Published in final edited form as:

Prog Neuropsychopharmacol Biol Psychiatry. 2015 January 2; 0: 66–74. doi:10.1016/j.pnpbp.2014.08.005.

Chronic alcohol exposure differentially affects activation of female locus coeruleus neurons and the subcellular distribution of corticotropin releasing factor receptors

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Abstract

Understanding the neurobiological bases for sex differences in alcohol dependence is needed to help guide the development of individualized therapies for alcohol abuse disorders. In the present study, alcohol-induced adaptations in (1) anxiety-like behavior, (2) patterns of c-Fos activation and (3) subcellular distribution of corticotropin releasing factor receptor in locus coeruleus (LC) neurons was investigated in male and female Sprague-Dawley rats that were chronically exposed to ethanol using a liquid diet. Results confirm and extend reports by others showing that chronic ethanol exposure produces an anxiogenic-like response in both male and female subjects. Ethanol-induced sex differences were observed with increased c-Fos expression in LC neurons of female ethanol-treated subjects compared to controls or male subjects. Results also reveal sex differences in the subcellular distribution of the CRFr in LC-noradrenergic neurons with female subjects exposed to ethanol exhibiting a higher frequency of plasmalemmal CRFr. These adaptations have implications for LC neuronal activity and its neural targets across the sexes. Considering the important role of the LC in ethanol-induced activation of the hypothalamo-pituitary-adrenal (HPA) axis, the present results indicate important sex differences in feed-forward regulation of the HPA axis that may render alcohol dependent females more vulnerable to subsequent stress exposure.

Keywords

locus coeruleus; chronic alcohol; sex differences; CRFr; c-Fos

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1. Introduction

Multiple lines of evidence indicate that chronic exposure to ethanol impacts stress-integrative circuitry, including activation of the hypothalamic-pituitary-adrenal (HPA) axis (Huang et al. 2010; Lovallo 2006; Vilpoux et al. 2009). Acute exposure to ethanol produces anxiolytic-like responses (File and Seth 2003; Varlinskaya and Spear 2006) and activates the HPA axis similar to physiological stress (Richardson et al. 2008). Prolonged ethanol exposure produces an anxiogenic-like response that is accompanied by dysregulation of the HPA axis as evidenced by adrenal hypertrophy and blunted corticosterone responses (King et al. 2006). As a stress-sensitive region that provides the major source of norepinephrine (NE) to the forebrain, the locus coeruleus (LC) has been shown to play an important role in ethanol-induced activation of the HPA axis (Selvage 2012). Along with catecholaminergic projections from other brainstem nuclei, LC neurons project to the primary hypothalamic regulator of HPA function, the paraventricular nucleus of the hypothalamus (PVN), where NE is released to activate corticotropin releasing factor (CRF) neurons (Dunn et al. 2004; Lee et al. 2011), which then stimulate the HPA axis hormone cascade (Helmreich et al. 2001).

Stress and CRF have been implicated in the neuronal dysregulation associated with ethanol withdrawal, and promoting sustained intake as well as reinstatement of ethanol seeking behavior (Breese et al. 2005; Le et al. 2000; Martin-Fardon et al. 2010). The LC is profoundly impacted by stress and its dysregulation can increase the risks of both ethanol dependence and the potential for relapse (Morilak et al. 2005). Intra-gastric delivery of ethanol to non-handled, freely moving rats increases LC neuronal activity, as measured by accumulation of c-Fos, a marker of recent neuronal activation, supporting the notion that acute ethanol induces activation of LC neurons (Kolodziejska-Akiyama et al. 2005; McClintick et al. 2013; Lee et al. 2011). Additionally, administration of ethanol intracerebroventricularly normally stimulates adrenocorticotropin (ACTH) release from the PVN; however, when the LC was lesioned, release was potentiated (Selvage 2012), indicating a direct role for the LC in ethanol induced HPA axis activation.

Pharmacologic and behavioral interventions for recovering alcoholics are often aimed at stress reduction and management of negative affect (Franck and Jayaram-Lindstrom 2013; Health 2011). In support of this, CRF receptor (CRF_r) antagonists are being investigated as potential therapeutics for the treatment of alcohol abuse disorders, showing efficacy at blocking binge-drinking and relapse precipitated by stressful events (Baldwin et al. 1991; Lowery and Thiele 2010). However, the mechanism underlying this potential efficacy is currently not well understood. Our prior work demonstrates that CRF_rs are differentially localized to the plasma membrane or cytoplasmic compartments of LC dendrites under conditions of stress (Bangasser et al. 2010a; Reyes et al. 2008), which has significant consequences for the activity of noradrenergic neurons. Furthermore, the CRF_r system has been proposed as a model of sex dependent signaling. In males, the CRF_r localizes intracellularly following CRF administration or exposure to a stressor, using a process involving recruitment of β -arrestin (Valentino et al. 2012). In females, an increased plasmalemmal distribution of CRF_r is observed after stress and this involves deactivation of

the β -arrestin pathway (Bangasser et al. 2010b). Sex differences in CRFr signaling are hypothesized to confer differences in coping ability.

