CHRONIC STRESS AND SEX AS MEDIATORS OF THE BASOLATERAL-CENTROMEDIAL AMYGDALA CIRCUIT AND ITS RESPONSE TO ACUTE ETHANOL

by

Sean Gainey

A Thesis

Submitted to the Faculty of Purdue University

In Partial Fulfillment of the Requirements for the degree of

Master of Science

Department of Psychology at IUPUI

Indianapolis, Indiana

May 2020

THE PURDUE UNIVERSITY GRADUATE SCHOOL
STATEMENT OF COMMITTEE APPROVAL

Dr. Marian L. Logrip, Chair

Department of Psychology

Dr. Christopher C. Lapish

Department of Psychology

Dr. Anthony J. Baucum II

Department of Biology

Approved by:

Dr. Jesse C. Stewart

ACKNOWLEDGMENTS

I would first like to thank my thesis advisor, Dr. Marian Logrip of the Department of Psychology at Indiana University-Purdue University Indianapolis. No issue was too big or too small for Dr. Logrip to address, and there was never too little time to explain the theory behind a solution. She was invaluable as a source of information and encouragement through the setup of the electrophysiology equipment that made this work possible.

I would also like to thank Dr. Christopher Lapish of the Department of Psychology and Dr. A.J. Baucum of the Department of Biology. I am grateful for the contextual knowledge of Dr. Lapish, the technical advice of Dr. Baucum, and the commentary of both professors on the writing of this thesis.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Anxiety disorders are the most common class of mental disorders in the United States, and they both promote and exacerbate disorders of substance abuse. Mounting evidence of sex differences in the relationship between anxiety disorders and alcoholism supports the potential existence of an anxiety-dependent vulnerability to alcohol abuse in women compared with men. One potential point of overlap in the physiological systems involved in anxiety response and reward processing is the amygdala. Here, a model of chronic stress in rodents was employed to probe changes in the electrophysiological and biochemical properties of the amygdala at a post-stress baseline and during a post-stress first exposure to alcohol. Electrophysiological data revealed that neurons in the centromedial amygdala were more responsive to stimulation in the basolateral amygdala in females compared with males, but a history of chronic stress altered the female response to match that of males with or without a history of chronic stress. Protein analysis of postsynaptic glutamatergic receptor expression and phosphorylation in the amygdala did not indicate any differences based on sex or exposure to stress or alcohol. These data demonstrate a sex difference in stress-induced alterations in amygdala circuitry and indicate a potential role for this circuitry in the comorbidity of anxiety disorders and alcoholism.

INTRODUCTION

Anxiety disorders are more common in the U.S. than any other class of mental disorders. The lifetime prevalence rate is 31.6% of the total population above age 13, including 37.3% of women compared to 25.6% of men (Kessler et al., 2012; Kessler et al., 2002). Terminology in the literature is mixed in part because of reorganization of disorder classification in the most recent revision of the Diagnostic and Statistical Manual of Mental Disorders (DSM), but these statistics regarding the general group of disorders including post-traumatic stress disorder (PTSD), specific phobia, generalized anxiety disorder, separation anxiety disorder, panic disorder, and agoraphobia have remained consistently high over time. The higher prevalence of diagnosis of anxiety disorders in women compared to men is well-documented. Furthermore, women with an anxiety disorder have a higher likelihood to be diagnosed with a second disorder than men and experience a greater illness burden from anxiety disorders (McLean et al., 2011). It may be the case that women have higher vulnerability for pathological responses to stress in general. For example, women exposed to trauma meeting criteria for PTSD are diagnosed at a rate of 20.4%, while only 8.2% of men exposed to trauma are diagnosed (Kessler et al., 1995), although the contribution of differences in help-seeking to this discrepancy remains unclear.

Alcoholism also has high lifetime prevalence rates with 12.5% for dependence according to DSM-IV criteria and 29.1% for alcohol use disorder (AUD) according to DSM5 criteria (Grant et al., 2015; Hasin, Stinson, Ogburn, & Grant, 2007). Terminology in the anxiety-alcohol literature varies on terms of alcohol misuse as well, and terms are presented here as they appear in their primary sources. Regardless of classification and terminology, comorbidity between anxiety disorders and alcoholism exceeds even what would be predicted based on their high individual prevalence rates: PTSD alone is found in 26.2% of women and 10.3% of men with AUDs but is diagnosed in only 7.8% of the general population (Sonne et al., 2003). Traumatic or chronic stress also lead to higher rates of chronic anxiety and depression in alcoholics than the general population (Mons & Beracochea, 2016).

Alcohol abusers are at higher risk for the development of an anxiety disorder, but the reverse is also common. A prominent example is combat-exposed veterans diagnosed with PTSD. This group exhibits higher rates of multiple measures of alcohol misuse compared to combat veterans without a diagnosis (Jacobson et al., 2008). The shared stress exposure with

differing PTSD and alcohol abuse outcomes could indicate an underlying vulnerability to both conditions exacerbated by traumatic stress. Sex differences in directionality are seen here as well, as women more often report PTSD predating alcohol abuse than men (Sonne et al., 2003). Women diagnosed with both PTSD and AUD report drinking to cope with stress more than men, and heavy-drinking women generally report greater stress-induced cravings for alcohol than men (Lehavot et al., 2014; Hartwell and Yar, 2013). Differences in stress responsivity related to alcohol affect long-term outcomes for alcoholics, as stress is a major risk factor for relapse (Sinha, 2012). Comorbidity between behavioral outcomes of anxiety and alcoholism implicate some underlying neurocircuitry that produces vulnerability to both conditions. However, while current pharmacological treatments have improved the duration of abstinence for some patients, they have not improved long-term relapse rates (Schuckit, 2009).

The amygdala presents a potential point of pathological overlap between anxiety and alcoholism due to its critical roles in consolidation of fear, expression of anxiety, and processing of reward (Janak & Tye, 2015). The amygdala is divided into several nuclei including the basolateral amygdala (BLA) and central amygdala (CeA). Much of the input to the amygdala flows into the BLA, is processed through circuitry within and between the subdivisions of the amygdala and flows out from the CeA (Duvarci & Pare, 2014). The BLA receives sensory input from the sensory cortical regions and further stimulus information from thalamus and hippocampus. The CeA sends output processed within the amygdala to the bed nucleus of the stria terminalis, hypothalamus, pons, and medulla (Sah et al., 2003). Part of processing circuitry within the amygdala includes excitatory glutamatergic projections from the BLA to the primarily GABAergic neurons of the lateral (CeL) and medial (CeM) subdivisions of the CeA (Shackman et al., 2016). Excitement of the CeL inhibits the CeM, where most of the GABAergic projection neurons that carry amygdala output are located, so overall output is largely a result of the balance between the strength of BLA-CeL and BLA-CeM connections (Tye et al., 2015, Calhoon & Tye, 2015).

This circuitry plays roles in processing both positive and negative external stimulus information. The amygdala as a whole and the CeA in particular have long been studied as mediators of acquisition and expression of conditioned fear (Davis, 1992, LeDoux et al., 1988). Fear acquisition is associated with a functional CeL, and fear responses are primarily associated with the CeM (Ciocchi et al., 2010). The role of the amygdala in reward processing has been

examined more recently, and appetitive behaviors have been shown to be mediated by distinct populations of BLA projections to the CeA (Kim et al., 2017). Because the amygdala plays roles in both positive and negative stimulus learning and response, the role of the amygdala can be considered processing of valence, thereby encoding the intensity with which a positive or negative stimulus should be sought or avoided (O'Neill et al., 2018). The overlapping roles of the amygdala in appetitive and aversive behaviors suggest its potential to contribute to disorders involving addiction and anxiety (Gilpin, Herman, & Roberto, 2015).

Differences in amygdala processing can be innate or induced by life experience with positive and negative stimuli. Induction of alcohol dependence in rats increases GABA release in the CeA, and acute exposure to ethanol decreases excitability of CeA neurons more in alcohol-dependent rats than in naïve rats (Roberto et al., 2004; Roberto et al., 2004). Electrophysiological recordings in slices from naïve rats show a blunting of alcohol's effects on BLA-CeM excitability in female rats, compared to males (Logrip, Oleata, & Roberto, 2016). Forced abstinence reverses some chronic ethanol-induced neuroadaptations, but some persist long after ethanol exposure ends, suggesting a possible contribution to persistent alcohol use disorders (Roberto et al., 2006).

CeM output may be regulated by more than intra-amygdala circuitry, however. The CeA receives direct inhibitory and excitatory input from multiple cortical regions (Janak & Tye, 2015; McDonald et al., 2006). An increase in CeM output, therefore, could be the result of changes in sensitivity to external inputs, intra-amygdala inputs, or both. Stress has been shown to promote lasting hippocampal changes through adaptations in α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor trafficking (Krugers, Hoogenraad, & Groc, 2010). AMPA and N-methyl-D-aspartate (NMDA) receptors are both glutamate receptors spread throughout the brain. AMPA receptors mediate fast excitatory responses, while NMDA receptors facilitate longer term changes in excitability related to synaptic plasticity (Pin and Duvoisin, 1995). In these experiments, I investigated whether life experiences were altering amygdala processing through changes in AMPA-mediated excitability of the CeM to determine whether excitability was altered in the long term regardless of the state NMDA receptor-mediate plasticity.

AMPA receptors are composed of four subunits of four different subunit types, GluA1, GluA2, GluA3, and GluA4. A dimer of GluA2 is present in in most AMPA receptors with a

dimer of any of the other 3 types, the most common of which is GluA1 (Nakanishi, 1992). Phosphorylation of the GluA1 subunit is one of the most common posttranslational modifications regulating excitability of AMPA receptors. Phosphorylation of the serine 845 site on the GluA1 subunit of AMPA receptors (pS845) is believed to increase trafficking of AMPA receptors to the synapse (Mao, Diaz, Fibuch, & Wang, 2013). Phosphorylation of the serine 831 site on the GluA1 subunit (pS831) is associated with increased probability of opening and increased open duration (Lee et al., 2013). These two phosphorylation sites provide two possible mechanisms by which CeM output may be elevated in response to input from all excitatory projections, which could produce inappropriately large stress responses to general sources of stress.

