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Enhancement of the hypothalamic-pituitary-adrenal axis but not cytokine responses to stress challenges imposed during withdrawal from acute alcohol exposure in Sprague-Dawley rats

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Abstract

Rationale—Alcohol withdrawal is associated with reduced activity, increased anxiety, and other signs of distress.

Objective—The goal of the current studies was to determine whether acute ethanol exposure would alter hypothalamic-pituitary-adrenal (HPA) axis reactivity and cytokine responses to stress challenges imposed during the withdrawal period.

Methods—Male Sprague-Dawley rats were intubated with 4g/kg of ethanol to simulate acute binge-like ethanol intake. After characterizing the blood ethanol concentrations (BECs; Experiment 1), exploratory activity in a novel environment was explored at 10, 14 and 18 hr after ethanol (Experiment 2) to characterize altered activity patterns indicative of withdrawal. In Experiment 3, rats were exposed to footshock during withdrawal to examine whether prior ethanol exposure would alter cytokine and HPA axis responses to stress. Experiments 4–5 investigated HPA axis sensitivity and gene expression changes during restraint imposed during withdrawal.

Results—Prior ethanol exposure produced a period of stress hyper-reactivity evidenced by an enhanced HPA axis response (increased corticosterone and adrenocorticotrophic hormone) observed during withdrawal. While this hyper-reactivity in response to two different stress challenges (novel environment and restraint) was accompanied by profound behavioral changes indicative of withdrawal, no alterations in cytokine changes evoked by stress were observed.

Conclusions—Taken together, these findings provide support for the hypothesis that alcohol withdrawal enhances HPA axis reactivity to stress challenges, though not likely not as the result of heightened inflammatory signaling, and may have implications for understanding the mechanisms by which stress impacts relapse drinking in humans.

Keywords

Ethanol; Stress; HPA axis; Corticosterone; ACTH; Cytokine; Paraventricular nucleus; Alcohol; Intragastric; Rat

Alcoholism affects approximately 17.6 million people in the United States (NIAAA 2007) and while ethanol withdrawal occurs in about 15–20% of primary care and hospitalized patients (Mayo-Smith 1997), some form of withdrawal occurs in 50% of people who consume 2 or more alcoholic beverages (Swift and Davidson 1998). Withdrawal symptoms are associated with negative mood (De Witte et al. 2003; Bayard et al. 2004) and can range

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from hypertension, sweating, tremor, anxiety, agitation, seizures, hallucinations, and disorientation to delirium tremens, with severe cases even leading to death (Monte et al. 2010). In contrast, the term ‘hangover’ typically refers to withdrawal symptoms incurred by a single ethanol exposure in rodents (Varlinskaya and Spear 2004) or an acute bout of drinking in humans (Wiese et al. 2000).

Withdrawal symptoms and/or hangover peak as ethanol is eliminated from the body and wane over several days (Becker 2000; Swift and Davidson 1998). Symptoms of withdrawal in animal models have been well characterized and similar signs are observed in humans. For example, animals undergoing ethanol withdrawal will display tail stiffness, muscle spasms, body rigidity (Friedman 1980); exacerbation of seizures (e.g., Becker 2000; Finn and Crabbe 1997; Friedman 1980); avoidance of bright, open spaces (e.g., File et al. 1992; Koob and Britton 1996); decreased social activity (Varlinskaya and Spear 2004); and hypoactivity across a range of behavioral tasks (Logan et al. 2010; Overstreet et al. 2004; Zhang et al. 2007). Though a variety of ethanol administration procedures are often employed in rodent models, the symptoms of withdrawal do not appear to vary as a function of route of ethanol administration per se. Instead, it is commonly thought that the magnitude of the withdrawal response relates predominantly to the quantity and duration of ethanol exposure (Becker 2000).

Ethanol withdrawal itself can be a stressful event (Shaham et al. 2003; De Witte et al. 2003; Adinoff 1994; Adinoff et al. 1991), as evidenced by activation of the hypothalamic-pituitary-adrenal (HPA) axis. People undergoing acute ethanol withdrawal have higher levels of cortisol, and the magnitude of the increased cortisol response appears to be proportional to the severity of the withdrawal (De Witte et al. 2003; Roberts and Keith 1995). It has also been observed that high levels of corticotropin releasing hormone (CRH) can affect withdrawal severity (Menzaghi et al. 1994). For instance, intracerebral administration of a CRH antagonist blocked the enhanced stress sensitivity seen during ethanol withdrawal (Menzaghi et al. 1994). Although the relationship between stress and alcohol consumption remains controversial, there is limited evidence to suggest that the withdrawal period may also be an important window of vulnerability for relapse (for review see Sinha 2001). For example, imposing a stress challenge (5-min personalized stress imagery) on abstinent alcoholics increased alcohol craving, with patients more prone to stress-induced craving also drinking more alcohol after leaving in-patient treatment (as described in Breese et al. 2005a). Similarly, exposure to novel stressors, such as footshock, has been shown to increase ethanol-seeking behavior in abstinent rats (Le et al. 1998). Given the potential for enhanced stress sensitivity during ethanol withdrawal, one goal of the following series of studies was to characterize a simple model of ethanol exposure that could be used to investigate possible alterations in reactivity to later stress challenges, where both HPA axis and cytokine responses to stress could be examined during withdrawal from an acute ethanol challenge.

