Binge-pattern alcohol exposure during puberty induces sexually dimorphic changes in genes regulating the HPA axis.

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Running Title: Effects of binge alcohol exposure during puberty

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Key Words: hypothalamus, puberty, AVP, CRH, Corticosterone,
ABSTRACT

Maternal alcohol consumption during critical periods of fetal brain development leads to devastating long-term consequences on adult reproductive physiology, cognitive function, and social behaviors. However, very little is known about the long-term consequences of alcohol consumption during puberty, which is perhaps an equally dynamic and critical period of brain development. Alcohol abuse during adulthood has been linked with an increase in clinically diagnosed anxiety disorders, yet the etiology and neurochemical mechanisms of alcohol-induced anxiety behavior is unknown. In this study, we determined the effects of binge ethanol exposure during puberty on two critical central regulators of stress and anxiety behavior: corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP). Our results showed that ethanol increased plasma CORT levels in both sexes, yet binge-treated animals had significantly lower CORT levels than animals exposed to a single dose suggesting that the hypothalamo-pituitary-adrenal (HPA) axis habituated to the repeated stressful stimuli of ethanol. Binge ethanol exposure also significantly increased CRH and AVP gene expression in the PVN of males, but not females. Overall, our results demonstrate that binge ethanol exposure during puberty changes the central expression of stress related genes in a sex-specific manner, potentially leading to permanent dysregulation of the HPA axis and long-term behavioral consequences.
INTRODUCTION

Alcohol abuse during adolescence is a growing fundamental health concern in the United States. According to the U.S. Department of Health and Human Services, boys on average have had their first drink before age 11, and girls before age 13; with the overall statistics showing that 41% of teenagers have had their first drink by age 14. Underage drinkers typically adopt a “binge” pattern of alcohol consumption, defined by the National Institute on Alcohol Abuse and Alcoholism (NIAAA) as heavy, episodic drinking in which enough alcohol is consumed in one sitting to bring the blood alcohol concentration above 0.08 g % (55). During adolescence, significant neural remodeling occurs as evidenced by changes in cortical gray matter (22, 29, 37), neurogenesis (40), and increased synaptic connectivity (14, 49, 57), raising the possibility that alcohol consumption during this critical period can lead to long-term neurobiological and behavioral defects.

One neurological system that undergoes extensive plasticity during pubertal development is the hypothalmo-pituitary-adrenal (HPA) axis (46). Under normal physiological conditions, an acute psychological or physical stressor activates the HPA axis. Hypothalamic corticotrophin-releasing hormone (CRH) stimulates adrenocorticotrophic hormone (ACTH) release from the anterior pituitary which in turn, causes the release of adrenal glucocorticoids. This sequence of events sets up a negative feedback system whereby increased circulating glucocorticoid levels serve to inhibit the additional release of hypothalamic CRH. Importantly, alcohol consumption can alter the expression of genes involved in mediating the stress response of the HPA axis. For instance, Rivier and Lee (1996) demonstrated that acute ethanol (EtOH) administration to adult rats increased the transcriptional activity of hypothalamic CRH and arginine vasopressin (AVP) neurons, as measured by increased CRH and AVP heteronuclear (hn) RNA following a single
dose of EtOH (44). In addition, the release of ACTH from the pituitary gland in response to EtOH was blocked when CRH and AVP antagonists were used concomitantly (44). These results show that acute EtOH exposure activates the HPA axis and the effects of EtOH are likely mediated by both CRH and AVP.