Many different animal models of stress have been developed for a variety of research purposes. Application of the stress hormone corticosterone to tissue is a valuable tool in electrophysiological experiments for investigating acute stress response in circuits such as the BLA-CeM pathway. In vivo models, however, have the advantage of including the full endogenous stress response. The hypothalamic-pituitary-adrenal (HPA) axis, for example, increases circulating levels of multiple hormones, any one or combination of which might promote lasting neuroadaptations. Corticosterone in rodents, or primarily cortisol in humans, is elevated by HPA axis activity in response to stress, and CeA circuitry involving corticotropin-releasing factor has been shown to play a role in fear learning in mice Sanford et al., 2017). In vivo stress models ensure that natural stress-induced adaptations are present regardless of which stress response components are responsible. Moderate repeated footshocks in rodents have been used acutely to measure conditioned fear associated with anxiety and chronically to model anxiety and symptoms of depression and PTSD (Bali & Jaggi, 2015; Whitaker, Gilpin, & Edwards, 2015).

Use of a chronic, in vivo model of stress to probe amygdala circuitry with molecular tools such as slice electrophysiology is a powerful but underutilized combination due to the labor demand of chronic behavioral preparation of individual subjects staggered over time. Use of this combination has produced valuable findings, including that alcohol and corticosterone exposure increase BLA excitability in slice recordings (Karst et al., 2010; Läck et al., 2007) and that repeated sessions of cued footshocks can model the lasting effects of stress exposure on behavioral measures of alcohol consumption (Logrip & Zorrilla, 2012). Further exploration of the electrophysiological consequences of chronic stress would be a significant addition to

research on anxiety and alcoholism and could better inform models such as direct application of stress hormones and ethanol for research of human conditions. Here I use a chronic footshock model of stress to measure changes in electrophysiological properties of the direct BLA-CeM pathway and changes in general CeA excitability as measured by AMPA receptor expression and modification. A simplified diagram of this circuitry overlaid on images captured during electrophysiological recording can be seen in Figure 1. Animals are shipped in adulthood to minimize early-life stress, such as that of shipping and acclimating to new facilities, as early-life stress can produce lasting effects that might obscure the effects of stress during adulthood (Gutman and Nemeroff, 2003). These adult, alcohol-naïve animals are subjected to a model of chronic stress that includes repeated daily sessions of cued, unpredictable footshocks in an inescapable environment. This models humans with trauma exposure in adulthood that precedes alcohol abuse, as is often the case in combat veterans.

I hypothesize that this model of chronic stress will produce physiological changes that establish vulnerability to future alcohol abuse, and that these changes will be greater in females compared to males. Specifically, I hypothesize that this will be shown by an increase in the magnitude of BLA-induced CeM excitation that is sensitive to a reduction during alcohol exposure. The proposed mechanism for this change in sensitivity is an increase in synaptic AMPA receptor expression in the CeM and an increase in phosphorylation-promoted trafficking and phosphorylation of AMPA receptors that is greater in females relative to males.

MATERIALS AND METHODS

General Design

Both experiments utilized a modified chronic stress model previously used to investigate the long-term behavioral effects of stress on alcohol self-administration (Logrip and Zorrilla, 2012). In this model, adult, alcohol-naïve animals are singly housed and subjected to 3 consecutive days of unpredictable, inescapable, light-cued footshock sessions before further testing. Experiment 1 probed the effects of this stress history on glutamatergic BLA-CeM projections by recording single CeM neuron responses to evoked BLA activity at a basal level and in the presence of ethanol. Experiment 2 investigated postsynaptic properties of CeA neurons by using Western blots to measure postsynaptic AMPA receptor expression and phosphorylation and the effect of acute ethanol exposure on those measures.

Subjects

A total of 72 adult Wistar rats (Envigo, Indianapolis, IN) were used with 24 (12 male) used in Experiment 1 and the remaining 48 (24 male) used in Experiment 2. Rats were received at 8-9 weeks of age and allowed a minimum of one week to acclimate to the temperature- and humidity-controlled vivarium on a 12-hour light cycle (lights on at 8 am). All rats were pair housed on arrival and split into single housing 24 hours before stress exposure. Rats were housed in standard transparent plastic rat cages with pine sawdust bedding, wire lids, and standard rat chow and water available ad libitum. All experiments were approved by the Institutional Animal Care and Use Committee at Indiana University-Purdue University Indianapolis according to the National Institutes of Health Guide for Care and Use of Laboratory Animals.

Apparatus

Footshock administration sessions were controlled and recorded with custom software and performed in 16 standard rat operant chambers enclosed in sound-attenuating boxes (Med Associates, St. Albans, VT). Chambers were equipped with a house light, stainless steel side walls, transparent Plexiglas front and back walls and ceiling, and a bedding tray under metal bar flooring connected to a shock delivery box. An infrared camera (Swann Communications, Santa

Fe Springs, CA) was affixed to the ceiling of the sound-attenuating box outside the operant chamber. Separate chambers in separate rooms were used for male and female footshock administration.

All brains were sliced with a VT1200S vibratome (Leica Biosystems, Inc., Buffalo Grove, IL). Electrophysiological data were collected using a custom setup with a CV-7B head stage, a Multiclamp 700B microelectrode amplifier, and an Axon Digidata 1550A digitizer (Molecular Devices, San Jose, CA). Recordings were collected using Clampex version 10.7.03 (Axon Instruments, San Jose, CA). Stimulation was produced by a DS3 isolated current stimulator (Digitimer, Ft Lauderdale, FL). Electrode micropipettes were made from 1.5 mm outer diameter, 1.12 mm inner diameter borosilicate capillary tubes with filaments and were pulled on a P-1000 micropipette puller (Sutter Instrument, Novato, CA). Slices were visualized with 4x and 40x magnification through a DIC infrared filter on a microscope connected to imaging software on the recording computer (Nikon, Melville, NY). Synaptic protein was isolated via centrifuge (Thermo Fisher, Waltham, MA). Western blots were run on Bio-Rad TGX gels and transferred to PVDF membrane (Bio-Rad, Hercules, CA). Membranes were quantified with a fluorescence scanner and Image Studio software (Li-Cor Biosciences, Lincoln, NE).

Stress History Model

Experiments 1 and 2 each used the same stress history model. Rats were split into single housing and handled for several minutes to establish familiarity with the experimenter. Rats were randomly assigned to either a stress history group or a control history group. Stress history sessions began at least 24 hours after handling and were administered in the first half of the light cycle. During each session, 60 footshocks were delivered on a variable interval between 11 and 50 seconds for a total session time of approximately 30 minutes. Each footshock was cued by a 5 second illumination of the house light. After 4.5 seconds of house light illumination, a 0.5 second, 0.4 mA footshock was delivered through the metal bar floor and co-terminated with the house light cue. The session ended after delivery of the 60th footshock, and the rat was returned to its home cage and transferred back to the vivarium.

A history was established for each animal with one session per day for three consecutive days. Animals in the stress history group for each experiment would receive footshocks as described above. Animals in the control group would go through the same sessions, but power

was disconnected from the shock delivery box. Infrared cameras recorded the sessions to verify effective footshock delivery. After each use the bedding tray was cleaned and refilled and the chamber walls, ceiling, and floor were cleaned with quatricide cleaning wipes (PDI Healthcare, Orangeburg, NY). In cases where animals with a control history had sessions on the same day as animals with a stress history, the control sessions were conducted separately and prior to stress sessions in addition to using sound-attenuating boxes and cleaning the chambers between uses to further avoid confounds from sounds or smells produced by animals receiving footshocks.

Estrous Cycle Synchronization

Estrous cycles of female rats were synchronized to reduce variability from fluctuations in circulating hormones. 10 µg/mL luteinizing hormone-releasing hormone (LHRH) was dissolved in a vehicle of 0.5 µg/µL 0.1 N acetic acid in phosphate buffered saline. Synchronization required two separate 200 µL subcutaneous injections of LNRH at 3 and 8 hours into the light cycle of female rats on the day of the first footshock session to mimic proestrus, which should time the diestrus phase for the day of sacrifice (Oglivie & Rivier, 1997). Males were similarly injected with the vehicle to control for the stress of subcutaneous injections.

Slice Preparation

Approximately 24 hours after final footshock session, rats were removed from the vivarium and lightly anesthetized with 5% isoflurane and 95% air delivered through a large nosecone inside a hood. The rat was rapidly decapitated, and its brain was dissected and dropped into an oxygenated (95% O2, 5% CO2), ice-cold slurry of cutting solution containing, in mM: 206 sucrose, 2.5 KCl, 1.2 NaH2PO4, 26 NaHCO3, 5 glucose, 5 HEPES, 0.5 CaCl2, and 7 MgCl2. The process from the beginning of anesthesia to putting the brain in cutting solution typically took less than 2 minutes. Coronal slices were prepared at a thickness of 300 µm while submerged in oxygenated, ice-cold cutting solution using a vibratome. Slices containing both BLA and CeA were trimmed to size and placed onto a wire mesh suspended in a small beaker of oxygenated (95% O2, 5% CO2) artificial cerebrospinal fluid (aCSF) containing, in mM: 130 NaCl, 3.5 KCl, 2.0 CaCl2, 1.5 MgSO4, 1.25 NaH2PO4, 24 NaHCO3, and 10 glucose.

Experiment 1

Rats were assigned to the stress history group or control history group and received the appropriate stress exposure as described above. Slices were prepared as described above and allowed to recover for 30 minutes with the beaker maintained at 32°C via warming bath, followed by 30 minutes at room temperature. The beaker was continuously oxygenated throughout the experiment after slice preparation was complete. Slices were individually transferred to a tissue slice chamber (Warner Instruments, Hamden, CT) mounted under a microscope with room temperature oxygenated aCSF continuously gravity-perfused across the slice at approximately 2 mL per minute and removed by a vacuum line. The slice rested on a wire mesh with the top and bottom exposed to aCSF and was held down with a small piece of platinum wire.