We have recently described sex differences in the response to ethanol in the central nucleus of the amygdala (CeA), a region known to send CRF projections to the LC (Retson and Van Bockstaele 2013; Van Bockstaele et al. 1998). The CeA exhibited sex differences in patterns of c-fos activation, with fewer alcohol-induced activated neurons in males compared to females. Further, after a swim stress, both male and female ethanol treated subjects exhibited enhanced neuronal activation patterns when compared to control subjects. Considering the strong link between stress, anxiety and vulnerability to relapse (Sinha et al. 2011; Breese et al. 2011), the present study sought to examine ethanol-induced anxiety-like phenotypes across the sexes, and to examine the impact of ethanol and sex on the neuronal activity of the LC. In addition, we tested the hypothesis that the distribution of CRFr in the LC varies by sex following chronic ethanol exposure. Understanding the impact of ethanol on CRFr signaling and the influence of sex may help fill a gap in our knowledge regarding ethanol-induced adaptations in NE systems that may impact HPA activity, and this may contribute to the development of targeted therapies that will have an increased efficacy in aiding recovery from alcohol abuse.

2. Methods

2.1 Animals

Adult male and female Sprague-Dawley rats (Harlan Sprague-Dawley, Inc., Indianapolis, IN) weighing between 100–150g at the start of the experiments were housed in individual cages in a dedicated room at the Thomas Jefferson University animal facility. Animals were maintained on a 12 hour light-dark cycle with access to food and water, or under experimental conditions, a liquid diet (Lieber and DeCarli 1982). Ethanol treated animals and controls were littermates that were matched-pair fed a calorically equivalent liquid diet that averages an ethanol intake of 12–14 g/kg/day for 14 days. Ethanol treated animals received 36% of total calories as ethanol while control animals received maltose-dextrin (the remaining calories consisted of 11% from carbohydrates, 35% fat, and 18% protein, Bio-Serv, Frenchtown, NJ). Ethanol concentrations in the liquid diet were ramped up stepwise over a period of 4 days prior to administering the full ethanol diet for 14 days. Animal handling and diet administration was the task of trained animal technicians experienced in working with this animal population (Covarrubias et al. 2005; Dippold et al. 2013). All experiments and sacrifice were performed at the same time of day to minimize any changes in ethanol levels across the day, and all behavior experiments were conducted in the same facility to ensure all animals were exposed to the same environment. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Thomas Jefferson University, and were in accordance with the NIH's Guide for the care and use of laboratory animals. At time of euthanasia blood alcohol content was obtained to insure animals had an active blood alcohol content at times of experiment and sacrifice, and all female animals underwent vaginal cytology to determine estrus phase.

2.1 Elevated Zero Maze

The elevated zero maze (EZM) evaluates natural rodent behavior where there is a drive to explore new environments that conflicts with defensive hiding behaviors (Pellow et al. 1985). The advantage of the EZM is in its circular design which eliminates any ambiguity in whether an animal is in an open or closed section of the maze. Behaviors such as time spent in the open arms of the maze, head dips over the side of the platform, and standing in the closed arms of the maze are indicative of exploration. These behaviors are increased with anxiolytic administration, and conversely decreased with anxiogenic drugs (Shepherd et al. 1994). All animals were exposed to an EZM for assessment of anxiety-like behaviors as previously described on day 12 of treatment (Carvalho et al. 2010b; Ambrose-Lanci et al. 2010). As a modified version of the elevated plus maze, the EZM is used extensively to examine anxiety like phenotypes in rodents (Braun et al. 2011), particularly after a pharmacological intervention (Kulkarni et al. 2007). Anxiolytics, including the benzodiazepine diazepam, increase the amount of time animals spend in the open arms of the maze, and the amount of time engaged in exploratory behaviors (Shepherd et al. 1994). In contrast, anxiogenic interventions increase the time spent in the closed arms of the maze, and decrease exploratory behaviors (Kulkarni et al. 2007). Briefly, the EZM apparatus consists of a circular black plastic platform of 120cm diameter that is elevated 70cm above the ground. The apparatus is divided into four equal quadrants with sections from 0–90° and 180–270° being ‘open’ and surrounded by a 1cm high apparatus edge, while quadrants from 90–180° and 270–360° are ‘closed’ and surrounded on inner and outer track boundaries by 27cm high black plastic walls. Animals were always placed into the apparatus in the same orientation at the center of the 90–180° closed arm. Between subjects, the EZM was cleaned with an ethanol solution and allowed to dry. All testing was done in the same room, at the same time of day, with identical lighting conditions. All trials were 10 minutes in duration, and videotaped ($n = 14$ for male control (MCtrl) and male ethanol (MEtoh), and 18 for female control (FCtrl) and female ethanol (FEtoh) groups). Behavioral analysis was performed with the AnyMaze software (Stoelting, Wood Dale, IL) allowing for measures of speed, distance, time spent in open or closed arms, head dips (defined by the animal extending its head over the bounds of the apparatus in an open arm), and standing behaviors (both front paws were lifted off the maze, and the animal reared onto its hind limbs).