Arginine vasopressin is a multifunctional neuropeptide critical for mediating both stress and anxiety responses (17, 56). First discovered as antidiuretic hormone (ADH), AVP is synthesized in the PVN, supraoptic nucleus (SON), bed nucleus of stria terminalis (BST), amygdala (AMY) and suprachiasmatic nucleus (SCN). Neurons that synthesize AVP in the parvocellular division of the PVN (pPVN) project to the median eminence and facilitate the synergistic actions of AVP and CRH following stressful stimuli by enhancing ACTH and glucocorticoid release (15). Several lines of evidence suggest that acute or chronic alcohol exposure might lead to increased displays of anxiety behavior. Correlative studies have demonstrated that over 50% of patients with alcohol dependency also have anxiety-related or depression-related psychiatric disorders (13), and these types of disorders are often associated with an abnormal stress response. Interestingly, women alcoholics have a higher incidence of clinically diagnosed anxiety disorders compared to men alcoholics (19, 59), possibly due to the inherent sex differences in the stress responses of the HPA axis (3, 25). Further, repeated episodes of acute ethanol (EtOH) exposure increased anxiety behaviors in peripubertal animals, as measured by increased latency to explore a novel object in the retention based passive avoidance task (38). Direct effects of EtOH on AVP gene expression in the hypothalamus is one possible mechanism by which EtOH could alter anxiety behaviors and in fact, acute EtOH administration has been shown to increase the amount of AVP hnRNA in adult rats (44). Taken
together, these studies show that AVP is an important modulator of normal HPA axis function which can be disrupted by alcohol exposure.

Our current understanding of how EtOH exposure, especially a binge-pattern of EtOH exposure, affects the brain during pubertal development is very limited. In this study, we tested the hypothesis that a binge-pattern of EtOH exposure during pubertal development stimulates the stress response by increasing circulating levels of corticosterone (CORT) and upregulating CRH and AVP mRNA levels in the PVN of the hypothalamus in male and female rats. Our results showed a striking sex difference in the response to binge EtOH exposure for most parameters measured. Overall, the results from our study confirm that a binge-pattern of EtOH exposure during puberty significantly alters the expression of genes important for regulating the HPA axis and might lead to a higher risk of developing anxiety disorders in adulthood.

MATERIALS AND METHODS

Animals

Male and female Wistar rats were purchased from Charles River Laboratories (Wilmington, MA) at weaning (postnatal day (PND) 23) and allowed to acclimate to the new environment for 7 days after arrival. This study used the well-established model for peri-adolescence in rodents (28). All treatments began on PND 37 and continued until PND 44, which is defined as peri-pubertal (28). Animals were housed on a 12:12 light/dark cycle with lights on at 07.00h. Food and water were available ad libitum and all procedures were approved by the Loyola University Medical Center Institutional Animal Care and Use Committee (IACUC).

Binge Exposure Paradigm and Treatment Design.
After one week of daily handling, the animals were randomly assigned to either 1) untreated control (N = 16), 2) saline-treated control (N = 16), 3) acute EtOH (N = 16, 3g/kg/day), or 4) binge EtOH (N = 16, 3g/kg/day) groups. The animals in the binge-pattern EtOH exposure received an intraperitoneal (IP) EtOH injection every morning for 3 consecutive days, followed by 2 days of saline injections, and then injected for additional 3 days with EtOH. This binge-pattern exposure paradigm has been used previously to mimic the pattern of binge alcohol consumption in adolescents (27) and it has been shown that this IP route of alcohol administration in juvenile animals does not yield statistically different blood alcohol concentrations (BAC) compared to oral gavage (54). Importantly, the animals were given alcohol in the morning. It has been previously shown that morning alcohol administration does not interfere with normal feeding behavior and does not result in body weight differences between alcohol-treated and control animals, thus eliminating the need for pair-fed controls (7, 8). All other groups were treated as follows: 1) untreated = daily handling only; saline = one IP saline injection each day for 8 consecutive days; acute EtOH = one IP saline injection each day for 7 consecutive days, followed by one IP EtOH (3g/kg) injection on day 8. On the last day of treatment (day 8), animals were euthanized by rapid decapitation 60 min. after the final injection.