A bipolar electrode was positioned in the BLA with a motorized manipulator arm while viewing the slice at 4x magnification (Figure 1). A 2-6 MOhm resistance micropipette was filled with internal solution containing 1 mg/mL biocytin and, in mM: 145 potassium-gluconate, 10 HEPES, 1 EGTA, 2 Mg2+-ATP, 0.3 Na2+-GTP, and 2 MgCl2. The micropipette was inserted into the recording head stage with the attached AgCl2-coated silver wire extending into the internal solution. It was then lowered into the bath using a second motorized manipulator arm while under slight positive pressure applied via syringe. Potential cells in the CeM were identified visually at 40x magnification. The recording pipette was moved into contact with a target cell until a stable gigaohm seal was achieved, and the membrane was ruptured to achieve a stable patch using light syringe suction. Patches were considered suitable for recording if they maintained a stable access resistance below 25 MOhm.

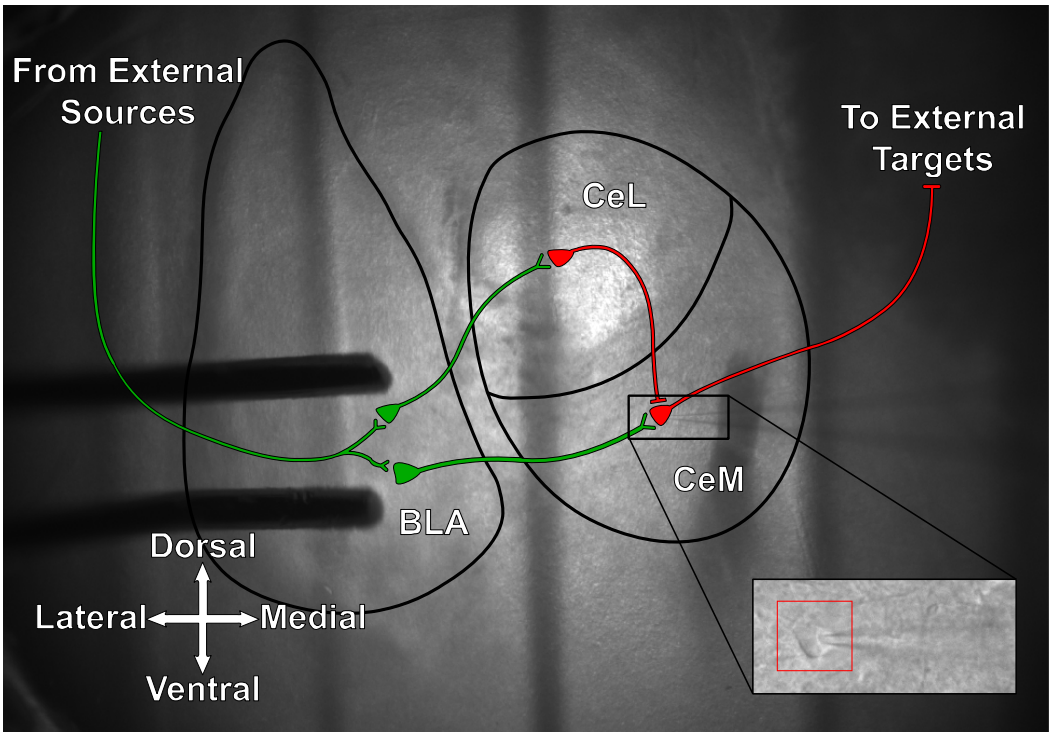

Figure 1. Simplified circuitry in BLA-CeM stimulated recordings. A bipolar electrode (left side) stimulates the BLA, as would the excitatory inputs it receives from external regions. Stimulated BLA glutamatergic neurons (green) project into the CeM. A

Cells were held in a voltage clamp configuration at -70 mV to nearly mimic resting membrane potential. Because the clamp should approximate resting membrane potential, a current of greater than 60 pA was considered an indicator a poor patch or an unhealthy cell, so recordings were limited to cells with less than 60 pA of current while clamped at -70 mV. Glutamatergic currents were pharmacologically isolated by including 30 µM GABAA receptor blocker bicuculline (Tocris Biosciences, Bristol, UK) and 1 µM GABAB receptor blocker CGP 55845 (Sigma Aldrich, St. Louis, MO) in the aCSF. Voltage response and input/output curves were produced to confirm the patch and determine an appropriate stimulation to consistently produce a sub-threshold excitatory postsynaptic current (EPSC) (Logrip, Oleata, & Roberto, 2017). The appropriate intensity was determined by finding the minimal stimulation to generate a response in the patched cell and the maximal stimulation before producing an action potential and choosing the median intensity between the two. Custom programming initiated a 3 ms stimulation at the determine intensity in the BLA once every 20 seconds while recording current in the patched CeM cell. After 10 minutes of recording baseline responses, 2.14 µL/mL 95%

ethanol was added to the aCSF for a concentration of 36 mM ethanol. After allowing 6 minutes of wash onto the slice and perfusion through it, the recording continued for another 10 minutes. The last 5 minutes of recording before application of alcohol was used as the baseline period, and the first 5 minutes after alcohol wash-on was used for the alcohol period. These periods were chosen based on the number of patches that did not remain stable through the entire 10 minutes of alcohol recording and choosing a baseline period of equal length. A validation recording was performed to confirm that the responses were primarily produced by glutamatergic AMPA receptor activation by adding NMDA receptor antagonist 2-amino-5-phosphonopentanoic acid (AP5) at 40 μM to rule out NMDA contribution to observed responses followed by AMPA receptor antagonist 6-Nitro-2,3-dioxo-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX) at 50 μM to confirm the contribution of glutamatergic AMPA receptor activation (AP5 and NBQX from Tocris Biosciences, Bristol, UK).

Experiment 2

Separate rats from Experiment 1 were assigned to the stress history group or control history group and received the appropriate stress exposure as described above. Half of the subjects from each sex and stress history group were selected randomly for an acute ethanol exposure group, and the remaining subjects were assigned to a control group. Slices were prepared as described above and incubated in a warming bath at 32°C for 60 minutes with the aCSF oxygenated (95% O2, 5% CO2) for the full duration. For slices in the acute ethanol group, 2.14 μL/mL 95% ethanol was added to the beaker to achieve a 36 mM concentration for the final 10 minutes of the 60-minute incubation. No addition was made to the aCSF of slices in the control group.

At the end of the incubation period, slices were removed from the beakers, and tissue punches containing the CeM were removed and frozen at -80° C. Postsynaptic AMPA receptors make up a relatively small portion of the total mass of a neuron. Tissue punches from all slices from each animal were pooled into a single sample to produce protein for quantification. A previously-described fractionation procedure was used to take advantage of AMPA receptor location in the postsynaptic density to enrich the samples with postsynaptic membrane (Hallet, Collins, Standaert, & Dunah, 2008). Synaptic enrichment also differentiates between functional synaptic receptors and typically inactivated perisynaptic receptors waiting to be trafficked in for

use in the synapse. In brief, the tissue was homogenized and lysed in the presence of protease and phosphatase inhibitors (Roche) in a lysis buffer containing, in mM: 320 sucrose, 10 Tris base, 5 NaF, 1 Na3VO4, 1 EDTA, and 1 EGTA. The homogenized lysate was separated via centrifuge at previously determined speeds to separate components of the tissue with greater mass than postsynaptic membrane-containing vesicles. Pellets underwent hypoosmotic lysis in a buffer containing, in mM: 35.6 sucrose, 10 Tris base, 5 NaF, 1 Na3VO4, 1 EDTA, and 1 EGTA, and protease and phosphatase inhibitors. The final high-speed spin of 20 minutes at 25,000 x G produced a pellet enriched with postsynaptic membrane separated from the lower density components in the supernatant. The pellet was used as the sample material for Western blot analysis.

While fractionation enriched samples with postsynaptic membrane, the final yield was still low enough to require Western blot gels with high capacity (30 µL) wells. 10 lane, 8-16% gradient TG gels (Bio-Rad, Hercules, CA) were used with 5 µg protein per sample. Bio-Rad Protein Assay kits were used to determine protein concentration in each sample for matching protein loads. Samples were distributed across gels such that 1 sample per group ran on each gel to minimize the impact of differences between runs. Samples were run through gradient gels in Invitrogen running boxes at 200 V for 1 hour and transferred to PVDF membrane in Bio-Rad transfer boxes at 35 V for 22 hours. The running buffer contained 25 mM Tris base, 192 mM glycine, and 0.1% SDS, and the transfer buffer contained 12.5 mM Tris base, 96 mM glycine, and 1% methanol Primary antibodies for pS831, pS845 (Cell Signaling Technology, Danvers, MA), and total GluA1 expression (Millipore Sigma, Burlington, MA) were incubated on separate membranes. Fluorescent secondary antibodies were quantified on a fluorescence scanner, and total protein load was assayed with coomassie staining.

Data Analysis

All data were analyzed using SPSS v25.0.0.2 (IBM, Armonk, NY) and graphed with GraphPad Prism v8.0.2 (GraphPad Software, Inc., La Jolla, CA). Significance was set at an α-value of 0.05. Outliers were identified by mean +/- 3 standard deviations, and assumptions of normality and homogeneity of variance for analysis of variance (ANOVA) tests were conducted with residuals using the Shapiro-Wilk's normality test and Levene's test, respectively. Data were found to be normally distributed ($p > 0.05$) and satisfied homogeneity of variance ($p > 0.05$)

unless otherwise stated. Significant interactions in the ANOVA were followed up with pairwise comparisons, and α-values were Bonferroni corrected as appropriate. Because of a priori hypotheses about the effects of sex and history, non-significant interactions were followed up with pairwise comparisons where indicated.

Electrophysiological data were quantified using Clampfit v10.7.0.3 (Axon Instruments, San Jose, CA). Stimulated responses were manually quantified individually without any processing and binned by minute (3 stimulations per minute). To quantify a response, baseline current was determined using the mean current flow in the 5 ms period prior to the stimulation artifact (see Figure 2). Individual stimulated responses were identified as the lowest downward deflection of the first drop in current following the stimulation artifact and subtracting the baseline current as determined by the mean of the 5 ms period before the stimulation artifact (see Figure 2). Evoked responses were binned by minute (3 stimulations per minute). Data from the last 5 minutes of the 10-minute initial baseline period were used to calculate the cell's baseline response, and the first 5 minutes after ethanol wash-on were used to calculate the cell's response in the presence of ethanol. A repeated measures ANOVA was conducted using sex and history (stress or control) as between-subjects measures and phase (baseline or 36 mM ethanol) as the within-subjects measure. To test for group differences in evoked baseline response, a two-way ANOVA was conducted with sex and history as the between-subjects factors. To test for group differences in the effects of ethanol on evoked responses, a two-way ANOVA was conducted on the average ethanol response as a percentage of the average baseline response, again using sex and history as the between-subjects factors. Independent-samples t-tests were run to determine whether ethanol phase responses as a percentage of baseline responses were significantly different from baseline.

