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Amygdalar neuronal plasticity and the interactions of alcohol, sex, and stress

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Introduction

Statistics from the National Library of Medicine indicate that 17.6 million American adults either have a problem with alcohol or are alcoholics (Alcoholism 2011), with an estimated societal and health cost of over \$180 billion annually (Harwood 2000). While statistically there are more men suffering from alcoholism, there is an increasing body of evidence to support a greater impact of alcohol exposure on females as compared to males (Hommer 2003). The National Institute on Alcohol Abuse and Alcoholism states that 5.3 million women in the United States use alcohol in a way that “threatens their health, safety and general well-being,” and that the problems that plague women drinkers are equal to, or greater than, those that affect men (Alcoholism 2008). Compared to men, over the same timeframe and with comparable alcohol use women experience a higher incidence of liver disease and cardiomyopathy (Thurman 2000; Fernandez-Sola et al. 1997), enhanced motor and cognitive impairment following exposure to alcohol (Ceylan-Isik et al. 2010; Nixon et al. 2002), and have a 50 to 100 percent higher death rate due to accidents, suicides, and health problems (Alcoholism 2008). However, despite this data women remain an understudied population, particularly in pre-clinical work. This study sought to identify neurobiological bases for the sex differences in alcohol dependence in stress-integrative circuits to potentially lead to improved individualized therapies.

A large body of work has focused on acute exposure to alcohol at the beginning of the addiction cycle, and on later stages of alcohol addiction such as withdrawal and relapse. Gaps still exist however, in our understanding of the transition to dependence, which is widely thought to be facilitated by changes in the brain's closely intertwined reward circuitry and stress systems. While stress and reward normally cause adaptive responses, dysregulation of these systems results in maladaptive long-term changes. Increasing evidence exists for alcohol's effects on neuronal alterations occurring not only at a cellular

level, but a molecular and epigenetic one as well (Leshner and Koob 1999; Koob and Le Moal 2001; Ponomarev 2013).

With transcription and translation underlying these alterations, a useful approach employs the identification of immediate early genes (IEGs), such as c-Fos (Ryabinin 2000; Vilpoux et al. 2009). C-Fos is a nuclear protein that heterodimerizes with the c-Jun transcription factor, and this complex can then bind to and alter expression of genes containing AP-1 regulatory elements (Sng et al. 2004). An advantage to using c-Fos in neuronal activation studies is its quick induction and return to baseline levels, allowing for a snapshot view of neuronal activity. Further, c-Fos has been used extensively to map neuronal activation patterns in the context of acute, chronic, and withdrawal from alcohol use (for review, see Vilpoux et al. 2009), in addition to other drugs of abuse and stress (Erb et al. 2005; Boutillier et al. 1991; Sabban and Kvetnansky 2001; Zlebnik et al. 2013; Vilpoux et al. 2009). In contrast, the protein FosB can provide a 'long-exposure' image of neuronal activation. FosB is a stable form of the IEG FosB, which can persist for weeks to months, even after cessation of an intervention (Chen et al. 1997; Li et al. 2010; Nakabeppu and Nathans 1991). FosB accumulates with repeated neuronal activation, and can be seen after chronic stressors, chronic drug use, and repeated exposure to rewarding stimuli (for review see McClung et al. 2004) (McClung et al. 2004). Thus, in combination with detection of c-Fos, an increased understanding of the role of FosB will provide insight into the changes taking place with chronic alcohol use.

Several lines of evidence indicate that following chronic alcohol exposure there is a change in frontal-limbic interactions that alter the hypothalamic-pituitary-adrenal axis (HPA) responses (Lovallo 2006). One component of the amygdalar complex, the central nucleus of the amygdala (CeA), is also thought to play an influential role in the regulation and maintenance of alcohol drinking behavior (McBride 2002; Funk and Koob 2007). Increases in neuronal activation are seen after acute alcohol use and in withdrawal, while chronic alcohol administration results in lesser activation, indicating adaptation (Vilpoux et al. 2009). Further, the amygdala is implicated in addiction-related plasticity (Kalivas and O'Brien 2008; Clapp et al. 2008), and a variety of gene expression changes have been observed in the CeA in the context of chronic alcohol use and withdrawal, including receptor and signaling alterations (Obara et al. 2009; Hansson et al. 2008; Freeman et al. 2013). Previous studies have shown that the CeA is enriched with corticotropin releasing factor (CRF) containing neurons, a neurohormone that plays a pivotal role in the stress response (Cassell and Gray 1989; Cassell et al. 1986; Gilpin 2012), and enkephalin (ENK) containing neurons, an endogenous opioid involved in reward (Randall-Thompson et al. 2010; Criado and Morales 2000). While both ENK and CRF are known to colocalize with GABA in the CeA, there is little to no overlap between the two peptides (Day et al. 1999; Veinante et al. 1997). Alterations in the endogenous opioid system are observed following exposure to alcohol, and ENK and its receptor (the delta opioid receptor; DOR), are thought to play a role in alcohol's effects (Mendez and Morales-Mulia 2008). ENK is known to be involved in the rewarding actions of alcohol (Tseng et al. 2013), and it may play a role in promoting and stimulating intake (Chang et al. 2010). In support of this, an acute dose of ethanol was shown to increase c-Fos immunoreactivity in ENK neurons of the CeA, and

high levels of ENK is a predictor of increased alcohol consumption (Karatayev et al. 2010; Criado and Morales 2000).

Dysregulation of the CRF system is observed in both stress and affective disorders (Koob and Zorrilla 2010), and stress promotes sustained alcohol use and the reinstatement of alcohol seeking behaviors (Breese et al. 2005; Le et al. 2000; Martin-Fardon et al. 2010). CRF receptor (CRFr) antagonists are being evaluated therapeutically for the management of alcoholism (Lowery and Thiele 2010; Huang et al. 2010), and when administered directly into the CeA block binge drinking behaviors in mice (Lowery-Gionta et al. 2012), in addition to efficacy at blocking relapse-like behaviors precipitated by stressful events (Lowery and Thiele 2010; Baldwin et al. 1991; Nielsen et al. 2004). Further, it has been established that differences exist between males and females in CRFr trafficking in stress-responsive brainstem regions, which may play a role in an altered vulnerability to stress in females (Bangasser et al. 2010; Bangasser and Valentino 2012). Thus, with ENK playing a role in the early stages of the addiction cycle and initiation of dependence, and CRF exhibiting a role during maintenance of intake and relapse, the present study sought to investigate how chronic ethanol exposure coordinately regulates these peptidergic circuits. Overall, this work examines the consequences of alcohol on CeA activation both at baseline and after a stressor, and identifies the phenotype of the activated neurons to elucidate the role of CRF and ENK in mediating neuronal activity following stress under alcohol dependent conditions across the sexes.

Materials and Methods

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 pair-fed a calorically matched liquid diet. Treated animals received 36% of total calories as ethanol while control animals received a diet in which ethanol calories were replaced by maltose-dextrin (the remaining calories consisted of 11% from carbohydrates, 35% fat, and 18% protein, Bio-Serv, Frenchtown, NJ). Animals receiving the ethanol-containing diet averaged an ethanol intake of 12–14 g/kg/day. Ethanol concentrations in the liquid diet were ramped up stepwise over a period of 4 days (2 days ethanol-free liquid diet, 2 days 22% of calories as ethanol), prior to animals receiving the full strength ethanol diet, which was then administered for 14 days. Animal handling and diet administration was the task of trained animal technicians experienced in working with alcoholic animals (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 swim stress 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 was taken from all animals and used for the measurement of CORT. Blood alcohol content (BAC) was obtained to insure animals had an active BAC at times of experiment and sacrifice, and all female animals underwent vaginal cytology to determine estrus phase. All CORT (n=17 MCtrl, n=16 MEtoh, n=20 FCtrl, n=22 FEtoh) and BAC (MEtoh = 13, FEtoh = 18) measures were performed by the Thomas Jefferson University clinical laboratories. After sacrifice, adrenal and brain weights were measured (n=12 for all groups).

Behavioral Measures

The forced swim test (FST), a robust stressor, was performed using methods previously described (Reyes et al. 2008; Waselus et al. 2009). Briefly, rats were individually placed into cylindrical glass tanks filled with water to a depth of 30cm, such that their tails were not able to touch the bottom of the tank. Swim trials lasted 15 minutes and at the conclusion rats were removed, towel dried, and placed in a warming cage containing a heating blanket (approximately 37° C). Rats were sacrificed one hour after completion of swim trials. Data is presented for 20 male and 24 female rats, 4 animals were omitted (3 MCtrl, 1 MEtoh) for being removed from the FST tanks prior to the completion of the testing period. FST trials began at 07:00, such that animals would still be under the influence of ethanol during the FST and at time of sacrifice, and multiple trials were conducted simultaneously through the use of multiple tanks. Control animals were brought to the same room and handled briefly. Time spent engaged in each of three behaviors; immobility (only the movements necessary to keep head above water), swimming (change in position of at least one quarter the circumference of cylinder), or climbing (active movements with forepaws in and out of water, directed against cylinder walls) was recorded. Data was quantified manually by an observer blinded to the animal's gender and study condition.

