

HPA Axis Activation by Ethanol Dependence in Adult and Adolescent Rats

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ABSTRACT

Alcoholism is a disorder marked by cycles of heavy drinking and chronic relapse, and adolescents are an age cohort particularly susceptible to consuming large amounts of alcohol, placing them at high risk for developing an alcohol use disorder. Adolescent humans and rats voluntarily consume more alcohol than their adult counterparts, suggesting that younger consumers of alcohol may be less sensitive to its aversive effects, which are regulated by the function of the hypothalamic-pituitary-adrenal (HPA) stress axis. While HPA axis dysfunction resulting from ethanol exposure has been extensively studied in adult animals, what happens in the adolescent brain remains largely unclear. In this study, chronic injections of ethanol was used to model alcohol dependence in adult and adolescent rats, and post-withdrawal anxiety behaviors were measured using light-dark box testing. Furthermore, corticosterone (CORT) release during treatment and after withdrawal was measured by collecting fecal and plasma samples from adults and adolescents. It was found that adults, but not adolescents, exhibit significant anxiety-like behavior following chronic ethanol withdrawal. Additionally, while the process of chronic ethanol treatment elicits an increase in day-by-day CORT release in both adults and adolescents, significantly sustained levels of CORT were not observed during withdrawal for either age group. Moreover, it was found that adults experience a longer-lasting CORT increase during chronic treatment, suggesting a larger and more robust period of dysfunction in the HPA axis for older consumers of alcohol. These results highlight CORT and glucocorticoids in general as a potential therapeutic target for treatment for alcoholism, especially that which has an onset during adolescence.

INTRODUCTION

Alcoholism is a disorder marked by cycles of heavy drinking and chronic relapse, and it is a prevalent condition in many populations of the world. In the United States alone, 7.0% of adults over the age of 18 and 2.8% of adolescents aged 12 to 17 reported having an alcohol use disorder in some capacity in 2013 (SAMHSA 2013). While addiction to alcohol is considered to be a complex problem determined by various psychological and physiological components, many theoretical models for alcohol abuse and dependence consider stress as a significant contributor to the initiation and continuation of alcohol use as well as relapse (Brady & Sonne, 1999). For example, negative mood and stress has been found to be positively correlated with increased levels of craving, and highly stressful life events independent of alcohol use history increase the risk of subsequent relapse (Cooney et al., 2007; Brown et al., 1990). As in human alcoholics, animals that are made alcohol-dependent using chronic exposure to high alcohol levels also exhibit uncontrolled excessive intake during withdrawal (Valdez et al., 2002; O'Dell et al., 2004; Sommer et al., 2008). Thus, developing pharmaceutical strategies that target stress-related relapse by way of using animal models has become a priority in research regarding treatments for alcoholism.

Alcohol, the HPA Stress Axis, and Anxiety

The hypothalamic-pituitary-adrenal (HPA) stress axis is a key regulatory network that links the brain with the body's physiological and behavioral responses to stress, including stress relevant to alcohol addiction. Function of the HPA axis is mediated by the activity of corticotropin-releasing factor (CRF) in the paraventricular nucleus (PVN) of the hypothalamus, which is one of three major structures of the HPA axis, along with the anterior lobe of the pituitary gland and the cortex of the adrenal gland (Sarnyai et al., 2001; Smith & Vale, 2006).

CRF synthesized and released into the bloodstream by the PVN binds to CRF₁ receptors on the anterior pituitary gland and induces the release of adrenocorticotrophic hormone (ACTH) into the systemic circulation. This release of ACTH subsequently stimulates the adrenal cortex to synthesize and release glucocorticoids – corticosterone in rodents, and cortisol in humans. These glucocorticoids are of particular importance in an organism's stress response not only because they exert effects on multiple organs and tissues, but also because they mediate proper HPA axis activity through a negative feedback mechanism by inhibiting further CRF and ACTH release (Adinoff et al., 1998). Thus, in a healthy individual, properly functioning negative feedback allows the stress response mediated by the HPA axis to eventually taper off.

Alcohol abuse and dependence have been correlated with alterations in the activity and function of the HPA axis. Clinical studies observing the effects of alcohol use in human alcoholics have demonstrated HPA axis hyporeactivity after 4 weeks of abstinence from alcohol, and this lower level of HPA axis activity not only increases the risk of early relapse, but is also more likely to be present in an individual with a family history of alcoholism (Kiefer et al., 2006; Adinoff et al., 2005). Similarly, in previous studies in rodents, it has been shown that experimenter-administrated alcohol stimulates the HPA axis, but this response is blunted upon chronic alcohol exposure, suggesting that exposure to a stress induces a selective tolerance over time (Allen et al., 2011; Richardson et al., 2008; Lee & Rivier, 1997). Furthermore, high circulating levels of glucocorticoids resulting from chronic alcohol exposure also lead to increased sensitization of extrahypothalamic stress systems like the CRF₁ receptors in the extended amygdala, which mediate behavioral stress responses during withdrawal (Koob 2010). However, the use of glucocorticoid receptor (GR) antagonists such as mifepristone has been shown to block escalated alcohol drinking during chronic alcohol exposure and withdrawal,

suggesting that HPA axis activity with respect to glucocorticoids is likely to be involved in the development of alcohol dependence (Vendruscolo et al., 2012).

Elevated anxiety is a characteristic symptom of alcohol withdrawal, and it is mediated by CRF activity, as evidenced by a reversal of withdrawal-related anxiety upon administration of a CRF antagonist into the central nucleus of the amygdala (Rassnick et al., 1993; Baldwin et al. 1991). Other extrahypothalamic CRF-containing structures like the bed nucleus of the stria terminalis exhibit increased CRF neurotransmission during ethanol withdrawal; however, upon subsequent ethanol intake, these increases in CRF activity are actually reduced (Olive et al., 2002). These observations of reduced CRF activity-mediated anxiety-related behaviors upon ethanol intake during withdrawal, along with increased excessive consumption during withdrawal, have led to a negative reinforcement model of alcohol dependence. Negative reinforcement entails the use of alcohol in order to self-medicate and alleviate the negative physiological and emotional symptoms induced by withdrawal, such as anxiety, dysphoria, irritability, and sleep disturbances (Edwards & Koob, 2010).

Adolescent Alcohol Consumption and Sensitivity

Adolescence is a particularly vulnerable developmental period with respect to substance use and substance use disorders, and this is observed cross-culturally and for the majority of addictive substances, including alcohol. A considerable amount of research has been done with respect to the effects of adolescent alcohol exposure on the propensity to drink alcohol in adulthood, much of which suggests that drinking during adolescence tends to contribute to increased alcohol intake as an adult. Clinical studies with human subjects have shown that alcohol consumption during adolescence has a tendency to lead to alcohol abuse and dependence during adulthood. For example, Hingson et al. (2006) found that relative to respondents who

began drinking at 21 years or older, those who began drinking before the age of 14 were more likely to experience alcohol dependence, especially within 10 years after having their first drink. Comparable results have also been observed using rat models of alcohol dependence; for example, Maldonado-Devincci and colleagues (2010) reported that voluntary alcohol intake by adult rats was found to be enhanced by repeated administration of binge drinking during adolescence. However, there is relatively little information known about the effects of early alcohol exposure on the adolescent brain as opposed to later effects in adulthood, and it is a research area worth pursuing especially when trying to understand motivations behind adolescent drinking.

Consumption of alcohol typically begins during adolescence, and human adolescents as well as those of other animal species demonstrate elevated levels of voluntary drinking compared to adults (Doremus et al., 2005). Increased consumption during adolescence may be explained by findings from studies in rats suggesting that younger consumers of alcohol are less sensitive than their adult counterparts to a number of undesirable effects of alcohol that are normally taken as cues for moderating and restricting intake, such as ethanol-induced social inhibition, sedation, and motor impairment (Varlinskaya & Spear, 2002; Draski et al., 2001; White et al., 2002). Another way by which adolescent rats have been shown to be less sensitive to intoxicating and impairing effects of ethanol is through the use of alcohol-related conditioned taste aversions. Adolescents need larger doses and number of pairings of a novel taste and ethanol to develop an aversion towards that taste (Anderson et al., 2008). Because adolescents are not experiencing aversive effects to the same extent as adults, this raises the question of how relevant negative reinforcement is as a model for alcohol dependence in adolescents, considering that there is a relative lack of need to alleviate negative symptoms by drinking compared to adults.