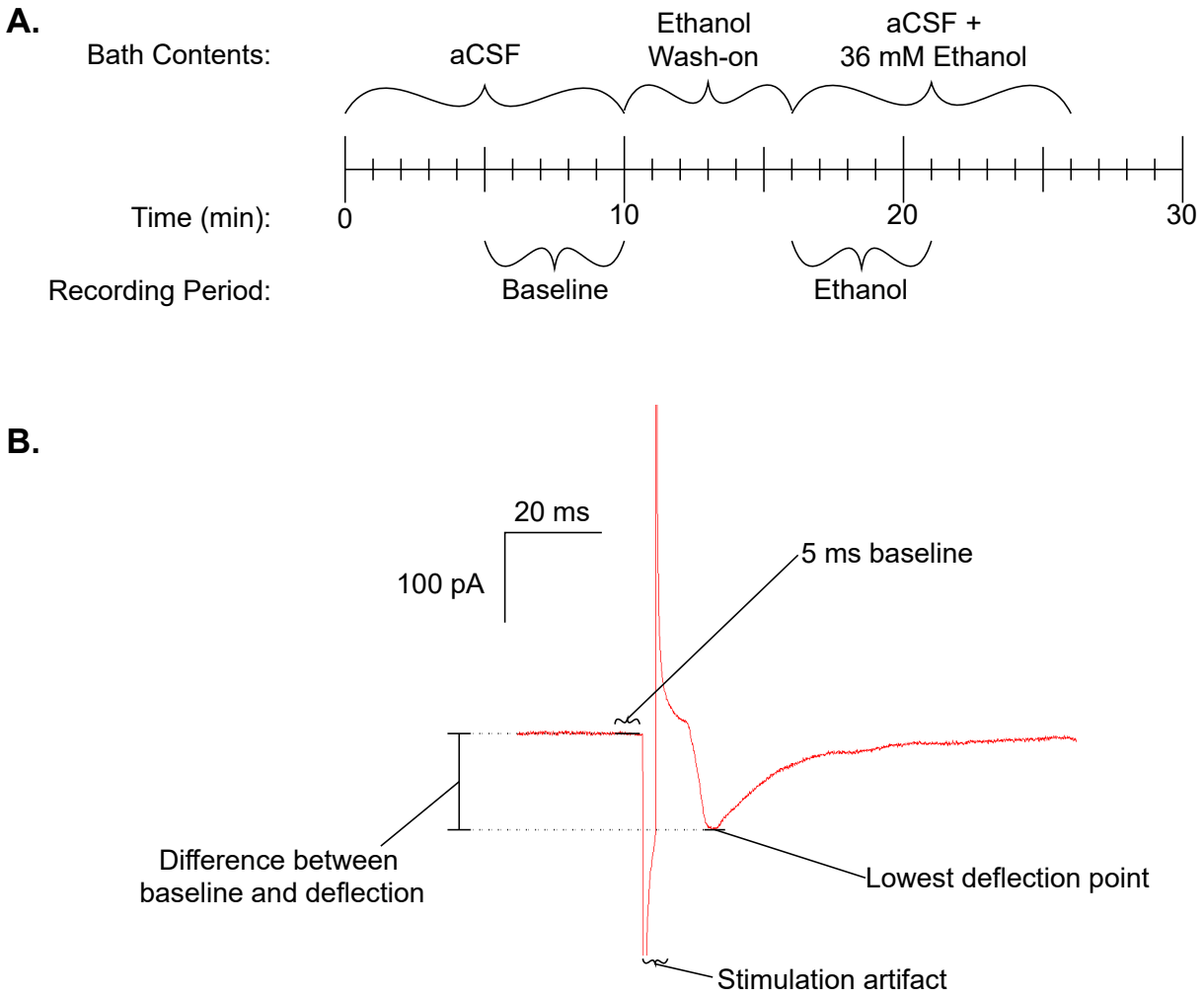

Figure 2. Recording session timeline and eEPSC quantification. A. Timeline of a typical recording session for one cell. After characterizing the cell and determining the optimal stimulation intensity for subthreshold EPSCs, the timeline for the session began (0 minutes). 3 stimulations were administered per minute for the next 26 minutes. During the first 10 minutes, the bath included only aCSF and GABA receptor blockers. The bath was switched to aCSF with blockers and 36 mM ethanol and allowed 6 minutes to wash onto and through the slice while recording continued. Recording continued for another 10 minutes. The last 5 minutes of aCSF were used to quantify the cell's baseline response, and the first 5 minutes of aCSF + 36 mM ethanol were used to quantify the cell's response in the presence of ethanol. B. Diagram of a single stimulation and response and its quantification. The mean current in pA before the stimulation artifact was used as the cell's baseline current flow while clamped at -70 mV. EPSC magnitude was taken as the difference between the current induced by BLA stimulation and the baseline current flow, which was calculated as the different between the lowest deflection point and the 5 ms baseline period.

Western blot membrane scans were quantified with Image Studio Lite v5.2.5 (Li-Cor Biosciences, Lincoln, NE) and normalized to total protein load based on coomassie staining

quantified with ImageJ v1.5j9 (National Institutes of Health, USA). The male, control history rat with acute aCSF (no ethanol) treatment was considered the control sample. Graphs used values that were normalized to this control sample on each gel for comparison between gels. Group differences in protein expression were analyzed with three-way ANOVAs using non-normalized values with sex, history, and acute aCSF or 36 mM ethanol treatment as between-subjects factors and the male, control history, aCSF treatment sample on each gel as a covariate. Follow-up comparisons were conducted as indicated.

RESULTS

Experiment 1: Electrophysiological Recordings in the CeM

Cells from 6 different animals from each sex and stress history group were recorded. Cells in 4 recordings died before the ethanol wash on period was completed and were excluded from analysis. Placements for all recordings can be seen in Figure 3, and representative traces can be seen in Figure 4. One recording from a female stress rat was identified as an outlier in responses during baseline and ethanol exposure based on residuals testing of a repeated measures ANOVA. Inspecting the raw data from this recording further indicated that the cell had an unstable access resistance and may have partially resealed during the experiment. This recording was removed from the dataset. After its removal, several recordings that had previously been identified as outliers no longer met that criteria. All analyses were performed with and without the outlier, and its removal did not change whether any measures were found to be significant; the corrected p-value was 0.028. After concluding the recording for experimental data for several cells, AP5 and NBQX were added to the aCSF to verify that the responses observed during the recording were primarily produced by glutamatergic activity at AMPA receptors (Figure 5).

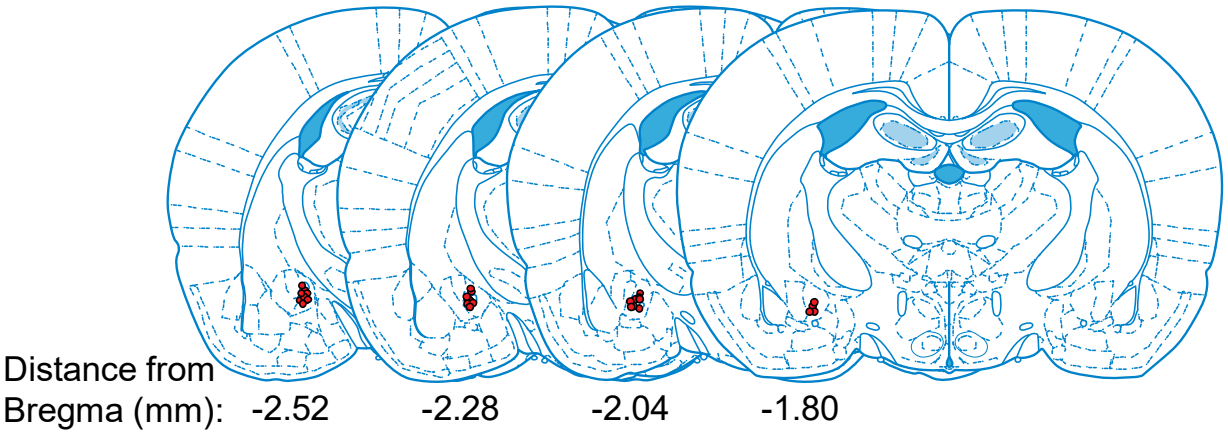

Figure 3. Placement diagram for whole cell recordings.The location of each recording in the CeM was saved and plotted (indicated by red dots).