Immunohistochemistry

For immunohistochemistry, rats were deeply anesthetized via exposure to isofluorane (Vedco, St. Joseph, MO) in a specialized holding cage, then transcardially perfused via the ascending aorta with heparin followed by a 2% paraformaldehyde and 3.75% acrolein (Electron Microscopy Sciences, Fort Washington, PA) mixture in 0.1M phosphate buffer (PB; pH 7.4). The brains are then collected and stored in the paraformaldehyde and acrolein mixture until sectioning. Sections through the rostro-caudal extent of each brain were collected at 35 µm using a vibrating microtome and serial sections through the CeA were processed for fluorescence as described.

Fluorescence

Sequential sets of tissues through the CeA were placed in 1% sodium borohydride in 0.1M PB for 30 minutes in order to remove any remaining aldehydes. Tissues were then incubated in 0.5% bovine serum albumin (BSA) and 0.25% triton X-100 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 antibodies against c-Fos made in rabbit (1:9000, CalBiochem), FosB made in rabbit (Cell Signaling), CRF made in guinea pig (1:8000, Peninsula Labs), or ENK made in mouse (1:1500, Fitzgerald), as appropriate. Proteins were

visualized using FITC conjugated donkey anti rabbit (1:200), TRIT conjugated donkey anti guinea pig (1:200), and Cy5 conjugated donkey anti mouse (1:200, all secondary antibodies from Jackson ImmunoResearch, West Grove, PA). Control tissues were processed in parallel with the omission of primary antibodies. Tissues at matched rostro-caudal levels were photographed for quantification with an Olympus BX51 (Tokyo, Japan), and captured with Spot Advanced Software (Diagnostic Instruments Inc, Sterling Heights, MI). For

FosB quantification, n=6 MCtrl, n=4 FCtrl, and n=5 MEtoh and FEtoh. For c-Fos quantification the stress(−) group contained n=5 MCtrl, MEtoh, and FEtoh, and n=4 FCtrl animals. The stress(+) group contained n=6 of all conditions.

Quantification

Serial sections through the extent of the CeA were used for c-Fos, FosB, and peptide analysis. The CeA is a component of the amygdalar complex with variable morphology along its rostral-caudal extent that, according to the Paxinos and Watson Rat Brain Atlas (Paxinos 1997), extends approximately from bregma −1.80 to −3.30. Six non-overlapping sections were identified that covered the rostral-caudal extent of the CeA, in order to obtain a representative sample. To achieve a reproducible sample, the six sections were matched across each animal and compared to the Paxinos and Watson Rat Brain Atlas as a reference (Paxinos 1997). Specifically, rostral-caudal levels represented in plates 25–30 were selected for the analysis. Quantification was performed in these non-adjacent sections, which prevented double counting of neurons. The CeA was then divided into three subgroups based on rostral-caudal morphology. CeA sections at Bregma −1.80 and −2.20 (corresponding to plates 25–26) were denoted as rostral CeA (rCeA), Bregma −2.20 and −3.10 (sections corresponding to plates 27–29) were denoted mid-CeA (mCeA), and Bregma −3.10 (composed of a section corresponding to plate 30) was denoted caudal CeA (cCeA). Further, within the CeA, three subdivisions have been identified – medial (CeM), lateral (CeL), and capsular (CeC). To best describe the nature of CeA activation, labeled cells have been quantified as being located in the CeM, CeL, or CeC utilizing the distinctions set forth in the Paxinos and Watson Rat Brain Atlas, and based on their location within the CeA at the rostral-caudal level of the section being analyzed (Paxinos 1997). The number of labeled cells per region is presented by average number of cells for all animals $\pm$ SEM, or by percentage of total labeled cells for all animals $\pm$ SEM, as denoted.

Statistical Analysis

Averages and standard errors for all groups were computed, and presence of significant interactions were examined with a two way or three way analysis of variance (ANOVA), with gender, treatment, an stress serving as independent factors, or with the addition of subregion, CeA extent, or time serving as repeated measure where indicated. Significant interactions were further examined as described, using additional post hoc ANOVAs or Student's t-tests (unpaired, two-tailed) to detail findings. Significance was set to $p < 0.05$ for all comparisons, and all statistical analysis was performed using IBM SPSS Statistics software version 19.

Results

Analysis of BAC, brain and adrenal weights, and CORT levels

Blood was collected at time of euthanasia and blood alcohol content (BAC) levels were quantified in all subjects. To examine the effects of stress on gender, a 2-way crossed ANOVA was conducted. A significant main effect of gender was observed in BAC levels [$F(1,28)=10.31$, $p=0.003$] as was a main effect of stress condition [$F(1,28)=9.37$, $p=0.005$]. The interaction between factors, however, was not significant ($p=0.36$). In the stress(−) group, male ethanol treated subjects (MEtoh) had an average BAC of 275.33 ± 83.44 mg/dl and female ethanol treated subjects (FEtoh) had an average of 124.17 ± 32.89 mg/dl. The stress(+) group had average BACs of 129.64 ± 28.75 mg/dl for MEtoh animals and 46.42 ± 20.62 mg/dl for FEtoh.

With respect to adrenal weights (figure 1A), overall analysis performed using two factor ANOVA (gender and treatment) resulted in a significant two way interaction [$F(1,44)=10.27$, $p=0.003$]. Significant main effects of ethanol treatment ($p=0.001$), and gender ($p=0.007$) were also seen. To further understand the interaction, a post hoc analysis using a Student's t-test (unpaired, two-tailed) revealed that while female adrenal weights were similar across treatment (0.0458 ± 0.003 g for female control subjects (FCtrl) and 0.0462 ± 0.004 g for FEtoh), male adrenal weights were increased after ethanol treatment (t-test, $p=0.002$), with male control subjects (MCtrl) displaying adrenals weighing 0.0328 ± 0.006 g, while MEtoh animals averaged 0.0470 ± 0.012 g. Brain weights were also measured in all groups and no significant difference was observed between sexes or following ethanol treatment [$F(1,44)=0.001$, $p=0.975$].

A three-way ANOVA (crossing gender, treatment, and stress) did not reveal a significant overall interaction on CORT levels (figure 1B). A significant interaction of treatment and stress was however observed [$F(1,66)=40.37$, $p=0.002$], and in addition to a significant main effect of stress [$F(1,66)=19.03$, $p=0.034$]. Consistent with the previous literature, in the stress(−) group, ethanol exposed animals had slightly higher CORT levels than their control counterparts (MCtrl 3.43 ± 0.71 mcg/dl, MEtoh 4.55 ± 0.86 mcg/dl, FCtrl 4.13 ± 1.12 mcg/dl, FEtoh 7.08 ± 1.05 mcg/dl). Additional post hoc analysis with Student's t-test revealed MCtrl and MEtoh groups had similar CORT levels, but a significant difference could be seen between FCtrl and FEtoh groups ($p=0.040$). In the stress(+) conditions, control animals had similar CORT levels to the stress(−) groups (MCtrl 3.99 ± 0.45 mcg/dl, FCtrl 4.53 ± 0.96 mcg/dl), while the ethanol treated animals had lower, but not significant, CORT readings (MEtoh 3.52 ± 0.83 mcg/dl, FEtoh 2.94 ± 0.97 mcg/dl). To further examine the main effect of stress, a series of post hoc t-tests were performed to compare stress(−) to stress(+) subjects of the same treatment and gender. A significant difference was found between FEtoh stress(+) and stress(−) subjects, with the CORT levels of the FEtoh treated animals being significantly lower after the FST ($p=0.002$).

Finally, vaginal cytology was conducted at time of euthanasia for all female subjects. Of the FCtrl subjects, 10 were in diestrus (DE), 4 in proestrus (PRO), and 5 in estrus (EST). Of the FEtoh subjects, 13 subjects were in DE, 2 in metestrus (ME), 2 in PRO and 5 in EST. The phase of the estrus cycle was found to have no significant interaction with stress, treatment,

or overall, on CORT levels, and no significant interaction with stress on BAC. In the experiments examining c-Fos levels there were 15 animals in DE, 2 in ME, and 4 in EST, and when pairwise comparisons were made between estrus phases, no significant differences were observed. In studies examining FosB activation patterns 6 animals were in DE or ME, and 3 in EST, again no significant interactions were seen with varying estrus phase. Finally, in the study detailing swim stress response 15 animals were in DE, 5 in PRO and 4 in EST. No significant interaction between estrus phase and treatment group was noted.