Prior work from our laboratory and others suggests that cytokine expression may be influenced by ethanol. In humans, the brain tissue of chronic alcoholics has been found to have higher levels of monocyte chemoattractant protein-1 (MCP-1), a chemokine, as well as increased microglial activation in the midbrain, ventral tegmental area (VTA), and cingulate cortex compared with healthy donors (He and Crews 2008). In rodent models, we recently demonstrated that the expression of Interleukin-1 β (IL-1 β) and Interleukin-6 (IL-6) in brain tissue was profoundly influenced by an acute dose of ethanol (4g/kg i.p.), with IL-6 exhibiting peak expression during acute intoxication and IL-1 β gene expression, in contrast, peaking during withdrawal from acute ethanol exposure (unpublished data). Moreover, Valles et al. (2004) examined cytokine expression in rat cortex and cultured astrocytes following exposure to chronic ethanol, with IL-1 β and cyclooxygenase-2 (COX-2; an enzyme

triggered by inflammatory stimuli) expression up-regulated by the long-term ethanol exposure. The impact of ethanol administration on basal cytokine expression is important in the present context because cytokines have been shown to be an important mechanism by which the HPA axis becomes sensitized (Johnson et al. 2002). Since exposure to stress alone (e.g. footshock) has been shown to increase cytokine expression (Deak et al. 2005; Blandino et al. 2006; Blandino et al. 2009), it was hypothesized that cytokine responses evoked by stress would be exacerbated during acute withdrawal as a potential mechanism to explain the enhanced HPA axis response often observed during this period.

To examine this possibility, a single 4g/kg i.g. dose was chosen, with blood ethanol concentrations (BECs) reaching approximately 175–225 mg/dL (Brasser and Spear 2002). Though this dose is on the high end of what is defined as binge exposure by NIAAA (NIAAA 2010), these levels are well within the range of BECs that are often used in rodent models, which can reach 400–450 mg/dL with an i.p. injection (Doremus et al. 2003; Varlinskaya and Spear 2004). As such, these studies began with a detailed characterization of the BEC curve produced by a 4g/kg dose (i.g.) of ethanol (Experiment 1). The timing of peak withdrawal-related behaviors (evidenced by reduced locomotor activity in an open field) was then assessed at several time points after ethanol clearance (Experiment 2). Experiment 3 examined whether prior ethanol exposure would differentially alter HPA axis and cytokine responses evoked by footshock (80 shocks at 1.0 mA) in the paraventricular nucleus of the hypothalamus (PVN), bed nucleus of the stria terminalis (BNST), adrenal, and liver. The PVN and adrenal were chosen because of their role in the HPA axis; the BNST was examined because of its role in coordinating behavioral responses to stress (Walker and Davis 1997) and evidence that the BNST, especially the dorsal portion, is involved in withdrawal-related anxiety (Huang et al., 2010). The liver was selected due to its role in processing ethanol as well as its sensitivity to ethanol effects, such as increased IL-6 expression (Schaffert et al. 2010). The goal of Experiment 4 was to more thoroughly examine the time-course of HPA axis reactivity by imposing restraint stress (60 min) during the withdrawal period, while Experiment 5 (also using restraint stress; 15 minutes) investigated whether alterations in HPA axis reactivity observed during withdrawal would be seen in cytokine expression changes as well.

General Methods

Subjects

Adult male Sprague-Dawley rats (300–350 g) were purchased from Harlan (Frederick, Maryland) and given two weeks to acclimate prior to experimentation. Colony conditions were maintained at $22 \pm 1^\circ\text{C}$ with 14:10 light:dark cycle (lights on 06:00–20:00 h). Animals were pair-housed in standard Plexiglas bins and had *ad libitum* access to food and water. In all experiments rats were handled for 3–5 min for two days prior to experimentation. For all studies, the time of injection was varied so that dependent measures would be collected at the same time of day, since most of the dependent measures examined herein are known to show circadian fluctuations. All experimental procedures were approved by the Institutional Animal Care and Use Committee at Binghamton University and animals were treated in accordance with the National Institutes of Health's guidelines for animal care (NIH Publication No.: 80–23, revised 1996).

Tissue collection and RNA extraction

Tissue was harvested through rapid decapitation (unanesthetized) and trunk blood collected into EDTA-coated Vacutainers containing 50 μL of aprotinin (Sigma). Plasma was separated through refrigerated centrifugation within 30 minutes. Plasma was aliquoted and stored at -20°C until time of assay. Brains were quickly collected, placed in ice-cold saline for

approximately 1 min, sliced into 2 mm sections, and then placed in RNAlater. Brains were stored at 4°C for 24 hours and then moved to -20°C until structures (PVN and BNST) were microdissected. Peripheral tissues (liver and adrenal) were quickly harvested and dissected on a cold plate, preserved in RNAlater, stored at 4°C for 24 hours, and then moved to -20°C until the time of RNA extraction.