2.3 Immunohistochemistry

For immunohistochemistry, rats were deeply anesthetized via exposure to isoflurane (Vedco, St. Joseph, MO) in a specialized holding cage, then transcardially perfused via the ascending aorta with heparin followed by a 2% formaldehyde and 3.75% acrolein (Electron Microscopy Sciences, For Washington, PA) mixture in 0.1M phosphate buffer (PB; pH 7.4). The brains are then collected and stored in the formaldehyde and acrolein mixture until sectioning. Sections through the rostro-caudal extent of each brain were collected at 35 μ m using a vibrating microtome and serial sections through the LC were processed for electron microscopy or fluorescence as described, and detailed in previous studies by our lab and others (Waselus et al. 2009; Reyes et al. 2011).

2.4 Fluorescence

Sequential sets of tissues through the LC were placed in 1% sodium borohydride in 0.1M phosphate buffer (PB) for 30 minutes in order to remove any remaining aldehydes. Tissues were then incubated in 0.5% bovine serum albumin (BSA) in 0.1M Tris buffered saline (TBS; pH of 7.6) for 30 minutes. Tissues were then extensively rinsed in 0.1M TBS and incubated overnight in a cocktail of c-Fos raised in rabbit (1:9000, CalBiochem, Billerica, MA) or tyrosine hydroxylase (TH) raised in mouse (1:1000, Immunostar Inc., Hudson, WI). Proteins were visualized using rhodamine isothiocyanate (TRITC) conjugated donkey anti-rabbit (1:200, Jackson ImmunoResearch, West Grove, PA), and TRITC conjugated donkey anti-mouse (1:200, Jackson ImmunoResearch). Control tissues were processed in parallel with the omission of primary antibodies. Two tissue sections at matched rostro-caudal levels representing the rostral LC (bregma -9.68) and the mid-LC (bregma -10.04) were photographed for visualization and quantification of c-Fos with an Olympus BX51 (Tokyo, Japan), and captured with Spot Advanced Software (Diagnostic Instruments Inc, Sterling Heights, MI) n = 5 for MCtrl, MEtoh, and FEtoh, n = 4 for FCtrl. The number of labeled cells is presented by group average $\pm$ standard error of the mean (SEM).

2.5 Western Blotting

Western blotting procedures were performed as described in previous studies (Carvalho et al. 2010a). Fresh tissue punches from the LC were homogenized in a solution of RIPA lysis buffer containing 1% phenylmethylsulfonyl fluoride (PMSF), 1% sodium orthovanadate and 1–2% protease inhibitor (n=3 for MCtrl, MEtoh, FCtrl, n=2 for FEtoh). SoftMax Pro was used to determine the concentrations of protein extracted from each sample via a bicinchoninic acid assay (BCA). Samples were loaded at equal protein concentrations on a 4–12% tris-glycine gel (Life, Carlsbad, CA), and transferred to Immobilon-FL transfer membranes (Milipore, Bedford, MA) for 2 hours at 25 volts. Membranes were then incubated with antibody cocktails against c-Fos made in rabbit (1:1000, CalBiochem, Billerica, MA) and beta actin made in mouse (1:1000 Protein tech, Chicago, IL) overnight at 4°C. Tissues were washed and secondary antibodies goat anti-rabbit or mouse (1:15,000 and 1:20,000 respectively, Li-Cor, Lincoln, NE) at a wavelength of either 680 or 800, were applied for 45 minutes. Blots were scanned on a Li-Cor Odyssey CLx and analyzed with Li-Cor Image Studio.

2.6 Electron Microscopy

Tissues were processed for electron microscopy as previously described by our laboratory (Scavone et al. 2011; Reyes et al. 2008; Reyes et al. 2006). Briefly, alternate sections through the LC were processed for CRFr and TH (n=5 for MCtrl and FCtrl, n=6 for MEtoh and FEtoh). Tissues were placed in 1% sodium borohydride in 0.1M PB (pH 7.4) for 30 minutes in order to remove any remaining aldehydes then then incubated in 0.5% BSA in 0.1M TBS (pH 7.6) for 30 minutes. Tissues were then extensively rinsed in 0.1M TBS and incubated overnight in a cocktail of CRFr raised in rabbit (1:1000) or TH made in mouse (1:1000, Immunostar Inc) as appropriate. TH was then visualized with immunoperoxidase labeling via biotinylated donkey anti-mouse antibodies (1:1000) for 30 minutes, followed by an avidin-biotin incubation for 30 minutes (ABC kit, Vector Laboratories, Burlingame, CA),

and visualization with a 5-minute reaction in 3,3'-diaminobenzidine (DAB; Sigma-Aldrich Inc., St. Louis, MO) and hydrogen peroxide in 0.1 TBS.