Blood alcohol concentration (BAC) measurements:
Trunk blood samples were collected into heparinized tubes, centrifuged at 3000 rpm for 10 min at 4°C; and plasma stored at -20°C. Blood alcohol levels were determined by measuring the change in absorbance at 340 nm following enzymatic oxidation of EtOH to acetylaldehyde (Point Scientific Alcohol Reagent Kit). Assay range is 0 to 400mg/dl and inter and intraassay CV = 1.78% and 3.34%, respectively.
Hormone measurements:

Plasma levels of testosterone (T) and estradiol (E2) were measured using commercially available EIA kits (Cayman Chemical Company, Ann Arbor, MI) according to manufacturer’s instructions. Corticosterone (CORT) was measured using radioimmunoassay (RIA). Briefly, blood samples were collected into heparinized tubes, centrifuged at 3000 rpm for 10 min at 4°C and plasma stored at -20°C. For the testosterone EIA kit, the range of detection was between 3.9 – 500 pg/ml and for the estradiol kit the range of detection was 8.6 – 4000 pg/ml. Intraassay CVs were 2.2% and 6.1% for testosterone and estradiol, respectively. To measure CORT, 3H-CORT (PerkinElmer, Waltham, MA) and rabbit CORT antiserum (MP Biomedicals, Solon, OH) were used to perform RIA. On the first day of the procedure, a standard curve was prepared using CORT-standards (4-PREGNEN, 11b, 21-DIOL-3,20-DIONE-Steraloids, Inc). Appropriate sample tubes were filled with 100 µl of a sample, 100 µl of antibody solution (in 0.1% gel PBS), and 100 µl diluted radioactive tracer (10-12000 cpm). Tubes were incubated overnight at 4°C. On the second day, all tubes were filled with 100 µl of 0.5% gel PBS, 1 ml Dextran-Coated Charcoal (DCC), and 1 ml 0.01 M PBS. Samples were centrifuged at 3000 rpm at 4°C for 15 min and the supernatant was collected into scintillation vials. Radioactivity was measured in each vial for 1 min. using Packard liquid scintillation counter. Levels of CORT in unknown samples were interpolated based on the standard curve. Assay range is 0 to 500ng/ml and inter and intraassay CV = 2.98% and 5.96%, respectively.

Tissue collection and qRT-PCR:

After sacrifice, brains were rapidly collected and frozen using isopentane and stored at
-80°C until further processing. Frozen brains were sectioned at 200 μm on a freezing microtome and the paraventricular (PVN) and supraoptic (SON) nuclei were microdissected using a 0.75 mm Palkovit’s brain punch tool (Stoelting Co., Wood Dale, IL) Total RNA isolation was performed on sonicated tissue samples using Trizol reagent (Invitrogen Inc., Carlsbad, CA) according to the manufacture’s directions. Following RNA isolation, samples were treated with DNA-free (Applied Biosystems/Ambion, Austin, TX) according to manufacturer’s instructions to eliminate genomic DNA contamination. Subsequently, 0.4μg total RNA was reverse transcribed using the First Strand Synthesis SuperMix for qRT-PCR (Invitrogen Inc., Carlsbad, CA). Total RNA samples subjected to first strand cDNA synthesis reaction minus reverse transcriptase enzyme were used as controls for transcript dependency of the PCR reaction. Roche FastStart SYBR Green Master Mix was added to AVP specific upper and lower AVP primer (0.25 μM final concentration; 5-CGCAGTGCCCACCTATGCTC; 3-AGGAAGCAGCCCAGCTCGTC), CRH primer (0.25 μM final concentration; 5-CTGGGGAACCTCAACAGAAG; 3-GGTGGAAGGTGAGATCCAGA) and OXY primer (0.25 μM final concentration; 5-CTTGGCCTACTGGGTCTGAC; 3-GGGCAGGTTGCTTCTCCCTCT). Then, 2 µL cDNA templates were added to triplicate reactions performed in a 96 well plate. Thermal reactions conditions were 95 °C for 10 min, then a repeated cycle of 95°C for 30s, 60°C for 30s, and 72°C for 30s with SYBR green fluorescence detection at the end of each 72°C step. Samples were then subjected to thermal melting curve analysis to verify that the Tm of the product is consistent with the calculated Tm based on sequence with continuous fluorescence detection to 95°C. Quantification of the target gene expression was achieved by extrapolating from standard curve of known concentrations ran simultaneously in the same plate.. Prior to extrapolation of absolute numbers using the standard
curve, all samples were normalized to the hypoxanthine guanine phosphoribosyl transferase 1 (HPRT) housekeeping gene. The hypoxanthine phosphoribosyl transferase 1 (HPRT) gene was used as an internal control in these studies due to its constitutive expression in all cells and its relatively low expression levels. The low levels of expression make it an ideal internal control for highly sensitive quantification of genes that might be expressed at low levels within the cell. In addition, our preliminary survey of several different housekeeping genes showed that HPRT was unaffected by ethanol treatments both in vivo and in cell culture (unpublished data).