Total cellular RNA was extracted from tissue as described previously (Blandino et al. 2009; Hueston et al. 2011). Total RNA yield and purity were determined using the Experion Automated Electrophoresis System (Bio-Rad). Synthesis of cDNA for was performed on 0.1–1.0 µg of normalized total RNA using the QuantiTect® Reverse Transcription Kit (Cat No. 205313, Qiagen, Valencia, CA) which included a DNase treatment step. cDNA amplification was performed in a 20 µL reaction consisting of 10µl IQ SYBR Green Supermix (Bio-Rad), 1 µL primer (final concentration 250 nM), 1 µL cDNA template, and 8 µL RNase-free water run in duplicate in a 96 well plate (BioRad) and captured in real-time using the iQ5 Real-Time PCR detection system (BioRad). The housekeeper gene, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), was measured in all experiments, though it was treated as a separate target gene (and displayed as a separate target in Table 2). In order to assess alterations in gene expression, therefore, GAPDH and all other targets of interest were adjusted relative to an ultimate control group within each experiment. Thus, instead of a $2^{-\Delta\Delta CT}$ transformation (Livak and Schmittgen 2001), gene expression was calculated utilizing a $2^{-\Delta CT}$ transformation of data, with the home cage vehicle-exposed animals serving as the baseline level of gene expression to which all other experimental groups were compared. Primers were designed using the NCBI primer blast (Table 1).

Measurement of Corticosterone (CORT)

Our lab has routinely measured and reported plasma CORT concentrations via radioimmunoassay (RIA) over the past decade. Since the antibody used for measurement of CORT in this RIA is no longer commercially available, CORT measures were performed via enzyme immunoassay (EIA) in some of these studies. Though baseline levels and relative treatment effects were comparable across experiments where different assays were employed, peak values were notably different across experiments. For this reason, comparisons of peak values across experiments should not be made. It is noteworthy to mention, however, that our peak values for stressor exposure are comparable to those reported previously with RIA (e.g., Deak et al. 2005) or EIA (Hueston et al. 2011).

Quantitative determination of plasma CORT levels was assessed by a commercially available CORT EIA kit (Assay Designs; Ann Arbor, MI) for Experiments 2 and 3. Samples were diluted 1:30 and heat inactivated to denature endogenous corticosteroid binding globulin (CBG) by immersion in 75°C water for 60 min, which produces a much more reliable and uniform denaturation of CBG than the enzyme cleavage step provided by the kit (unpublished observations). The ELISA had a sensitivity of 27.0 pg/ml and inter-assay and intra-assay coefficients of 5.65% and 3.15% respectively.

For the RIA (Experiments 4 and 5), total plasma CORT levels were measured using rabbit antiserum (antibody B3-163; Endocrine Sciences, Tarzana, CA, USA) as in our previous work (Deak et al. 2003; 2005). This antiserum has very low cross reactivity with other glucocorticoids and their metabolites. The intra-assay coefficient was 6.14%. Using a sample volume of 20 µl, the assay sensitivity was 0.5 µg/dl.

ELISA for Adrenocorticotrophic hormone (ACTH)

Quantitative determination of plasma ACTH levels was assessed by a commercially available ACTH ELISA kit (MD BioSciences; St Paul, MN) according to manufacturer's

instructions. The ACTH assay was run on the first thaw of the aliquoted blood. The ACTH assay had a sensitivity of 0.46 pg/ml, an inter-assay coefficient of 9.05%, and an intra-assay coefficient of 4.51%.

Blood ethanol concentrations (BECs)

All BECs were determined in 5 µl aliquots using an Analox AM-1 alcohol analyzer (Analox Instruments, Lunenburg, MA). The machine was calibrated every 15 samples using a 100 mg% industry standard, with BECs recorded in mg/dL (mg%). The floor of assay sensitivity using the Analox is ~12–15 mg/dL, as evidenced by background BEC measurements obtained from rats never exposed to ethanol. As such, measurements at or below those observed in vehicle-injected rats should be interpreted as zero values.

Drugs

Ethanol (95%, VWR International, West Chester, PA) was diluted fresh daily with tap water to make a 20% (v/v) solution. Ethanol injections were administered i.g. at 4 g/kg. Vehicle intubations consisted of equivolume tap water and cage mates always received identical drug treatments.

Experimental Methods and Results

Experiment 1: Blood Ethanol Concentration Curve

The first experiment sought to establish peak BECs as well as clearance of ethanol following a 4g/kg dose of ethanol. Rats (n=8 per group; N=24 total) were injected (i.g.) with 4 g/kg ethanol (20%) at 1900 h (one hour prior to lights off) and placed back in their home cages. Animals were split into three groups such that blood was collected from each subject three or four times - but never in consecutive hourly sampling - in order to limit the amount of blood taken from each rat in a 24 hour period (typically 50 µl or less). Cage mates were sampled at the same time points to avoid any potential effects of isolation, with each rat removed from its home cage, placed into a restraint tube, blood collected via the tail clip method, and then immediately replaced back into its home cage. Blood collections occurred at 0, 1, 2, 3, 4, 5, 6, 7, 8 and 14 hrs post-injection.

Results

In order to determine peak BECs as well as clearance from ethanol, results were analyzed with a one-way ANOVA and post-hoc tests performed using a Fisher's LSD test. As expected, an effect of time on blood ethanol concentration was found [$F(9, 107) = 43.9$ $p < 0.001$]. Post-hoc tests revealed that hours 1 through 8 were significantly different from baseline (hour 0), while hour 14 was not (Figure 1), indicating that ethanol had fully cleared by 14 hr. All other time points were significantly different from hour 3, indicating hour 3 was the peak of BECs following this 4g/kg i.g. intubation of ethanol.