Furthermore, these experimental results are strongly suggestive of stress playing a differential role in adolescent versus adult consumption of alcohol, and it brings up the question of how extensively the HPA axis stress-response resulting from alcohol exposure is different in adolescents compared to adults.

Despite the fact that withdrawal-induced craving has been well-established as a motivator for chronic alcohol use in adults, withdrawal-induced drinking has not been investigated as extensively in adolescents. Preliminary studies in our lab indicate that both adolescents and adults chronically treated with ethanol experience spontaneous withdrawal behaviors (tail rigidity, vocalizations when handled, ventromedial distal limb flexion, abnormal posture/gait, and tremors) to a significantly greater extent compared to control animals after 18 hours of abstaining from ethanol. However, 4 days following chronic ethanol exposure, adults treated with ethanol demonstrated significantly higher levels of post-withdrawal anxiety compared to controls, whereas adolescent animals did not show this effect of treatment. This observation makes withdrawal-related anxiety an unlikely motivator for subsequent drinking in adolescents. One objective of this study is to assess whether or not post-withdrawal anxiety is present in adolescents at an earlier time point during withdrawal.

Attenuation of HPA axis activity at the level of glucocorticoid (specifically CORT) release in younger compared with older animals has been widely reported after exposure to a variety of drugs of abuse, such as cocaine and morphine. In humans, there is genetic evidence that a diminished cortisol response to alcohol is correlated with a higher risk for developing alcoholism (Schuckit et al., 1987). Additionally, it has been observed in rats that following a one-time acute ethanol challenge, the CORT response exhibited an increasing trend from adolescence through adulthood (Silveri & Spear, 2004). Furthermore, age-related differences in

CORT response to acute ethanol injections have also been shown to be sex-specific, with the most apparent difference being in females (Willey et al., 2012). While these results help elucidate the nature of CORT responses in adolescents following acute ethanol treatment, sparse research has been done with respect to age-related differences in CORT release following chronic ethanol exposure and withdrawal. This study seeks to investigate such differences, as they may help with understanding the neurological mechanisms underlying the pathology of alcoholism, especially those that differ across age groups or develop over time. Furthermore, this study aims to test the hypothesis that adult rats experience a more robust CORT response during chronic ethanol treatment and withdrawal compared to adolescent rats, reflecting a heightened HPA axis response to ethanol relative to adolescents.

Fecal Steroid Radioimmunoassay as a Method of Quantifying HPA Activity

When quantifying hormone levels in animal models, researchers have traditionally collected and utilized plasma samples as the main method for analysis. However, the process of collecting blood from rodents is a rather limited technique in that these animals have small blood volumes, restricting the frequency and volume at which blood samples can be collected during a given study. Additionally, daily blood collections themselves are stressful for animals and may result in inadvertent HPA axis dysfunction unrelated to ethanol dependence. Thus, one way by which this study seeks to characterize age-related differences in CORT release resulting from ethanol exposure on a day-to-day basis is through the use of daily fecal sample collection, rather than plasma collection, in order to assess significant changes in CORT levels. While fecal CORT assays have previously been used on laboratory rodents, the context for these assays has primarily been within the realm of circadian rhythms and cycling (Cavigelli et al., 2005). As

such, this study is among the first to validate fecal steroid radioimmunoassay as a viable method for assessing stress hormone levels during alcohol treatment.

METHODS

Animals

Male and female adult (PN 60-63) and adolescent (PN 28-30) Sprague-Dawley rats ordered from Charles River Laboratories in Raleigh, N.C. were used for the experiments described below. All animals were given a week to acclimate to laboratory housing facilities prior to any experimental procedures to minimize any stress response that may have been induced by factors unrelated to the experiment. Animals were maintained in a temperature- and humidity-controlled room on a 12/12-hour light/dark cycle during all experiments and given ad libitum access to water and standard rat chow from within the cages. Care of all animals was carried out in accordance with Duke University's Institutional Animal Care and Use Committee.

Rat Model for Alcohol Dependence

Alcohol dependence was modeled using a 4 day chronic treatment paradigm comprised of intraperitoneal (IP) injections administered three times per day at approximately 0900, 1200, and 1600 hours for a total of 12 doses. Experimental animals received 1.5 g/kg doses of ethanol (14.25% v/v 200-proof ethanol in deionized water mixture), and control animals received a vehicle injection of 0.9% saline at each of these time points. For the purposes of this study, animals were considered to be undergoing ethanol withdrawal at 18 hours following the final injection at 1600. The relatively moderate 1.5 g/kg ethanol dose amount was chosen in part based on previous reports in the literature assessing the effect of dose size on various alcohol-induced effects (Walker and Ehlers 2009; Lê and Israel 1994). Animal weights were recorded prior to the 0900 injection daily.

Light/Dark Box Testing

Light/dark box testing was used in order to quantitatively assess the extent to which ethanol withdrawal affects anxiety-related behaviors in adult and adolescent rats. This test was performed 18 hours following the final dose of ethanol treatment using Hamilton-Kinder activity monitors (dimensions: 40 x 40 x 40 cm) containing plastic “dark” inserts (20 x 40 x 40 cm) in one half of the apparatus. Incandescent lamps in the testing room were used to provide roughly 85-125 lx for the light half of the apparatus, and a small opening on the “dark” insert allowed free movement between the two halves. Corncob bedding was used to line the floors of both halves of the apparatus.

Each animal was initially placed in the dark section and subsequently allowed to move freely for 15 minutes, and Motor Monitor software was used to measure locomotion. Anxiety-related measures, such as time spent in the light compartment and latency to enter the light compartment, were assessed along with regular locomotor responses, such as total distance traveled. Furthermore, each 15 minute session was divided into 15 second intervals. Following light/dark box testing, animals were returned to their home cages.

Plasma CORT during Withdrawal

In order to assess the extent to which HPA axis activity at the level of CORT release differs between adult and adolescent rats following an 18-hour withdrawal period from chronic ethanol exposure, separate cohorts of animals were administered the 4-day ethanol treatment paradigm mentioned earlier. 18 hours following the final dose of ethanol, animals were sacrificed following anesthesia with isoflurane, and samples of trunk blood were collected. Circulating levels of CORT in plasma was assessed via radioimmunoassay (Corticosterone Double Antibody RIA kit, MP Biomedicals, Santa Ana, CA), and for the purposes of eliminating

blood alcohol concentration (BAC) as a potential confounding variable, BAC was measured using an Analox GL6 analyzer. The sacrifice was performed live in order to minimize the potentially stressful effects of anesthetization for the animals. Furthermore, because baseline CORT levels are strongly influenced by the stage of estrus cycle a female rat is in at a given point in time (Atkinson & Waddell, 1997), vaginal lavage was done on all of the adult female animals after sacrifice in order to help determine the stage of estrus that each female was in at the time point of their sacrifice.

Daily Fecal CORT Release during Ethanol Treatment

In order to characterize patterns of CORT release resulting from chronic ethanol treatment on a day-by-day basis in adult and adolescent rats, fecal pellets from separate cohorts of singly-housed animals were collected and stored in plastic vials at -80°C . These fecal samples were collected 3 days prior to the first day of ethanol treatment as a baseline level of measurement, every day of treatment at 1 hour following the last injection, and after 18 hours into ethanol withdrawal. Two months after the end of the experiment, these samples were thawed and crushed to a fine powder using a Magic Bullet blender, and 0.5 g of each sample was weighed out and suspended in 5 mL of 80% ethanol. After centrifugation at 2500 rpm for 20 minutes, the supernatant was removed and dried by means of air evaporation. Lastly, the remaining sample was reconstituted with 1 mL of a buffer solution, and the MPBio Corticosterone Double Antibody RIA kit was used to measure fecal CORT concentrations in ng/mL.