Tissues were prepared for CRFr visualization through an immuno-gold silver enhancement. Tissues were washed extensively and incubated in goat anti-rabbit IgG conjugated to 1nm gold particles (Amersham Bioscience Corp, Piscataway, NJ) for 2 hours. Tissues were then rinsed in the 0.2% gelatin-phosphate buffered saline (PBS) and 0.8% BSA buffer, and 0.1M PBS, sequentially. Incubation then occurred in a mixture of 2% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA) in 0.01M PBS for 10 minutes, followed by washes in 0.01M PBS and 0.2M sodium citrate buffer (pH 7.4). Silver enhancement of the gold particles was performed with a silver enhancement kit (Amersham Bioscience Corp.) wherein the optimal enhancement times were empirically determined for each experiment, with a range between 8–10 minutes. After enhancement, tissues were washed in 0.2M citrate buffer and 0.1M PB, then incubated in 2% osmium tetroxide (Electron Microscopy Sciences) in 0.1M PB for one hour, followed by washes in 0.1M PB. Dehydration of tissues was then performed in a series of ascending concentrations of ethanol, followed by dehydration propylene oxide. Finally, tissues were embedded in Epon 812 between sheets of aclar (Electron Microscopy Sciences). Upon hardening, tissues were photographed with a light microscope (Olympus) and then sectioned at 75nm with a diamond knife (Diatome, For Washington, PA) on a Leica Ultracut (Leica Microsystems, Wetzlar, Germany). From this, sections were collected onto copper grids (Electron Microscopy Sciences), and examined with an electron microscope (Morgagni Fei Company, Hillsboro, OR), with digital images being captured by an AMT advantage HR/HR-B CCD camera system (Advance Microscopy Techniques Corp, Danvers, MA). At least 10 copper grids containing 5 to 7 thin sections were collected from at least two separate embedded tissue sections from each animal. Criteria for considering a dendrite immunolabeled was defined by the presence of two or more silver particles in a profile. For each animal 150 images from the LC were photographed, and the number of dendritic profiles analyzed per animal ranged from 111–278, with an average of 180 profiles per animal. Images were quantified using Image J, and figures were created using Adobe Photoshop to crop and adjust brightness and contrast.

2.7 Statistical Analysis

Averages and SEM for all groups were calculated, and analysis was performed using a two way analysis of variance (ANOVA) with sex and treatment serving as factors. Additional post-hoc testing was performed with Student's t-tests (unpaired, two-tailed) to detail findings where denoted. IBM SPSS Statistics software version 22 was used for all analyses and significance was set to $p < 0.05$ for all comparisons.

3. Results

3.1 Chronic ethanol exposure induces an anxiogenic response irrespective of sex

Statistical analyses of behavioral data were carried out using IBM SPSS Statistics Version 22, with a multivariate analyses, and presented with the corresponding SEM. When examining distance traveled using the EZM, significant main effects were observed between control and ethanol treated groups [$F(1,60)=29.72$, $p < 0.001$], and across sex [$F(1,60)=4.89$,

$p < 0.05$]. Ethanol treated animals traveled less distance, with females traveling a greater distance across both control and ethanol treatment groups. The average distance for MCtrl was 38.65 ± 4.08 m for MEtoh 22.12 ± 2.58 m, FCtrl 47.30 ± 2.22 m, and FEtoh 28.00 ± 3.81 m (Figure 1A). Similarly, when examining mean speed there were significant main effects of treatment [$F(1,60) = 29.65$, $p < 0.001$] and sex [$F(1,60) = 4.91$, $p < 0.05$]. Mean speed for MCtrl was 0.064 ± 0.007 m/s, MEtoh 0.037 ± 0.004 m/s, FCtrl 0.078 ± 0.004 m/s, and FEtoh 0.047 ± 0.006 m/s.

When considering the percentage of time spent in the open arms, a two way ANOVA (sex and treatment) revealed significant main effects of treatment [$F(1,60) = 16.31$, $p < 0.001$] and sex [$F(1,60) = 6.37$, $p < 0.05$]. MCtrl animals spent $30.6 \pm 4.8\%$ of time in the open arms with FCtrl spending $42.9 \pm 2.7\%$, MEtoh subjects $19.4 \pm 2.5\%$, and FEtoh subjects $25.2 \pm 3.9\%$ of the test in the open arms (Figure 1B). Post-hoc testing with a Student's t-test revealed that MEtoh subjects spent significantly less time in the open arms compared to controls (t-test, $p < 0.05$), and that FEtoh subjects spent significantly less time in the open arms compared to the FCtrl group (t-test, $p = 0.001$). While the difference between the MEtoh and FEtoh groups was not significant, the FCtrl group spent a significantly higher percentage of time in the open arms compared to the MCtrl group (t-test, $p < 0.05$).

A two way ANOVA comparing sex and treatment revealed a significant overall interaction between treatment group and sex with respect to the average number of head dips [$F(1,60) = 210.83$, $p < 0.05$], with the CtrlM group averaging 12.93 ± 2.21 , the EttohM averaging 4.36 ± 0.75 , CtrlF at 21.44 ± 2.24 , and EttohF at 5.56 ± 0.96 . Post-hoc testing with a Student's t-test indicated that the MEtoh and FEtoh groups exhibited this behavior significantly less than their control counterparts (t-test, $p = 0.001$ for males and $p < 0.001$ for females). No differences were observed between the MEtoh and FEtoh group; however, the FCtrl subjects exhibited this behavior significantly more than the MCtrl group (t-test, $p < 0.05$). This indicates that ethanol treated animals were less likely to explore the maze, with the female control group being most exploratory overall (Figure 1C).