Experiment 2: Behavioral characterization of withdrawal following 4 g/kg ethanol

Results from Experiment 1 demonstrated clearance of the 4g/kg ethanol bolus at 14 hrs. The goal of Experiment 2, therefore, was to examine behavioral patterns in an activity chamber at several time points following ethanol clearance in order to identify a possible peak withdrawal. Rats (n=8–10 per group, N=34 total) were administered 4 g/kg ethanol or tap water 10, 14, or 18 hrs prior to placement in locomotor activity chambers. To minimize animal use, a single group of vehicle-injected rats were used, where 2–4 rats were tested at each time point and collapsed into a single vehicle-injected control for analysis. At 900 h (10, 14 or 18 hrs post-intubation), rats were placed in locomotor activity chambers for 15

min. Immediately afterwards, rats were removed from the chambers, sacrificed, and trunk blood collected for ACTH, CORT, and BEC analysis.

Locomotor activity testing was conducted in six identical acrylic chambers (40 x 40 x 30 cm; Accuscan Instruments, Columbus, OH, USA). Each chamber was surrounded by a 15 × 15 infrared photocell array interfaced with a computer using the Versamax and Versadat programs (Accuscan Instruments). Though the programs tabulated numerous variables related to locomotor behavior, our analyses focused on: total distance traveled (as a gross index of exploratory activity); number of center entries and time spent in the center quadrant (an index of anxiety); and rest time. Overhead lights were on in the room, but dimmed. The data were aggregated across the 15 min session for all measures, but were then expanded into a detailed analysis of five, 3-min bins to clarify the time-course of behavioral activity.

Results

Data were analyzed using a one-way ANOVA with Dunnett's tests utilized for post-hoc analyses. Significant main effects of ethanol injection were observed on total distance traveled [$F(3, 29) = 8.838, p < 0.01$], the number of center entries [$F(3, 29) = 99.89, p < 0.001$], and rest time [$F(3, 29) = 10.14, p < 0.001$]. Post hoc analyses revealed that, regardless of testing time, rats injected with ethanol showed significantly decreased total distance traveled (Figure 2b) and center entries (Figure 2d) relative to vehicle controls. Though time spent in the center quadrant was relatively low overall (~20 sec in controls; data not shown), this measure was reduced in a manner similar to center entries, indicating that rats ambulated through the center quadrant but did not tend to stop there. Rest time was significantly increased at all time points relative to vehicle-injected controls (Figure 2f). Together, these behavioral alterations indicate a relatively stable withdrawal period that ranged from approximately the time of ethanol clearance (Brasser and Spear 2002; current Figure 1) through 18 hrs post-injection. To look more closely at the anxiogenic measures, total distance was covaried with center entries and time. When total distance was added as a covariate for both measures, no significant effect was found, therefore suggesting that reduced locomotor activity accounted for the bulk of the anxiety-like behavior observed in this task.

In order to detail the rats' activity during the 15 min test, several variables were examined across 5 3-min bins (total distance, center entries, and rest time) and analyzed with repeated measures ANOVAs. These analyses were deemed important since activity patterns often show time-dependent changes even within short behavioral tasks (e.g., Deak et al. 2009). For total distance, there was a main effect of group and time [$F(3, 29) = 11.92, p < 0.01$; $F(4, 116) = 29.21, p < 0.01$, respectively], but no interaction (Figure 2a). Center entries had a main effect of group [$F(3, 29) = 9.951, p < 0.01$], but not of time, nor was there an interaction. (Figure 2c). For rest time, we observed a main effect of group [$F(3, 29) = 8.630, p < 0.01$] and time [$F(4, 116) = 26.45, p < 0.01$], but no significant interaction (Figure 2e).

For plasma measures, a significant effect of ethanol administration was found on BECs [$F(3, 29) = 3.078, p < 0.05$], ACTH [$F(3, 29) = 4.852, p < 0.01$], and CORT [$F(3, 29) = 6.084, p < 0.01$]. Post-hoc tests revealed that BECs relative to the control animals were only significantly different at 10 hrs ($p < 0.05$) (Figure 3a). Visual inspection of the data indicated that this effect was largely driven by 2 rats in which BECs were notably above control levels, whereas all other rats had reached BECs of approximately zero. Animals previously injected with ethanol and then exposed to a novel open arena during withdrawal also had significantly increased ACTH (Figure 3b) at 14 hours ($p < 0.01$) and increased CORT (Figure 3c) at all time points compared to vehicle controls (all p-values < 0.05).

Experiment 3: The effects of withdrawal on cytokine responses evoked by footshock

Prior work from our laboratory and others has shown that footshock increased the expression of IL-1 β in the hypothalamus (Deak et al. 2003; Blandino et al. 2009), and that blockade of IL-1 receptors prevented the sensitization of the HPA axis produced by stress (e.g. inescapable tailshock; Johnson et al. 2002). Because Experiment 2 reported an augmentation of the HPA axis response following exposure to a novel environment during the withdrawal period (14 hr), we hypothesized that footshock exposure might also evoke an enhanced cytokine response in the PVN during withdrawal from acute ethanol exposure. Several other measures (IL-6 as a comparator cytokine; c-fos as a marker of cellular activation; and GAPDH as a stable housekeeper) were also examined, as were other key brain structures (BNST), peripheral organs (liver), and endocrine glands (adrenals).