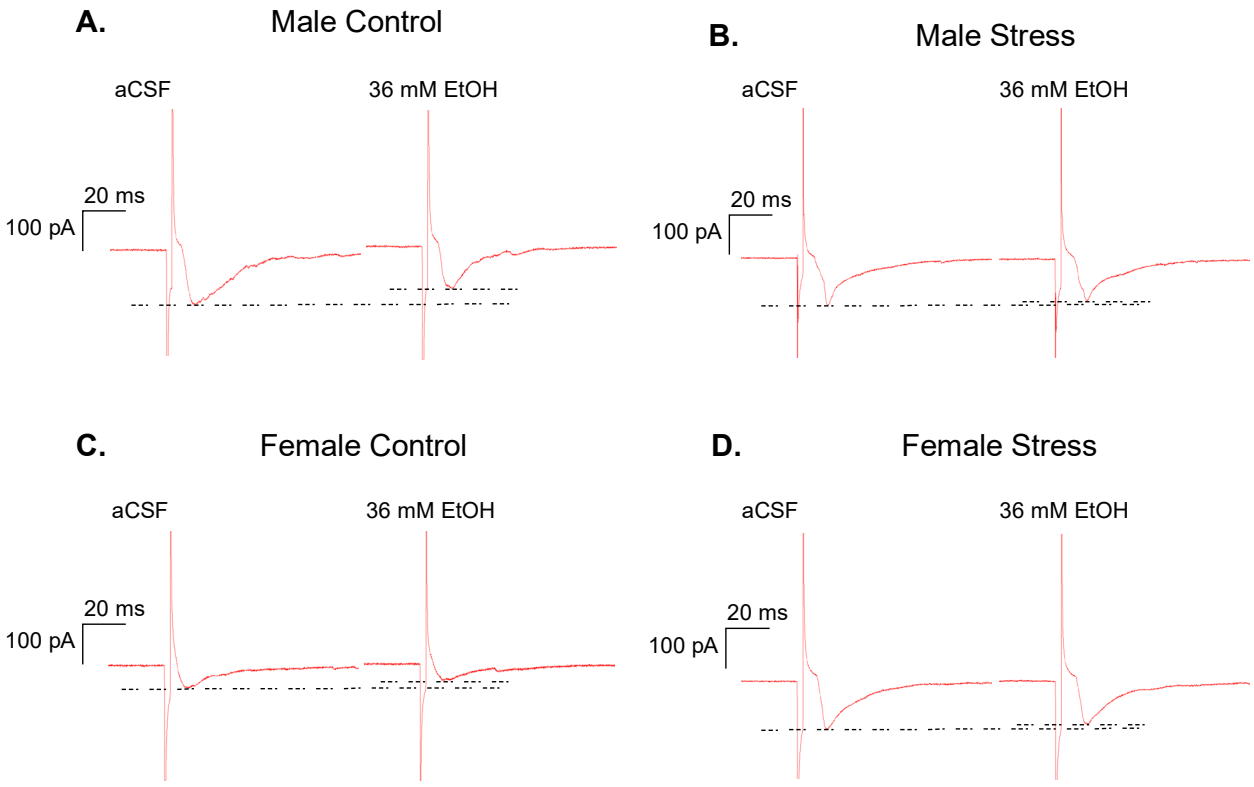

Figure 4. BLA-evoked subthreshold EPSCs in representative CeM neurons. A. Representative traces for a single neuron from a Male, Control History rat during the baseline aCSF phase and during the 36 mM Ethanol phase. Evoked EPSCs were quantified as the difference

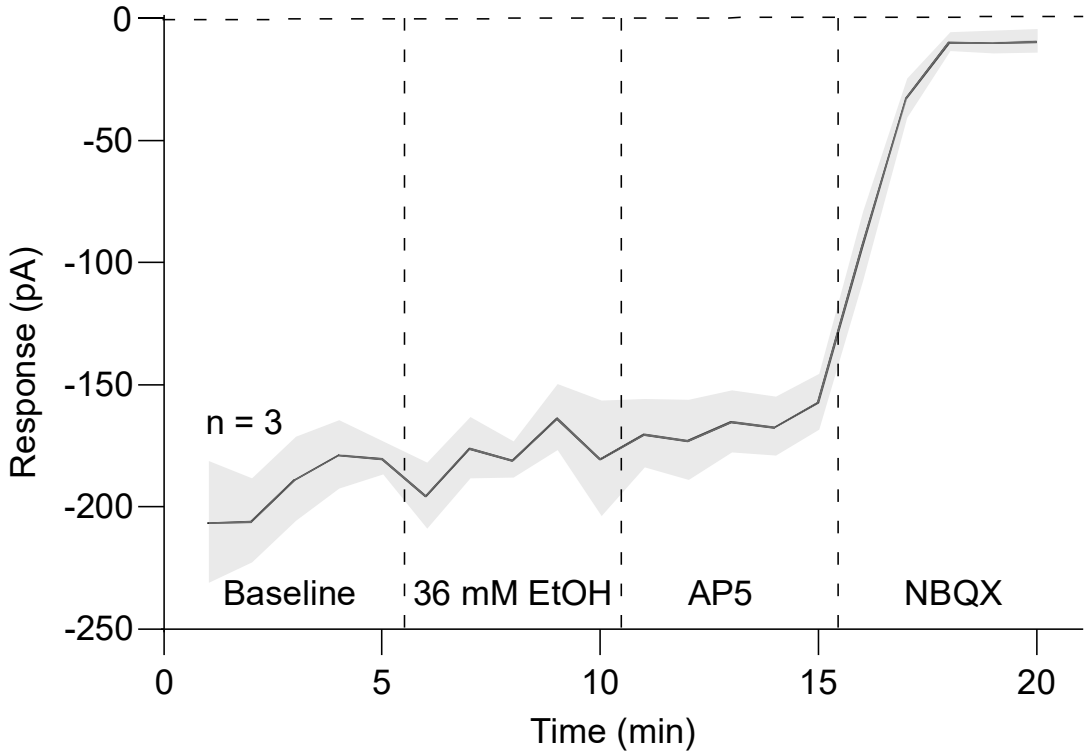

Figure 5. Verification of evoked EPSCs as AMPA receptor-mediated. Following several stable recordings, NMDA receptor antagonist AP5 was washed onto the slice to verify that the immediate responses had negligible NMDA receptor contribution. AMPA receptor antagonist NBQX was then washed on, blocking all responses and verifying that observed EPSCs were primarily AMPA receptor-mediated. Values are means +/- SEM.

Evoked Responses During Baseline and Ethanol Phases

A repeated measures ANOVA was conducted for BLA- evoked CeM EPSCs using sex and history (stress or control) as between subjects factors and phase (baseline time period or 36 mM ethanol time period) as the within subjects factor. Residuals were found to be normally distributed and data satisfied homogeneity of variance ($p > 0.05$). The ANOVA indicated no significant 3-way interaction between sex, history, and phase and no significant 2-way interactions between sex and phase or history and phase ($p > 0.05$). A significant 2-way interaction between sex and history was indicated, $F(1, 31) = 5.346$, $p = 0.028$, $\eta 2 = 0.147$ (Figure 6). This is the interaction that was supported by removal of the outlier, reducing its p-value from 0.050 to 0.028. No main effects of sex or history were found, but phase was significantly different between baseline and ethanol time periods, indicating that ethanol did reduce EPSC magnitude overall, $F(1, 31) = 7.631$, $p = 0.010$, $\eta 2 = 0.198$.

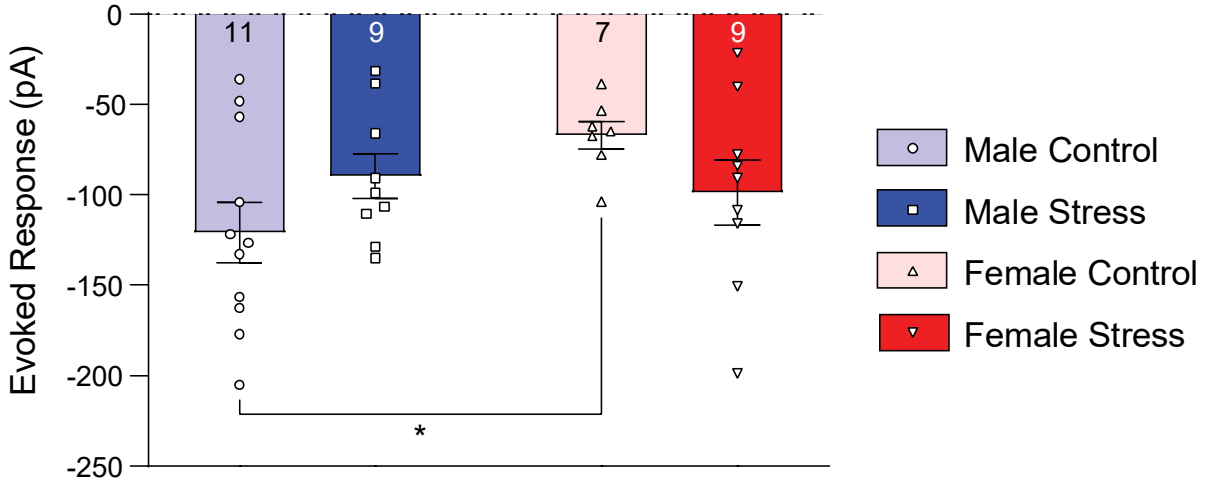

Figure 6. BLA-evoked CeM responses in the presence of aCSF. Male rats showed no significant difference in BLA-evoked CeM EPSCs between those with a control history and those with a stress history. Female rats had a non-significant trend toward a higher response magnitude for those with a stress history compared to controls ($p = 0.061$). There was a significant 2-way interaction between sex and history during the baseline period ($p = 0.018$) with female control responses reduced compared to male controls ($p = 0.021$). Error bars indicate SEM. * $p < 0.05$.

A 2-way interaction between sex and history indicated that baseline eEPSC magnitude decreased in males but increased in females (Figure 6). Pairwise comparisons for the 2-way interaction between sex and history revealed that the reduction in males was not statistically significant, $F(1, 31) = 1.661$, $p = 0.207$, nor was the increase in females, $F(1, 31) = 3.775$, $p = 0.061$. Pairwise comparisons for history revealed that males with a control history had a significantly different response from females with a control history, $F(1, 31) = 20.185$, $p = 0.018$, primarily driven by males having a greater EPSC magnitude compared to females during the baseline period, a mean difference of 53.791 pA, CI 95% [8.736, 98.846], $p = 0.021$ (Figure 6).

Reduction in Evoked Responses in the Presence of 36 mM Ethanol

A two-way ANOVA was conducted with group means for evoked response in the presence of ethanol as a percentage of baseline response. No outliers were identified, and residuals were found to be normally distributed using Shapiro-Wilk's normality test ($p > 0.05$), and there was homogeneity of variances according to Levene's test ($p = 0.625$). There was no significant interaction between sex and history, $F(1, 32) = 0.040$, $p = 0.843$. Due to a priori hypotheses, pairwise comparisons were conducted for sex and history. There were no significant differences

between males and females with a control history, $F(1, 32) < 0.001$, $p = 0.991$, or a stress history, $F(1, 32) = 0.076$, $p = 0.785$, and there were no significant differences between males with a control history vs a stress history, $F(1, 32) = 1.347$, $p = 0.254$, or between the means of females with a control history vs a stress history, $F(1, 32) = 0.589$, $p = 0.448$. There were no significant main effects of sex, $F(1, 32) = 0.034$, $p = 0.855$, or history, $F(1, 32) = 1.810$, $p = 0.188$. Independent-samples t-tests indicated no significant reduction during the ethanol phase for male stress, $t(8) = 0.551$, $p = 0.597$, female control, $t(6) = 2.016$, $p = 0.090$, or female stress groups, $t(7) = 0.751$, $p = 0.477$, but there was a significant reduction for the male control group, $t(10) = 3.184$, $p = 0.010$ (Figure 7). Independent-samples t-tests indicated a significant reduction during the ethanol phase overall for males, $t(19) = 3.211$, $p = 0.005$, but not for females, $t(14) = 1.761$, $p = 0.100$, and overall for subjects with a control history, $t(17) = 3.686$, $p = 0.002$, but not for subjects with a stress history $t(16) = 1.277$, $p = 0.220$ (Figure 8).