Amplification efficiency of all target and reference genes were equivalent as determined by measuring ΔCt (Ct_{target} – Ct_{ref}) over a range of dilutions and calculating absolute value of the slope (log cDNA dilution vs ΔCt). Total copies were determined by generating an HPRT standard curve within each PCR reaction, using 10-fold serial dilutions ranging from 1 × 10 to 1 × 10^7 copies of pcDNA3.1 vector plasmids containing rat HPRT gene (plasmid constructs generously provided by Dr. Phong Le, Loyola University Chicago). Samples without cDNA acted as negative controls. Expression of CRH, AVP, or OXY was compared by determining the ratios of copies/μl of the transcript to copies/μl of HPRT in individual samples.

Statistical Analysis

One-way ANOVA was used to test for differences between treatment groups within sexes followed by Dunett t-test if one-way achieved significance. Untreated or saline groups were each chosen as controls in subsequent Dunett t-tests. Between sex variations were determined using two-way ANOVA followed by Tukey’s post-hoc analyses. One-way ANOVA with repeated measures was used to analyze body weight data throughout the course of treatments. A p-value of less than 0.05 was considered to be significant.
RESULTS

*EtOH exposure did not alter final body weight or growth pattern during puberty.*

Body weight was measured every-other day during the duration of treatments to determine whether EtOH altered normal growth patterns during pubertal development. There was no significant difference in body weight among groups for males or females at any point during the 8-day treatment regiment (Fig. 1).

*Blood alcohol concentrations following acute and binge EtOH exposure.*

Blood alcohol concentrations (BAC) were measured on the final day of treatment (day 8) 60 min. following injections. There were no statistically significant differences in BAC levels between acute and binge-treated animals in either sex (Fig 3) and, as expected, untreated and saline treated groups were below the limit of detection for the assay. These values are consistent with the defined BAC threshold of binge drinking. In females, there was a strong trend towards lower BAC for the binge-pattern EtOH exposure groups compared with acute, suggesting that the metabolism of EtOH was altered with repeated dosing (Fig. 2B). However, as this result was not statistically significant more studies would need to be performed to verify that this occurred.

*Effects of binge alcohol exposure on gonadal steroid hormone levels.*

To determine the effects of binge EtOH exposure during puberty on circulating gonadal steroid hormone levels, testosterone (T) and 17β-estradiol (E2) were measured on the last day of treatment. Binge, but not acute, EtOH treatment significantly decreased plasma T levels in males compared to untreated and saline groups (Fig 3A; p<0.001). Testosterone levels in the
untreated, saline, and acute groups were consistent with normal levels for peripubertal rats (11). On the other hand, EtOH had no effect on E2 levels in females (Fig 3B). Average E2 levels were similar among females in all groups, indicating that they were in similar stages of sexual maturity (24.76 ± 4.84, 25.34 ± 7.11 and 19.25 ± 2.95 pg/ml in saline, acute EtOH and binge EtOH groups, respectively). Pubertal onset was also verified in female rats by monitoring vaginal introitus. First day of vaginal introitus was 33.84 ± 0.17 days of age for all groups, verifying that all female animals had achieved pubertal onset at least four days prior to the first day of EtOH administration.