Ethanol-induced effects on FosB expression in amygdalar subdivisions: identification of neuronal phenotypes

The CeA is a subdivision of the amygdalar complex with varied subnuclei along its rostral-caudal extent. Extending from approximately Bregma -1.80 to -3.30 , three subdivisions have been described – medial (CeM), lateral (CeL), and capsular (CeC) (Paxinos 1997). Further, the CeA can be divided into subregions of rostral (rCeA), mid (mCeA), and caudal (cCeA). Figures 2 and 3 present the average $\pm$ SEM total number of FosB IR neurons for male and female, control and ethanol treated animals, in addition to the average total number of FosB IR neurons in each subdivision and subregion. Figure 2 shows sections through the CeA illustrating differences in the levels of FosB IR between groups. A two factor ANOVA resulted in a significant overall interaction between gender and treatment [$F(1,16)=9.41$, $p=0.007$]. There was also a significant main effect of treatment [$F(1,16)=27.449$, $p<0.001$] and in all cases, the ethanol-treated animals had more FosB IR labeling than their control counterparts with MEtoH animals exhibiting the greatest number of labeled neurons. To further analyze the effect of ethanol treatment on FosB IR, post hoc Student's t-tests were performed and indicated that the MEtoH group had significantly more FosB IR ($p<0.001$) than controls, but that this difference was not significant between FEtoH and FCtrl groups. When comparing across genders, the FCtrl group displayed significantly higher numbers of FosB IR neurons compared to the MCtrl group ($p<0.001$), and while MEtoH animals expressed larger numbers of FosB IR than the FEtoH group, this was not significant.

When examining medial, lateral, and capsular subdivisions of the CeA (figure 3), overall analysis of division was performed using a three factor ANOVA (crossing treatment, gender, and subdivision, with subdivision taken as a repeated measure). This resulted in a significant main effect of subdivision [$F(1,16)=36.04$, $p<0.001$], and a significant interaction of subdivision and treatment [$F(1,16)=7.14$, $p=0.017$]. To further examine the effect of ethanol exposure, a two way ANOVA was performed for male or female subjects (treatment and subdivision as a repeated measure) and a significant interaction of treatment and subdivision was observed in males, but not females [$F(1,9)=6.025$, $p=0.036$]. Examination of FosB IR neurons by rostral-caudal extent yielded a significant interaction between extent, gender, and treatment [$F(1,16)=5.301$, $p=0.035$]. To examine the effect of ethanol treatment, a two way ANOVA was performed for male or female subjects (treatment and extent as a repeated measure) and a significant interaction of treatment and extent was observed [$F(1,9)=56.040$, $p<0.001$] in males, but not females.

FosB IR neurons that were co-labeled with CRF or ENK were seen throughout the rostral-caudal extent of the CeA and in all three subdivisions as detailed in table 1, and shown in figure 4. As expected from the distributions of ENK and CRF, the largest numbers of dual IR neurons were present in the mCeA, and CeL and CeC subdivisions. Analysis was performed using a two factor ANOVA (gender and treatment) which noted no overall significant interaction. Within genders, there was an increase in FosB/CRF dual IR in the ethanol treated groups, with the largest difference being present between MCtrl and MEtoh groups. When post hoc analysis was performed to compare treatment groups of each gender using Student's t-test, a significant difference was observed ($p < 0.001$) between the MEtoh and MCtrl groups, while the difference between FCtrl and FEtoh groups was non-significant. MCtrl and FCtrl groups were also compared using a Student's t-test to examine differences in FosB/CRF dual IR and a significant difference was noted between genders ($p = 0.008$), with the females exhibiting a higher level of FosB/CRF dual IR at baseline. The role of ENK was also examined with FosB, and overall analysis was performed using a two factor ANOVA (gender and treatment) which showed a non-significant interaction between gender and treatment, but a main effect of treatment [$F(1,16) = 5.757$, $p = 0.029$]. To further examine the effect of treatment, t-tests were performed between control and treated subjects of each gender as a post hoc analysis. A significant increase in dual ENK/ FosB labeling was seen between MCtrl and MEtoh groups ($p = 0.041$), while the increase in labeling between FEtoh and FCtrl groups was not significant ($p = n.s.$).

Ethanol-induced effects on swim behavior

Subjects were exposed to a 15 min forced swim test, and the amount of time spent exhibiting three behaviors (immobility, climbing and swimming) was recorded (figure 5). The FST was also divided into three periods of 5 minutes (m) to examine changes in behaviors over time. Overall, when performing a two way ANOVA (treatment and gender), a significant two way interaction was seen in amount of time spent exhibiting the swimming behavior [$F(1,39) = 4.326$, $p = 0.044$]. Of the 15m, the MCtrl group spent 679.6 ± 23.2 seconds (s) swimming, the MEtoh group spent 565.1 ± 19.2 s, the FCtrl group spent 669.0 ± 16.0 s and the FEtoh group spent 632.1 ± 16.4 s. A three way ANOVA was performed to examine the effect of FST time period on swimming behavior (gender, treatment, and time, with time as a repeated measure), and a main effect of time could be seen [$F(1,39) = 33.036$, $p < 0.001$] with a non-significant overall interaction (figure 5A). To better describe the effect of time on swim behaviors, each time point was analyzed with a two way ANOVA (gender and treatment). The 0-5m swim had a non-significant overall interaction. The 5-10m swim showed a significant main effect of treatment [$F(1,39) = 12.830$, $p = 0.001$], with the ethanol groups spending less time engaged in swimming compared to controls. When performing post hoc Student's t-tests, there was no significant difference between MCtrl and FCtrl animals, with a significant decrease noted for MEtoh compared to MCtrl swim times ($p = 0.008$), and a trend toward a significantly lower swim time of FEtoh than the FCtrl group ($p = 0.06$). The 10-15m swim also showed a significant main effect of treatment [$F(1,39) = 10.870$, $p = 0.002$]. Performing post hoc t-tests to compare groups showed no difference between MCtrl and FCtrl groups, or between FCtrl and FEtoh groups, but a significant decrease in swim behaviors of MEtoh vs MCtrl groups ($p = 0.008$).

To examine climbing behaviors (figure 5B) a two way ANOVA (treatment and gender) was performed which showed no overall interaction between treatment and gender, but did show a main effect of treatment [$F(1,39)=5.502$, $p=0.024$]. Of the 15m, the MCtrl group spent 88.3 ± 14.2 s climbing, the MEtoH group spent 132.1 ± 22.5 s, the FCtrl group spent 96.2 ± 9.1 s, and the FEtoH group spent 124.4 ± 14.1 s. A three way ANOVA was performed to examine the effect of FST period on climbing behavior (gender, treatment, and time, with time as a repeated measure), and a main effect of time could be seen [$F(1,39)=163.71$, $p<0.001$] with a non-significant overall interaction. To better describe the change in climbing behaviors over time, each time block was analyzed with a two way ANOVA (gender and treatment). The 0-5m climb had a non-significant overall interaction, while the 5-10m climb had a non-significant overall interaction and a main effect of treatment [$F(1,39)=5357.255$, $p<0.001$]. Post hoc Student's t-test were performed, and it was seen that MEtoH subjects spent significantly more time climbing than MCtrl subjects ($p=0.001$), as did FEtoH subjects compared to controls ($p=0.012$). When examining climbing behaviors between 10-15m, again, no overall interaction was present, but a significant main effect of treatment was seen [$F(1,39)=5.056$, $p=0.030$]. Here, the MEtoH group again spent significantly more time engaged in climbing than their control counterparts ($p=0.048$), while no difference was seen between FEtoH and FCtrl or between control groups.

Finally, floating behaviors were examined (figure 5C). A two way ANOVA (treatment and gender) was performed which showed no overall interactions, but a main effect of treatment [$F(1,39)=5.056$, $p=0.030$]. Of the 15m, the MCtrl group spent 133.3 ± 22.2 s floating, the MEtoH group spent 202.0 ± 27.5 s, the FCtrl group spent 134.0 ± 17.0 s, and the FEtoH group spent 142.8 ± 16.6 s floating. To further investigate this behavior a three way ANOVA was performed (gender, treatment, and time, with time as a repeated measure). This showed no significant overall interactions, but a main effect of time [$F(1,39)=31.210$, $p<0.001$]. To better describe the change in floating behaviors over time, each time block was analyzed with a two way ANOVA (gender and treatment). No significant overall interactions were seen in any of the three time blocks, but a significant effect of treatment could be seen in the 10-15m period [$F(1,39)=4.882$, $p=0.033$]. Post hoc testing was performed to compare between treatment conditions in the 10-15m block. There was no significant difference between FEtoH and FCtrl groups ($p=ns$), and while MEtoH subjects trended towards higher average float times than controls, this difference was not significant ($p=.059$).

Ethanol and stress-induced effects on c-Fos expression in amygdalar subdivisions: identification of neuronal phenotype

Overall analysis performed using three factor ANOVA resulted in a significant three way interaction (gender, treatment, and stress) [$F(1,35)=4.184$, $p=0.048$] in numbers of c-Fos IR neurons present in the CeA. Table 2 details the average $\pm$ SEM numbers of cFos IR neurons by subdivision, or extent for control and ethanol treated, male and female, and swim(+) or swim(-) subjects. To explain the source of the interaction, a post hoc analysis was performed via two factor ANOVA (treatment and gender) for the stress(-) and stress(+) groups. In the stress(-) condition (figure 6) a significant interaction of gender and treatment could be seen [$F(1,15)=4.664$, $p=0.047$] in c-Fos IR. Further post hoc testing was performed with a Student's t-test to examine the differences in gender and revealed that males had

similar levels of labeling regardless of treatment, while female ethanol treated subjects had significantly more c-Fos IR than controls ($p=0.032$). When comparing across genders, control animals had similar levels of labeling while FEtoH animals had significantly more c-Fos IR than MEtoH animals (t-test, $p=0.008$).

