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Immediate and prolonged effects of alcohol exposure on the activity of the hypothalamic-pituitary-adrenal axis in adult and adolescent rats

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Abstract

Alcohol stimulates the hypothalamic-pituitary-adrenal (HPA) axis. Part of this influence is likely exerted directly at the level of the corticotropin-releasing factor (CRF) gene, but intermediates may also play a role. Here we review the effect of alcohol on this axis, provide new data on the effects of binge drinking during adolescence, and argue for a role of catecholaminergic circuits. Indeed, acute injection of this drug activates brain stem adrenergic and noradrenergic circuits, and their lesion, or blockade of $\alpha 1$ adrenergic receptors significantly blunts alcohol-induced ACTH release. As alcohol can influence the HPA axis even once discontinued, and alcohol consumption in young people is associated with increased adult drug abuse (a phenomenon possibly mediated by the HPA axis), we determined whether alcohol consumption during adolescence modified this axis. The number of CRF-immunoreactive (ir) cells/section was significantly decreased in the central nucleus of the amygdala of adolescent self-administering binge-drinking animals, compared to controls. When another group of adolescent binge-drinking rats was administered alcohol in adulthood, the number of colocalized *c-fos*-ir and PNMT-ir cells/brain stem section in the C3 area was significantly decreased, compared to controls. As the HPA axis response to alcohol is blunted in adult rats exposed to alcohol vapors during adolescence, a phenomenon which was not observed in our model of self-administration, it is possible that the blood alcohol levels achieved in various models play a role in the long-term consequences of exposure to alcohol early in life. Collectively, these results suggest an important role of brain catecholamines in modulating the short- and long-term consequences of alcohol administration.

Keywords

Alcohol; hypothalamic-pituitary-adrenal axis; adolescent rats; brain stem catecholamines; central nucleus of the amygdala; locus coeruleus lesion; anti-DBH saporin toxin

It is by now widely accepted that alcohol exerts effects on a large array of biological events, and that this influence depends, at least in part, on the dose of the drug, the mode (route) and

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duration of exposure, whether delivery is investigator-controlled or involves self-administration, and whether it is measured immediately after drug exposure or following a drug-free period. In this chapter, we will review the effects of alcohol on the rodent hypothalamic-pituitary-adrenal (HPA) axis, introduce new data on the effects of binge drinking in adolescent animals upon the HPA axis and raise a novel hypothesis that alcohol may exert some of its effects on the HPA axis via actions on catecholamine modulatory actions of the HPA axis.

Stimulatory effect of alcohol on the HPA axis

Mechanisms

We presented an overview of the stimulatory effect of alcohol on the HPA axis in an earlier review (Rivier, 1996) and while considerable progress has been made in our understanding of the mechanisms through which this influence is exerted, the basic concept that it primarily involves endogenous corticotropin-releasing factor (CRF) from the paraventricular nucleus (PVN) of the hypothalamus remains unchanged (Rivest and Rivier, 1994; Rivier et al., 1984; Rivier and Lee, 1996). The role played by CRF depends on two types of receptors, CRFR1 and CRFR2 (DeSouza, 1995; Perrin et al., 1993), and the question therefore arises of what CRF receptor type was involved in the effect of alcohol? We first reported that alcohol was able to up-regulate the type 1 receptor (Lee and Rivier, 1997a), and that blockade of these receptors interfered with the HPA axis response to stressors (Rivier et al., 2003). Further support for the importance of CRFR1 in our models came from the finding that mice that lacked this receptor displayed a significantly blunted adrenocorticotropin hormone (ACTH) response to alcohol (Lee et al., 2001). More recent work has focused on investigating whether alcohol directly influences the CRF gene, the site of action of this influence, and the potential role of intermediates. Evidence for a direct role of alcohol on CRF expression was obtained by using hypothalamic cells and the NG108-15 cell line which showed that this drug up-regulated the CRF promoter, and the CRF gene through cAMP/PKA-dependent pathways (Li et al., 2005). Our current hypothesis is that these mechanisms are important in the *in vivo* response of the HPA axis to alcohol, but that depending on the model used (such as doses, route and frequency of drug delivery), several other modulating factors may also play a role.

i. Cytokines—It is well known that cytokines, proteins that are involved in the early events of immune activation, stimulate the HPA axis (Rivier, 1993). When reviewing the findings of the effect of cytokines on the HPA axis (Rivier, 1993; Turnbull and Rivier, 1995), we concluded that injection of pro-inflammatory cytokines such as interleukins [(IL), IL-1 and IL-6], tumor-necrosis factor α (TNF α), and endotoxin [lipopolysaccharide (LPS)], caused dose-related increases in plasma ACTH and corticosterone levels. Specifically, IL-1 stimulates the release of CRF from the median eminence, and the finding that CRF antibodies block the ACTH response to this cytokine, suggests that it is mediated through endogenous CRF (Sapolsky et al., 1987). Indeed, the ability of LPS to activate PVN CRF neurons further supports this concept (Lee et al., 1995). Furthermore, a review of potential interactions between alcohol and cytokine secretion (Martinez et al., 1992) lead to the concept that alcohol might release cytokines, which led our laboratory to investigate the hypothesis that the stimulatory effect of alcohol on the HPA axis might, at least in part, depend on endogenous cytokine release. In particular, we explored the effects of acute pretreatment with alcohol upon the release of ACTH, corticosterone and pro-inflammatory cytokines (TNF α and IL-6) following endotoxicity (LPS administration), and found that the drug increased the corticosterone, but not the ACTH response to LPS (Rivier, 1999). We also reported that prolonged exposure to intermittent alcohol vapors decreased the ACTH as well as hypothalamic nitric oxide (NO) and cytokine responses to LPS injection (Seo et al.,