Effect of Previous Ethanol Exposure on CORT Release after an Acute Ethanol Challenge

In order to assess the way by which the influence of prior chronic ethanol exposure on CORT release differs between adolescents and adults, adolescent and adult rats were

administered chronic treatments of either ethanol or saline using the 4-day paradigm outlined earlier. 18 hours following the final injection of the 4-day treatment, an acute challenge of one 1.5 g/kg injection of ethanol was administered to both ethanol-dependent and ethanol-naïve animals. All animals were sacrificed one hour after receiving the 1.5 g/kg acute challenge of ethanol and trunk blood samples were collected. For the sacrifice, animals were decapitated without anesthesia in order to minimize the potentially stressful effects of anesthesia. Circulating levels of CORT in plasma was assessed via radioimmunoassay (Corticosterone Double Antibody RIA kit, MP Biomedicals, Santa Ana, CA).

Statistical Analysis

Age-related differences, sex-related differences, and ethanol treatment effects in plasma CORT levels were tested for using three-way ANOVA tests with NCSS 2007 software. Furthermore, two-way ANOVAs (sex x ethanol treatment, age x ethanol treatment, sex x age) were also used for interaction effects. Age-related differences, sex differences, and ethanol treatment effects were considered significant at $p < 0.05$. For the fecal CORT levels, a repeated measures ANOVA was used with day of treatment as the within-subjects factor, and age and treatment as between-subject factors. Finally, Grubbs' test for outliers was used to determine if any of the data points collected were outliers, and any points that were identified as such were excluded from statistical analysis. GraphPad Prism 6 was used for graphical representation and interpretation, and data are presented as group mean \pm standard error of the mean (SEM).

RESULTS

Blood Alcohol Concentration during Withdrawal

Blood alcohol concentration (BAC) of plasma samples collected at 18 hours into ethanol withdrawal was not significantly affected by sex, age, or treatment (**Figure 1, 2**), indicating that BAC is controlled for across all experimental groups.

Light/Dark Box Testing: Measures of Locomotion

Ethanol treatment had a significant effect on average basic locomotion for both adult and adolescent animals ($F(1,92) = 22.86, p < 0.001$, **Figure 3, 4**), and two-sample t-tests found that this significant effect of treatment occurred in male adults and adolescents, as well as female adolescents. However, ethanol treatment did not have a significant effect on the average distance travelled in the dark portion of the light/dark box for adults or adolescents (**Figure 5, 6**). It was also observed that of the 900 seconds that light/dark testing was done, both adult and adolescent animals spent more time in the dark compartment of the box (**Figure 7, 8**). There was also a significant effect of treatment on time spent in the dark for the adult females ($F(1,92) = 6.09, p < 0.02$).

While these measures are well-established indicators of locomotive behavior in rats, average distance travelled in the dark may be a more reliable measure of a rat's locomotion, considering that animals spent more time in the dark during overall testing. Females exhibited more locomotion ($F(1,92) = 17.66, p < 0.001$) and greater distance travelled in the dark ($F(1,92) = 7.48, p < 0.01$) compared to males in both groups, a well-known sex difference (Valle 1970).

Light/Dark Box Testing: Measures of Anxiety

There was a significant effect of treatment on the average latency to enter the light compartment of the light/dark box, a reliable anxiety-related behavior, for adult animals ($F(1,92)$

= 12.15, $p < 0.001$, **Figure 9**). Ethanol-treated male and female adults took significantly longer times to enter the light compartment relative to control adults. However, no such significant effect of treatment on latency was observed in adolescent animals (**Figure 10**). A two-way ANOVA test also demonstrated a significant interaction effect between age and treatment ($F(1,92) = 5.18$, $p < 0.05$). Ethanol treatment had a significant effect in female adults, with ethanol-dependent animals spending significantly less time in the light zone than control animals ($F(1,92) = 5.85$, $p < 0.02$, **Figure 11**). This effect of treatment was not seen in the average distance travelled in the light compartment for adult males or adolescents of either sex (**Figure 12**).

Plasma CORT during Withdrawal

Chronic ethanol treatment did not have a significant effect on the concentration of CORT in plasma of adult or adolescent animals (**Figure 13, 14**). However, there was a significant effect of sex ($F(1,95) = 9.82$, $p < 0.01$) within the animals that were treated with ethanol, with female adults and adolescents exhibiting higher levels of plasma CORT compared to male adults and adolescents.

Daily Fecal CORT Release during Ethanol Treatment

A repeated measures ANOVA (within-subjects factor: time; between-subjects factor: age) found that there was a statistically significant effect of time on the average concentration of fecal CORT for ethanol-treated adolescent and adult rats alike ($F(1,16) = 7.06$, $p < 0.001$, **Figure 15**). For adults, the maximum average fecal CORT concentration was observed at the end of the 6th day of the experiment, which was Day 3 of injections (mean = 178 ng/mL), and these elevated CORT levels were sustained for the remaining days of the experiment. On the other hand, the adolescents exhibited the maximum concentration of average fecal CORT on the 5th

day of the experiment, which was Day 2 of injections (mean = 136 ng/mL). Furthermore, not only did the average fecal CORT in adolescents peak earlier relative to that of adults, but this peak quickly dropped back down to near-baseline levels by the end of the experiment.

Effect of Previous Ethanol Exposure on CORT Release after an Acute Ethanol Challenge

An acute challenge of 1.5 g/kg of ethanol resulted in significantly lower CORT concentrations in the plasma of animals that had received a previous 4-day chronic treatment of ethanol compared to ethanol-naïve animals that had only been receiving vehicle injections of saline prior to the acute challenge. Both adults and adolescents demonstrated a significant effect of treatment ($F(1,52) = 41.88, p \approx 0.00$, **Figure 16, 17**). A two-sample t-test also revealed that there was a significant effect of sex within ethanol-naïve adolescents, with females having significantly lower circulating CORT in plasma compared to males ($p < 0.05$).

DISCUSSION

Age-related Differences in Post-Withdrawal Anxiety

A previous study done by our lab found that adolescent and adult rats chronically treated with ethanol displayed significantly robust spontaneous withdrawal symptoms (vocalizations upon handling, tail rigidity, and abnormal posture/gait) compared to control animals 18 hours following the final dose of treatment. However, when post-withdrawal anxiety was assessed after 4 days of abstinence from ethanol, only ethanol-treated adults had demonstrated significantly higher anxiety-like behaviors, such as increased latency to enter and spending less time in the light compartment of a light/dark box, while there was no significant effect of treatment in adolescents. Thus, in order to assess whether 4 days was too extensive of an abstinence period to detect withdrawal-related anxiety in ethanol-treated adolescents, light/dark testing was performed in this study at 18 hours after the final dose of treatment, the same time at which

spontaneous withdrawal had been assessed. Despite the shorter duration of the withdrawal period, a significant effect of treatment on anxiety-related behaviors was observed in adult animals only. The fact that adolescent animals treated with chronic ethanol exhibited significantly robust spontaneous withdrawal symptoms, but not anxiety-related behaviors, at the same point in time following the final dose of ethanol strongly suggests that these two categories of behaviors are mediated by neural mechanisms that are somewhat separated and distinct. These observations also imply that there may be age differences in how strongly various light/dark test measures are associated with anxiety-like behavior. For example, Arrant et al. (2013) found that total locomotive activity as well as distance travelled in the dark compartment statistically loaded onto the anxiety-related factor in adolescents. With this in mind, the results obtained in this study could potentially suggest that adolescents also experience withdrawal-related anxiety, since ethanol treated adolescents exhibited significantly less locomotive activity compared to controls.

When comparing these results with other studies that have examined post-withdrawal anxiety in ethanol-treated adolescent rats, some aspects of these findings are in agreement with the literature while other aspects are contradictory. For example, Doremus et al. (2003) reported that at 18 hours following an acute 4 g/kg ethanol challenge, adult but not adolescent animals demonstrated evidence of anxiety in a maze task during acute ethanol withdrawal. Despite the use of a different behavioral test and the implementation of an acute challenge rather than chronic treatment, these results are congruous with what was observed in this study: adolescents tend not to demonstrate anxiogenic responses to ethanol withdrawal, whereas adults do so to a significant extent. Similarly, Slawecki and Roth (2004) observed a lack of enhanced anxiety-related behaviors in adolescent animals that were chronically exposed to ethanol vapor over the course of 12-14 days and assessed after 5 or 12 days of withdrawal with an open field test.