In all cases, stress(+) subjects (figure 7) exhibited an increased number of activated neurons compared to the stress(-) condition. Among the stress(+) condition groups, a two way ANOVA (treatment and gender) did not reveal a significant overall interaction [$F(1,20)=1.071$, n.s.], however a main effect of treatment could be seen [$F(1,20)=5.047$, $p=0.036$]. Further post hoc testing was performed with a Student's t-test to examine the differences in gender and showed that males exhibited a trend toward c-Fos IR increase after ethanol treatment ($p=0.077$), while there was no difference between stress(+) female treated and control groups. When compared across genders using a post hoc Student's t-test, stress(+) FCtrl subjects had more c-Fos IR than MCtrl subjects ($p=0.045$), while there was no difference between MEtoH and FEtoH groups.

To identify differences in c-Fos IR between CeA subdivisions (CeM, CeL, and CeC, as seen in figure 8) overall analysis of subdivision was performed using four way ANOVA (gender, treatment, stress, and subdivision, with subdivision taken as a repeated measure). This resulted in a significant main effect of subdivision [$F(1,35)=17613$, $p<0.001$]. To further examine the effect of stress, division analysis was performed within stress(-) and stress(+) groups. In the stress(-) groups, using a two factor repeated measures ANOVA (treatment and subdivision, with subdivision as the repeated measure) for male subjects, no difference in c-Fos IR was observed, while in females a difference in labeling was seen through a significant interaction between subdivision and treatment [$F(1,7)=7.208$, $p=0.031$]. When examining the stress(+) condition, while there was a main effect of subdivision in males [$F(1,10)=6.960$, $p=0.025$] there was no overall interaction between subdivision and treatment, and no differences were seen in females.

Examination of c-Fos IR neurons by rostral-caudal extent with a four factor ANOVA (gender, treatment, stress, and extent as a repeated measure) yielded a significant four way interaction [$F(1,34)=4.537$, $p=0.040$]. In the stress(-) condition these interactions were dissected using a 3 way ANOVA (gender, treatment, and extent as a repeated measure) post hoc, which showed a significant 3 way interaction [$F(1,14)=8.257$, $p=0.012$]. Further post hoc ANOVAs (treatment and extent) showed no difference in extent labeling between male treatment groups, and a significant difference in extent between FCtrl and FEtoH subjects [$F(1,7)=12.068$, $p=0.010$]. In the stress(+) condition males and females exhibited a main effect of extent [$F(1,10)=48.047$, $p<0.001$ for males and $F(1,10)=72.240$, $p<0.001$ for females] but no difference in either sex between treatment conditions.

c-Fos IR neurons that were co-labeled for CRF or ENK were present throughout the rostral-caudal extent of the CeA, and in all three subdivisions as detailed in table 3 and shown in figure 9. With respect to c-Fos and CRF IR co-labeling, a three factor ANOVA (gender, treatment, and stress) revealed a significant three way interaction [$F(1,35)=4.328$, $p=0.045$]. The stress(+) group was examined via ANOVA for treatment and there was a significant increase in dual IR of the stress(+) ethanol treated groups [$F(1,24)=7.540$, $p=0.011$]. In the

stress(–) condition, while the FEtoH group had the highest level of dual labeling, a post hoc Student's t-test revealed that this was not significantly higher than the FCtrl group ($p=n.s.$). When examined by CeA subdivision (CeM, CeL and CeC), a significant main effect of subdivision could be seen across groups (gender, treatment, stress, and subdivision as a repeated measure) [$F(1,35)=37.729$, $p<0.001$], with the largest number of labeled neurons appearing in the CeL and CeC subdivisions. When examined by rostral-caudal extent there was also a main effect of extent (gender, treatment, stress, and extent as a repeated measure) [$F(1,35)=87.318$, $p<0.001$] with the mCeA extent containing the most numerous dual IR labeling, paralleling the distribution of CRF in the CeA.

When c-Fos and ENK dual labeled neurons were examined there was a trend toward a significant interaction (stress, gender, and treatment) between all groups [$F(1,35)=2.981$, $p=0.093$] (figure 9). When examining MCtrl and FCtrl groups, there were increases in c-Fos/ENK dual IR neurons after the FST in both sexes, and MEtoH animals showed a larger increase in c-Fos/ENK IR than seen with MCtrl groups. When examined by CeA subdivision all groups expressed the largest number of c-Fos/ENK dual IR neurons in the CeL and CeC subdivisions. A way four ANOVA (gender, treatment, stress, and subdivision as a repeated measure), revealed a significant interaction between CeA subdivision and stress [$F(1,35)=13.327$, $p=0.001$], and a significant main effect of division [$F(1,35)=72.720$, $p<0.001$]. When considering rostral-caudal extent, a four way ANOVA (gender, treatment, stress, and extent as a repeated measure) revealed a significant interaction between rostral-caudal extent and stress [$F(1,33)=20.027$, $p<0.001$], and a significant main effect of extent [$F(1,33)=34.876$, $p<0.001$]. The largest numbers of dual IR were seen in the mCeA region, as parallels the distribution of ENK in the CeA.

Discussion

The present study evolves the existing literature detailing effects of chronic ethanol exposure on stress-integrative circuitry, and demonstrates important sex differences in neuronal activation of a limbic pathway. Using the chronic ethanol feeding paradigm, comparisons were made between long term and short term neuronal activation patterns within subdivision of the CeA, and along its rostral-caudal extent. Further, the effect of an acute stress on neuronal activation in ethanol treated subjects of both genders revealed important differences.

Methodological Considerations

The identification of regional subdivisions within the CeA is complex, with some studies utilizing the boundaries proposed by Swanson (1998) (Swanson and Petrovich 1998; Day et al. 1999; Marchant et al. 2007) and others utilizing those of the Paxinos and Watson Rat Brain Atlas (1997) (Fu and Neugebauer 2008; Lam and Gianoulakis 2011; Spannuth et al. 2011; Paxinos 1997). Here, subdivisions of the CeA were based on the Rat Brain Atlas of Paxinos and Watson (1997), which corresponds with previous work from our group as well as other studies focused on sex differences and the effect of ethanol on neural circuits (Retson and Van Bockstaele 2013; Gilpin et al. 2012; Karanikas et al. 2013).

Although every effort was made to survey a large sample of the CeA, it is likely that the number of c-Fos and FosB containing neurons have been underestimated. Furthermore, the numbers of CRF and ENK neurons may have been underestimated as animals were not treated with colchicine, an axonal transport blocker sometimes used to increase peptide visualization (Swanson et al. 1983), since this would have impacted neuronal activation patterns. Importantly, this study is the first to detail the distribution of CRF and ENK CeA neurons expressing markers of neuronal activation in the context of chronic ethanol use and stress, with considerations of sex differences.

As an increasing number of studies examine the interplay between sex hormones and alcohol, the impact of circulating hormones in freely cycling female animals must be considered. Many examples can be seen where differences exist between ovariectomized animals and freely-cycling counterparts. For example, ovariectomized animals exhibited decreased ethanol intake in 2-bottle choice studies (Ford et al. 2002), and show reduced responses to the rewarding effects of ethanol (Torres et al. 2014). When allowed to cycle freely, no differences were observed across the estrus cycle in a choice paradigm, but when cycle was pharmacologically determined, an effect of estrus phase was present (Roberts et al. 1998). These varied findings reinforce the role of sex hormones in differential alcohol usage and sequelae, as well the importance of considering freely cycling subjects in studies. In the present work, when subjects were examined for phase of the estrus cycle, there was no significant difference between phases across groups. While the effects of the estrus cycle are primed for future studies, these data are consistent with previous studies showing a lack of effect of estrus cycle on stress responses (Guo et al. 1994; Rivier 1999; Bland et al. 2005; Babb et al. 2013), a lack of effect of estrus cycle on the aversive effects of ethanol (Torres et al. 2014), and no change across estrus phase in ethanol use in freely cycling rats (Ford et al. 2002; Roberts et al. 1998).

Alcohol and sex effects on BAC and CORT levels

The present study provides evidence that levels of CORT were higher in ethanol treated animals when compared to controls; however, following exposure to the FST, CORT levels were lower in the ethanol treated animals suggesting a possible attenuation of the HPA axis in this group. Some studies suggest that HPA axis dysfunction is a risk factor for future alcohol use disorders (Schepis et al. 2011; Sher 2007), while other studies found that alcoholics with attenuated stress responses were more likely to relapse (Clapp et al. 2008; Obara et al. 2009). Combined with data showing increased adrenal weight in the ethanol treated subjects, these data suggest that chronic treatment may act as a physiological stressor. A large body of evidence exists supporting the notion that alcohol acts as a physiological stressor and that chronic use and relapse vulnerability involves dysregulation of the HPA axis (Stephens and Wand 2012; Sher 2007; Errico et al. 2002; Junghanns et al. 2003; Junghanns et al. 2005), and further that abstinence leads to blunting of the stress response (Junghanns et al. 2003; Errico et al. 2002; Junghanns et al. 2005).