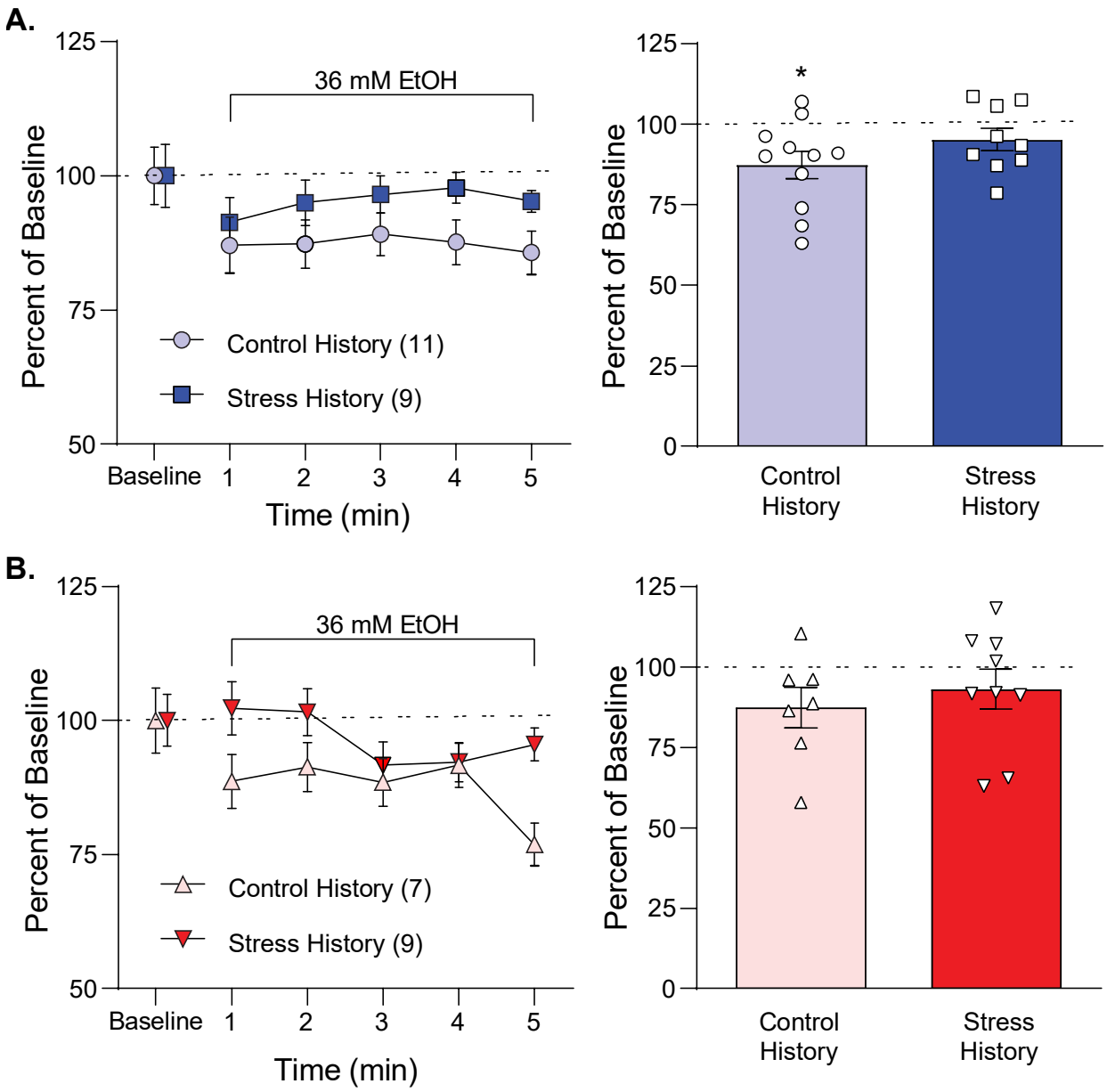

Figure 7. BLA-CeM eEPSCs during ethanol exposure relative to baseline. A. Time course of responses for males during the ethanol phase, shown as a percentage of the average baseline response. Male controls had a significant reduction in eEPSC magnitude during the ethanol phase ($p = 0.010$). B Time course of responses for females during the ethanol phase shown as a percentage of the average baseline response. Error bars indicate SEM. * $p < 0.05$.

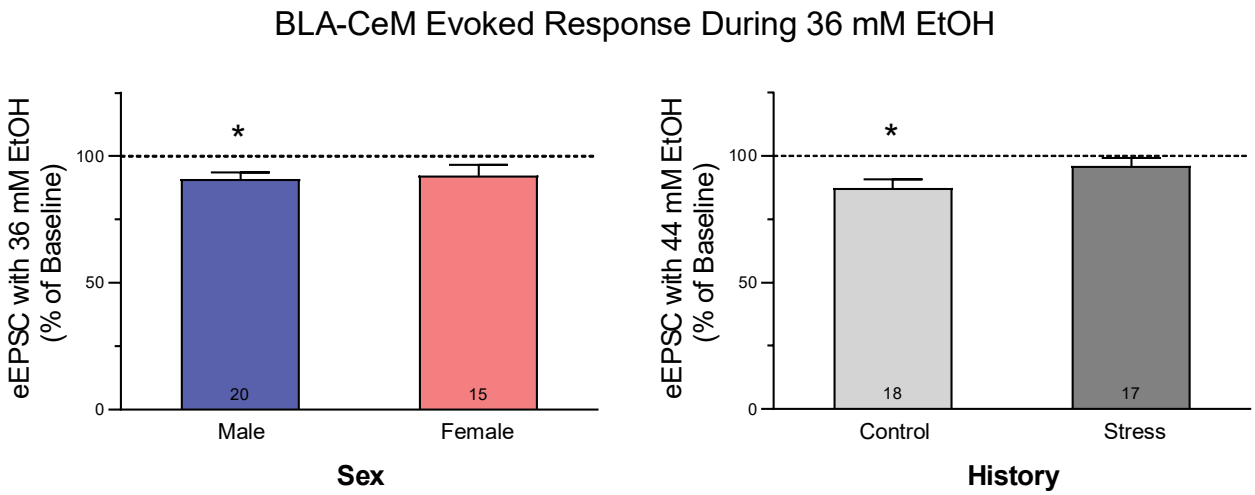

Figure 8. Ethanol-induced reductions in eEPSCs by sex and stress history. Males showed a significant reduction during the ethanol phase ($p = 0.005$), but females did not. Animals with a control history showed a significant reduction during the ethanol phase ($p = 0.002$), but animals with a stress history did not. Error bars indicate SEM. * $p < 0.05$.

Experiment 2: AMPA Receptor Expression and Posttranslational Modification in the CeM

6 samples were excluded from total GluA1 analysis; 3 samples returned negative fluorescence values, and 3 were identified as outliers. No samples were excluded from pS831 analysis, but 6 samples could not be calculated as a ratio of phosphorylated to total GluA1 because of the excluded GluA1 samples. 24 samples returned negative fluorescence values for pS845 and could not be interpreted; a full explanation is detailed in section 3.2.4.

Total GluA1 AMPA Receptor Subunit Expression

A three-way ANOVA was conducted to determine the effects of sex, stress history, and acute alcohol treatment on total GluA1 expression, using the value for the male, control history, aCSF treatment sample on each gel as a covariate. There was no significant three-way interaction between sex, history, and treatment, $F(1, 33) = 0.492$, $p = 0.488$, or two-way interactions between sex and history, $F(1, 33) = 0.025$, $p = 0.875$, between sex and treatment, $F(1, 33) = 0.594$, $p = 0.446$, or between history and treatment, $F(1, 33) = 0.001$, $p = 0.978$ (Figure 9). There were no significant main effects of sex, $F(1, 33) = 0.589$, $p = 0.448$, history, $F(1, 33) = 0.112$, $p = 0.740$, or treatment, $F(1, 33) = 0.483$, $p = 0.492$. This indicates that total GluA1 expression was

not dependent on the sex of the animal, the exposure to a history of chronic stress, or the effects of acute ethanol exposure.