However, in contrast with the findings from this study, there was also a lack of enhanced anxiety-related behaviors in adult animals as well. These findings from Slawecki and Roth (2004) could be reconciled by taking into consideration the fact that their study used a longer abstinence period before testing for anxiety, thus clearing out whatever mechanism was needed to mediate an enhanced anxiety response.

CRF activity strongly mediates anxiety and anxiety-related behaviors, so anxiety resulting from withdrawal may not be as prominent in younger animals because the HPA axis along with other extrahypothalamic CRF-containing structures may still be undergoing development and refinement in adolescents relative to adults. Wills et al. (2010) found that compared to adults, adolescents demonstrate increased CRF protein levels and higher CRF cell counts within the PVN and CeA at baseline levels. Furthermore, adolescent rats undergoing withdrawal from an ethanol diet required higher doses of CRF in order to elicit sensitization of withdrawal-induced anxiety compared to adults, suggesting that adolescent rats may be less sensitive to the anxiogenic effects of CRF. A separate study investigating the effect of voluntary alcohol exposure during adolescence on CRF cells found a reduced cell count in the CeA in adulthood, but not in the bed nucleus of the stria terminalis (Karanikas et al., 2013). Taking the results of these studies together, it is strongly suggestive that the adolescent brain is less responsive to CRF compared to the adult brain, and this could be what lessens adolescents' sensitivity to aversive effects of drinking, facilitating increased consumption of ethanol relative to adults. Future studies looking at developmental changes between adolescence and adulthood that lead to increased sensitivity to CRF could be insightful in pinpointing anxiety-related pharmacological targets for treating alcohol dependence.

One final implication of adolescents not exhibiting withdrawal-induced anxiety is that a negative reinforcement model of alcohol dependence may not be as applicable for adolescents as it is for adults. If adolescents do not experience anxiety during withdrawal, then the motivation behind consuming more alcohol is likely not for self-medicating against the anxiogenic effects of alcohol withdrawal.

Effect of Chronic Ethanol Withdrawal on CORT Release

Based on the finding that ethanol-dependent adults but not adolescents demonstrated withdrawal-related anxiety 18 hours following the last dose of treatment, it was expected that circulating CORT in the plasma of ethanol-dependent adults would be significantly higher relative to adolescents at the same point in time. Unexpectedly, however, ethanol treatment did not have a significant effect on circulating CORT regardless of age, suggesting a lack of correlation between CORT release and anxiety-related behaviors during ethanol withdrawal.

Studies in the literature investigating the relationship between alcohol withdrawal and HPA axis function have shown that abstinence from alcohol consumption leads to elevated glucocorticoid levels that reflect increased HPA axis activity during withdrawal (Heilig et al., 2010; Rose et al., 2010). At first impression, this trend seems to be in direct opposition to what was observed in this study – that withdrawal from ethanol treatment does not significantly alter circulating CORT – but Borlikova et al. (2006) reported findings that could explain this observed lack of effect of treatment. Rats that were chronically treated with an ethanol diet for 24 days continuously showed elevated CORT levels during withdrawal, but a separate group of ethanol diet rats that were given interspersed withdrawal periods during the 24 days of ethanol exposure did not show elevated CORT levels during withdrawal. Even though the animals in this study were not treated with ethanol over such a length of time, they did undergo interspersed periods of

abstinence between each day of treatment, and consequentially, these repeated instances of withdrawal could have actually blunted HPA axis function, leading to circulating CORT levels that were not significantly higher than that of control animals. This interpretation supports the well-established phenomenon that cycles of chronic intermittent alcohol exposure leads to HPA axis hypoactivity over time (Allen et al., 2011; Lopez et al., 2010; Richardson et al., 2008; Lee & Rivier, 1997). Moreover, the fact that this effect was observed in both adolescents and adults supports the idea of there being similarities in patterns of glucocorticoid release following alcohol withdrawal across age groups.

The results obtained from this study are also somewhat contradictory to previously established links between CORT and anxiety in the literature. For example, Shepard et al. (2000) reported enhanced CRF mRNA levels in the CeA as well as enhanced anxiety-like behavior during an elevated plus-maze task in adult rats that were given crystalline CORT implants, suggesting that elevated glucocorticoids may increase anxiety by inducing CRF expression in the CeA. However, it could also be possible that chronic ethanol exposure leads to alteration and disruption of any influence that CORT has on CRF-mediated anxiety behaviors, hence why it was observed in this study that CORT was not significantly affected by treatment in adults, despite the fact that they exhibited enhanced anxiety-related behaviors.

It is also important to note that both female adults and adolescents treated with chronic ethanol exhibited significantly higher levels of circulating CORT compared to their male counterparts. This observation is congruent with previous findings of adult females exhibiting higher plasma CORT levels than adult males following withdrawal from ethanol (Willey et al., 2012; Silveri & Spear, 2004), suggesting that older females respond to drug stimuli with higher CORT secretion than males. However, other works have shown that females demonstrate

consistently larger CORT responses compared to males only in adulthood (Ogilvie & Rivier, 1996). This leads to the possibility that perhaps the younger female rats used in this study may have experienced their first estrus by the time of sacrifice and plasma collection, since a significant sex difference was also observed in circulating CORT of adolescent animals. Thus, the design of this study could have been improved if vaginal lavage was performed on female adolescents along with adults.

Age-Related Differences in Daily CORT Release during Chronic Ethanol Treatment

Because ethanol treatment did not have an effect on plasma CORT in adult or adolescent animals undergoing withdrawal, it was then hypothesized that there were differences in CORT release over the course of chronic ethanol treatment itself, in order to explain why adults but not adolescents exhibited withdrawal-related anxiety behaviors. There was indeed an observable difference in the onset of maximum fecal CORT during the course of ethanol treatment. For adolescents, maximum CORT release was reached at Day 2 of injections, while for adults, maximum CORT release was reached at Day 3. Furthermore, while adults showed sustained elevated levels of CORT throughout the remaining days of ethanol treatment relative to baseline measurements, adolescents demonstrated a subsequent decrease back down to near-baseline levels of CORT by the end of the 4 day treatment paradigm. These results suggest that adults experience a more robust and longer-lasting increase in CORT release, and thus, increased HPA axis activation, during chronic exposure to ethanol compared to adolescent animals. Furthermore, these results suggest that adolescents may have some mechanism that allows for more efficient or quicker turn-around of HPA axis activity in response to a chronic stressor compared to adults. Conversely, ongoing development and enhanced plasticity that is

characteristic of the adolescent brain may also explain how fecal CORT in adolescents rose and fell so quickly relative to adults.

The possibility that adolescents have a more flexible and adaptable HPA axis-mediated stress response to chronic ethanol exposure contradicts previously reported findings of adolescent rats demonstrating a delayed rise and a more prolonged CORT release to stressors than adult rats (McCormick & Mathews, 2007). Additionally, other studies have found that prolonged release of CORT in adolescents is due to the incomplete maturation of negative feedback systems, as opposed to reduced sensitivity to ACTH in the adrenal cortex (Gomez et al., 2002). However, the stressors that elicited these contradictory findings were for the most part physical stressors, including receiving foot shocks and being restrained, and not chronic exposure to ethanol. A unique property of ethanol as an HPA axis stressor could be the reason why adolescents demonstrated a more temporary CORT elevation, rather than a more prolonged elevation, relative to adults in this study.