Differences in BAC levels were observed across sex and stress conditions. It should be noted that lower BAC levels observed in the stress(+) group may have resulted from the duration between swim and euthanasia, as animals were not exposed to ethanol during this

period. There were no significant differences in brain weights, indicating that while physical dependence had been achieved, and neuronal loss may be present, that the atrophy seen in after long-term alcohol use in humans was not present (Krill and Halliday 1999).

Ethanol-induced effects on neuronal activation

A large body of work exists describing changes in neuronal activation patterns following acute ethanol exposure or following withdrawal in male animals. Acute administration delivered either intraperitoneally (i.p.) or via vapor chambers increases c-Fos expression in the CeA (Canales 2004; Ryabinin et al. 1997; Criado and Morales 2000). Similarly, withdrawal from an ethanol containing liquid diet induces c-Fos expression in the CeA both in rats and mice (Moy et al. 2000; Olive et al. 2001; Knapp et al. 1998). Following chronic treatment (14+ days) in male rats (Ryabinin et al. 1997) or self-administration in mice (Bachtell et al. 1999), increases in c-Fos expression in the CeA are also observed. However, compared to acute use, levels of c-Fos are reduced after chronic exposure. This observation led to the hypothesis that neuronal circuitry initially activated by ethanol undergoes habituation following chronic exposure (Chang et al. 1995; Ryabinin and Wang 1998).

Although the precise functional role of the long term neuronal activation marker FosB remains unclear, a large body of work indicates that FosB is increased in the brain following exposure to stress (Nikulina et al. 2008), is expressed in limbic circuits following exposure to rewarding stimuli (Berton et al. 2007), and confers behavioral resilience (Vialou et al. 2010a; Vialou et al. 2010b). Increased levels of FosB are seen in the amygdala after social defeat (Nikulina et al. 2008) and following exposure to drugs of abuse such as cocaine. It is hypothesized that drugs of abuse aberrantly activate the FosB system, causing an increase in FosB which then increases the rewarding effects of a drug. McClung and Nestler (2003) described gene expression changes that occurred with cocaine use in an inducible mouse model of FosB expression. The authors saw a shift in FosB effects where short term expression caused a reduced cocaine preference, while long-term expression led to an increased preference for cocaine, hypothesizing a role for FosB in the molecular transition that occurs between acute and chronic usage (McClung and Nestler 2003; McClung et al. 2004).

Comparing FosB and c-Fos activation patterns may provide some important insight into the transition from acute exposure to maladaptive changes seen under chronic conditions in males and females. While male ethanol treated subjects exhibited the greatest number of

FosB neurons compared to all other groups, c-Fos expression levels were similar in male ethanol and control groups. As evidence exists for the role of FosB as a repressor of c-Fos (Renthall et al. 2008), the high levels of FosB in males may underlie the decreased c-Fos expression. It is thus tempting to speculate that this increased activity in the MEtoH animals on a long term scale, contrasted to recent activity patterns similar to those seen in controls, is a component of the mechanism behind habituation seen in previous works after chronic treatment, as presented in figure 10. As increased FosB expression is associated with increased sense of reward, this may also reflect an increased sensitivity to rewarding stimuli in ethanol treated males, a characteristic that is associated in humans with an increased alcohol use (McClung et al. 2004). This is further consistent with human subjects research

indicating a greater number of males vs females afflicted with alcohol use disorder (Alcoholism 2011).

In female subjects, FCtrl animals show more FosB IR than their MCtrl counterparts, indicating an increased level of neuronal activation in the CeA at baseline, which adds to the growing body of knowledge about the subtle inherent differences between male and female brains (Cosgrove et al. 2007). Although the female ethanol-treated group exhibited approximately 20% more FosB than control animals, the increase in FosB expression was substantially smaller than seen between male groups (figure 10). Lower levels of FosB expression in the FEtoH group may indicate alterations in neuronal activity patterns at baseline compared to the higher levels of FosB seen in males, and if FosB is indeed repressing c-Fos expression, the reduced FosB may underlie the increased c-Fos seen here in females at baseline (Renthal et al. 2008). When examining short term activation patterns, FEtoH subjects exhibited almost twice as many c-Fos containing neurons as controls. Comparing the FEtoH treated group to animals exposed to the FST, the FEtoH stress(–) group showed only 13% lesser activation than stress(+) FCtrl animals, and more c-Fos immunoreactivity than stress(+) MCtrl animals. This indicates that chronic ethanol exposure has a substantial impact on the baseline CeA activation of female animals, and this adaptation is similar to neuronal activation in response to stress, reinforcing the role of chronic ethanol as a stressor. If the CeA is not habituating to chronic ethanol exposure at the same level as is seen in male animals, and continues at an increased level of activation with mild perturbations, the effects could be widespread and may contribute to the differential pathologies seen between males and females. Chronic activation of the neuronal stress systems are well associated with downstream consequences. For example, it is known that CeA sends direct CRF or dynorphin innervations to the noradrenergic locus coeruleus (LC), and the serotonergic dorsal raphe nucleus (DRN), both of which are heavily implicated in the dysregulation behind stress, addiction, and affective disorders (Retson and Van Bockstaele 2013; Van Bockstaele et al. 1996). If this continued increased activation in females represents a dysregulation of the stress systems, it may elucidate one mechanism by which alcohol use disorders and affective disorders have high rates of comorbidity, and the adaptations seen after chronic alcohol use may in part underlie the differences seen between genders.

Ethanol induced effects on the behavioral and neuronal activation response to stress

Behavioral differences between ethanol and control groups were seen in the FST. Ethanol treated animal of both sexes spent more time engaging in climbing behaviors which are indicative of an active coping response, a behavioral difference that may be linked to increased levels of FosB IR. While the interplay between FosB and stress is still under investigation, one hypothesis about its actions in a non-manipulated context is that it is responsible for adaptive aspects of the response to stress, and the opposition of learned helplessness (Berton et al. 2007; McClung et al. 2004). This increase in active coping behaviors with increased FosB seen in ethanol treated animals compared to controls may result in findings here that ethanol treated animals spent a significantly greater amount of time engaging in climbing behaviors when exposed to swim stress. While no significant difference was seen in ethanol treated animals in the amount of time spent immobile, the

MEtoh group exhibited this behavior more than any other condition despite exhibiting the highest levels of FosB. While the increase in immobility is consistent with the role of ethanol as a stressor, it is also possible that this behavior is consistent with a decrease in activity of ethanol treated animals noted in other ongoing behavioral studies.

As expected, animals in the control fed group who were exposed to the FST had higher levels of c-Fos IR than control stress(–) subjects, furthering the role of the CeA in the stress response (Ventura-Silva et al. 2013). When comparing MCtrl and FCtrl neuronal activity levels after the FST, FCtrl animals had 25% more c-Fos IR than MCtrl subjects, indicating a greater impact of stress activation in the CeA of control female vs male animals. These findings regarding baseline differences are in agreement with previous studies showing differences between genders in stress sensitivity of the LC, a region that receives dense CeA projections (Curtis et al. 2006; Retson and Van Bockstaele 2013; Van Bockstaele et al. 1996), and the knowledge that women are disproportionately impacted by stress-related affective disorders (Solomon and Herman 2009).

Comparing ethanol exposed and control animals after the FST, the ethanol exposed animals had higher levels of c-Fos IR. This indicates that chronic exposure alters the response to stress as seen by increased neuronal activation in the CeA. Interestingly MEtoh and FEtoh subjects have similarly robust neuronal responses to stress. This is in contrast to the control responses where females showed more c-Fos IR than males. In addition, MEtoh subjects have the largest number of c-Fos IR neurons of any group after the FST exposure, showing almost 4 times as much IR after stress. This indicates that while there may be neuronal habituation at baseline after ethanol exposure, that this group is particularly reactive to stressors, and that the CeA circuitry alterations in males at baseline may be separate from the circuitry activated after stress.

Regional differences in neuronal activation

The CeA receives a multitude of projections from other areas of the amygdala like the basolateral region (Pessoa 2010), and from diverse areas of the brain such as the thalamus, cortex and hippocampus (Parsons and Ressler 2013; Ressler 2010). The CeA also sends diverse outputs of CRF and the endogenous opioid dynorphin to regions like the LC and DRN (Retson and Van Bockstaele 2013), and sends gabaergic outputs to regions like the ventral tegmental area and lateral hypothalamus (Koob 2008). While the CeA in aggregate is a well-studied region, the functions, connections, and phenotypes of its subdivisions are a continued source of investigation, with the existence of multiple methodologies for defining subdivisions (Paxinos 1997; Swanson and Petrovich 1998). Here, we add to the knowledge about CeA subdivisions through the observation that differences in CeA regional activation were seen under varying conditions. In this study, the greatest amount of FosB was seen in the CeC of both sexes. It is known that the CeC contains cocaine-amphetamine-regulated transcript (CART) which has known roles in stress and reward (Cheng et al. 2011), and is seen here as well as in other studies to be highly immunoreactive for ENK (Chieng et al. 2006). The increased activation of this region in a long-term setting may thus be related to the effects of alcohol on the reward systems.