2004). Additionally, another study in our laboratory showed that alcohol did not release proinflammatory cytokines in the brain, and also provided evidence of a functional interaction between a nuclear factor- κ B (NF- κ B) -dependent pathway and alcohol in stimulating the rat HPA axis activity that involved independent roles of corticosterone and ACTH (Lee and Rivier, 2005). More recently, Glover and colleagues (2009) found that the suppression of cytokine/chemokine production due to alcohol occurred regardless of corticosterone levels, as demonstrated by comparing sham and adrenalectomized (ADX)/placebo mice. These authors concluded from their research using ADX mice administered a catecholamine antagonist (nadalol) that the suppression of cytokine production was not caused by the excess stress responses associated with alcohol administration. While still somewhat inconclusive, collectively these results do not support a primary role of endogenous cytokines in modulating the HPA axis response to alcohol.

ii. Catecholamines—In acute alcohol models, the DNA binding protein NF- κ B appears important for the activation of the rat HPA axis (Lee and Rivier, 2005), as does the release of the unstable gas NO (Seo and Rivier, 2003) which is known for its ability to activate the HPA axis (Kim and Rivier, 2000; Lee et al., 1999). Recently, we investigated the effect of alcohol on catecholamines, which also stimulate this axis (Dunn and Swiergiel, 2008; Kiss and Aguilera, 2000). The main noradrenergic brain region is the locus coeruleus (LC), which has widespread efferent projections that supply norepinephrine throughout the central nervous system (Reyes et al., 2005; Valentino and Van Bockstaele, 2008). Furthermore, the noradrenergic brain stem projections join in the ventral noradrenergic bundle to innervate the hypothalamus (and presumably the PVN) and basal forebrain [for review see (Koob, 2008)]. While the direct projections of the LC to the PVN are considered sparse (Cunningham and Sawchenko, 1988; Sawchenko and Swanson, 1982), they nevertheless mediate the HPA axis responses to various stressors (Day, 2005; Reyes et al., 2008; Valentino et al., 1998; Wittmann, 2008), possibly through innervation of the prefrontal cortex (Al-Damijji and White, 1992; Dayas et al., 2001; Radley et al., 2008). In view of these considerations, we hypothesized that alcohol would stimulate the LC, and found that the intragastric (ig) injection of the drug indeed up-regulated *c-fos* signals in this brain stem region (Lee et al., 2011). This finding, as well as previous research in other laboratories [for review see (Koob, 2008)], provides strong evidence that alcohol is able to activate noradrenergic circuitry. The functional importance of this response for the overall HPA axis activation by alcohol was then demonstrated with two approaches. First, we compared the stimulatory effect of ig-administered alcohol (4.0 g/kg) in sham or electrolytic LC-lesioned adult rats, and report here that these lesions abolished the expected rise in plasma ACTH levels (Fig. 1). The second approach was to investigate the role of the A1-A2/C1-C3 medullary cell groups, another catecholamine-rich group of cells that provides important input to the PVN (Douglas, 2005; Reyes et al., 2005; Rinaman, 2001; Sved et al., 2002). We were particularly interested in this area because it conveys visceral information to the PVN (Cunningham et al., 1990) and represents important homeostatic centers that contribute to the catecholaminergic innervation of the PVN (Mejias-Aponte et al., 2009). Lesion of the A1-A2/C1-C3 cell groups was done by microinfusion of anti-dopamine β -hydroxylase (DBH)-saporin and was completed according to published protocols [see for example (Blessing et al., 1998; Estacio et al., 2004; Madden et al., 1999; Rinaman and Dzmura, 2007; Rohde and Basbaum, 1998; Schiltz and Sawchenko, 2007; Wrenn et al., 1996)]. Anti-DBH-saporin is known to destroy both noradrenergic (A1-A2) and adrenergic (C1-C3) cell groups, so DBH or phenylethanolamine N-methyltransferase (PNMT) antibodies can be used to detect this aminergic lesion. However, because of our interest in the noradrenergic medullary projection to the PVN, we assessed DBH cells in the A1-A2 and C1-C3 regions. Compared to results obtained in rats that did not receive anti-DBH-saporin, changes induced by the toxin and measured 2 h after alcohol (EtOH) injection were: (a) a significant decrease

in the number of DBH-positive fibers in the PVN (Lee et al., in press), indicating the loss of catecholaminergic input to this hypothalamic region; (b) a significant ($P < 0.05$) decrease in the number of DBH-ir cells in the A1/C1, A2/C2, and C3 regions of the brain stem (Fig. 2); and (c) a decreased number of PVN CRF-ir cells (Sham: 79.6 ± 8.1 , Lesion: 56.4 ± 5.9 , $P < 0.05$). We know that the role played by medullary nuclei varies with PVN regions, as A1 nuclei project to the magnocellular division of the PVN (mPVN) while a subset of C1 neurons primarily project to the parvocellular division of the PVN (pPVN) (Sawchenko et al., 1996; Sawchenko and Swanson, 1982). Even though the type of lesion we used (anti-DBH saporin toxin) cannot differentiate A1 or C1, and we, therefore, cannot dissociate between the influence of these two regions, the HPA axis response to alcohol is thought to involve the pPVN (Rivier, 1996; Rivier and Lee, 1996). Future studies are therefore needed to further clarify the role of the A1-A2/C1-C3 regions in alcohol models.

Having demonstrated the overall importance of brain stem noradrenergic projections to the PVN in modulating the HPA axis response to acute alcohol administration, we then investigated the identity of the receptors involved. The bulk of the stimulatory catecholaminergic innervation to the central limb of the HPA axis is provided by the adrenergic receptor type α [references in (Dunn and Swiergiel, 2008; Herman and Cullinan, 1997; Kiss and Aguilera, 2000)], and subtype α_1 receptors have been localized in the endocrine PVN (Day et al., 1999; Williams and Morilak, 1997). However, the effectiveness of their blockade depends on the type of stressors used [see for example (Douglas, 2005; Douglas et al., 2005; Kiss and Aguilera, 2000; Pardon et al., 2003; Stone and Zhang, 1995; Williams and Morilak, 1997)], which pointed to the importance of determining their role in our model. In our laboratory, we found that blockade of adrenergic receptors type α , but not β , significantly blunted the ACTH response to NO donor 3-morpholino-sydnominine (SIN-1) (Seo et al., 2003). Collectively, these findings indicate that not only is alcohol able to activate catecholaminergic brain stem circuitries, but also these pathways modulate the HPA axis response to alcohol showing a novel mechanism for mediating the neuroendocrine effects of alcohol in the rodent.