Rats (n=6/group, N=24 total) were injected with either ethanol or vehicle at 1900 h and then replaced in their home cage for 12 hours. At that point, rats were either placed in the shock chamber (for a complete description of the shock apparatus, see Hueston et al. 2011) for approximately 2 hours where 80 shocks were received (5 sec each, 90 sec variable ITI, 1.0 mA) or remained in their home cage as non-stressed controls. We have previously demonstrated that transfer to the footshock chamber to control for novelty does not affect hypothalamic IL-1 β (Deak et al. 2003). Immediately following the 80th shock or at a similar time point for controls (i.e., 14 hr post-injection) animals were killed and brain and trunk blood collected.

Results

Analysis with a 2x2 ANOVA showed a significant effect of footshock on CORT [$F(1, 20) = 388.1, p < 0.001$], but there was no effect of prior ethanol exposure, nor was there an interaction. There was no significant effect on BECs, indicating that BECs had completely cleared in all rats (Table 2). In the PVN, there were no effects on GAPDH or IL-6 gene expression. IL-1 β and c-fos gene expression were significantly increased in response to footshock as evidenced by the main effect of stress condition [IL-1 β : $F(1, 20) = 7.101, p < 0.05$; c-fos: $F(1, 20) = 14.81, p < 0.01$]. However there was no main effect of prior ethanol exposure nor was there a significant interaction for IL-1 β or c-fos. In the BNST, there was no effect of stress, ethanol administration, or significant interaction terms for GAPDH, IL-1 β , or IL-6. While c-fos gene expression was increased in the BNST in response to footshock [$F(1, 19) = 16.97, p < 0.01$], ethanol exposure produced a paradoxical decrease in c-fos expression in the BNST [main effect of ethanol treatment: $F(1, 19) = 5.17, p < 0.05$]. However, the interaction between stress and ethanol treatments on c-fos expression in the BNST failed to reach statistical significance (see Table 2).

In the adrenal glands, there was no effect of ethanol exposure or stress on GAPDH or IL-1 β gene expression. There was a significant effect of ethanol exposure on COX-2 gene expression [$F(1, 20) = 5.3, p < 0.05$], but there was no effect of footshock nor was there a significant interaction between stress and ethanol treatment on COX-2 in the adrenal gland. In the liver, there were no effects on GAPDH, IL-1 β , or COX-2 gene expression (see Table 2).

Experiment 4: Examination of the HPA axis response to restraint imposed during withdrawal

Contrary to what we had hypothesized, in the previous experiment neither cytokine expression nor the HPA axis response to footshock was exacerbated in rats experiencing withdrawal from acute ethanol compared to controls. Since footshock is a relatively intense stress challenge, however, use of this stressor raised concerns about whether a biological ceiling effect had been achieved—a concern supported by our prior work indicating that

plasma CORT levels in response to footshock (80 shocks, 1.0 mA) are essentially identical to those produced by severe metabolic challenges (Deak et al. 2005). As a result, a more mild stress challenge was used in this experiment, perhaps permitting greater sensitivity for discriminating CORT differences across groups. Loose restraint in a Plexiglas tube was chosen as a mild/moderate stress challenge. Rats ($n=8-10/\text{group}$, $N=18$ total) were injected with ethanol or saline at 1900 h. 14 hours later, animals were placed in restraint tubes for 60 min. Blood was taken via the tail clip method immediately (0 min, baseline), 15, 30, and 60 min after stressor onset, as well as 60 min after removal from restraint (120 min group).

Results

Repeated measures ANOVAs were used except for in the analysis of total CORT, where a one-way ANOVA was used. BECs were measured only at the 0 and 60 min time points to verify ethanol clearance. As expected, there was no main effect of ethanol exposure or time on BEC, nor was there an interaction (Figure 4a). There was a main effect of ethanol exposure [$F(1, 15) = 13.24$, $p < 0.01$] and of time [$F(4, 60) = 45.52$, $p < 0.001$] on CORT. Though this interaction was not significant (Figure 4b), planned comparisons were targeted at the 15 min time-point because of the apparent enhancement of corticosterone observed in Experiment 2. These analyses showed that ethanol-exposed rats had significantly higher CORT levels in response to restraint stress than water-treated controls ($p < 0.01$). Furthermore, the restraint-induced increase in total CORT (over the entire 2 hr sampling period) was significantly higher in the ethanol-withdrawn animals relative to vehicle-injected controls [$F(1, 15) = 13.46$, $p < 0.01$] (Figure 4c).

Experiment 5: Gene expression changes following 15-min of restraint imposed during withdrawal

The goal of this study was to examine gene expression changes in the PVN of rats that might account for the enhanced HPA axis response to stress (novel environment or restraint) observed during withdrawal. In addition to examining plasma measures (BECs, ACTH, and CORT), we examined c-fos in the PVN as a measure of cellular activation and 3 key cytokines (IL-1 β , IL-6, TNF) that have been shown to impact HPA axis function (Turnbull and Rivier 1999). This was a logical mechanism to pursue since preliminary data from our laboratory (unpublished) suggests that ethanol exposure alters cytokine gene expression in brain, and other studies have shown that cytokines produce sensitization of the HPA axis (e.g., Johnson et al. 2002). Rats ($n=8-10$ per group, $N=34$ total) were injected with ethanol or vehicle at 1900 h and 14 hours later were placed in restraint tubes for 15 minutes or remained in their home cages. Immediately upon removal from restraint tubes, animals were killed and tissue/blood was collected as described previously.