A two way ANOVA (sex and treatment) revealed a significant main effect of treatment for standing behaviors [$F(1,60) = 51.53$, $p < 0.001$]. Control animals exhibited standing behaviors significantly more often than ethanol animals, with the MCtrl group standing an average of 8.07 ± 1.16 times and FCtrl animals averaging 11.00 ± 0.94 standing behaviors, while the MEtoh group stood 3.14 ± 0.64 times and FEtoh group similarly stood 3.39 ± 0.65 times on average (Figure 1D). Post-hoc Student's t-tests revealed no difference between the MCtrl and FCtrl groups, or between MEtoh and FEtoh groups, however significant differences were observed between MEtoh and MCtrl groups ($p = 0.001$), and between FEtoh and FCtrl groups ($p < 0.001$).

3.2 c-Fos expression in LC after chronic ethanol exposure

The immediate early gene c-Fos was used to visualize neuronal activity. Labeled neurons could be seen in both TH containing neurons of the LC core, and in the peri-LC (Figure 2). A two way ANOVA was performed to examine the interaction between sex and treatment in number of c-Fos labeled neurons. A trend was seen toward an overall interaction between factors [$F(1,15) = 4.396$, $p = 0.053$]. When examining this further with a series of post-hoc

Student's t-tests, significantly more c-Fos labeled LC neurons could be seen in the FEtoH group (129.2 ± 18.9) compared to the FCtrl group (38.5 ± 26.3 ; t-test, $p < 0.05$). No difference was seen between the MCtrl (67.4 ± 30.3) and MEtoH groups (59.8 ± 15.7 ; t-test, $p = \text{ns}$). When comparing across sex, there was no significant difference between the MCtrl and FCtrl groups, while the FEtoH group had significantly more c-Fos labeling than the MEtoH group (t-test, $p < 0.05$).

Levels of the immediate early gene c-Fos were also analyzed via western blot as a ratio to beta actin (Figure 3). A two way ANOVA (sex and treatment) was performed and resulted in a significant overall interaction between factors [$F(1,11) = 31.536$, $p = 0.001$]. Similar to cell count results, a series of post-hoc Student's t-test revealed a significant increase in c-Fos protein in the FEtoH group compared to the FCtrl group ($p < 0.01$), and significantly more c-Fos protein in the FEtoH group than the MEtoH group ($p < 0.01$).

3.3 Sex differences in CRFr localization following ethanol exposure

Immunogold-silver labeling for CRFr was seen both in the cytoplasmic compartment of dendrites and on their plasma membrane (Figure 4). As noted in previous studies, CRFr were observed presynaptically on axon terminals as well as in perikarya (Waselus et al. 2009). While data for only dendrites is presented here, changes in the distribution of CRFr in other neuronal structures represents an important area for future investigation.

To analyze the subcellular distribution of CRFr in cytoplasmic versus plasmalemmal compartments, a two way ANOVA comparing sex and treatment revealed a significant overall interaction between these factors [$F(1,18) = 13.639$, $p < 0.01$]. The MCtrl group had an average cytoplasmic to total receptor localization ratio of 0.540 ± 0.034 , the MEtoH group had a ratio of 0.616 ± 0.020 , the FCtrl group had a ratio of 0.608 ± 0.026 , and the FEtoH group had a ratio of 0.518 ± 0.022 , (Figure 4). To further explicate the observed interaction, a series of post-hoc Student's t-tests were performed. Comparing the MCtrl and MEtoH groups, the MEtoH group had a significantly higher cytoplasmic to total ratio of CRFr than the MCtrl group (t-test, $p < 0.05$) indicating that CRFr were more frequently localized to cytoplasmic compartments of dendrites. When examining the female subjects, the FCtrl group had a significantly higher CRFr ratio than the FEtoH group (t-test, $p < 0.05$) indicating a greater frequency of distribution of CRFr on the plasma membrane.

For profiles where a synapse was identifiable, synaptic associations of plasma membrane localized particles was recorded. For MCtrl subjects, $13.07 \pm 3.38\%$ of plasma membrane associated particles were synaptically associated, in MEtoH subjects $16.06 \pm 6.68\%$, in FCtrl subjects $21.91 \pm 5.61\%$, and in FEtoH subjects $38.38 \pm 6.47\%$. A two way ANOVA (sex and treatment) yielded no significant overall interaction between groups, however a main effect of sex was seen [$F(1,18) = 6.901$, $p < 0.05$], with female subjects having increased synaptic associations. A post-hoc Student's t-test showed that the FEtoH group exhibited a trend toward increased synaptic associations compared to the FCtrl group (t-test, $p = 0.093$), and that in ethanol treated groups there was a significantly higher number of synaptic associations observed in FEtoH subjects than MEtoH subjects (t-test, $p < 0.05$).