Site(s) of action

A central question in understanding the mechanism through which alcohol α activates the HPA axis is the identification of the site(s) of action of the drug. A longstanding issue has been, in particular, whether alcohol primarily acted on the PVN, or if it (also? mainly?) activated corticotropes in the anterior pituitary through a direct (i.e., CRF-independent) mode of action. As mentioned above, there is no doubt that alcohol can up-regulate PVN gene transcription (Rivier and Lee, 1996), thereby causing the release of CRF to the pituitary. This effect is observed regardless of whether the drug is administered systemically or directly into the brain (Lee et al., 2004), which supports the hypothesis that it represents an alcohol-specific effect, and is not mediated through peripherally dependent mechanisms. Is ACTH secretion induced by alcohol also strictly mediated at the hypothalamic level, or does it include a direct influence of alcohol on the corticotropes? To address this question, we relied on a ribonuclease protection assay to measure pituitary transcripts for the ACTH precursor proopiomelanocortin (POMC) in rats with or without immunoneutralization of endogenous CRF, and found that alcohol was unable to exert a stimulatory effect upon these transcripts in the absence of CRF (Lee et al., 2004). These results provided strong evidence that the HPA axis response to alcohol does not represent a direct influence of the drug on the pituitary, but requires CRF. We further demonstrated that alcohol primarily acted on the hypothalamus (and not the pituitary) by showing that intracerebroventricular (icv) injection of alcohol at doses that did not reach the periphery, released ACTH (Lee et al., 2004). These results isolate alcohol's influence on activating the HPA axis in the brain, instead of via an action outside the brain.

Long-term effects of alcohol

Adult animals

A very intriguing aspect of the regulation of the HPA axis is its ability to be altered by an initial stressor delivered weeks or even months earlier. Depending on whether the stressors are homo- (alcohol only) or heterologous (alcohol and footshock), previous activation can result in habituation or facilitation of the subsequent response [see ref. in (Dallman, 2005; Lee and Rivier, 1997b; Rivier and Vale, 1987)]. A long-term influence can even be observed after one initial treatment, as indicated by the ability of a single injection of the pro-inflammatory cytokine IL-1 β to prolong the ACTH response to a second challenge presented up to 3 weeks later (Kentner and Pittman, 2010; Schmidt et al., 1995). Altered PVN CRF gene expression and changes in pituitary responsiveness to steroid feedback are thought to play a role, though other mechanisms might also intervene. We have reported that while rodents respond to an initial exposure to alcohol with a significant activation of their HPA axis (Rivier and Lee, 1996), this response tends to become blunted during prolonged treatment regardless of whether the drug is delivered according to an experimenter-controlled protocol (Rivier et al., 1990) or self-administered protocol (Lee and Rivier, 1993; Rivier, 1995). In a procedure in which the initial and subsequent exposure to alcohol was discontinuous, we also observed that three consecutive daily injections of alcohol also blunted the HPA axis response to a second drug challenge up to 12 (Lee and Rivier, 1997b) or even 24 (Lee and Rivier, 2003) days later, and that the initial age of the animals did not appear to alter this phenomenon (Lee and Rivier, 2003). These results raised the hypothesis that such blunting of the HPA response with repeated alcohol exposure might play a role in the development of alcohol abuse, as individuals who had “lost” the ability to activate their HPA axis might attempt to regain this response with increasing doses of alcohol. While this is a plausible and intriguing hypothesis, it remains untested.

Adolescent animals

An alternative hypothesis regarding the role of the HPA axis in the vulnerability to addiction is that early exposure may sensitize the HPA axis. To test this hypothesis, more recently we turned to a different model, i.e., exposure to alcohol during adolescence. Indeed, as adolescence is a time when the brain is still developing and when alcohol drinking is initiated (Substance Abuse Mental Health Service Administration (SAMHSA), 2004), it is of great importance to better understand both the mechanisms through which this drug influences the young brain (and to identify mechanisms that might be different from those operative in adults), and to investigate the short- and long-term consequences of alcohol exposure in the adolescent brain.

In a recent review, Spear and Varlinskaya (2010) reported that adolescents appear to be more sensitive than their adult counterparts to the positive rewarding effects of alcohol, and are also less sensitive to its aversive properties. The authors further suggested that these sensitivities to alcohol may be exacerbated by a previous history of alcohol or stress exposure as well as by genetic vulnerabilities, thus leading to a relatively high usage of alcohol during adolescence, and perhaps an increased likelihood for the manifestation of abuse disorders. The effects of alcohol exposure during adolescence are quite diverse and may involve impairment of neurogenesis (Morris et al., 2010), and changes in the mesolimbic dopaminergic and glutamatergic systems (Pascual et al., 2009), all of which could contribute to increased alcohol intake in the adult rat (Pascual et al., 2009). For example, Truxell and colleagues (2007) reported that prior juvenile (post-natal day (PND) 22–28), adolescent (PND 30–34) or adult (PND 60–64) experience with alcohol affected future alcohol consumption. In the same study, when comparing juveniles with adults, they showed that the former were predisposed to consume solutions with high alcohol

concentrations (30% v/v), and that blood alcohol levels (BALs) obtained during the initial exposure influenced subsequent alcohol preference (Truxell et al., 2007). Other groups examined the factors that influence elevated alcohol consumption in adolescent rats compared to adult rats, and found that young animals have an even greater intake under a number of test conditions [i.e., isolate-housing versus pair-housing, type of sipper tube, caloric value of solution, prior experimental perturbations (Doremus et al., 2005)]. In addition, voluntary alcohol intake by adult male and female rats was enhanced by repetitious binge alcohol administration during adolescence (Maldonado-Devincci et al., 2010a). Collectively, these observations suggest that adolescent rats may spontaneously drink more than adults, and that drinking during adolescence can influence the propensity to drink alcohol in adulthood.