It is important to note that results from this fecal sample study should be taken with some degree of caution. The sample size that was used for this experiment consisted of 8 adolescent animals and 8 adult animals, which is relatively small, limiting the generalizability of experimental results. Additionally, all of the animals used in this experiment received chronic ethanol treatment, meaning that there were no positive controls (i.e. animals receiving a vehicle injection) represented in the experimental design. Thus, the observed elevation in CORT may have resulted partially due to the stress associated with receiving multiple injections rather than ethanol itself. One final drawback of this experiment was the necessity for the animals to be housed individually so that fecal samples may be collected accurately and precisely. Prior studies looking at effects of drugs other than alcohol on rats have shown that the act of single-housing

animals may lead to the enhancement of impaired HPA axis function as well as increased sensitivity to the drug after withdrawal (Turner et al., 2014). Consequentially, single-housing the animals may have led to inadvertent enhancement of CORT release in this study. However, this study is among the first to use fecal steroid assays to quantify CORT release during chronic ethanol exposure, and because there was a significant effect of time observed, this method holds promise for use in future related studies.

Effect of Previous Ethanol Exposure on CORT Release after an Acute Ethanol Challenge

From earlier parts of this study, it was observed that there was an increase in CORT release over the duration of chronic ethanol treatment for both adults and adolescents, but these elevated CORT levels were not sustained in either age group following 18 hours of withdrawal. Thus, it was then hypothesized that animals treated with chronic ethanol would have significantly less CORT released in plasma upon receiving an acute ethanol challenge compared to those that were previously given chronic saline injections (ethanol-naïve). It was found that adolescent and adult animals that had been chronically exposed to ethanol showed significantly lower plasma CORT levels at 60 minutes following the acute ethanol challenge compared to animals that were previously ethanol-naïve, supporting the proposed hypothesis. These results suggest that exposure to a chronic stressor leads to an HPA axis response to a future acute stressor that is blunted in both adults and adolescents. The lack of age-related differences in the way by which chronic stress affects HPA axis activity in response to an acute stressor is indicative of a shared mechanism between adolescents and adults that allow for the attenuation of HPA axis function after being subjected to repeated instances of stressors.

There are prior studies that have observed congruent results of adult animals demonstrating a blunted HPA axis response to an acute stress challenge after having been

exposed chronically to ethanol, by means of dampened CORT and ACTH release (Boyd et al., 2010; Richardson et al., 2008; Weiss et al., 2001). On the other hand, studies investigating effects of adolescent chronic exposure on the stress response during a subsequent acute challenge in adolescent animals are very limited in the literature. Experimental designs of these studies using animals in adolescence typically involve chronically exposing animals to ethanol during prenatal development or adolescence and not examining any effects until the animal has reached adolescence or adulthood, respectively. However, such studies have also demonstrated that prior exposure to chronic ethanol does lead to a blunted HPA axis-mediated stress response to an acute challenge at a chronologically later time, by way of decreased plasma ACTH, plasma CORT, and mRNA expression of various HPA axis receptors (Logrip et al., 2013; Van Waes et al., 2006). Thus, the results obtained in this study fit the overall narrative of initial chronic exposure to ethanol contributing to a dampened HPA axis-mediated stress response when receiving subsequent acute challenges of ethanol. An implication that can be drawn from these findings is how important it is to target and combat alcohol dependence as early in development as possible so as to minimize the impact it has on the ability of the HPA axis to respond to later stress and to reduce the risk of developing an alcohol use disorder.

One surprising outcome of this study was the significant difference in plasma CORT between ethanol-naïve adolescent males and females, with females displaying significantly lower levels of CORT compared to males. Because neither of these experimental groups had had prior exposure to ethanol and because sex-related differences in hormone release typically begins post-adolescence, it had originally been predicted that both sexes of adolescent control animals would demonstrate roughly equal levels of circulating CORT. This observation may have been a result of human error during the CORT radioimmunoassay protocol, or it could be an indicator that

extraneously older adolescents were used in this study, as evidenced by the observed sex-related difference in HPA axis function.

Future Directions

In summary, this study uniquely characterized age-related differences in HPA axis activity resulting from alcohol withdrawal in adult and adolescent rats from the vantage point of CORT release. It was found that adults, but not adolescents, exhibit significant anxiety-like behavior following chronic ethanol withdrawal. Additionally, while the process of chronic ethanol treatment elicits an increase in day-by-day CORT release in both adults and adolescents, significantly sustained levels of CORT were not observed during withdrawal for either age group. Moreover, this study found that adults experience a longer-lasting CORT increase during chronic treatment, suggesting a larger and more robust period of dysfunction in the HPA axis for older consumers of alcohol. These results highlight CORT and glucocorticoids in general as a potential therapeutic target for treatment for alcoholism, especially that which has an onset during adolescence.

Further investigation of possible mechanisms that regulate age-related differences in withdrawal-induced anxiety from chronic alcohol would be a worthwhile trajectory of research. In order to elucidate a more complete picture of HPA axis function, the same experiments done in this study could be replicated, but CRF or ACTH release during withdrawal would be measured instead. Specifically within the context of CRF activity, *in vivo* experimentation could be done through the use of microdialysis to determine CRF efflux in the CeA, a measure of the level of activity of CRF neurons projecting from the PVN of the hypothalamus to the CeA. Recent research findings have also implicated the activity of the neuroimmune system as a significant influence on anxiogenic-related behaviors, so further pursuit of age-related

differences in the expression of various cytokines and chemokines resulting from alcohol dependence and withdrawal could also draw insightful conclusions.

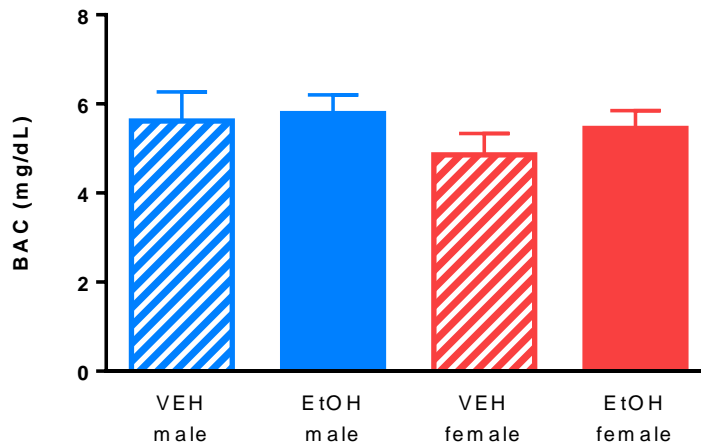
Average BAC at 18 Hrs Withdrawal: Adults

Figure 1. Average blood alcohol concentration (mg/dL) in blood of adult rats following 18 hours of withdrawal (mean + SEM). There was no significant effect of treatment or sex. N = 9, 15, 10, 17, respectively.

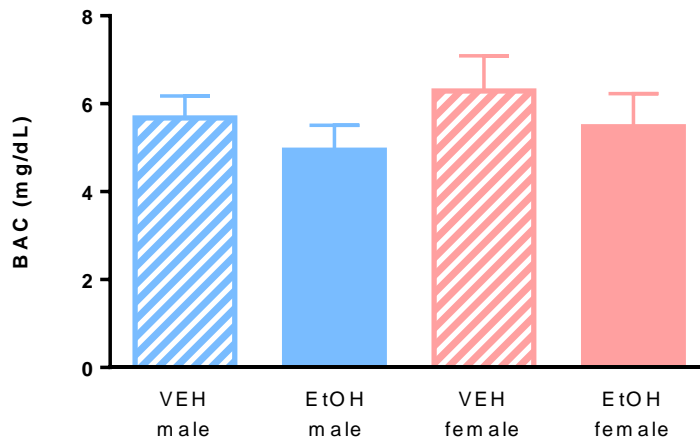
Average BAC at 18 Hrs Withdrawal: Adolescents

Figure 2. Average blood alcohol concentration (mg/dL) in blood of adolescent rats following 18 hours of withdrawal (mean + SEM). There was no significant effect of treatment or sex. N = 9, 14, 8, 13, respectively.