4. Discussion

The present study confirms and extends reports by others showing that chronic ethanol exposure, administered via a liquid diet, produces an anxiogenic-like response in rats (File and Seth 2003; King et al. 2006). At a circuitry level, ethanol-induced sex differences were observed with increased c-Fos expression in LC neurons of female ethanol treated subjects compared to controls or male subjects. Results also reveal sex differences in the subcellular distribution of the CRFr in LC-noradrenergic neurons with female subjects exposed to ethanol exhibiting a higher frequency of plasmalemmal CRFr. These neuronal adaptations have implications for feed-forward regulation of the HPA axis, potentially rendering females more vulnerable to subsequent stressors (Rivier et al. 1984).

4.1 Methodological considerations

The delivery of ethanol used in the present study has several advantages compared to other studies. Although increases in c-Fos expression have been shown in the LC of rats exposed to ethanol (Chang et al. 1995; Thiele et al. 1997), whether this increase was due to the effect of ethanol itself or because of mode of administration has not been clear. When administered intraperitoneally, ethanol can induce cytokine release due to local inflammation and investigator handling of animals can result in increased levels of stress. It is well known that c-Fos increases in the LC following exposure to a stressful stimulus (McDevitt et al. 2009). Here, the mode of ethanol delivery via a liquid diet eliminates the stress of handling the animals, as well as inflammatory responses or stress following injection.

The impact of the estrous cycle on the distribution of CRFr was examined by assessing the timing of the female rats' estrous cycle at the time of perfusion using vaginal cytology. No significant differences were observed in behavior or CRFr localization when comparing rats across the estrous cycle. However, the effects of estrous are nuanced and an important avenue for future investigation. For example, Larkin et. al reported that acute intracerebrovascular ethanol administration in female rats stimulated ACTH secretion in females most strongly in pro-estrus or estrus, which correlated with an increase in LC activation, indicating that ethanol administration in this paradigm stimulated the HPA axis more robustly when the effects of estradiol were highest; however, they also determined that estradiol was not the only cause of this effect (Larkin et al. 2010).

While estrous cycle considerations should continue to be examined in future studies, the lack of effect seen here is consistent with other chronic studies (Ford et al. 2002; Roberts et al. 1998). While every effort is made to take a representative sample of the LC for examination of neuronal activity, the number of observed c-Fos immunoreactive (IR) cells presented here represents an underestimation, as raw numbers have been presented from slices through the mid and rostral LC rather than using this data to present an extrapolation of labeling throughout the nucleus.

When examining the findings of immunoelectron microscopy studies, pre-embedding labeling techniques are well established to allow optimal preservation of ultrastructural morphology while maintaining minimal loss of antigenicity during embedding, and silver intensification of immunogold particles is well established to permit visualization of the

distribution pattern of cellular components. Analysis of tissue beneath the tissue-Epon interface was conducted to ensure maximum detection of immunogold-silver labeled particles.

4.2 Chronic ethanol exposure induces an anxiety-like phenotype

While the anxiolytic effects of acute ethanol and the anxiogenic effects of withdrawal are well established (Koob and Le Moal 2005), the present study extends these findings by demonstrating a transition to anxiogenesis during chronic alcohol exposure. Previous work has shown that chronic ethanol use causes adaptations and alterations in the HPA axis that are long lasting and impact the behavior of males and females differently when the ethanol stimulus is removed (McClintick et al. 2013; Becker 2012; Ford et al. 2002). The present results fill a gap in knowledge by increasing our understanding of the temporal nature of this transition. It is well accepted that acute alcohol administration induces anxiolytic effects (File and Seth 2003; Varlinskaya and Spear 2006). However, over the course of chronic administration, it is hypothesized that desensitization to the anxiolytic effects of ethanol occurs, resulting in a transition to anxiety-like behaviors (King et al. 2006). Consistent with this, Fukushima et al. described an increase in locomotion following administration of acute ethanol compared with a decrease in exploratory behaviors after chronic administration (Fukushima et al. 2012). In the present study, ethanol-treated animals spent less time engaged in all three measured exploratory behaviors in the EZM: time in the open arms, head dips, and standing, when compared to their control counterparts. These results are consistent with a previous study examining the effects chronic ethanol vapor exposure on Wistar and Wistar-Kyoto rats (Getachew et al. 2008). In that study, animals were exposed to ethanol vapor daily for 7 or 14 days, and when behavioral measures were taken, ethanol exposed animals were shown to have increased depressive and anxiety-like behaviors on an elevated plus maze. of an increased anxiety-like phenotype in ethanol-treated subjects even before withdrawal is induced.