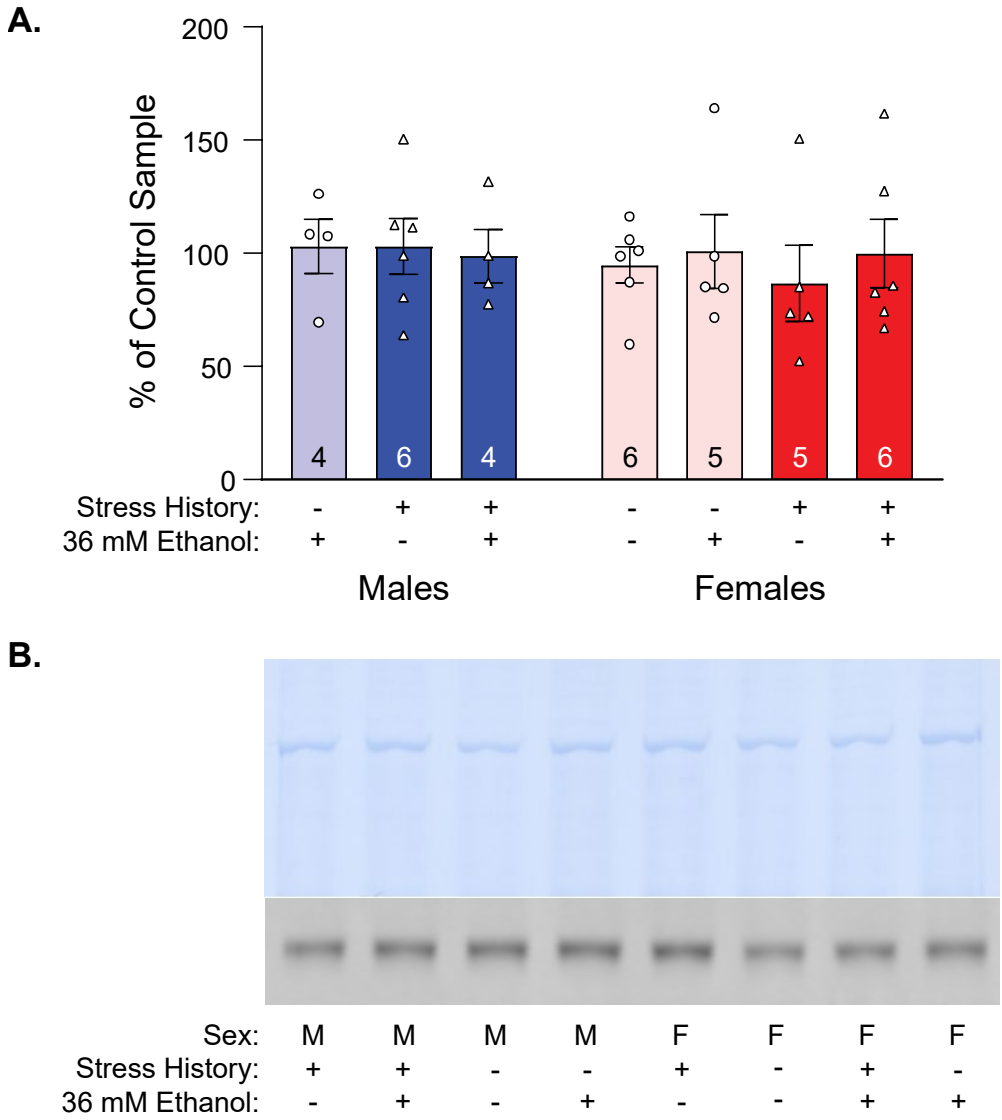

Figure 9. Total synaptic GluA1 expression in the CeA. A. A 3-way ANOVA revealed no significant main effects or interactions on total synaptic GluA1 subunit expression in response to chronic stress or acute ethanol exposure. Error bars indicate SEM. B. Representative image for GluA1 quantification.

Phosphorylated S831 on GluA1 Receptor Subunits

A three-way ANOVA was conducted to determine the effects of sex, stress history, and acute ethanol treatment on pS831, using the control sample on each gel as a covariate. There was a non-statistically significant trend toward a three-way interaction between sex, history, and

treatment, F(1, 39) = 3.768, p = 0.059. There were no significant two-way interactions between sex and history, F(1, 39) = 0.824, p = 0.370, between sex and treatment, F(1, 39) = 0.068, p = 0.795, or between history and treatment, F(1, 39) = 0.370, p = 0.581 (Figure 10). There were no significant main effects of sex, F(1, 39) = 0.314, p = 0.578, history, F(1, 39) = 2.017, p = 0.164, or treatment, F(1, 39) = 0.248, p = 0.621. This indicates that the concentration of GluA1 AMPA receptor subunits phosphorylated at S831 was not dependent on sex, chronic stress, or acute ethanol exposure, but there was a trend toward an interaction between all three factors.

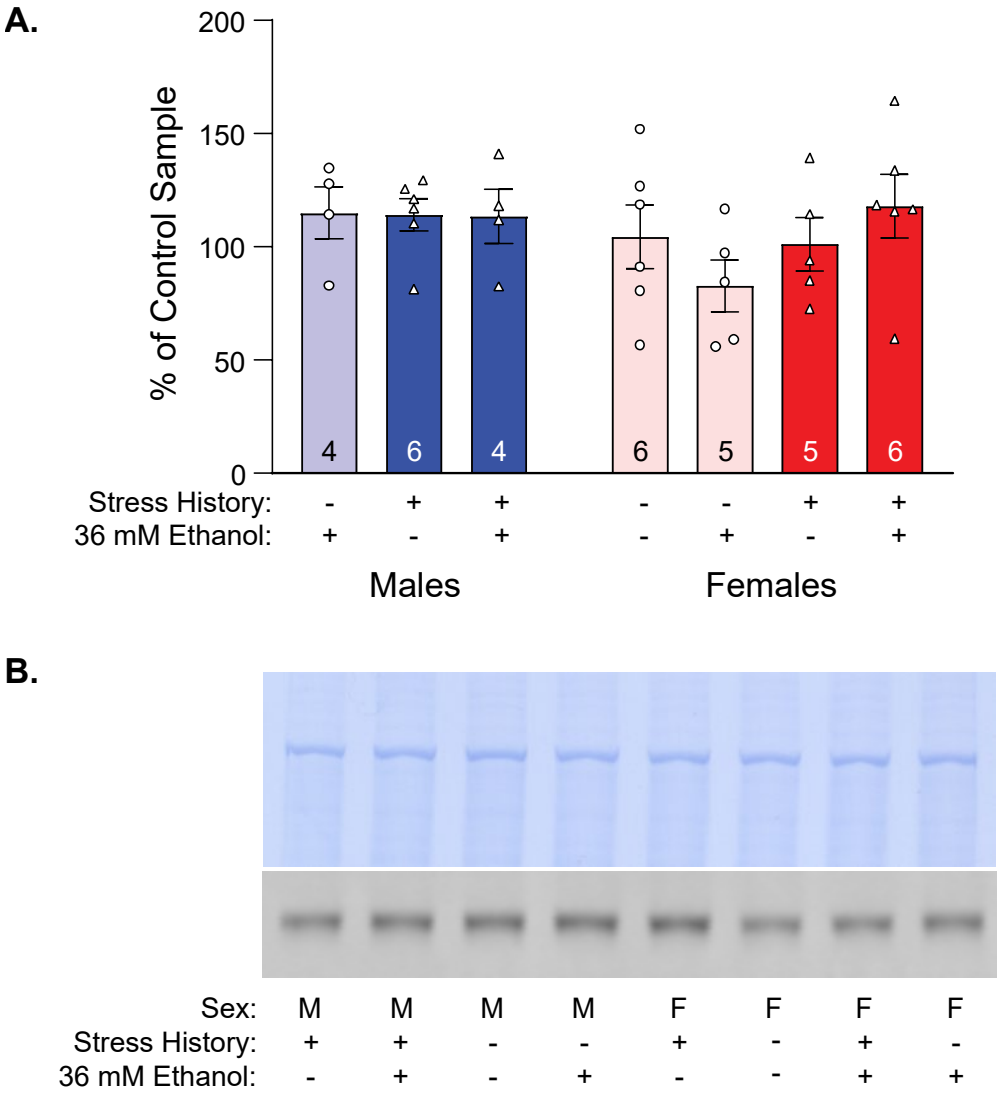

Figure 10. Phosphorylation of synaptic GluA1 at S831 in the CeA. A. A 3-way ANOVA indicated no main effects or interactions between chronic stress or acute ethanol exposure on phosphorylation of synaptic GluA1 subunits at the S831 phosphorylation site. Error bars indicate SEM. B. Representative image for pS831 quantification.

Proportional Phosphorylation of S831

Because the ratio of phosphorylated protein to total protein can also indicate molecular adaptations, another three-way ANOVA was conducted to examine group differences in the ratio of pS831 to total GluA1 expression, again using the control sample as a covariate. There was no significant three-way interaction between sex, history, and treatment, $F(1, 33) = 1.542$, $p = 0.223$, or two-way interactions between sex and history, $F(1, 33) = 1.243$, $p = 0.273$, between sex and treatment, $F(1, 33) = 2.666$, $p = 0.112$, or between history and treatment, $F(1, 33) = 0.116$, $p = 0.603$ (Figure 11). There were no significant main effects of sex, $F(1, 33) < 0.001$, $p = 0.993$, history, $F(1, 33) = 1.977$, $p = 0.169$, or treatment, $F(1, 33) = 0.267$, $p = 0.609$. This indicates that the proportion of GluA1 receptor subunits phosphorylated at S831 was not dependent on sex, chronic stress, or acute ethanol exposure.

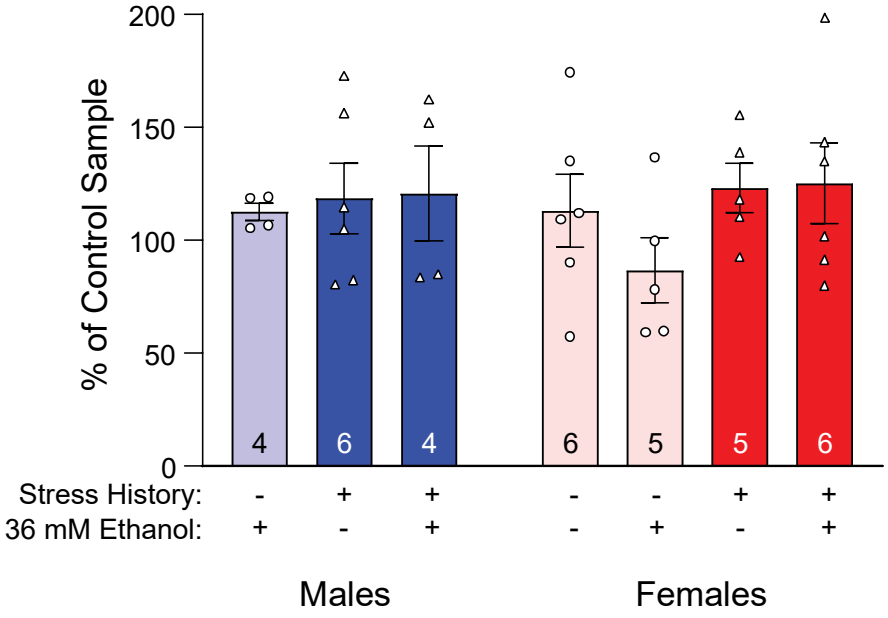

Figure 11. Percentage of total synaptic GluA1 phosphorylated at S831 in the CeA. S831-phosphorylation of GluA1 subunits as a percentage of total synaptic GluA1 expression was assessed by a 3-way ANOVA, which indicated no significant interactions or main effects of chronic stress history or acute ethanol exposure. Error bars indicate SEM.