Other age-dependent effects of alcohol include the fact that binge drinking by adolescent rats appears to cause more differential brain damage than in adults (Crews et al., 2000). In addition, long-lasting changes in functional brain activity have been observed following a relatively brief exposure to high levels of alcohol during a period corresponding to parts of adolescence [for review see (Ehlers and Criado, 2010)]. The use of high doses of alcohol in chronic intermittent alcohol injections administered to adolescents produces a variety of effects, including some that have long-lasting consequences (Silvers et al., 2003). Specifically, rats have decreased weight gain, increased metabolic rates, and also develop a tolerance to the cognitive impairing and hypnotic effects of alcohol. In another study, after six weeks of drinking that began in late adolescence, Evrard and colleagues (2006) found alcohol-induced morphological changes in astrocytes and neurons, and observed that after a 10-week abstinence period, the drinking cessation only partially improved the alterations caused by adolescent drinking. There is also evidence of age-related differences in sedative response (sleep time and righting reflex) and cortical electroencephalogram (EEG) measurements following acute alcohol administration to adolescent and adult rats. In particular, alcohol differentially effected power in EEG bands of adolescent rats that were not influenced in adults, and adolescent rats had higher BALs and a significantly shorter sleeping time after regaining the righting reflex than adults (Pian et al., 2008). In a symposium on adolescent vulnerabilities to chronic alcohol or nicotine exposure (Barron et al., 2005), it was suggested that adolescent exposure to chronic alcohol may alter brain development (in terms of motor function and memory formation) in long-lasting ways. In the same symposium, vapor exposure to alcohol in adolescence was reported to decrease N1 amplitude of event-related potentials (ERPs) in the cortical region six weeks after termination of alcohol exposure and it was suggested that alcohol exposure may cause long-term learning and memory deficits. Overall, recent findings point to a major influence of exposure to alcohol during adolescence upon long-term brain development and function (Table 1).

Stress may also play a differential role in adolescent versus adult drinking. For example, Brunell and Spear (2005) examined the effect of stress on self-administration of a sweetened alcohol solution in pair-housed adolescent and adult rats, and found that the young animals were more sensitive to the interactions of alcohol and stress than adults, and that chronic stress disrupted the elevated alcohol intake in pair-housed adolescent rats but not in adults. Also, adaptation to a footshock stressor was disrupted in young, but not older animals (Brunell and Spear, 2005). Two studies from the same laboratory examined the influence of age when alcohol drinking was initiated, in relation to alcohol deprivation and stress, upon the long-term self-administration of alcohol in both sexes (Fullgrabe et al., 2007; Siegmund et al., 2005). In terms of stress-induced drinking, the authors found that both male and female rats that were exposed to alcohol during adolescence with long-term continuation into adulthood may be more susceptible to stress-induced alcohol consumption in adulthood

(Fullgrabe et al., 2007; Siegmund et al., 2005). Adolescents, therefore, seem to be more susceptible to the effects of stress on alcohol drinking.

While the HPA axis stress-response system is expected to be altered by alcohol in adolescent animals as it is in adults [though whether the drug is self-administered or investigator-controlled likely plays a role (Ogilvie et al., 1997)] at present there is no specific information in this regard with the exception of a recent study performed in our laboratory (see below). We know that HPA axis function in adolescence, particularly in response to stressors, is different from that of adults in that adolescents display a prolonged activation of HPA function following a stressful event (McCormick and Mathews, 2007); although the effects may be stressor- and sex-specific, adolescent exposure to stressors may alter adult cognitive performance and behavioral reaction to drugs (McCormick and Mathews, 2007). In another study by the same group, the programming of adult learning and memory in relation to adolescent development and HPA function was investigated, and the authors found that in contrast to the effects of chronic stress on adult cognitive function, a remodeling of the developing nervous system can occur following exposure to glucocorticoids released from the HPA axis during adolescence. Ultimately, this can produce enduring changes in adult cognitive function (McCormick and Mathews, 2010). Recent findings from our laboratory indicate that administration of an alcohol challenge (4.5 g/kg, ig) in adulthood following exposure to intermittent alcohol vapors during adolescence (PND 40 mean $\pm$ SEM BAL = 212.8 $\pm$ 5.69 mg %) blunted PVN CRF messenger RNA (mRNA) expression and significantly increased in the number of PNMT-ir cells/brain stem section in the C2 area in comparison to air-exposed controls (Allen et al., 2011). These studies suggest that stressors can influence adolescent HPA axis function, which can point to a potential similar influence by alcohol as alcohol itself is considered a stressor since it activates the HPA axis.

We recently performed a series of experiments aimed at providing a mechanistic understanding of the short- and long-term effects of adolescent binge drinking on the HPA axis, with an emphasis on the brain structures that might be involved in drug self-administration and anxiety. As noted above, the central circuits known to regulate the HPA axis include the PVN of the hypothalamus, the limbic regions involved in anxiety, and the adrenergic and noradrenergic brain stem regions that provide catecholamine inputs to the hypothalamus. Alcohol exposure alters CRF activity in the PVN (Rivier et al., 1984; Rivier et al., 1990) and the amygdala (Funk et al., 2006), with reported increases in PVN neuronal activity (Lee et al., 2000), as well as changes in CRF release from the central nucleus of the amygdala (CeA) in response to stressors (Pich et al., 1993; Wills et al., 2010). In this regard, it is important to distinguish between the response of a brain structure to alcohol itself, and the consequence of an initial alcohol exposure on the HPA axis' ability to respond to an additional, subsequent stressor. For example, we had reported that the PVN neuronal response to mild electro-footshocks (Lee et al., 2000) or an ig alcohol challenge (Ogilvie et al., 1997) was decreased immediately following footshock or alcohol exposure. Additionally, as discussed above, we also observed a significant blunting of the HPA axis for several days, if not weeks, following an initial alcohol treatment. These results prompted us to investigate the consequences of exposing adolescent rats to supersac (3% w/v glucose and 0.125% w/v saccharin) or supersac + alcohol in a self-administration paradigm (binge drinking) on the effects upon the adolescent brain (Group A), or the adult stress-response to shocks (Group B; all animals were given two 0.35 mA shocks/min for 30 min) or alcohol injection (Group C; all animals were administered 4.0 g/kg alcohol ig). The protocols were as follows: the animals were provided standard rodent chow and water *ad libitum*, except during the first three self-administration training sessions (PND 25–27) when water was not offered. All operant chamber procedures were conducted during the dark cycle of a 12 h light/12 h dark cycle with lights off at 1800. For the operant chambers and the development