4.3 Neuronal activity is altered in ethanol treated females

The present study is the first to describe LC activation in female chronically ethanol exposed animals and to show that this group has an increased level of activity compared to controls or male ethanol-treated counterparts. Previous studies have used immediate early genes to examine patterns of neuronal activity in male rodents following chronic ethanol administration as well as during withdrawal (Vilpoux et al. 2009). Acute administration of ethanol activates LC neurons irrespective of sex (Kolodziejska-Akiyama et al. 2005; Lee et al. 2011; McClintick et al. 2013). In male animals, however, differences in neuronal activation have been reported across different alcohol delivery paradigms. Acute administration and withdrawal both result in increased neuronal activity, while chronic administration does not (Vilpoux et al. 2009; Chang et al. 1995). For example, in a study by Chang et al., an acute i.p. dose of ethanol induced c-Fos immunoreactivity in the LC; however, when ethanol was administered for 17–24 days the c-Fos response was no longer present (Chang et al. 1995). Taken with other studies, this decrease in activity over a chronic exposure timeframe has led to the hypothesis of an adaptation to chronic ethanol exposure in male LC neurons (Vilpoux et al. 2009).

The findings of increased c-Fos in the female ethanol treated subjects may indicate that the neurons of ethanol exposed females are not adapting in the manner that has been seen in males, potentially rendering them more vulnerable to future stressors. This finding is consistent with research showing sexual dimorphism in the LC that has been hypothesized to contribute to sex differences in the stress response (Bangasser et al. 2011). The lack of habituation seen after ethanol exposure is in agreement with a previous study examining LC response after a stressor. The previous work noted that females did not exhibit a plateau of LC activity after a stressor and continued to respond to CRF administration, a characteristic not seen in the LCs of male rats (Curtis et al. 2006). The hypothesized lack of habituation is further in agreement with the finding here of an increase in CRFr on the plasma membrane of ethanol exposed female subjects, as previous studies noting this CRFr localization pattern after a stressor have posited that this may render the LC activated by continued CRF signals (Bangasser and Valentino 2012), which may explain the increased c-Fos IR seen here. The findings here may help evolve our understanding of the sexual dimorphism seen in alcohol use disorders, as dysregulation of the HPA axis is noted particularly in human female alcoholics (Silva and Madeira 2012).

4.4 CRFr localization patterns indicative of a stress response

Genetic and pharmacological models are increasingly linking the CRF system with neuronal and behavioral dysregulation induced by chronic ethanol use (Franck and Jayaram-Lindstrom 2013; Funk et al. 2007; Lowery and Thiele 2010; Hwang et al. 2004). Marchigian Sardinian alcohol-preferring rats, an animal model bred for its propensity to binge drink, have been found to exhibit an upregulation of the CRFr system (Ciccocioppo et al. 2006). Studies utilizing a CRFr knock out mouse model found that repeated stressors had a greater effect on the ethanol consumption of wild type mice compared to knock-outs, indicating a role for CRFr in stress-induced consumption of ethanol (Pastor et al. 2011). Further, pharmacological intervention into the CRFr system is particularly effective in mediating withdrawal and relapse. In dependent animals, CRFr antagonist administration reduces ethanol self-administration (Funk et al. 2007), and CRFr and CRF peptidergic antagonists have been shown to mediate ethanol withdrawal induced anxiety (Koob 2008; Baldwin et al. 1991; Koppaka et al. 2012). CRF antagonists that were systemically administered during the abstinent period following chronic ethanol exposure have also been shown to potentiate the response to a restraint stressor, with subjects receiving the antagonist exhibiting more exploratory behaviors in an elevated plus maze than their counterparts (Breese et al. 2005; Valdez et al. 2003).

Prior studies from our group have shown that following agonist administration or stress exposure, CRFr exhibit a re-distribution from the plasma membrane to intracellular portions of LC neurons in males (Reyes et al. 2006; Reyes et al. 2008), and a re-distribution to the plasma membrane in the LC of females (Bangasser et al. 2010a; Bangasser and Valentino 2012). Further studies showed that female rats retain their sensitivity to CRF after stressors, as seen through a lack of the response plateau to increased CRF that is present in their male counterparts (Curtis et al. 1999; Valentino et al. 2012), and these previous studies have put forth the hypothesis that increased plasma membrane distributions of CRFr in females after a stressor are responsible for the lack of attenuation (Bangasser and Valentino

2012; Curtis et al. 2006). If CRFRs are exhibiting an increased plasma membrane distribution after ethanol exposure in females, and CRFRs are shown to modulate the post-exposure stress response, it would then be hypothesized that the feed-forward effects of alterations of the CRFR system after ethanol exposure may render the LC-NE system of females particularly vulnerable to subsequent stress (Figure 5). Indeed, one of the pharmacological interventions for alcoholism currently in human trials is Verucerfont, a CRFR antagonist. This intervention is being tested in anxious, recently abstinent, alcoholic women, and initial testing indicated efficacy at reducing cravings in this female population after stressful stimuli (Clinicaltrials.gov 2013).