Results

All data were analyzed using 2x2 ANOVAs and post-hocs conducted with Fishers LSD tests. When BECs were examined, no significant main effects of ethanol exposure or restraint were observed, nor was there a significant interaction of these factors (Figure 5a). While exposure to restraint significantly increased ACTH levels [$F(1, 30) = 14.79$, $p < 0.001$], no significant main effect of ethanol pre-exposure was observed, with the interaction term approaching statistical significance ($p < 0.10$; Figure 5b). Restraint significantly increased CORT levels [stress main effect: $F(1, 26) = 410.4$, $p < 0.001$] and there also was a significant interaction between ethanol pre-exposure and restraint [$F(1, 26) = 16.10$, $p < 0.001$]. Post-hoc tests revealed that CORT was significantly increased by restraint, but with this stress-induced elevation in CORT significantly greater for rats experiencing ethanol withdrawal (Figure 5c) compared to controls.

In the PVN, restraint stress increased c-fos gene expression as expected [$F(1, 28) = 14.69$, $p < 0.001$], although the interaction between restraint and ethanol pre-exposure approached but did not achieve statistical significance (Figure 5d). No other significant main effects or interactions were observed for the other PVN measures (GAPDH, TNF α , IL-1 β , or IL-6; Figure 5e-h, respectively). These data support the results of Experiment 3 showing that cytokine responses to stress probably do not account for the enhanced stress-induced HPA axis response observed during withdrawal from acute ethanol exposure.

Discussion

In the current experiments, when rats were given an acute large dose of ethanol (4g/kg), BECs mimicked a night of heavy drinking (~225 mg/dL) and profoundly influenced exploration of a novel environment during the post-ethanol clearance period. Furthermore, exposure to a single, intragastric intubation of ethanol resulted in a highly reproducible and profound enhancement of the HPA axis response to stress, with this response observed after either exposure to a novel environment (Experiment 2) or restraint (Experiment 4, 5). Taken together, these data suggest that augmentation of the HPA axis response during ethanol withdrawal may be a universal response to most stress challenges, rather than a peculiarity of one unique stressor. Though visually appearing modest in nature, the ethanol withdrawal-induced enhancement of CORT levels following stressor exposure was approximately a 25% increase in CORT (~6 μ g/dl). Since prior work has shown significantly greater corticosteroid receptor occupancy with changes of this size (Deak et al. 1999), this effect is likely functionally relevant.

Importantly, the findings presented here complement chronic ethanol models because our work demonstrates that enhancement of the HPA axis response to stress challenges (such as restraint or novelty) imposed during withdrawal occur in the *absence* of increased basal CORT. Though much of the literature examining behavioral changes and stress reactivity after ethanol exposure has utilized chronic ethanol administration procedures (e.g. Majchrowicz 1975; Rivier et al. 1990; Becker et al. 1997; Breese et al. 2004; Breese et al. 2005b), there is some concordance between findings from these chronic models and our acute exposure paradigm. For instance, several studies examining stress responsivity after chronic ethanol exposure have demonstrated augmented stress reactivity during withdrawal, with restraint stress increasing anxiety-like behavior in ethanol-exposed rats relative to stressed controls not given ethanol (Breese et al. 2005b; Rylkova et al. 2009; Valdez et al. 2003; but see also Zhao et al. 2007). Similarly, handling-induced convulsions (HIC), were also found to be enhanced (higher severity and duration) after repeated withdrawals from chronic intermittent ethanol vapor exposure when compared to mice that only experienced a single withdrawal (Becker et al. 1997). Additionally, when exposed to a pharmacological stressor (CRH administration), ethanol vapor-exposed rats exhibited a higher ACTH response to a lower dose of CRH during ethanol withdrawal when compared to controls, with these withdrawn rats also showing higher basal CORT and ACTH levels (Rivier et al. 1990). Thus, it is possible that the effects observed in the present studies may represent an earlier stage of HPA axis alterations that ultimately, with greater alcohol exposure and repeated cycles of withdrawal, transform into pathological stress responses which manifest as increased basal CORT and/or impaired HPA axis shutoff.

While ethanol-withdrawn animals in our model exhibited what could be initially interpreted as anxiogenesis (i.e. significantly decreased center time and entries in the open field compared to controls), covariate analyses did not reveal the reduced locomotor activity (primarily expressed as increased huddling behavior) to be separable from measures of anxiety. Although these results do not necessarily exclude anxiogenesis as a component of ethanol withdrawal-related behavioral changes, they do serve as a cautionary note regarding

extrapolation from altered activity patterns to purported psychological aspects of withdrawal.