of binge-like self-administration, male rats were trained to lever press for alcohol using a variation of the previously used saccharin free-choice operant conditioning paradigm, using the same operant alcohol self-administration components (Ji et al., 2008). This paradigm previously produced pharmacologically relevant BALs (84.0 mg %) that are as high as the BAL criterion for binge drinking in humans (National, Institute on Alcohol Abuse and Alcoholism, 2004).

In all groups, the binge-drinking animals ($n = 6$) with the highest BALs were compared to 6 supersac controls. Immediately after a binge session (1800, 2000, 2200 or 2400) on PND 40–42, the overall mean $\pm$ SEM (range) BAL ($n = 18$) for all three groups was 81.7 ± 6.3 (32.7 – 139.7) mg %. There was no group difference in the weights of the control or binge-drinking rats in adolescence or in adulthood (Table 2). In adolescent rats (Group A), the number of CRF-ir cells per section was significantly ($P=0.0133$) decreased in the CeA of binge drinking animals, compared to controls. However, there was no effect on the number of colocalized *c-fos*-ir and CRF-ir cells/section in the CeA (Fig. 3, $P>0.05$). Similarly, there was no statistical significance ($P>0.05$) between controls and binge drinking animals in any other parameter measured during adolescence (Tables 3–9). When adult rats that had consumed alcohol during adolescence were subjected to an emotional stressor in adulthood (footshocks, Group B), there were no significant differences ($P>0.05$) between the alcohol-exposed and control rats in terms of CRF peptide levels, CRF mRNA levels and catecholaminergic enzymes in any of the brain regions we examined (Tables 3–9). Overall, the most impressive finding was observed in response to an alcohol challenge in adulthood (Group C) in one of the three examined adrenergic brain stem regions that project to the hypothalamus. Specifically, we observed a significant ($P=0.0249$, Fig. 4) decrease in the number of colocalized *c-fos*-ir and PNMT-ir cells/brain stem section in the C3 area of adult rats administered an alcohol challenge (4.0 g/kg, ig) in adulthood that had previously self-administered alcohol during adolescence, compared to their supersac controls. In contrast, there were no changes in PNMT-ir cells/section in the C3 region (Fig. 4, $P>0.05$). Similarly, there were no group differences in all other parameters measured between controls and binge drinking animals (Tables 3–9). The novel finding of changes in the C3 region following administration of an alcohol challenge to adult animals that self-administered alcohol during adolescence may provide a rationalization for the functional characterization of this brain region in the adolescent binge drinking model.

Collectively, our results as well as those provided by others, indicate that adolescent rats tend to drink more alcohol than their adult counterparts (Truxell et al., 2007); that alcohol self-administration causes different effects in adolescent and adult rats [i.e., adolescent rats are likely to drink more in adulthood following exposure during adolescence (Maldonado-Devincci et al., 2010b; Pascual et al., 2009; Truxell et al., 2007)]; that drinking alcohol during adolescence has detrimental effects on brain development and function (Barron et al., 2005; Ehlers and Criado, 2010; Evrard et al., 2006; Pascual et al., 2009); and that it influences later response to stressors (Brunell and Spear, 2005). Here we show that adolescent self-administration, which resulted in relatively modest BALs, induced significant changes in the catecholaminergic C3 region, but did not alter the adult HPA axis response to an acute challenge (alcohol injection or footshocks). In contrast, we recently reported a significant blunting of the HPA axis in response to an alcohol challenge in adult rats that had been exposed to alcohol vapors during adolescence, a phenomenon that was not observed in our model of self-administration (Allen et al., 2011). It is, therefore, possible that the amount of alcohol to which the animals are exposed plays a role in the long-term consequences exerted by this drug because BALs of the vapor-exposed group were over two times greater than those of rats that self-administered alcohol. However, in the alcohol vapor-exposed group, a significant increase in the number of PNMT-ir cells/brain stem section in the C2 area was found (Allen et al., 2011). The changes found in the C3 and C2

brain stem regions of rats that self-administered alcohol or were subjected to alcohol vapors during adolescence, respectively, suggests that central catecholamines may represent important targets as well as mediators of the effects of alcohol on the HPA axis.

Amygdalar CRF has been suggested to play a role in the aversive and anxiogenic effects of stress and/or alcohol withdrawal (Koob, 2003). For example, Zorilla and colleagues (2001) had shown an initial decrease in CRF-like content in the CeA of alcohol-dependent rats followed by a prolonged increase during six weeks of alcohol withdrawal. Similarly, a progressive increase in CRF-ir was found in the CeA of alcohol-dependent rats during withdrawal (Pich et al., 1995). These findings supported the importance of the amygdala in conveying the emotional component of the stress response (Pich et al., 1995). Another study of alcohol self-administration found that, in alcohol-dependent animals, excessive alcohol consumption was reversed by systemic administration of a CRF1 antagonist and intracerebral administration of a peptide CRF antagonist, DPhe 12–41, into the CeA (Funk and Koob, 2007; Funk et al., 2006), and also could be reversed by administration of a CRF2 agonist into the CeA (Funk and Koob, 2007). The results of our adolescent binge drinking study pointed to a slight, but significant, blunting effect on the number of CRF-responsive cells in the CeA of adolescent rats. It is possible that these animals experienced the effects of alcohol withdrawal as a stressor, as evidenced by the disrupted CRF activation in the CeA when they were sacrificed the morning after their last nightly binge session. The combined results of all of these experiments suggest that the limbic regions involved in stress and anxiety might be influenced by adolescent exposure to alcohol, and that the consequences of alcohol withdrawal on the CeA may be different from that of adults. In view of the fact that binge drinking during adolescence may exert short- and long-term effects on the central circuits known to regulate the HPA axis and the stress-response through the adrenergic brain stem regions that provide catecholamine inputs to the hypothalamus and the limbic regions, future studies are required to further investigate the role of the limbic system and the catecholaminergic brain stem regions, in modulating the immediate as well as future consequences of binge drinking during adolescence. One possibility is that in adolescents, the plasticity in the HPA axis is rapidly transferred to plasticity in limbic regions.