4.5 Implications for Individualized therapies

The impact of stress on the inability to maintain abstinence is well-established and supported with the statement by National Institute on Drug Abuse (NIDA) director G.R. Hanson that “Patients and treatment providers alike point to stress as the most common cause of relapse” (Hanson 2002). The present study points to the LC-CRFR system as a target of neuronal dysregulation in chronic ethanol exposure, and targeting this system may provide a basis for individualized therapies. For example, CRFR antagonists are currently being examined as a clinical intervention into the dysphoria involved in ethanol abstinence (Clinicaltrials.gov 2013, 2014). Findings presented here may indicate that CRFR antagonists will be more effective in women, where there is a stronger dysregulation of the CRFR system. This is reflected in the current clinical trial targeting CRFR antagonists in abstinent women with a strong anxiety phenotype (Clinicaltrials.gov 2013). In addition, the augmented LC activity observed specifically in females may suggest the potential for selective norepinephrine reuptake inhibitors (SNRIs) in management of alcohol abuse. Indeed, women diagnosed with depression have already been shown to be more responsive to SNRI therapy (Khan et al. 2005; Itoi and Sugimoto 2010; McEwen et al. 2012). In summary, further analysis of the neurobiological bases for sex differences in alcohol dependence can advance our understanding of their etiology and guide individualized therapies.

Acknowledgements

This project was supported by National Institute on Alcohol Abuse and Alcoholism grant AA021637 awarded to T. Retson, and National Institute on Drug Abuse grant DA009082 and DA020129 awarded to E. Van Bockstaele. The authors would like to thank M.A. Rubel and J.A. Yost for their ongoing discourse.

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Highlights

- Chronic ethanol exposure results in an anxiety-like phenotype by elevated zero maze
- LC activity, measured by c-Fos, is altered between sexes after chronic ethanol
- Sex differences are observed in the subcellular distribution of CRFRs in LC neurons

Figure 1.

Graphs illustrating metrics analyzed during a 10 min exposure to the elevated zero maze (EZM). A illustrates distance traveled. Significant main effects were observed for treatment ($p < 0.001$) and sex ($p < 0.05$), with ethanol treated subjects traveling less than controls and females traveling more than males. B illustrates the percentage of trial time spent in the open arms. Significant main effects of sex ($p < 0.05$) and treatment ($p < 0.001$) were seen, with ethanol treated animals spending less time in the open arms, and female control animals spending more time in the open arms than male control animals. C illustrates the average number of head dips. A significant interaction between sex and treatment was seen ($p < 0.05$), with control animals exhibiting this behavior more than the ethanol treated subjects, and female control subjects performing more of this behavior than male control subjects. D illustrates the average number of standing episodes exhibited during the trial. A significant difference was seen between treatment groups ($p < 0.001$) with controls animals exhibiting this behavior more often.

Figure 2.

Graphical depiction of c-Fos IR labeling in LC. A–D show representative labeling of c-Fos in males and females of both control and treatment groups. As shown in E, the LC of female ethanol treated subjects had significantly more c-Fos IR than controls ($p < 0.05$), while no difference was seen between male groups. F is a schematic from the Rat Brain Atlas of Paxinos and Watson (1998) illustrating the location of the LC, with a high magnification representation of the boxed area. G illustrates c-Fos labeling (red, arrows) in the peri-LC and LC core of a female ethanol treated subject, which can be localized in NE neurons as seen by tyrosine hydroxylase (TH; green). G' is a higher magnification representation of the boxed portion of G. 4v, fourth ventricle

Ratio c-Fos to BA protein

Figure 3.

Graph depicting a significant interaction in the level of c-Fos protein across sex and treatment. The female ethanol treated group had significantly more c-Fos protein than control counterparts ($p < 0.01$), while no difference was seen between male control and ethanol treated groups.

Figure 4.

Sections from control and alcohol-treated rats with immunogold-silver labeling for CRFr within the cytoplasm (arrows) and along the plasma membrane (arrowheads). Dendrite from a male control rat shows predominant localization along the plasma membrane (A), while CRFr labeling is increased in the cytoplasm following alcohol exposure (B). In contrast to control male rats, CRFr labeling is prominent in the cytoplasm (arrows) in control female rats (C), while following alcohol exposure, CRFr labeling is seen predominantly at the plasma membrane (arrowheads). A significant interaction between sex and treatment

($p < 0.01$) was observed with respect to the ratio of cytoplasmic to total CRFr labeling. Unlabeled axon terminals (ut) are shown contacting the CRFr-containing dendrites (A,B,D). m, mitochondria. Scale bars, 0.5 μ m.

Figure 5.

Schematic diagram depicting a working model of ethanol-induced adaptations in LC neurons. In male subjects chronic ethanol is activating the neural stress systems and increasing CRF release onto the dendrites of LC neurons. Upon CRFr activation, the receptor is internalized and deactivated, as proposed by previous studies looking at agonist-induced CRFr activity. This alteration in the CRFr system then decreases subsequent neuronal activation, and is accompanied by desensitization followed by habituation of the LC-NE-CRFr system to chronic ethanol exposure. In females, the working model posits that CRFr are not internalized as seen in males, and this increased number of CRFr on the plasma membrane is then hypothesized to promote repeated CRF signaling induced by chronic ethanol. This continued signaling may be responsible for the increased LC activation seen in the FEtoH group via c-Fos IR, and for the anxiety-like phenotype seen in females in the EZM. Further, the CRFr system dysregulation has implications for altered stress vulnerability seen in withdrawal and abstinence, making the CRFr system an attractive target for therapeutic investigation.