**Effects of acute and binge EtOH exposure on circulating CORT levels.**

To determine whether EtOH treatment activated the stress response, circulating CORT levels were measured using RIA. As expected from previous studies, the overall stress-induced CORT levels were significantly higher in females compared with males (Fig. 4, p<0.001). Moreover, in both males and females EtOH exposure significantly increased plasma CORT levels in both acute and binge-pattern EtOH treatment groups compared with saline and untreated animals (Fig 4). Interestingly, CORT levels were lower in the animals that were administered the binge-pattern EtOH paradigm compared to those that received a single does of EtOH (acute, P<0.05). This habituation effect was similar in both males and females (Fig. 4 A,B).

**Sex-dependent effects of binge alcohol exposure on CRH and AVP gene expression in the PVN.**

In order to investigate the effects of EtOH exposure during puberty on the expression of genes involved in stress and anxiety responses in males and females, CRH and AVP mRNA expression was measured using real-time quantitative RT-PCR. Overall, females had significantly higher
basal levels (untreated groups) of CRH mRNA than males (Fig. 5, p<0.001). However, binge, but not acute, EtOH exposure significantly increased the expression of CRH (Fig 4A, p < 0.05) and AVP mRNA (Fig 4B, p < 0.05) in the PVN of male animals only (Fig. 5 A,B). There were no differences between the expression of CRH and AVP mRNA in the acute EtOH exposure group compared to saline-treated indicating that the effect observed in the binge EtOH group is due to repeated EtOH exposure. By stark contrast, in females there were no significant differences among treatment groups in the expression of CRH and AVP mRNA in the PVN (Fig 5 C, D).

**EtOH exposure does not change the expression of AVP in the SON**

In order to confirm that the observed changes in the expression of AVP mRNA in PVN of male rats were not exclusively due to the reported diuretic effect of EtOH, we measured the expression of the AVP mRNA in the SON, a region of the brain involved in regulating fluid homeostasis. There were no differences between treatment groups in the expression of AVP mRNA in the SON in males (Fig 6A) or in females (Fig 6B) suggesting that the change in the AVP mRNA observed in the PVN reflected change in the population of AVP-expressing cells responsible for mediating stress and anxiety. Interestingly, baseline levels of AVP mRNA were significantly lower in females compared to males in the SON (Fig. 6B, p<0.001).

DISCUSSION

Our current understanding of how alcohol consumption affects the developing post-natal brain is severely limited. To date, specific molecular and neuroendocrine markers that are activated by alcohol during puberty have not been identified. Elucidating the neurobiological
targets of alcohol resulting from a binge pattern of consumption during adolescence is critical for understanding the long-term behavioral consequences and potential development of mental health disorders. Therefore, the goal of this study was to investigate the effects of binge-pattern EtOH exposure during puberty on the expression of specific neuroendocrine factors that mediate stress and anxiety including CRH and AVP. Our data reveal the novel findings that peripubertal binge-pattern, but not acute, EtOH exposure increased the expression of CRH and AVP mRNA in the PVN of peri-pubertal male rats, yet had no effect in peri-pubertal females. Data presented here indicate that the effects of alcohol on the HPA axis are sex-specific and dependent on repeated high dose exposures. Moreover, CORT levels were increased significantly in response to both acute and binge EtOH exposure paradigms, suggesting that EtOH is a potent activator of the HPA axis during pubertal development. Notably, there was no sex difference in the CORT response to EtOH and under binge-pattern exposure conditions the animals appeared to habituate to the effects of the stressor. Lastly, we have shown that binge-pattern EtOH exposure decreased sex steroid hormone levels in male, but not female, rats and that the observed effects of EtOH on AVP gene expression were unlikely due to the diuretic effects of EtOH but instead, specific for the population of AVP-expressing neurons that regulate stress and anxiety.

Acute and binge-pattern EtOH exposure increased plasma CORT levels in our study, confirming previous reports that EtOH is a potent activator of the stress response in adults. Ogilvie and Rivier (1997) showed that acute EtOH administration increased CORT levels which peaked at 15 min. post EtOH injection and were sustained until 1.0 h after the injection (35). However, we showed that plasma CORT levels in the binge EtOH groups were significantly lower compared to the acute EtOH exposure groups, suggesting that the HPA axis habituated to the stressful stimuli of multiple doses of EtOH. Also, females had higher plasma CORT levels
after EtOH exposure compared to males indicating that females were more sensitive to EtOH administration than males. These data are in agreement with multiple studies demonstrating that there are gender differences in responsiveness to alcohol with females tending to achieve higher CORT levels compared to males given the same dose of EtOH (35, 42).