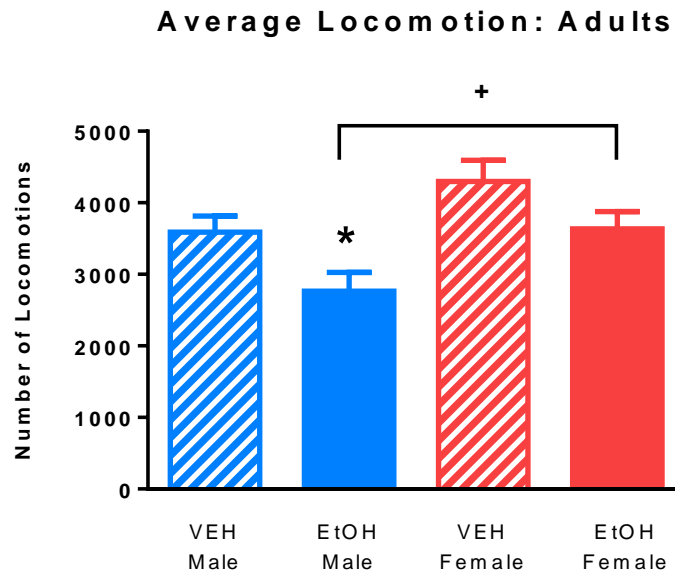


Figure 3. Average number of locomotions during light/dark box testing in adults (mean + SEM). There was a significant effect of sex and treatment. Females performed significantly more locomotions compared to males. Ethanol-treated males performed significantly fewer locomotions compared to control male animals. N = 14, 18, 13, 16, respectively (* = $p < 0.05$; + = sex difference, $p < 0.001$).

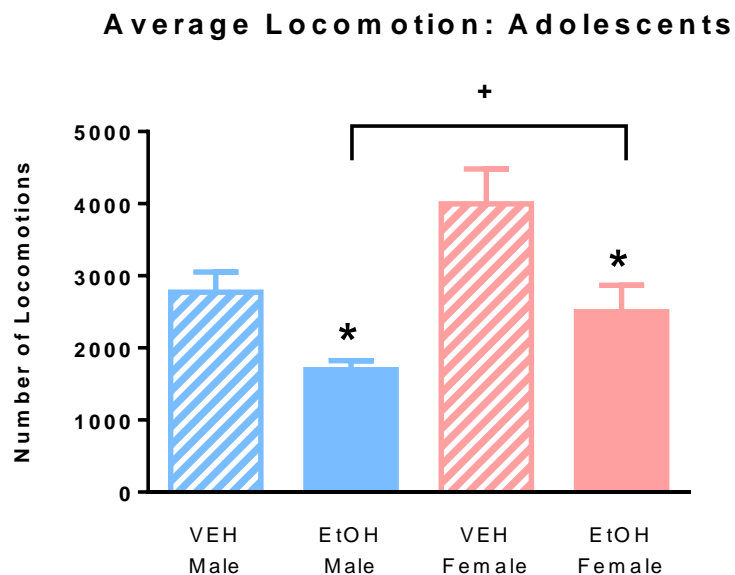


Figure 4. Average number of locomotions during light/dark box testing in adolescents (mean + SEM). There was a significant effect of sex and treatment. Females performed significantly more locomotions compared to males. Ethanol-treated animals performed significantly fewer locomotions compared to control animals. N = 8, 10, 6, 7, respectively (* = $p < 0.001$; + = sex difference, $p < 0.001$).

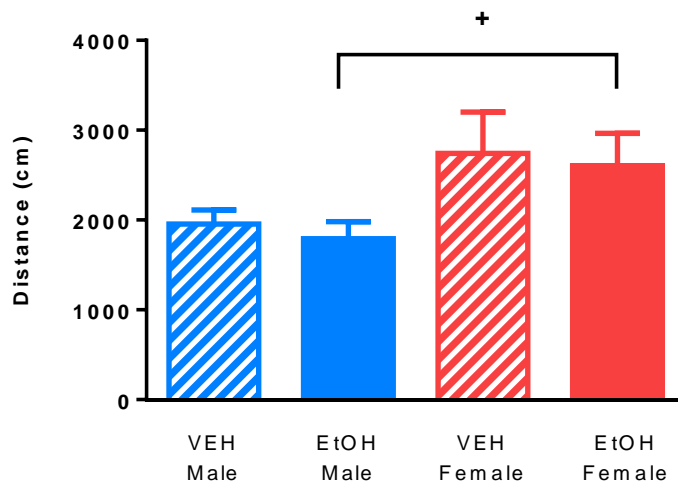
Average Distance Travelled in Dark: Adults

Figure 5. Average distance travelled (cm) in the dark compartment of the light/dark box by adults (mean + SEM). There was no effect of treatment. However, females travelled significantly greater distances compared to males. N = 14, 18, 13, 16, respectively (+ = sex difference, $p < 0.01$).

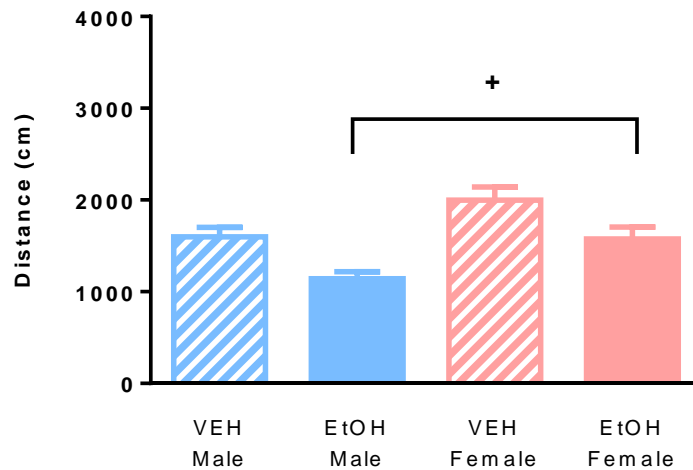
Average Distance Travelled in Dark: Adolescents

Figure 6. Average distance travelled (cm) in the dark compartment of the light/dark box by adolescents (mean + SEM). There was no effect of treatment. As was the case for adults, females travelled significantly greater distances compared to males. N = 8, 10, 6, 7, respectively (+ = sex difference, $p < 0.01$).

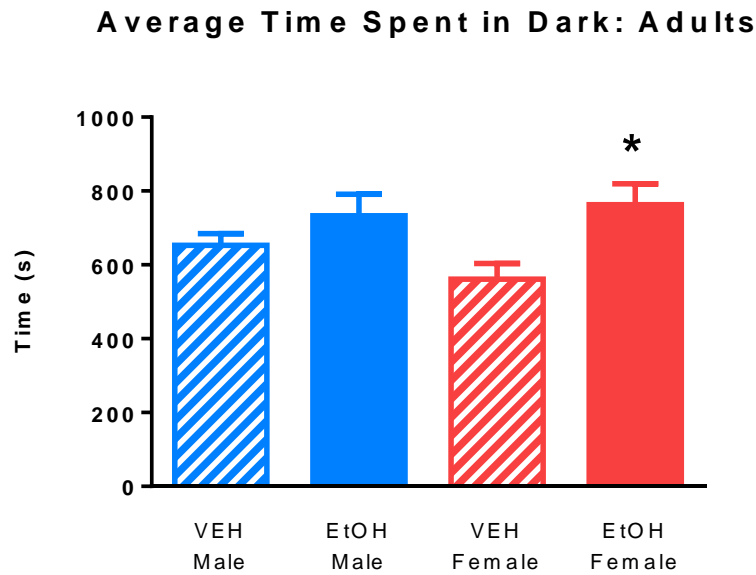


Figure 7. Average time (s) spent in the dark compartment of the light/dark box in adults (mean + SEM). There was a significant effect of treatment in females, with ethanol-dependent animals spending significantly more time in the dark zone. N = 14, 18, 13, 16, respectively (* = $p < 0.02$).