When examining activation patterns within the CeA after the FST, the MEtoH animals differed from the MCtrl group. The CeM and CeC of MEtoH animals had the largest increases in IR, in a pattern similar to those seen in female animals. When the distribution of c-Fos IR in the CeA was compared by examining IR in a given subdivision as a percentage of total activated neurons, differences in activity patterns could be seen between stress(–) and stress(+) groups (figure 11 A–D). In the MCtrl and MEtoH groups, after the FST a higher percentage of activated neurons were located in the CeM and CeC compared with non-stressed counterparts. In FCtrl animals, there was a similar proportional increase in the CeM after the FST, linking this region with the stress response across genders. The majority of CeA outputs to brainstem regions originate in the CeM (Cassell et al. 1986), and the CeM is thought to be the main CeA component in the response to conditioned fear (Duvarci et al. 2011). The largest portion of coordinately projecting neurons to the noradrenergic LC and serotonergic DRN were shown to be in the CeM (Retson and Van Bockstaele 2013; Van Bockstaele et al. 1996), potentially linking dysregulation of these monoaminergic circuits to the changes seen in the CeA after ethanol exposure and stress. This further illustrates the effect of chronic ethanol on alterations in neuronal circuitry, and shows that ethanol may activate different neuronal architecture than stress, potentially causing an altered susceptibility in males. In contrast, in the FEtoH group the proportional activation of CeA subdivisions was almost unchanged between stress(–) and stress(+) groups. This lack of divisional change, combined with the similar levels of c-Fos IR overall, and similar levels of dual ENK or CRF labeling, provides additional evidence for chronic ethanol activating neural circuitry similar to that of stress in female animals.

Neurochemical signature of activated neurons

We chose to examine the role of CRF and ENK, both of which have previously been shown to localize in the CeA (Cassell et al. 1986; Criado and Morales 2000; Gilpin 2012; Mendez and Morales-Mulia 2008), and have established roles in the stress response and alcohol related sequelae (Lowery and Thiele 2010; Mendez and Morales-Mulia 2008). Work has been done to look at the effect of CRF on the CeA, showing that ethanol and CRF increase CeA GABA release {Herman, 2013 #168; Roberto, 2010 #169}, but the role of CRF in neurons active in chronic exposure remains to be elucidated. Here we show evidence for a role of CRF and ENK systems in chronic ethanol exposure and describe differences in the activation patterns of the CeA CRF and ENK systems.

When examining neurons dual labeled for FosB and CRF, the MEtoH and FEtoH animals had similar levels of total neurons showing dual IR, and slightly higher numbers when compared to their control counterparts. When examined for percentage of dual IR compared to total FosB IR, the ethanol groups had only slightly smaller percentages than controls. This would indicate that at baseline the CRF system is playing a similar role in the CeA system of control and ethanol treated animals, and that this system is functioning similarly in males and females. ENK labeling in FosB IR neurons was higher in ethanol treated animals of both genders, with the ENK system again playing similar roles in both males and females at baseline. However, when examined by percentage of total FosB IR, MCtrl subjects exhibited the largest percentage of dual activated neurons at 27%. This proportionality larger number of ENK active neurons in the CeA in male control fed animals

compared to female control fed animals may indicate a higher baseline activation of the endogenous opioid system in male animals irrespective of ethanol treatment.

When examining neurons dual labeled for c-Fos and CRF IR the MCtrl, MEtoh and FCtrl groups had similar amounts of dual IR labeling, with the largest numbers of dual labeled cells present in the FEtoh animals. This would indicate an enhanced activation of the CeA CRF system in FEtoh animals at baseline. When examining the numbers of dual CRF/c-Fos IR neurons as a percentage of total c-Fos labeled neurons, however, the FEtoh group had a percentage of dual IR similar to all other groups. This would imply that increased CRF IR may be a component of the overall activation increase seen in the FEtoh group, rather than activation of solely this system.

Upon exposure to the FST, MCtrl and FCtrl subjects had relatively similar levels of dual IR labeling to stress(–) MCtrl and FCtrl groups, indicating that the FST did not preferentially activate CRF containing c-Fos neurons in control animals of either gender. When examining the effect of the FST on the ethanol exposed groups, the MEtoh and FEtoh groups had substantially more dual IR, with the MEtoh group having increased levels over the FEtoh group, and almost 3 times as many as the MEtoh animals not exposed to stress. This would indicate that the activation of the CRF system plays a particular role in stress in the MEtoh group. The FEtoh stress(–) and stress(+) groups had similar levels of CRF/c-Fos dual IR, revealing that CRF circuitry plays a role in FEtoh subjects at baseline, and this subset of neurons is equally active after a stressor. In addition, female stress(+) animals had similar distributions of labeling across subregions, pointing to an enhanced level of activation without a change in activation patterns. Taken together, this may further suggest that in the CeA of FEtoh subjects, ethanol is activating the CRF stress circuitry. When examined by percentages, groups of both genders exposed to the FST tended to have smaller percentages of CRF/c-Fos dual IR neurons of total activated neurons than their stress(–) counterparts, despite increases in overall numbers. This indicates that while CRF plays a substantial role in the activation seen after ethanol exposure and stressors, this system also relies on other phenotypes of neurons as well, identifying the need for further elucidation.

Previous studies show that acute use of ethanol activates ENK neurons in the CeA robustly (Criado and Morales 2000), while long-term ethanol administration diminishes this effect (Banks et al. 2003). Here, similarities were seen in the numbers of dual ENK/c-Fos IR neurons between MCtrl, MEtoh, and FCtrl groups, while FEtoh animals had almost twice as many dual IR neurons. When examined as a percentage of total c-Fos IR neurons however, all stress(–) groups had similar levels of ENK/c-Fos IR, between 36–39%. This reveals that the ENK system plays a substantial role in the neuronal activation patterns of the CeA at baseline, and a larger role than CRF. It also shows that while FEtoh animals had more IR neurons, that their percentage of dual IR was the same, indicating that other neuronal systems play roles in the increased activation as well.

All animals undergoing the FST had relatively similar levels of ENK/c-Fos IR, which were higher than those seen in the stress(–) condition for MCtrl, MEtoh and FCtrl groups. Furthering the role of ENK in the neuronal activation patterns seen after stressors, but pointing to a non-ethanol specific activation role for ENK in male subjects. FEtoh stress(+)

subjects had dual IR levels similar to those of the other treatment groups after the FST, which was slightly lower than seen in the FEtoH stress(–) subjects. Complementary to what was discussed for CRF in the FEtoH group, this similar level of neuronal activation of the ENK system in stressed and non-stressed subjects indicates its role in the female chronic alcohol induced neuronal circuitry. Since this phenotype looks similar to the one seen after a stressor it may demonstrate that in females chronic ethanol is activating the CeA stress circuitry, and further signals a dysregulation of the ENK circuitry in female animals after chronic ethanol exposure.

The role of ENK in the neuronal activity present in chronic ethanol exposed animals and in neurons activated after a stressor is additionally noteworthy for its localization within the CeA. In MCtrl, MEtoH and FCtrl animals, the distribution of dual IR was similar, with the majority of labeling present in the CeL. In FEtoH stress(–) animals and all stress(+) groups, the CeL and CeC had similar levels of dual IR, indicating a large amount of the observed increase in labeling occurred via increased activation of the CeC.

Conclusion

In the present study we detailed CeA activation patterns by subdivision and rostral-caudal extent, compared baseline to long-term activation patterns, and examined the effect of chronic ethanol exposure at baseline and after stress. Here we used FosB to demonstrate differences in neuronal activation patterns in the CeA between genders, showing that females have a higher level of CeA activation at baseline. However, when exposed to chronic ethanol there is an increased number of activated neurons in males versus females. When compared with c-Fos levels after handling at sacrifice, this pattern is altered, with male ethanol treated animals having similar c-Fos levels to controls, and female ethanol treated animals displaying robust activation. This switch may indicate that male animals habituate to the neuronal activation induced by ethanol exposure, while females do not, potentially elucidating a source of the sex differences prevalent in alcohol addiction. Next, we provide evidence for increased CeA activity in female animals compared to males after a stressor, and show that after chronic ethanol exposure, both genders react similarly, and more robustly, than controls. Upon further examination, similar distributions of neurons are activated after stress in female control and ethanol treated animals, which may demonstrate that ethanol is activating the same neuronal populations as stress. However, in males, different distributions of activated neurons are observed, which may point to ethanol engaging both stress systems and other CeA circuitry concurrently. The differences seen in neuronal activation of the CeA between males and females at baseline, after chronic alcohol exposure, and in their response to stress, may in part underlie the distinctive response seen between genders to alcohol use. Thus, this study points to a need for further investigations with considerations for gender, as this may lead to improved individualized therapies, and more targeted and effective treatment practices.