Acute alcohol consumption, chronic alcohol consumption, and withdrawal from alcohol dependency have all been associated with dysregulation of the HPA axis in adults (5, 26, 30, 32, 34, 43-45, 52). For instance, studies in adult rats have shown that acute alcohol administration increased circulating levels of ACTH (43). This effect was dependent upon the synergistic action of CRH and AVP, as pharmacological antagonism of the CRH and AVP receptors abolished the response (34, 45). Similarly, chronic alcohol treatment resulted in a 33% decrease in the number of AVP-expressing neurons in the suprachiasmatic nucleus (SCN) (30) and altered the diurnal rhythm in circulating glucocorticoids (52). AVP neurons in the SCN convey circadian information to the PVN and other extrahypothalamic brain nuclei. Moreover, the circadian expression of AVP in the SCN modulates the diurnal rhythm of circulating glucocorticoids (26). Our data, showing that EtOH administration alters CRH and AVP mRNA in peri-pubertal male rats, are consistent with the observations in adult animals. Notably in our study, a single dose of acute EtOH failed to increase CRH and AVP mRNA in the PVN despite inducing a dramatic increase in circulating CORT levels. These data suggest that the EtOH-induced upregulation of these genes occurs as a result of repeated EtOH exposure, as would be achieved with a binge pattern of alcohol consumption. It remains to be determined whether there are long-lasting behavioral or physiological deficiencies associated with these changes in gene expression. Nevertheless, there is a growing body of evidence to suggest that neurite remodeling, neurogenesis, and synaptic connectivity are prominent developmental events during
puberty (see review (49). Accordingly, it is logical to assume that repeated and excessive alcohol use during this critical stage of development could lead to permanent alterations in the morphological and/or neurochemical circuitry that might be organized during this time. These changes could in turn, have negative consequences on normal adult behavior patterns, as brain morphology and neurochemistry are intimately linked with behavior.

The neuropeptide AVP is a compelling candidate for mediating the underlying neuroendocrine basis of the sexually dimorphic effects of alcohol. First, central release of AVP from the PVN, BST, and AMY mediates a broad range of social behaviors and participates in modulation of the stress response. In both males and females AVP augments the stress response by acting synergistically with CRH which can be altered by a variety of factors including gonadal steroid hormones and alcohol (1, 4, 18, 20, 24, 31, 41). Moreover, AVP and the AVP receptors are differentially regulated by gonadal steroid hormones in a sex- and age-dependent manner (2, 36, 39, 50, 58). For example, AVP expression in the BST is greater in males than females (33), and this sex difference is abolished by castration and restored with T replacement; an effect that is accomplished mainly by T-derived 17β-estradiol (E2) (23). To date, little is known about the maturation of the central AVP system including the temporal, sex-specific, and hormonal influences that occur during pubertal development. Previously, we showed that AVP mRNA expression in the BST and AMY was differentially regulated in pre- and post-pubertal animals (36). Here, we have demonstrated prominent sex-dependent effects of binge-pattern EtOH administration on AVP mRNA levels during the pubertal transition. Our study showed that in males there was a significant increase in AVP mRNA following binge-pattern, but not acute, EtOH administration. Conversely, there were no effects on AVP in females using the same binge-pattern paradigm. It is important to note that although the binge-pattern EtOH
treatments significantly reduced testosterone levels, there was no effect on estradiol levels, suggesting that the EtOH effects on gonadal steroid hormones alone are not sufficient to explain these sex differences.