The added value of a mild stress challenge (exposure to a novel environment) in Experiment 2 provided the opportunity to examine stress-induced plasma CORT and ACTH concentrations, ultimately revealing significantly increased CORT levels in ethanol-exposed rats relative controls at all time points and ACTH levels significantly increased at 14 hours. Although the dissociation between ACTH and CORT at the 10 and 18 hr time points may be viewed as troubling to some, ACTH and CORT often do not follow an identical pattern of secretion (Bornstein et al. 2008). Indeed, the overall greater variability in plasma ACTH measures, pulsatility of ACTH secretion, and diverse mechanisms which drive CORT release could easily explain the dissociation between ACTH and CORT observed here (Ulrich-Lai et al. 2006; Pacak et al. 1995).

In Experiment 3, when rats were exposed to footshock during withdrawal from acute ethanol exposure, significant increases in c-fos in the BNST and IL-1 β in the PVN were observed, as has been previously reported with footshock (Deak et al. 2003; Arakawa et al. 2009; Blandino et al. 2006; Blandino et al. 2009). There was, however, no effect of prior ethanol administration on expression of these genes, suggesting that these effects are not differentially affected by withdrawal. Similarly, CORT levels were significantly increased as a result of footshock, yet unaffected by prior ethanol exposure. Since footshock is a highly aversive stress challenge, likely producing maximal drive to the HPA axis and potentially a ceiling effect on plasma CORT concentrations, this lack of ethanol withdrawal-induced HPA axis augmentation was not surprising in this instance. Restraint was then subsequently used as a stress challenge (Exps. 4 and 5) since it produces peak CORT changes about half of those observed by footshock (see Hueston et al. 2011 for a side-by-side comparison of plasma CORT levels using the same assay procedure), thereby yielding a more tractable paradigm for examining alterations in HPA axis responses to stress during ethanol withdrawal.

Indeed, in Experiments 4 and 5, our previous results were again replicated: ethanol-withdrawn rats exhibited significantly higher levels of CORT after restraint than controls. Beyond plasma and into the brain (Exp. 5), it was found that restraint significantly increased c-fos mRNA expression in the PVN, with no ethanol withdrawal effects observed for this measure. Unfortunately, 15 minutes post-restraint may not have been the ideal time to look at c-fos expression (Rivest and Rivier 1994; Hwang et al. 2007). Though greater sensitivity for detecting c-fos changes may have been achieved with in situ hybridization, this method is not sufficiently sensitive to detect stress-dependent changes in cytokines (Deak 2007)—the primary goal of this experiment. There were also no significant effects of restraint or ethanol on IL-1 β or IL-6 in the PVN in Experiment 5. Though collectively these findings do not reveal the specific mechanism by which ethanol withdrawal enhanced the HPA axis response to moderate stressors, they provide a third replication of this result and further suggest that inflammatory factors are likely not involved in that augmentation.

Regardless of the mechanisms involved, use of a single-intubation model of ethanol exposure to examine interactions between withdrawal and stress reactivity provides a reliable, clinically relevant, and highly tractable model for clarifying these mechanisms in future studies, many of which are already under way in our laboratory. Since the present studies did not involve any tests of alcohol consumption or the use of alcohol-dependent subjects, they should be viewed with caution when ascertaining how these findings might extrapolate to dependence-related issues such as relapse drinking. Ultimately (and with future studies), however, these findings may have significant implications for expanding our understanding of the relationship between ethanol consumption, stressor exposure and

relapse drinking (e.g., Breese et al. 2005a; Sinha 2001), providing insight into treatment of addictive processes.

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Fig. 1.

Rats were injected with 4 g/kg, 20% ethanol (i.g.) and tail blood was taken at 0 (baseline), 1, 2, 3, 4, 5, 6, 7, 8, and 14 hrs post-injection via the clip method in restraint tubes. Rats were split into three groups ($n=8$ per group) and had blood taken at approximately every third time point. Blood was centrifuged, plasma was separated, and plasma was run through the Analox AM1 to determine blood ethanol concentration. Peak intoxication was at 3 hrs, with ethanol no longer present in the blood by 14 hrs. * $p < 0.05$ when compared to baseline; ^ $p < 0.05$ when compared to 3 hours. The colored portion of the figure and text display the timing of stress challenges on an experiment-by-experiment basis relative to key experimental events (alcohol injection, clearance, and the expected period of withdrawal)

Fig. 2.

Rats ($n=8-10$ per group) were injected with 4 g/kg (i.g.) or vehicle 10, 14, or 18 hours prior to being placed in locomotor activity chambers (Accuscan Instruments) for 15 minutes (divided into 5 3-min bins): data was assessed longitudinally over the 15 min (left graphs: a,c,e) and also aggregated across the 15 min session (right graphs: b,d,f). Trace diagrams (far right) are provided to further demonstrate the path traveled across the 15 min session for vehicle injected controls (g) and rats injected with alcohol and tested 10, 14, or 18 hrs later (panels h, i, j, respectively). * $p < 0.05$ when compared to vehicle-injected controls

Fig. 3. Immediately after behavioral testing in Experiment 2, rats were killed upon removal from the activity chambers and blood was collected. Blood ethanol concentration (a), ACTH (b), and CORT (c) levels were measured. * $p < 0.05$ compared to vehicle injected controls

Fig. 4.