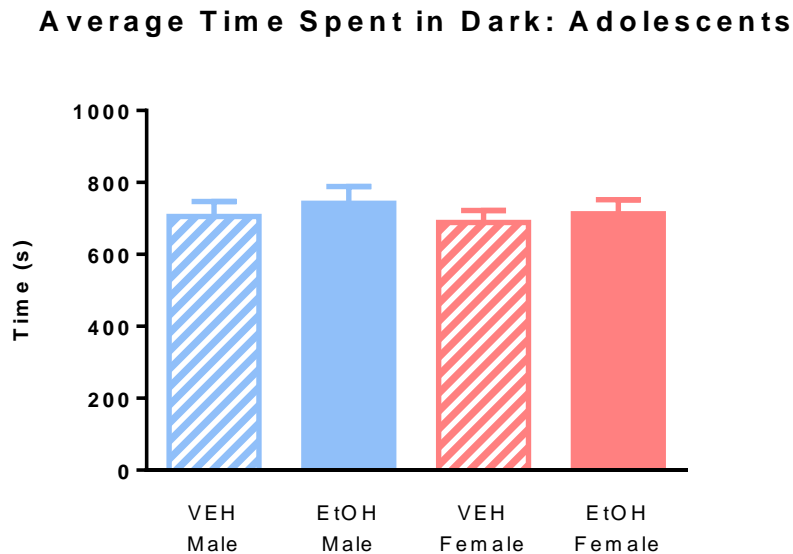


Figure 8. Average time (s) spent in the dark compartment of the light/dark box in adolescents (mean + SEM). Unlike the case for adults, treatment did not have a significant effect on the length of time spent in the dark zone. N = 8, 10, 6, 7, respectively.

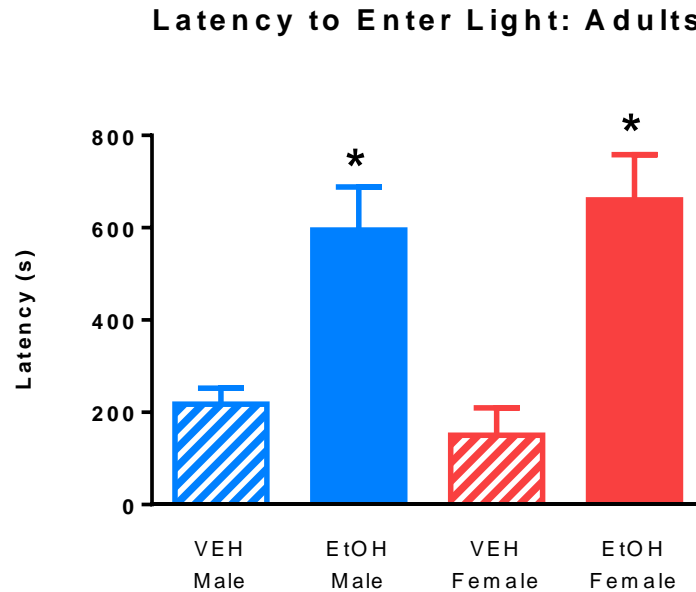


Figure 9. Average latency (s) to enter the light compartment during light/dark box in adults (mean + SEM). There was a significant effect of treatment, with ethanol-dependent animals waiting for longer lengths of time before entering the light zone. $N = 14, 18, 13, 16$, respectively (* = $p < 0.001$).

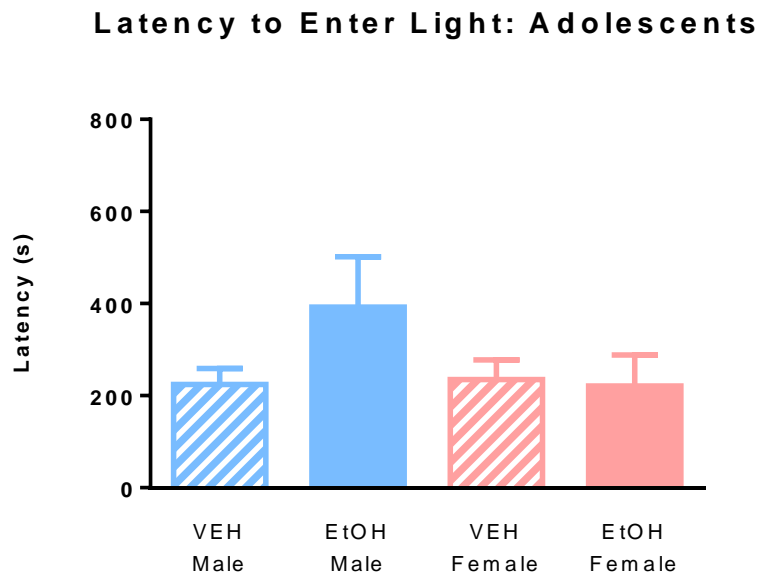


Figure 10. Average latency (s) to enter the light compartment during light/dark box in adolescents (mean + SEM). Unlike the case for adults, treatment did not have a significant effect on the length of time it took for adolescents to enter the light zone. $N = 8, 10, 6, 7$, respectively.

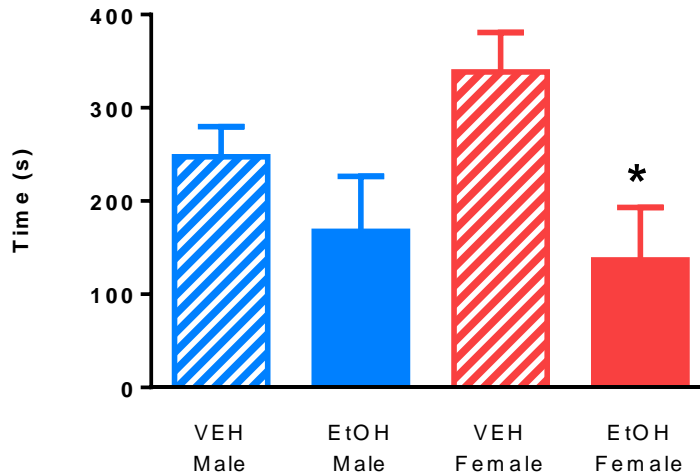
Average Time Spent in Light: Adults

Figure 11. Average time (s) spent in the light compartment of the light/dark box in adults (mean + SEM). There was a significant effect of treatment in females, with ethanol-dependent animals spending significantly less time in the light zone. N = 14, 18, 13, 16, respectively (* = $p < 0.02$).

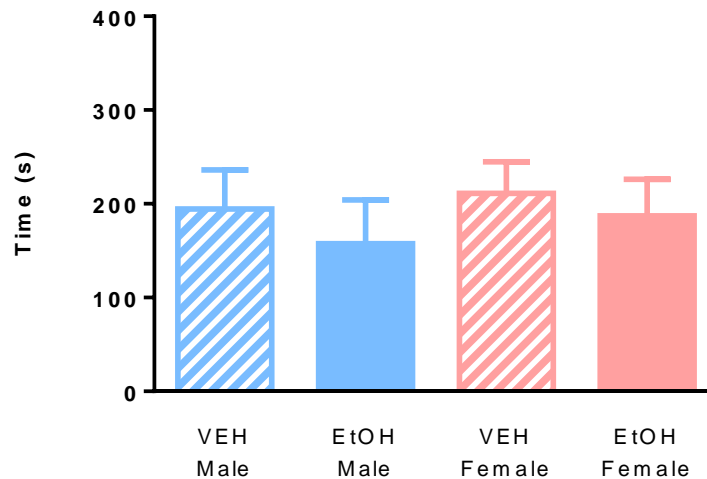
Average Time Spent in Light: Adolescents

Figure 12. Average time (s) spent in the light compartment of the light/dark box in adolescents (mean + SEM). Unlike adults, treatment did not have a significant effect on the length of time animals spent in the light compartment. N = 8, 10, 6, 7, respectively.

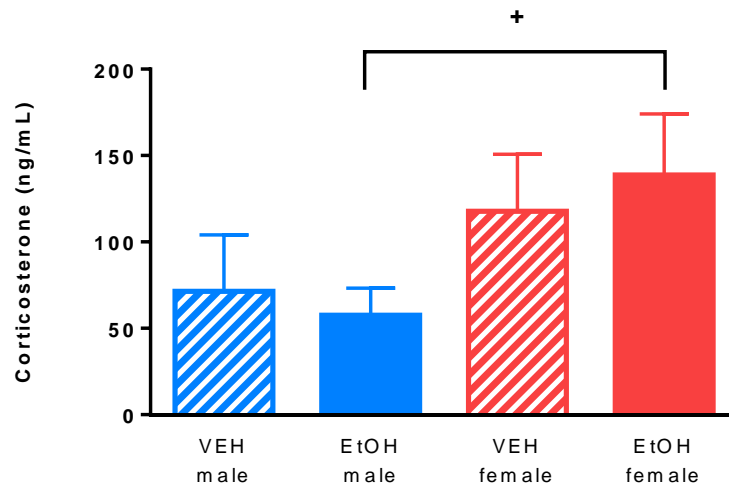
Circulating Corticosterone at 18 Hrs Withdrawal: Adults

Figure 13. Circulating corticosterone (ng/mL) in plasma of adult rats following 18 hours of withdrawal (mean + SEM). Treatment did not have a significant effect, but there was a significant effect of sex. Ethanol-dependent females exhibited significantly higher levels of corticosterone compared to males. N = 9, 15, 10, 17, respectively (+ = sex difference, $p < 0.01$).