Conclusions

While the new results presented are of great potential interest, at present the mechanisms that govern them remain unclear and are under current investigation. Nevertheless, the new findings include: (a) decreased number of DBH-ir cells in the A1/C1, A2/C2, and C3 regions of the brain stem and a decreased number of PVN CRF-ir cells following alcohol injection after brain lesion with anti-DBH saporin; (b) a blunting effect on CRF-ir cells in the CeA of adolescent rats that binge drank during adolescence; and (c) a decrease in the number of colocalized *c-fos* and PNMT-ir cells in the C3 region of the brain stem of adult rats subjected to an alcohol challenge in adulthood following binge drinking during adolescence. These outcomes suggest that interfering with the A1-A2/C1-C3 input to the PVN and the amygdala may be important in allowing these regions to respond to alcohol.

Overall, the data reviewed here indicate that as expected of any drug, alcohol exerts both short- and long-term effects and, that similarly to other stressors, prolonged consequences can be very long lasting. Hormones of the HPA axis modulate a vast array of biological responses, and receptors for these hormones are found in very diverse parts of the central nervous system. It is thus likely that by modifying the activity of this axis, alcohol will influence, and possibly compromise, the ability of the body to maintain or restore homeostasis and to coordinate appropriate behavioral responses in response to stressors. Our work also suggests that central catecholamines may represent important targets as well as mediators of the effects of alcohol on the HPA axis, and may have a key role in the

neuroplasticity of the HPA axis conveyed by alcohol in adults and adolescents. Further knowledge of the interaction of alcohol with the HPA axis and its mediators may lead to a better understanding of the etiology of stress in the development of alcohol abuse.

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Figure 1. Compared to rats without lesions, LC lesions abolish the ACTH response to alcohol (EtOH). Time = 0 indicates plasma ACTH levels (pg/mL) immediately prior to alcohol administration (4.0 g/kg, ig). Each point illustrates the means $\pm$ SEM of 5–7 rats. ★★: $P < 0.01$, when comparing EtOH/LC lesion vs. ACTH response in corresponding controls (EtOH only).

Figure 2.

(A) Lesions caused by the anti-DBH-saporin toxin significantly decreases the number of DBH-positive cells in the A1/C1, A2/C2, and C3 cell groups, compared to rats pretreated with the vehicle. Bright-field photographs through the A1/C1, A2/C2, and C3 areas of brain stem from sham controls (top) and lesion (bottom) rats challenged with alcohol (4.0 g/kg, ig). Images show a representation of the immunohistochemical techniques, which stained DBH-ir cells in brown (scale bar = 200 μ m). (B) Cell counts were obtained for DBH-ir positive cells in the A1/C1, A2/C2 and C3 areas of the brain stem. Mean $\pm$ SEM number of DBH-ir cells per section in the A1/C1, A2/C2 and C3 areas of sham and lesion rats injected with alcohol. $\star$: $P < 0.05$; $\star\star$: $P < 0.01$; $\star\star\star$: $P < 0.001$.

Figure 3.

Self-administered binge-like alcohol consumption during adolescence (PND 29–42) blunts the number of CRF immunoreactive (ir) cells in the CeA of adolescent rats (PND 43, Group A), but has no effect upon the number of colocalized *c-fos*-ir and CRF-ir cells. A: Double immunohistochemical procedure stained *c-fos* nuclei black and CRF cytoplasm brown. Display panels compare the two conditions and the pictures were taken with a 20X dry objective (scale bar = 10 μ m) and the inset a 40X dry objective; B: Mean $\pm$ SEM number of colocalized *c-fos* and CRF (top) or CRF (bottom) positive cells per section, $n = 6$ rats/group. ★★: $P < 0.01$.

Figure 4.

Self-administered binge-like alcohol consumption during adolescence (PND 29-42) decreases the number of colocalized *c-fos* and PNMT immunoreactive (ir) cells in the C3 region of the brain stem of adult rats subjected to a stressor (4.0 g/kg alcohol challenge via intragastric catheter, Group C), in adulthood (PND 70) but has no effect upon the number of PNMT-ir cells. A: Double immunohistochemical procedure stained *c-fos* nuclei black and PNMT cytoplasm brown. Display panels compare the two conditions and the pictures were taken with a 40X dry objective (scale bar = 10 μ m) and the inset with a 60X oil objective; B: Mean $\pm$ SEM number of colocalized *c-fos* and PNMT (top) or PNMT (bottom) positive cells per section, n = 6 rats/group. $\star$: $P < 0.05$.