Developmental changes in HPA axis reactivity have been well documented. Most striking is the observation that corticosterone and ACTH levels take much longer to return to baseline in juvenile compared to adult animals subjected to a variety of stressful stimuli (46, 53). It is well known that males and females achieve sexual maturity at different rates with respect to reproductive function, but whether there are sex differences in the rate of HPA axis maturation during puberty is unknown. The majority of studies investigating the maturation of the HPA axis during pubertal development have focused strictly on comparisons between the pre- and post-pubertal states, but not at time points in between (46). However, one study in human adolescents showed that there were sex differences in the cortisol response to a CRH challenge (51). In general, girls achieved peak cortisol levels later and had a slower return to baseline following a CRH challenge compared with boys (51), a difference which persisted throughout all Tanner stages of development. These data suggest that sex differences in HPA axis reactivity might not necessarily be due to a differential timing in HPA axis maturation between males and females, but rather due to inherent differences that are perhaps organized prior to pubertal onset. Recent evidence from Evuarherhe and colleagues (2009) support this possibility. In their study, 17β-estradiol inhibited the corticosterone-induced response to restraint stress in pre-pubertal female rats, but enhanced the response post-pubertal (21). Importantly, the adult response to restraint stress in the presence of 17β-estradiol was still enhanced regardless of whether the animals were ovariectomized prior to, or following, puberty. These data indicate that the maturation of the HPA axis in females might be programmed prior to pubertal onset and that it is independent of
circulating gonadal steroid hormones during pubertal development. At this time, there are no empirical data to suggest that the HPA axis matures at different rates in males compared to females and in fact, the evidence available from humans would predict that they likely mature at similar rates. However, we cannot rule out the possibility that the differences we observed might be due to discrepancies in the relative state of HPA maturation between males and females. Nevertheless, our data demonstrate a clear distinction between the effects of repeated EtOH exposure during the pubertal transition which might lend some insight into the time course for the development of the HPA axis between males and females. Further, our data have demonstrated that repeated EtOH exposure during the pubertal transition results in a dysregulation of the HPA axis akin to that of a chronic stressor in adults.

In this study, both males and females began receiving injection treatments on day 37 which were completed on day 44, falling within the defined peri-pubertal period for both males and females (30-45 days of age, (28). In general, female rats undergo pubertal onset (as measured by vaginal introitus) at 32-35 days of age, with regular estrous cyclicity and mature ova first occurring between 43 and 47 days of age (48). Unlike females, in which puberty is defined as the first day of vaginal introitus, males do not have a reliable external benchmark that defines pubertal onset, as the commonly used external marker of preputial separation is dependent upon increased T levels. The binge pattern alcohol exposure paradigm employed in our experiments did not inhibit feeding behavior or normal growth patterns that occur during pubertal development. Moreover, it allowed us to distinguish the pharmacological effects of EtOH from the effects of other nonspecific stressors, such as handling and injections. This binge EtOH exposure paradigm has been previously shown to be reliable for testing the effects of alcohol using an exposure pattern that is typical for adolescents (27). In addition, it has been
show that the IP injection of EtOH, employed in our paradigm, does not result in significantly different BAC compared to oral gavage in adolescent Wistar rats (54).

Although it was not possible to separate the parvocellular and magnocellular divisions of the PVN in our tissue punch sample preparations, we are confident that the EtOH-induced changes in AVP expression accurately reflect changes in the AVP-expressing neurons associated with regulation of the HPA axis, and not in those responsible for osmoregulation for the following reasons. First, we showed that there were no changes in the expression of the AVP mRNA in the SON, a region where AVP is primarily responsible for regulating fluid homeostasis. Second, there was no effect of EtOH in the acute-treated group and since fluid homeostasis is an immediate and not cumulative physiological response, any diuretic effect of EtOH would have been observed in the acutely treated groups. Third, if the effects of EtOH on AVP expression were due to an osmoregulatory effect, we would have expected to see a significant increase in the females, as well as the males. Finally, our data are in agreement with previous studies showing that there were no changes in AVP expression in the magnocellular division of the PVN 3.0 h after an acute EtOH administration (34).

