.

Presentation Abstract: Prenatal alcohol exposure can damage the developing brain, adversely affecting cognitive and emotional development. In fact, prenatal alcohol exposure may cause abnormalities in the hypothalamic-pituitary-adrenal (HPA) axis, and recent evidence indicates that choline influences genes important in the regulation of stress. The present study examined if alcohol exposure during the 3rd trimester equivalent affects stress responses and if this is modulated by postnatal choline supplementation. Using a rat model, this study included six treatment groups in a 3 (ethanol-exposed, sham control, non-intubated control) x 2 (choline, vehicle) design. It was hypothesized that alcohol would increase stress, whereas choline would reduce anxiety-related behaviors, particularly in alcohol-exposed subjects. Results suggest that choline selectively targets cognitive systems in the brain, and that choline's mitigation of fetal alcohol effects is not mediated by effects on stress.

Brougham, R. R., John, R., Sparks, L., Cogan, C., **Breit, K.,** Dietch, J., Oestricher, J., Ing, M., & Rogers, K. (2010). Photo aging, future time perspective and social smoking. Poster presented at the annual conference for the Association for Psychological Sciences, Boston, MA.

Presentation Abstract: The current study examines whether an educational intervention in combination with photo aging reduces young adults' intention to socially smoke, decreases willingness to smoke under certain conditions (e.g., stress), and decreases smoking behavior. Data provide support for a relationship between gender, future time perspective and photo aging, finding that women with low time discounting who received a photo aging example and education about the effects of smoking showed a significant decrease in intentions to smoke six weeks after the original questionnaire.

Breit, K., Wanstreet, J., & Kuchenbecker, S. (2008). Creativity: Self-reported limiting vs. encouraging parental behaviors and college-age students' major, social, and physical risk-taking behaviors. Poster presented at the annual conference for the Western Psychological Association, Irvine, CA.

Presentation Abstract: How does limiting or supporting a child's emerging creativity affect development? Allen, *et. al,* (2005) support that parental time invested is very beneficial, but specific qualitative aspects have not been explored. Using retrospective recall, 105 students at a small Southern California liberal arts university answered questions regarding social, educational, and physical risk-taking behaviors either in a classroom or online. Students' recalled support for creative endeavors was associated with increased overall life-satisfaction as measured by the Gallup Life Satisfaction Scale (2007) and additional self-report questions.

RESEARCH PUBLICATIONS IN PROGRESS

Powers, M. S., **Breit, K.,** & Chester, J. A. (2015). Assessment of the role of cannabinoid type 2 receptors in alcohol-reward related behaviors. *(Submitted to Alcoholism: Clinical and Experimental Research)*

Breit, K. R. & Thomas, J. D. (2015). Choline's mitigation of developmental alcohol exposure alterations are not mediated by effects on stress. *(Submitting to Alcoholism: Clinical and Experimental Research)*

TEACHING EXPERIENCE

Instructor at Purdue University January 2015 – May 2015

Introduction to Behavioral Neuroscience (Psy 222): Spring 2015

In charge of preparing and designing the entirety of the course, teaching the course, and managing a graduate teaching assistant.

Recitation Leader at Purdue University August 2014 – December 2014

Elementary Psychology (Hybrid Course; Psy 120): Fall 2014

Course Coordinator: Dr. Jill Gulker

In charge of teaching weekly recitations to supplement the online lectures for the course, helping students with material through office hours and emails, mentoring struggling students, and monitoring exams.

Teaching Assistant at Purdue University August 2014 – December 2014

Drugs and Behavior (Psy 428): Fall 2014

Faculty: Dr. Susie Swithers

In charge of preparing the classroom, helping students with material through office hours and emails, mentoring struggling students, grading exams to analyze students' grasp of the material, and instructing the class for specific topics.

Teaching Assistant at SDSU August 2010 – May 2012

Physiological Psychology (Psy 260): Fall 2010, Fall 2011

Faculty: Dr. Jennifer Thomas

Behavioral Neuroscience (Psy 360): Spring 2011

Faculty: Dr. Katherine Turner

Sensation and Perception (Psy 388): Spring 2012

Faculty: Dr. Tom Scott

In charge of preparing the classroom, helping students with material through office hours and emails, mentoring struggling students, grading exams to analyze students' grasp of the material, and instructing the class for specific topics.

Student Mentor August 2011 – July 2012

Center for Behavioral Teratology, Thomas Lab

Educated new students and organized research tasks for undergraduate research assistants

PROFESSIONAL SERVICE

Student Representative for the Psychology Safety Committee Fall 2013 – Spring 2015

Provided student perspective for the Purdue Psychology Safety Committee

Master's Committee Student Representative Fall 2011 – May 2012

Provided student perspective for the Psychology Master's Committee panel at SDSU

Master's Program Application Reader Fall 2011 – April 2012

Served as a student perspective for 2012 SDSU Master's program applications

CURRENT PROFESSIONAL MEMBERSHIPS

American Psychological Association
Research Society on Alcoholism
International Neuroethics Society

OTHER WORK EXPERIENCE

Disneyland Resort January 2007 – July 2010
Pageant Helper (Entertainment Department)
Entertainment/Character host and Parade Performer (Pixar Play Parade opening cast)
Graduate of the Disney College Program
Business and professional development intern

COMMUNITY INVOLVEMENT

Kappa Alpha Theta April 2008 – May 2010
Founding member of the Eta Sigma chapter at Chapman University
Recruitment Chairman 2009-2010
Skit Chairman 2009-2010
Choreographer for Skit Night 2008-2010

Volunteer Experience
Petal Pushers April 2006 – Present
Volunteer for CASA (Court-Appointed Special Advocates) April 2008 – May 2010
Disney VoluntEARS January 2007 – July 2010

ACADEMIC SCORES

Graduate Record Examination (GRE) Scores (Old Version – 2009)
Quantitative: 680
Verbal: 500
Writing: 6

REFERENCES

Dr. Julia A. Chester (Ph.D. advisor at Purdue University)
Email: jcheste@purdue.edu

Dr. Jennifer D. Thomas (M.A. advisor at San Diego State University)
Email: thomas3@mail.sdsu.edu

Dr. Amy L. Brewster (Research Collaborator and Neuroethics instructor at Purdue University)
Email: abrewst@purdue.edu