Table 1

Summary of different models of adolescent alcohol exposure on brain damage

Reference	Animal Model	Brain Damage/Alteration
(Crews et al., 2000)	<ul style="list-style-type: none"> Sprague Dawley [postnatal day (PND) 35] 4 day binge ethanol treatment (e.g., 4 days of 4 times per day 15% ethanol intragastrically, approximately 9–10 g/kg/day alcohol) in rats examined in adolescence 	<ul style="list-style-type: none"> Olfactory bulbs Frontal cortical olfactory regions Anterior portions of the piriform and perirhinal cortices
(Ehlers and Criado, 2010)	<ul style="list-style-type: none"> Sprague Dawley (PND 30–40) 10 day alcohol vapor exposure for 12 h/day. Assessments at 6–7 weeks following alcohol exposure (adulthood) 	<ul style="list-style-type: none"> Disturbances in waking electroencephalogram (EEG) and a reduction in the P3 component of the event-related potential Long-lasting reductions in the mean duration of slow-wave sleep episodes and the total amount of time spent in slow-wave sleep
(Silvers et al., 2003)	<ul style="list-style-type: none"> Sprague Dawley (PND 30) 5.0 g/kg alcohol intraperitoneally (ip) or saline every 48 hours for 20 days. Following completion of pretreatment and training, animals were tested after receiving an ethanol (1.0, 1.5, or 2.0 g/kg) injection 	<ul style="list-style-type: none"> Attenuated alcohol-induced spatial memory deficits in the Morris Water Maze Task
(Evrard et al., 2006)	<ul style="list-style-type: none"> Wistar (PND 45–50) Ethanol 6.6% (v/v) in drinking water for 6 weeks and studied after ending exposure or after a 10-week recovery period 	<ul style="list-style-type: none"> 5-HT immunoreactivity (ir) decreased in the mesencephalic dorsal raphe nucleus and recovered after abstinence In the three prosencephalic areas (CA1 hippocampal area, striatum and frontal cortex), astrocytes' cell area (GFAP-ir cells) increased after alcohol exposure and tended to return to normality after abstinence, while cytoplasmic astroglial S100b protein-ir, relative area of MAP-2-ir and Nf-200-ir fibers decreased, and later partially recovered In the striatum and frontal cortex, nitric oxide synthase-ir decreased only after abstinence
(Pian et al., 2008)	<ul style="list-style-type: none"> Wistar (PND 36) Administration of 0, 0.75 1.5 or 3.5 g/kg alcohol ip prior to EEG recordings 	<ul style="list-style-type: none"> 1.5 g/kg increased power in the 1–2 Hz band and decreased the power in the 32–50 Hz band in the parietal cortical region 1.5 g/kg also increased stability of the EEG power in the slow-wave frequency bands (2–4 Hz, 4–6 Hz, and 6–8 Hz) Significantly shorter sleeping time and higher blood alcohol levels after regaining reflex
(Barron et al., 2005) Research of White and Swartzwelder.	<ul style="list-style-type: none"> Sprague Dawley (PND 30) One 5.0 g/kg ip injection Chronic-intermittent ethanol (CIE, 5.0 g/kg ip, every 48 h for 20 days) 	<ul style="list-style-type: none"> Working memory impairments during an alcohol challenge (1.5 g/kg ip) The impact of acute ethanol on motor coordination remained unchanged before, two days after, and 16 days after completion of CIE treatment
(Barron et al., 2005) Research of	<ul style="list-style-type: none"> Sprague Dawley (PND 30–45) 10 to 14 days of intermittent (12 hr/d; mean blood alcohol level, 250 mg/dl) 	<ul style="list-style-type: none"> Increased parietal cortical 1 to 2 Hz and hippocampal 16 to 32 Hz peak frequency in the EEG.

Reference	Animal Model	Brain Damage/Alteration
Slawecki and Ehlers.	vapor inhalation, effects examined in adulthood	<ul style="list-style-type: none">• Decreased cortical N1 amplitude

Summary of different models of adolescent alcohol exposure on brain damage.

Table 2

Weights of adolescent binge-drinking rats and supersac controls.

Treatment Group	Adolescent Weight (PND 42)	Adult Weight (PND 69)
A		
Supersac Control Rats	197.2 $\pm$ 7.6	Not Applicable
Binge-Drinking Rats	210.8 $\pm$ 7.2	Not Applicable
B		
Supersac Control Rats	213.2 $\pm$ 6.4	390.8 $\pm$ 13.4
Binge-Drinking Rats	215.3 $\pm$ 6.9	406.8 $\pm$ 6.0
C		
Supersac Control Rats	202.5 $\pm$ 4.3	348.5 $\pm$ 5.4
Binge-Drinking Rats	198.3 $\pm$ 8.0	350.7 $\pm$ 14.0

No statistical difference was found between the mean $\pm$ SEM weight (g) of adolescent binge-drinking rats and their controls on the last night of self-administration (PND 42, adolescent) or the night before the experimental day (PND 69, adult), n = 6/treatment per group.

Table 3

Number of CRF-ir or *c-fos*-ir and CRF-ir colocalized cells in the CeA per treatment group.

Treatment Group	CRF-ir cells per section in CeA	<i>c-fos</i> -ir/CRF-ir cells per section in CeA
B		
Supersac Control Rats	30.28 ± 2.17	0.26 ± 0.07
Binge-Drinking Rats	31.04 ± 3.03	0.32 ± 0.05
C		
Supersac Control Rats	55.44 ± 3.4	1.0 ± 0.27
Adolescent Binge Rats	51.56 ± 4.27	0.53 ± 0.15

No effect was found in the number of colocalized *c-fos*-ir/CRF-ir cells in either adult groups that self-administered supersac only (control) or supersac + alcohol (binge drinking) during adolescence, n = 6/treatment per group. Group B was subjected to 0.35 mA electro-footshocks in adulthood and Group C was given a 4.0 g/kg alcohol challenge via intragastric catheter in adulthood.

Table 4

Number of CRF-ir or *c-fos*-ir and CRF-ir colocalized cells in the PVN per treatment group.

Treatment Group	CRF-ir cells per section in PVN	<i>c-fos</i> -ir/CRF-ir cells per section in PVN
A		
Supersac Control Rats	66.21 ± 5.57	0.55 ± 0.2
Binge-Drinking Rats	59.03 ± 4.34	0.49 ± 0.09
B		
Supersac Control Rats	44.28 ± 4.5	2.99 ± 0.45
Binge-Drinking Rats	37.62 ± 2.84	4.26 ± 1.08
C		
Supersac Control Rats	42.66 ± 9.4	7.12 ± 0.73
Binge-Drinking Rats	32.27 ± 4.8	7.66 ± 1.69

Self-administered binge-like consumption of alcohol during adolescence does not influence the number of CRF or colocalized *c-fos*/CRF immunoreactive (ir) cells in the PVN of adolescent (Group A) or adult rats subjected to different stressors in adulthood (Group B: 0.35 mA electro-footshock or Group C: 4.0 g/kg alcohol challenge via intragastric catheter), n = 6/treatment per group.