Alcohol is a potent neurotoxic agent that induces widespread cell death in the developing brain (12, 47). Exciting new data have revealed that AVP has an important neuroprotective role in the PVN. Chen and colleagues (2008, 2009) have shown that AVP prevented apoptosis in H32 cells, a cell line derived from the PVN, following serum deprivation (9, 10). These effects were mediated by mitogen-activated protein kinase (MAPK) activated Bad phosphorylation and by protein kinase C (PKC) α and β. These data raise the possibility that the physiological role of increased AVP following binge EtOH exposure in males is, in part, to provide cellular protection against repeated ethanol toxicity. In females, on the other hand, E2 is known to be
neuroprotective in addition to playing roles in modulating reproductive functions and anxiety responses (6, 16). Our study showed that there was no increase in AVP mRNA after binge EtOH exposure in females, possibly because there are different mechanisms for neuroprotection in males compared with females.

Taken together, we have identified specific neuroendocrine targets of EtOH in the adolescent hypothalamus following a binge pattern of EtOH exposure. Further, we have demonstrated that the effects of EtOH administration on genes critical for the developing HPA axis are sex specific. Sexually dimorphic patterns of addictive behavior emerge during adolescence and often persist in adulthood. Patterns of alcohol use during this developmental time period can also lead to permanent changes in brain function that often manifest in adulthood as psychological disorders, such as depression and anxiety. Overall, these data have identified potential genes involved in increased anxiety responses after EtOH exposure and might lead to a better understanding of the mechanisms involved in increased prevalence of anxiety disorders in females compared to males.

ACKNOWLEDGEMENTS

The authors would like to acknowledge funding sources NIH T32AA013527 and R21AA018398.

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Fig 1. Effects of ethanol treatment on body weight during pubertal development. Mean body weights of male (A) and female (B) animals untreated or treated with daily IP injections of saline, saline + one day EtOH (acute), or binge EtOH paradigm.

Fig 2. Effects of ethanol treatments on blood alcohol and corticosterone levels in male and female rats. Panel A: blood alcohol concentrations (BAC) 1.0 h post-injection in males (A) and females (B) treated with saline, acute EtOH or binge EtOH. Data expressed as mean alcohol mg/dl. * indicates statistically significant difference (P < 0.05).

Fig 3. Effects of ethanol treatment on gonadal steroid hormone levels. Plasma concentrations of testosterone (T) in male rats (A) and 17β-estradiol (E2) in female rats (B) 1.0 h after IP injection of saline, acute EtOH or binge EtOH treatments. Untreated animals received no injection. Data expressed as T or E2 pg/ml. * indicates statistically significant difference (P < 0.05).

Fig 4. Effects of ethanol treatment on circulating corticosterone levels. Plasma corticosterone (CORT) levels in peri-pubertal male (A) and female (B) rats 1.0 h after IP injection of saline, acute, or binge EtOH treatments. Untreated animals received no injection. Data expressed as mean CORT pg/ml of blood. Dissimilar letters indicate statistically significant difference among groups (P < 0.05).

Fig 5. Effects of ethanol treatments on CRH and AVP mRNA in the PVN. CRH and AVP mRNA expression in the PVN of peri-pubertal male (A, B) and female (C, D) rats treated with saline,
acute, or binge EtOH. Data expressed as mRNA copies/µg total RNA. * indicates statistically
significant difference (P < 0.05).

Fig 6. Effects of ethanol treatments on AVP gene expression in the SON of male and female rats.
AVP mRNA expression in SON of peri-pubertal male (A) and female (B) rats treated with
saline, acute, or binge EtOH. Data expressed as AVP mRNA copies/µg total RNA.
Figure A: Comparison of T (pg/ml) levels in untreated, saline, acute EtOH, and binge EtOH conditions. The untreated group shows the highest T levels, followed by saline, acute EtOH, and then binge EtOH, with the binge EtOH group showing a significant decrease (*).

Figure B: Comparison of E2 (pg/ml) levels in untreated, saline, acute EtOH, and binge EtOH conditions. All groups show similar E2 levels, with slight variations.
Auntreated  saline  acute EtOH  binge EtOH

AVP mRNA (copies/μg RNA)

untreated  saline  acute EtOH  binge EtOH

AVP mRNA (copies/μg RNA)