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Fig. 1.

A illustrates adrenal weights at time of euthanasia. A significant two way interaction between gender and treatment was observed ($p=0.003$, as indicated by the $‡$), with post hoc tests showing increases in adrenal weight seen between MCtrl and MEtoh subjects ($p<0.01$). B indicates average levels of CORT taken at time of sacrifice for all conditions. A significant interaction was observed in CORT levels between ethanol treatment and stress ($p=0.002$, as indicated by the $‡$), with post hoc tests showing significant differences in CORT levels between FCtrl and FEtoh groups at baseline, and a significant difference between FEtoh subjects at baseline or after exposure to a stressor

Fig. 2.

A–B, D–E: Photomicrographs taken at the level of the mid-CeA showing immunoreactivity for FosB, a marker of long-term neuronal activation. Immunoreactivity for FosB is illustrated by arrows. Panel C shows a schematic illustration adapted from Paxinos and Watson (1997) taken at the level of the mid-CeA indicating the medial subdivision (CeM), the lateral subdivision (CeL), and the capsular subdivision (CeC). The subdivisions are shown as overlays in the photomicrographs. The average number of FosB labeled neurons for each group is shown in panel F, and there was a significant interaction between gender and treatment ($p=0.007$, indicated by the ‡). Increases in the number of FosB immunoreactive cells can be seen in both male and female ethanol treated animals (B, E) compared to their control counterparts (A, D) with post hoc tests revealing that MEtoh subjects had significantly more FosB than controls (F), while FCtrl subjects had significantly more IR than MCtrl subjects

Fig. 3.

Graphical representation of the distribution of FosB-containing neurons in the CeA of control (Ctrl) and ethanol (Etoh)-treated male (M) and female (F) animals. There was a significant main effect of subregion ($p < 0.001$, as indicated by $\ddagger$) in FosB-containing neurons across CeA subdivisions (medial, lateral and capsular), and a significant interaction between subregion and treatment ($p = 0.017$, indicated by $\dagger$). While both male and female ethanol-treated animals had increased numbers of FosB labeled cells compared to their control counterparts, a significant interaction of treatment and subregion was observed in males ($p = 0.036$).

Fig. 4.

Photomicrographs showing dual immunocytochemical labeling for FosB and corticotropin releasing factor (CRF; A-A'') or methionine-enkephalin (ENK; B-B'') in the CeA taken in the CeL of male ethanol treated animals. Solid white arrows indicate neurons that are dual labeled for FosB and the peptide. Dual FosB and peptide IR was seen in all conditions, and throughout the rostral-causal extent of the CeA. When CRF was examined, a significant difference was seen between male ethanol and control groups ($p < 0.01$). For sections immunolabeled for ENK, a main effect of treatment was seen ($p < 0.05$), in addition to a significant difference between male control and ethanol groups ($p < 0.05$)

Fig. 5.

Analysis of times (in seconds) spent engaged in three behaviors during a 15 minute forced swim test. Swimming (A) represents an active behavior, climbing (B) represents an escape oriented behavior associated with active coping, and floating (C) represents a passive behavior. Asterisks represent time points where a significant main effect of treatment was present. There was a significant interaction of treatment and gender observed in time spent swimming ($p=0.044$, indicated by ‡). In climbing behaviors, a main effect of treatment was noted ($p=0.024$, indicated by †), and ethanol treated animals were observed to spend more time engaging in this behavior than their control counterparts during the latter 10 minutes of the trial. With respect to floating behaviors, a significant effect of treatment was seen in the last 5 minutes of the trials, with MEtoh animals spending the most time engaging in this behavior

Fig. 6.

A–B, D–E: Photomicrographs taken at the level of the mid-CeA showing immunoreactivity for c-Fos, a marker used to examine recent neuronal activation in subjects not exposed to the FST. Panel C shows a schematic illustration adapted from Paxinos and Watson (1997) taken at the level of the mid-CeA indicating the medial subdivision (CeM), the lateral subdivision (CeL), and the capsular subdivision (CeC). The subdivisions are shown as overlays in the photomicrographs, and immunoreactivity for c-Fos is indicated by arrows. The average number of c-Fos labeled neurons for each group is shown in panel F, where a significant interaction between gender and treatment can be seen ($p=0.047$, indicated by ‡). Increases in c-Fos immunoreactivity after chronic alcohol exposure can be seen most predominantly between control female (FCtrl: D) and ethanol treated female (FEtoh: E) groups, with FEtoh animals displaying a significantly increased number of activated neurons as seen by post hoc testing ($p=0.008$). Male (M) ethanol treated (Etoh) and control (Ctrl) groups had similar levels of c-Fos immunoreactivity.

Fig. 7.

A–B, D–E: Photomicrographs taken at the level of the mid-CeA showing immunoreactivity for c-Fos, a marker used to examine recent neuronal activation after exposure to the forced swim test. Panel C shows a schematic illustration adapted from Paxinos and Watson (1997) taken at the level of the mid-CeA indicating the medial subdivision (CeM), the lateral subdivision (CeL), and the capsular subdivision (CeC). The subdivisions are shown as overlays in the photomicrographs, and immunoreactivity for c-Fos is indicated by arrows. The average number of c-Fos labeled neurons for each group is shown in panel F next to the stress(–) groups for comparison. Among all groups a significant overall interaction was seen between gender, treatment and stress ($p=0.048$, indicated by ‡). Within stress(+) groups, a main effect of treatment was seen (indicated by #). Increases in c-Fos immunoreactivity after swim exposure can be seen most predominantly between male ethanol treated (MEtoh: B) and male control (MCtrl: A) groups, female ethanol (FEtoh: E) and control (FCtrl: D) groups displaying similar levels of c-Fos immunoreactivity. There was also a significant difference noted by post hoc testing between FCtrl and MCtrl stress(+) groups, while MEtoh and FEtoh groups had similar levels of c-Fos labeling

Fig. 8.

Graphical representation of the distribution of c-Fos containing neurons in the CeA of control (Ctrl) and ethanol (Etoh)-treated male (M) and female (F) animals at baseline (A), or after the forced swim test (B). Comparing across stress(-) and stress(+), a significant main effect of subdivision was observed between all groups ($p < 0.001$) in the number of c-Fos-containing neurons across CeA subdivisions (medial, lateral and capsular, indicated by † in A and B). Panel A represents the stress(-) conditions where in females, a significant interaction was present between division and treatment ($p = 0.031$). Panel B represents the stress(+) subjects where a main effect of division was present in males ($p = 0.025$)

Fig. 9.

Photomicrographs of dual labeling for c-Fos and corticotropin releasing factor (CRF; A-A'') or enkephalin (ENK; B-B'') taken in the CeL and CeC respectively of stress(+) males. Dual c-Fos and peptide IR was seen in both stress(+) and stress(-) conditions, and throughout the rostral-caudal extent of the CeA. Solid white arrows indicate neurons that are single labeled for c-Fos, while arrowheads indicate neurons that are dual labeled for c-Fos and the peptide. Asterisks indicate a neuronal process labeled for CRF that is in proximity to an activated neuron. When CRF was examined (C), a significant three way interaction was seen (gender, treatment, stress) for CRF/c-Fos dual IR ($p=0.045$, indicated by $\ddagger$), and a significant effect of treatment on CRF/c-Fos dual IR was seen in the stress(+) condition ($p=0.011$, indicated by $\dagger$). For sections immunolabeled for ENK (D), there was a trend towards an overall three-way interaction ($p=0.093$), and significant differences were observed across CeA subregions (medial, lateral, capsular), and stress condition ($p=0.001$, indicated by $\dagger$).

Working model for Δ FosB and c-Fos levels over the course of ethanol exposure

Fig. 10.

A–B illustrate the working model for levels of FosB and c-Fos over the course of ethanol exposure. A shows an increase in c-Fos with initial ethanol exposure, as seen in previous studies, that decreases over time as neurons habituate in the MEtoh group as proposed here. The repeated activation is thought to yield an increased FosB visible at sacrifice. B illustrates the hypothesis that FEtoh subjects have an increased initial CeA activation, though not to the same extent as the MEtoh group, that never fully habituates and reflects the higher baseline activity levels seen in the FCtrl group.

Change in c-Fos percentage by region in stress(+) compared to stress(-) groups

Fig. 11.

A–D CeA diagrams in A–D indicate the difference in activity patterns between stress(–) and stress(+) groups in CeA subdivision, when subdivision activation is compared as a percentage of total activity. While the FST alters activity patterns of the MCtrl, MEtoh and FCtrl groups, the FEtoh group shows a similar activity pattern with or without a stressor, indicating that the stress systems may be activated by chronic ethanol exposure in this group. M indicates medial, L indicates lateral, and C indicates capsular subdivisions of the CeA.

Table 1

Average number of neurons $\pm$ SEM that were co-immunolabeled for FosB and either CRF or ENK presented by treatment group in CeA subdivisions and rostral-caudal regions.