Table 5

CRF mRNA expression in the PVN and CeA per treatment group.

Treatment Group	CRF mRNA Expression in PVN (arbitrary units, % of control)	CRF mRNA Expression in CeA (arbitrary units, % of control)
A		
Supersac Control Rats	100.0 ± 10.56	100.0 ± 11.64
Binge-Drinking Rats	79.05 ± 7.73	98.9 ± 17.0
B		
Supersac Control Rats	100.0 ± 12.62	100.0 ± 18.37
Binge-Drinking Rats	94.96 ± 10.75	78.69 ± 7.65
C		
Supersac Control Rats	100.0 ± 3.24	100.0 ± 8.81
Binge-Drinking Rats	111.0 ± 7.22	113.8 ± 9.63

Self-administered binge-like consumption of alcohol during adolescence does not influence the expression of CRF mRNA (arbitrary units expressed as % of control) in the PVN or CeA of adolescent (Group A) or adult rats subjected to different stressors in adulthood (Group B: 0.35 mA electro-footshock or Group C: 4.0 g/kg ethanol challenge via intragastric catheter), n = 6/treatment per group, except n = 5 supersac control rats in Group B.

Table 6

Number of PNMT-ir or *c-fos*-ir and PNMT-ir colocalized cells in the C1 region of the brain stem per treatment group.

Treatment Group	PNMT-ir cells per section in C1	<i>c-fos</i> -ir/PNMT-ir cells per section in C1
A		
Supersac Control Rats	48.39 ± 5.5	1.44 ± 0.29
Binge-Drinking Rats	40.20 ± 5.41	1.06 ± 0.18
B		
Supersac Control Rats	22.89 ± 1.88	1.94 ± 0.32
Binge-Drinking Rats	21.16 ± 2.74	2.28 ± 0.47
C		
Supersac Control Rats	26.69 ± 2.17	5.72 ± 1.0
Binge-Drinking Rats	29.19 ± 2.51	6.36 ± 0.9

Self-administered binge-like consumption of alcohol during adolescence does not influence the number of PNMT or colocalized *c-fos*/PNMT immunoreactive (ir) cells in the C1 region of the brain stem of adolescent (Group A) or adult rats subjected to different stressors in adulthood (Group B: 0.35 mA electro-footshock or Group C: 4.0 g/kg alcohol challenge via intragastric catheter), n = 6/treatment per group.

Table 7

Number of PNMT-ir or *c-fos*-ir and PNMT-ir colocalized cells in the C2 region of the brain stem per treatment group.

Treatment Group	PNMT-ir cells per section in C2	<i>c-fos</i> -ir/PNMT-ir cells per section in C2
A		
Supersac Control Rats	15.61 ± 2.71	0.75 ± 0.14
Binge-Drinking Rats	10.61 ± 1.01	0.81 ± 0.15
B		
Supersac Control Rats	8.53 ± 0.69	0.28 ± 0.08
Binge-Drinking Rats	9.97 ± 0.41	0.5 ± 0.16
C		
Supersac Control Rats	9.89 ± 0.45	1.22 ± 0.3
Binge-Drinking Rats	10.39 ± 1.26	1.47 ± 0.21

Self-administered binge-like consumption of alcohol during adolescence does not influence the number of PNMT or colocalized *c-fos*/PNMT immunoreactive (ir) cells in the C2 region of the brain stem of adolescent (Group A) or adult rats subjected to different stressors in adulthood (Group B: 0.35 mA electro-footshock or Group C: 4.0 g/kg ethanol challenge via intragastric catheter), n = 6/treatment per group.

Table 8

Number of PNMT-ir or *c-fos*-ir and PNMT-ir colocalized cells in the C3 region of the brain stem per treatment group.

Treatment Group	PNMT-ir cells per section in C3	<i>c-fos</i> -ir/PNMT-ir cells per section in C3
A		
Supersac Control Rats	11.97 ± 1.49	0.87 ± 0.15
Binge-Drinking Rats	10.27 ± 1.41	0.88 ± 0.14
B		
Supersac Control Rats	5.58 ± 0.8	0.28 ± 0.06
Binge-Drinking Rats	6.67 ± 0.64	0.28 ± 0.08

No effect was found upon the number of PNMT-ir cells or colocalized *c-fos* and PNMT-ir cells in either group, n = 6/treatment per group. Group A was sacrificed immediately after adolescent binge-drinking and Group B was subjected to 0.35 mA electro- footshocks in adulthood.

Table 9

Number of DBH-ir or *c-fos*-ir and DBH-ir colocalized cells in the LC region of the brain stem per treatment group.

Treatment Group	DBH-ir cells per section in LC	<i>c-fos</i> -ir/ DBH -ir cells per section in LC
A		
Supersac Control Rats	64.63 ± 8.0	1.01 ± 0.28
Binge-Drinking Rats	49.13 ± 2.45	0.65 ± 0.21
B		
Supersac Control Rats	33.75 ± 2.63	6.72 ± 1.17
Binge-Drinking Rats	29.37 ± 2.95	8.80 ± 1.91
C		
Supersac Control Rats	42.56 ± 3.71	2.55 ± 0.39
Binge-Drinking Rats	44.61 ± 5.89	2.22 ± 0.68

Self-administered binge-like consumption of alcohol during adolescence does not influence the number of DBH or colocalized *c-fos*/DBH immunoreactive (ir) cells in the LC region of the brain stem of adolescent (Group A) or adult rats subjected to different stressors in adulthood (Group B: 0.35 mA electro-footshock or Group C: 4.0 g/kg alcohol challenge via intragastric catheter), n = 6/treatment per group.