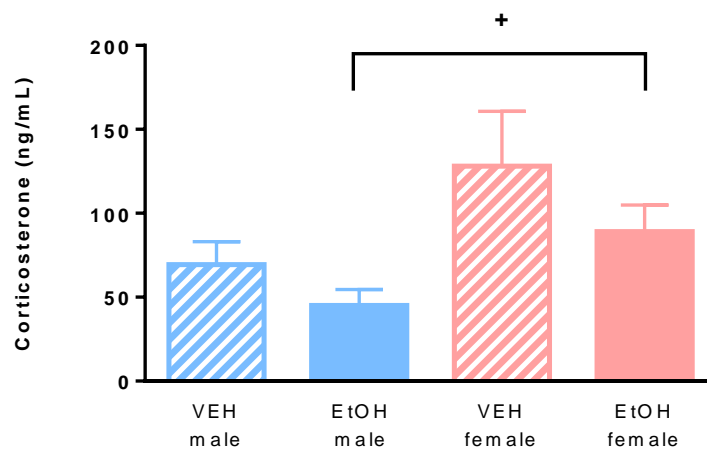
Circulating Corticosterone at 18 Hrs Withdrawal: Adolescents

Figure 14. Circulating corticosterone (ng/mL) in plasma of adolescent rats following 18 hours of withdrawal (mean + SEM). Treatment did not have a significant effect, but there was a significant effect of sex. Ethanol-dependent females exhibited significantly higher levels of corticosterone compared to males. N = 9, 14, 8, 13, respectively (+ = sex difference, $p < 0.01$).

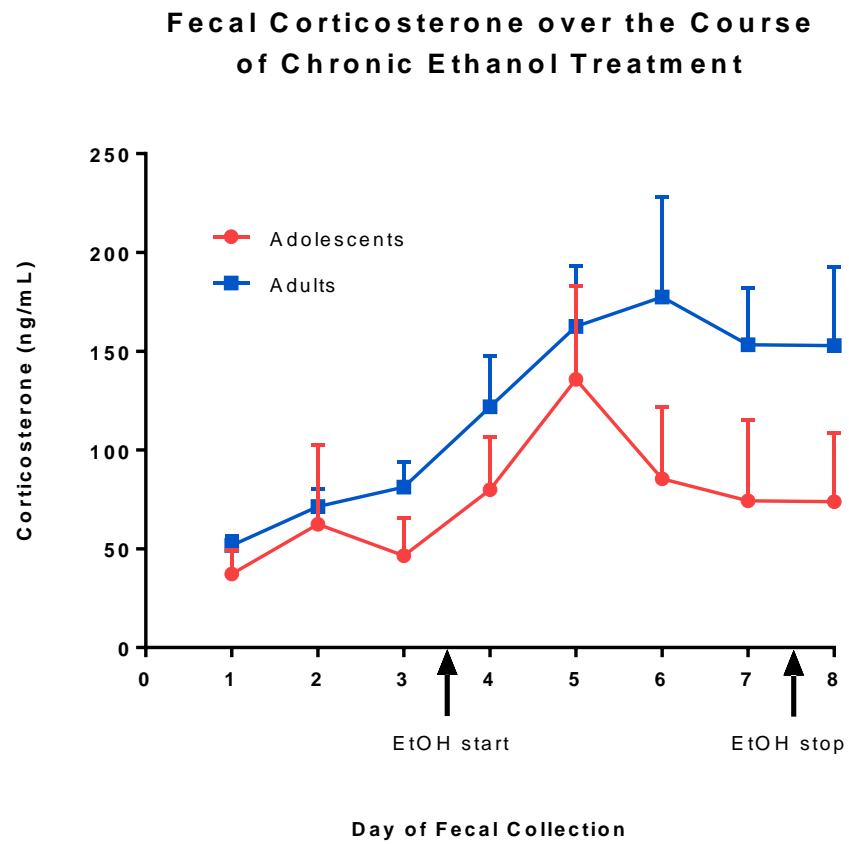


Figure 15. Average corticosterone levels in fecal samples of adult and adolescent rats over the course of a 4-day ethanol treatment (mean + SEM). There was a statistically significant effect of time on fecal corticosterone levels. $N = 8$ adults, 8 adolescents (repeated measures ANOVA: $p < 0.001$).

Circulating CORT 1 Hr After Acute EtOH Challenge: Adults

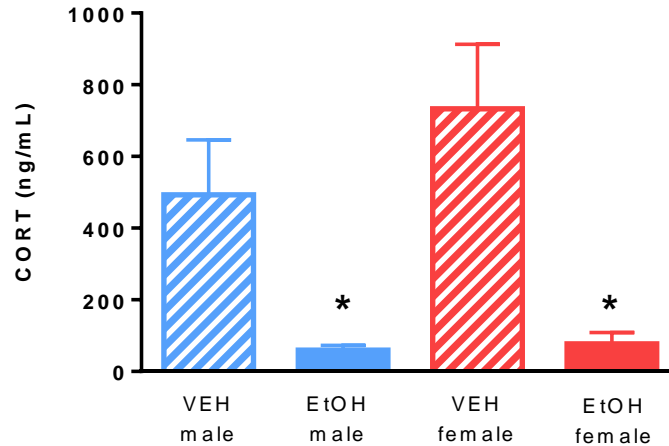


Figure 16. Circulating corticosterone (ng/mL) in plasma of ethanol-dependent and ethanol-naïve adult rats at 1 hour following an acute 1.5 g/kg ethanol challenge (mean + SEM). Previous exposure to ethanol had a significant effect; ethanol-dependent animals exhibited significantly higher levels of corticosterone compared to ethanol-naïve animals. N = 6, 6, 5, 6, respectively (* = $p \approx 0.00$).

Circulating CORT 1 Hr After Acute EtOH Challenge: Adolescents

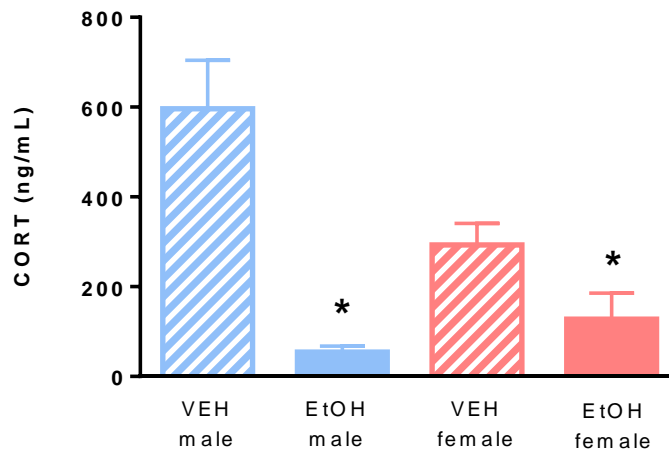


Figure 17. Circulating corticosterone (ng/mL) in plasma of ethanol-dependent and ethanol-naïve adolescent rats at 1 hour following an acute 1.5 g/kg ethanol challenge (mean + SEM). Previous exposure to ethanol had a significant effect; ethanol-dependent animals exhibited significantly higher levels of corticosterone compared to ethanol-naïve animals. N = 8, 8, 8, 5, respectively (* = $p \approx 0.00$). Interestingly, there was also a significant effect of sex between vehicle male and female adolescent animals ($p < 0.05$).

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