	M-Ctrl		M-Etoh		F-Ctrl		F-Etoh	
	CRF	ENK	CRF	ENK	CRF	ENK	CRF	ENK
Total FosB / CRF or ENK	95.2 $\pm$ 21.4	117.8 $\pm$ 22.5	150.2 $\pm$ 16.3	179.4 $\pm$ 13.9	129.0 $\pm$ 23.3	138.8 $\pm$ 18.4	146.8 $\pm$ 30.8	172.2 $\pm$ 22.6
Medial, Lateral, Capsular Subdivision								
CeM	25.2 $\pm$ 7.7	22.2 $\pm$ 3.7	39.2 $\pm$ 3.6	34.0 $\pm$ 6.3	38.5 $\pm$ 12.5	30.0 $\pm$ 5.1	34.2 $\pm$ 6.1	33.6 $\pm$ 4.7
CeL	38.5 $\pm$ 9.8	45.5 $\pm$ 9.8	53.8 $\pm$ 8.7	62.2 $\pm$ 4.6	53.8 $\pm$ 10.4	56.5 $\pm$ 5.8	60.0 $\pm$ 12.3	64.2 $\pm$ 11.5
CeC	31.5 $\pm$ 6.7	50.2 $\pm$ 10.5	57.2 $\pm$ 6.6	83.2 $\pm$ 9.6	36.8 $\pm$ 5.2	52.3 $\pm$ 11.8	52.6 $\pm$ 13.0	74.4 $\pm$ 8.8
Rostal, Mid, Caudal Region								
rCeA	27.7 $\pm$ 7.0	39.8 $\pm$ 6.8	46.2 $\pm$ 7.2	63.4 $\pm$ 7.0	35.0 $\pm$ 11.7	58.3 $\pm$ 16.5	50.8 $\pm$ 9.1	62.4 $\pm$ 10.7
mCeA	56.7 $\pm$ 13.3	63.7 $\pm$ 13.8	82.6 $\pm$ 7.2	94.6 $\pm$ 6.4	74.3 $\pm$ 14.5	60.8 $\pm$ 2.2	76.8 $\pm$ 18.6	88.6 $\pm$ 17.4
cCeA	10.8 $\pm$ 4.7	14.3 $\pm$ 4.2	21.4 $\pm$ 8.9	21.4 $\pm$ 5.8	19.8 $\pm$ 7.8	19.8 $\pm$ 6.0	19.2 $\pm$ 4.5	21.2 $\pm$ 2.8

Table 2
Average number of c-Fos IR neurons $\pm$ SEM present in the CeA subdivisions and rostral-caudal regions of all groups

	M-Ctrl stress(-)	M-Ctrl stress(+)	M-Etoh stress(-)	M-Etoh stress(+)	F-Ctrl stress(-)	F-Ctrl stress(+)	F-Etoh stress(-)	F-Etoh stress(+)
Total c-Fos IR neurons	183.6 $\pm$ 66.1	312.8 $\pm$ 21.4	141.2 $\pm$ 35.9	526.8 $\pm$ 106.6	192.8 $\pm$ 30.5	423.2 $\pm$ 43.3	369.6 $\pm$ 53.6	502.2 $\pm$ 57.6
Medial, Lateral, Capsular Subdivision								
CeM	34.8 $\pm$ 12.1	25.6 $\pm$ 7.0	82.4 $\pm$ 30.3	157.7 $\pm$ 20.8	39.8 $\pm$ 12.6	137.2 $\pm$ 8.7	129.4 $\pm$ 23.3	172.8 $\pm$ 21.9
CeL	82.4 $\pm$ 30.3	97.7 $\pm$ 11.2	62.8 $\pm$ 16.2	148.8 $\pm$ 27.6	74.3 $\pm$ 6.5	127.7 $\pm$ 17.9	129.4 $\pm$ 23.3	136.7 $\pm$ 17.2
CeC	66.4 $\pm$ 26.0	134.8 $\pm$ 9.9	52.8 $\pm$ 16.1	220.3 $\pm$ 60.4	78.8 $\pm$ 15.7	158.3 $\pm$ 20.3	139.8 $\pm$ 20.0	192.7 $\pm$ 25.8
Rostral, Mid, Caudal Region								
rCeA	37.4 $\pm$ 12.0	125.5 $\pm$ 11.9	34.0 $\pm$ 8.1	182.8 $\pm$ 30.7	52.3 $\pm$ 14.9	163.7 $\pm$ 14.9	134.6 $\pm$ 17.3	191.8 $\pm$ 28.6
mCeA	108.6 $\pm$ 40.7	146.3 $\pm$ 5.8	78.2 $\pm$ 40.7	283.2 $\pm$ 71.2	92.3 $\pm$ 16.6	198.5 $\pm$ 26.3	171.0 $\pm$ 28.2	223.5 $\pm$ 21.1
cCeA	37.6 $\pm$ 17.2	41.0 $\pm$ 9.9	29.0 $\pm$ 12.7	60.8 $\pm$ 6.8	48.3 $\pm$ 6.8	61.0 $\pm$ 7.3	64.0 $\pm$ 12.1	86.8 $\pm$ 11.2

Table 3
Average number of c-Fos IR neurons ± SEM co-labeled with CRF or ENK by CeA subdivisions and rostral-caudal regions for each treatment condition.

	M-Ctrl stress(-)	M-Ctrl stress(+)	M-Etoh stress(-)	M-Etoh stress(+)	F-Ctrl stress(-)	F-Ctrl stress(+)	F-Etoh stress(-)	F-Etoh stress(+)
c-Fos+CRF IR in Medial, Lateral, Capsular Subdivision								
Total c-Fos + CRFIR	54.6 ± 22.4	59.7 ± 7.1	44.0 ± 15.2	127.2 ± 19.4	62.5 ± 7.9	67.2 ± 7.1	104.8 ± 24.2	93.2 ± 14.2
c-Fos+CRF IR Rostral, Mid, Caudal Region								
CeM	7.8 ± 3.4	10.3 ± 2.5	6.4 ± 2.2	31.7 ± 6.9	11.8 ± 3.6	13.5 ± 1.9	28.6 ± 7.9	21.8 ± 4.3
CeL	29.4 ± 13.0	24.3 ± 3.0	24.8 ± 9.2	50.2 ± 9.5	27.5 ± 1.7	32.8 ± 4.9	40.6 ± 7.5	37.3 ± 5.7
CeC	17.4 ± 6.3	25.0 ± 4.3	12.8 ± 4.6	45.3 ± 7.3	23.3 ± 3.5	20.8 ± 2.0	35.6 ± 9.0	34.0 ± 6.4
c-Fos+ENK IR in Medial, Lateral, Capsular Subdivision								
rCeA	7.0 ± 2.9	14.3 ± 3.8	7.4 ± 2.4	36.5 ± 6.8	14 ± 5.3	11.7 ± 1.8	29.8 ± 6.3	19.5 ± 4.3
mCeA	36.6 ± 14.7	35.5 ± 2.7	27.6 ± 9.2	75.7 ± 12.8	32.5 ± 4.0	42.3 ± 6.6	50.4 ± 12.2	52.2 ± 7.6
cCeA	11.0 ± 5.5	9.8 ± 3.6	15.0 ± 5.9	15.0 ± 2.6	16.0 ± 1.4	13.2 ± 2.5	24.6 ± 6.7	21.5 ± 3.7
Total c-Fos + ENKIR	77.4 ± 31.8	91.2 ± 10.3	57.4 ± 16.7	128.3 ± 28.9	73.3 ± 8.4	121.0 ± 18.4	141.6 ± 35.7	132.2 ± 18.9
c-Fos+ENK IR in Medial, Lateral, Capsular Subdivision								
CeM	8.6 ± 4.0	11.5 ± 2.4	7.8 ± 2.5	16.0 ± 3.1	10.5 ± 3.4	18.8 ± 3.1	34.8 ± 12.8	19.5 ± 2.9
CeL	42.4 ± 17.5	41.8 ± 5.3	32.4 ± 10.1	50.0 ± 8.8	40.3 ± 2.7	55.2 ± 9.3	54.4 ± 13.0	52.2 ± 7.8
CeC	26.4 ± 10.9	37.8 ± 5.9	17.2 ± 5.2	62.3 ± 18.0	22.5 ± 2.9	47 ± 7.5	52.4 ± 10.8	60.5 ± 8.4
c-Fos+ENK IR Rostral, Mid, Caudal Region								
rCeA	12.8 ± 5.9	32.2 ± 5.6	13.8 ± 4.2	41.5 ± 8.2	15.75 ± 5.0	38.8 ± 3.5	48.8 ± 11.7	50.0 ± 9.2
mCeA	50.8 ± 19.8	49.3 ± 4.4	34.8 ± 10.48	73.2 ± 21.5	39.3 ± 7.6	62.8 ± 12.3	66.2 ± 19.1	59.3 ± 9.1
cCeA	13.8 ± 6.9	9.7 ± 1.9	14.7 ± 5.8	13.7 ± 1.8	18.3 ± 3.0	19.3 ± 4.6	26.6 ± 5.7	22.8 ± 2.3