Rats (n=8–10 per group) were injected with 4 g/kg ethanol (i.g.) or vehicle and 14 hours later were placed in restraint tubes for 60 min. Tail blood was collected via the clip method at 0 min (baseline), 15, 30 and 60 min. Rats were returned to their home cages and after 60 min had lapsed, rats were placed back in restraint tubes and blood was collected a final time for assessment of HPA axis shutoff. Blood was analyzed for blood ethanol concentration and CORT using an Analox AM1 and commercially available EIA, respectively. * $p < 0.05$ compared to vehicle injected controls

Fig. 5.

Rats were injected with 4 g/kg (i.g.) or vehicle and 14 hours later were placed in restraint tubes for 15 min. Immediately upon removal from restraint tubes, rats were killed via decapitation, brains were removed, and gene expression was measured using real-time RT-PCR. Trunk blood was also collected and BECs determined using an Analox AM1, with CORT measured via EIA, and ACTH measured with an ELISA. * $p < 0.05$ compared to both vehicle and ethanol injected groups; ^ $p < 0.05$ compared to vehicle injected restraint group

Table 1

Real-Time RT-PCR primer sequences and accession numbers

Target Gene	Accession #	Primer sequences
GAPDH ^a	BC059110	Forward: ATGACTCTACCCACGGCAAG Reverse: AGCATCACCCCATTTGATGT
IL-1 β ^b	NM_031512	Forward: AGGACCCAAGCACCTTCTTT Reverse: AGACAGCACGAGGCATTTT
IL-6 ^c	NM_012589	Forward: CCGGAGAGGAGACTTCACAG Reverse: CAGAATTGCCATTGCACAAC
TNF α ^d	NM_012675	Forward: GGGGCCACCAAGCTCTTCTG Reverse: CGACGTGGGCTACGGGCTTG
c-fos	NM_022197.2	Forward: CCAAGCGGAGACAGATCAAC Reverse: AAGTCCAGGGAGGTCACAGA
COX-2 ^e	YP_665632.1	Forward: ATCCTGAGTGGGATGACGAG Reverse: AGGCAATGCGGTTCTGATAC

^a Glyceraldehyde 3-phosphate dehydrogenase^b Interleukin-1 beta^c Interleukin-6^d tumor necrosis factor alpha^e cyclooxygenase-2

Table 2

Alterations in plasma corticosterone, blood ethanol concentration and gene expression among footshocked versus non-stressed rats

	Home Cage Control			Footshock	
	Saline	Ethanol	Saline	Ethanol	
Plasma	BEC ^a (mg/dL)	9.23 ± 1.82	24.42 ± 10.44	6.48 ± 0.94	15.90 ± 5.32
	CORT ^b (µg/dL)	0.68 ± 0.32	0.79 ± 0.44	33.02 ± 2.27	32.83 ± 2.29
PVN ^d (% of Control)	GAPDH ^c	117.19 ± 27.92	173.91 ± 43.10	175.31 ± 20.29	176.23 ± 52.81
	IL-1β ^e	105.57 ± 17.87	187.82 ± 45.28	425.90 ± 91.94	403.33 ± 196.89
	IL-6 ^f	100.35 ± 0.35	149.21 ± 49.78	168.74 ± 67.09	173.50 ± 62.24
	c-fos	135.64 ± 48.23	370.90 ± 74.91	2016.02 ± 428.51	1914.59 ± 880.85
BNST ^g (% of Control)	GAPDH	87.28 ± 18.19	78.95 ± 21.56	79.15 ± 17.20	17.93 ± 10.90
	IL-1β	81.50 ± 12.04	92.92 ± 17.99	85.36 ± 31.98	87.14 ± 9.75
	IL-6	93.41 ± 31.29	131.56 ± 52.74	134.70 ± 24.02	176.16 ± 37.73
	c-fos	107.01 ± 15.68	<i>87.67 ± 13.73</i>	325.06 ± 64.79	175.59 ± 29.73
Adrenal (% of Control)	GAPDH	115.32 ± 11.92	138.09 ± 9.29	110.59 ± 6.29	130.99 ± 10.06
	IL-1β	116.83 ± 27.42	153.86 ± 44.51	100.01 ± 12.46	83.04 ± 7.41
	COX-2 ^h	110.27 ± 20.62	<i>52.51 ± 12.09</i>	418.11 ± 174.46	<i>66.66 ± 24.26</i>
Liver (% of Control)	GAPDH	104.84 ± 13.57	169.00 ± 21.41	144.75 ± 12.76	165.37 ± 14.33
	IL-1β	127.58 ± 38.49	218.22 ± 24.99	164.26 ± 53.25	116.47 ± 19.41
	COX-2	104.83 ± 19.61	108.87 ± 22.08	76.91 ± 6.03	80.86 ± 32.14

Rats were exposed to footshock for 2 hrs, starting 12 hr after a 4 g/kg ethanol or saline challenge. Non-stressed controls that received either the saline or ethanol challenge remained in their home cage. All values are expressed as mean ± SEM, with blood ethanol concentrations expressed in mg/dL, corticosterone levels in µg/dL, and gene expression values expressed as a percentage of the control (home cage vehicle) group. Bold numbers indicate a main effect of footshock, while italics denote a significant main effect of prior ethanol exposure.

^a Blood ethanol concentration

^b Corticosterone

^c Glyceraldehyde 3-phosphate dehydrogenase

^d paraventricular nucleus

_e Interleukin-1 beta

_f Interleukin-6

_g bed nucleus of the stria terminalis

_h cyclooxygenase-2