Phosphorylated S845 on GluA1 AMPA Receptor Subunits

Membrane scan results for pS845 could not be resolved. Reported values were extremely low, with 24 returning negative fluorescence values from the membrane scans. Negative values on the membrane scan indicate less fluorescence than is detected on average in the background

of the scan. Because the background fluorescence is taken as zero, any findings resulting from an attempt at normalization would likely be unreliable, so all 24 negative values were excluded. This resulted in too few samples per group for meaningful statistical analysis. Total sample sizes can be seen in Table 1. Statistical analysis for pS845 is not included beyond this table.

Table 1. Counts of non-negative fluorescence results for synaptic pS845 GluA1. Phosphorylation of GluA1 subunits at the S845 site was quantified at extremely low levels. Many samples returned lower fluorescence values compared to background fluorescence of the membrane. The number of positive results, shown above, was too low for meaningful statistical analysis.

Sex	History	Treatment	# Positive Results
Male	Control	aCSF	4
		EtOH	0
	Stress	aCSF	2
		EtOH	2
Female	Control	aCSF	2
		EtOH	1
	Stress	aCSF	2
		EtOH	3

DISCUSSION

Overall Discussion

These experiments explored a gap in stress and alcohol abuse literature regarding the effects of chronic stress on glutamatergic input to the CeM and how that input responds to acute ethanol. Previous experiments established postsynaptic sex differences in activation by direct glutamatergic afferents from the BLA and how they respond to acute corticosterone and alcohol interaction (Logrip, Oleata, & Roberto, 2017). These experiments and their prior findings indicated a contribution of alcohol to postsynaptic IPSCs but did not indicate that the contribution was presynaptic based on a lack of change in paired-pulse facilitation (Roberto et al., 2005). Experiments here expanded on those findings by replacing acute stress hormone application with native responses from chronic in vivo stress exposure comparable to what has been used in behavioral research. Direct input from the BLA was assessed using whole-cell electrophysiology, while responsivity of the CeM to any source of glutamatergic input was assessed by quantification of AMPA receptor expression and phosphorylation. Electrophysiological data implied that exposure to a stress history erases a difference between males and females in CeM responsivity to direct BLA afferents. Protein analysis of the CeA indicated that this change is not likely due to changes in AMPA receptor expression or phosphorylation.

Sex Differences in the Effects of Stress and Ethanol on CeM Responsivity

Electrophysiological data indicated that direct BLA-CeM projections differ at baseline between males and females such that BLA-evoked EPSCs in the CeM have greater magnitude in males with a control history compared to females. However, an interaction between sex and stress history indicated that exposure to chronic stress reduced responsivity in males while increasing it in females, effectively equalizing the responsivity of the direct glutamatergic BLA-CeA projection (Figure 6). This is a pattern consistent with the idea that in the human literature it appears that prior trauma appears in most alcoholism cases in women while having no correlation for alcoholism in men.

Sensitivity of this circuitry to ethanol-induced reduction of EPSCs was also found to differ by sex. Control males showed a statistically significant reduction in response magnitude during ethanol exposure, but females did not. Neither sex with a history of chronic stress showed this reduction. When collapsed across sex and stress history, it was revealed that males overall showed a reduction during ethanol exposure regardless of stress history, as did animals with a control history regardless of sex (Figure 8). This demonstrated that not only is the sex difference in baseline responsivity lost in response to stress, but also that female sensitivity of this circuitry to the effects of acute ethanol differs from males in a manner that resembles the difference between animals with and without a history of chronic stress.

Lack of Contribution from Differences in Synaptic GluA1

Synaptic GluA1 subunit-containing AMPA receptors were not found to differ by sex, stress history, or ethanol exposure in overall expression or phosphorylation, nor were there any observed interactions between these factors. Tissue punches contained some non-CeM tissue, so changes specific to the CeM could be masked by concentrations elsewhere. The presence of GluA1 subunits in the synapse was directly analyzed with this protocol, but neither the total expression of GluA1 nor the expression of non-GluA1 subunit-containing AMPA receptors were assessed. Altered responsivity of synaptic AMPA receptors could be the result of changes in expression of AMPA receptor subunits other than GluA1, which would not be detected by this experiment. Total synaptic GluA2 subunit quantification could reveal whether the concentration of AMPA receptors at the synapse was elevated, as most AMPA receptors contain a GluA2 subunit dimer (Traynelis et al., 2010).

It could be the case that the quantity of GluA1 subunit-containing AMPA receptors did differ by sex or chronic stress history, but that those receptors were not present in the postsynaptic membrane and were concentrated in the intracellular scaffold of the postsynaptic density, which may have been lost during the fractionation process. Phosphorylation at the S831 site is associated with receptor activity and not trafficking (Lee et al., 2013), so the lack of difference in S831 phosphorylation does not provide any information about GluA1 subunits outside of the synaptic membrane. Receptors near to but not inserted in the synaptic membrane can contribute during long-term potentiation but are not present for immediate activity (Yang et al., 2008), and thus would not be captured by this design.

If it were the case that AMPA receptor expression were altered outside of the postsynaptic density, phosphorylation at the S845 site of GluA1 receptors would be of greater interest, as S845 phosphorylation is associated with GluA1 subunit-containing AMPA receptor trafficking to the synapse (Mao, Diaz, Fibuch, & Wang, 2013), but it was unable to be quantified with the methods utilized here. One potential explanation is phosphatase activity during the 60-minute experimental period. While care was taken to minimize cell death and maintain supportive conditions, tissue slices inevitably differ from their natural environment, and phosphatase activity may overcome phosphorylation. Phosphatase inhibitors in the aCSF could potentially help with this problem, but this is complicated by necessarily interrupting the balance of phosphatases with kinases. It could also be the case that poor quantification was due to experimental design. Western blot conditions for multiple antibodies commonly used in the lab have been refined since the experiment, some of which may affect detection of phosphorylated proteins. Taken at face value, these data indicate that changes in AMPA receptor-dependent EPSCs in the CeM are not the result of postsynaptic GluA1 subunit modifications. The observed differences in electrophysiological data could be the result of different AMPA receptor subunit composition or of presynaptic changes, although previous research has not indicated presynaptic contribution based on paired-pulse experiments (Roberto et al., 2005, Logrip, Oleata, & Roberto, 2017). These possibilities also apply to adaptations involving extra-amygdalar inputs to the CeM.

Implications for Sex Differences in Chronic Stress and Alcohol Interactions

The differences in baseline BLA-CeM connectivity have implications for the timeline of stress-induced neuroadaptations. Stress-related disorders are known to have time-dependent differences in expression of anxiety. For example, anxiety-like symptoms 2-4 weeks following trauma have been shown to be good indicators of future PTSD diagnosis (Shalev et al., 1997, Murray et al., 2002), but the same symptoms measured 1 week or less after trauma are not good indicators of long-term outcomes (Shalev, 1992; Murray et al., 2002). While some evidence suggests that men are generally more likely to self-medicate anxiety with alcohol abuse (Vesga-López et al., 2014), women diagnosed with both PTSD and AUD are more likely to use alcohol to cope with stress than men with the same diagnoses (Lehavot et al., 2014). These data, when taken with observed trends in trauma preceding alcohol abuse in women more than in men, may indicate that the time course of neuroadaptations may be a meaningful determinant of sex

differences in vulnerability. The electrophysiological observations from Experiment 1 demonstrate sex-dependent alterations in intra-amygdalar circuitry only 24 hours following cessation of chronic stress exposure, as well as stress-induced reduction in acute alcohol sensitivity within those circuits. These measures may change over time and could even reverse in effect if they are connected to the differential anxiety symptoms over time during the development of PTSD following trauma. Longer in vivo manipulation is needed to address these questions. Alternate stress models may also be important to pursue, as different types of trauma lead to different sex-dependent rates of PTSD development (Kessler et al., 1995). This model has been used for research on trauma such as combat exposure. Restraint may be more relevant to natural stress exposure, and observer stress models may be more relevant toward many occupational forms of trauma for jobs such as EMTs and first responders.

GABAergic Circuitry

The study of direct glutamatergic BLA-CeM connectivity necessitated a silencing of GABAergic activity. However, the high proportion of GABAergic interneurons in the amygdala implicate them as another potential source of relevant stress and reward-related neuroadaptations. Recent work focuses on differential cell types within GABAergic populations in the CeA and their relevance toward modulating amygdala output. Neurons containing protein kinase C-delta (PKCδ) in the CeL have been referred to as CeAON neurons, with PKCδ-negative neurons referred to as CeAOFF, due to their connection to promoting anxiety-like behaviors when activated (Janak & Tye, 2015; Amanda et al., 2012). Modifications to this circuitry may hold further relevance to the interaction of anxiety with alcohol due to the activation of GABA receptors by ethanol. Alterations in this circuitry would have been masked in these experiments due to the use of excitation from glutamatergic inputs, which required the silencing of GABA activity through GABA receptor blockade.

Alternative Sources of Changes in Excitability

Excitability can be mediated through AMPA receptors by means other than phosphorylation and without altering total AMPA receptor content. In the case of Q/R editing, GluA2 receptor subunits tetramerize based on their Q/R editing status such that only Q subunits

are tetramerized and trafficked to synapses (Greger et al., 2003). Difference in Q/R editing could, therefore, alter excitability by changing subunit composition (shifting it toward more or less GluA2 subunit inclusion) or by changing the number of trafficked subunits without altering the total number produced.

Any changes to subunit composition of AMPA receptors can affect excitability. AMPA receptors lacking GluA2 subunits are calcium-permeable, resulting in the potential for them to modulate plasticity by permitting a constant flow of calcium into the cell (Man, 2011). Although GluA2-containing AMPA receptors still leak calcium prior to editing at the Q/R site, GluA2 subunits are typically Q/R-edited in the human brain and therefore almost completely calcium-impermeable (Burnashev et al., 1992; Pachernegg et al., 2015). Calcium influx through GluA2-lacking receptors can lead to enhanced calcium influx through NMDA receptors, the combination of which may produce a complex system of elevated calcium levels in the cell (Man, 2011; Swanson et al., 1997). This suggests one potential mechanism by which subunit composition changes can alter excitability without changing the receptor's interaction with its ligand. Alternatively, all AMPA receptor subunits are spliced at the flip/flop site into one of two variants, of which the flip variant is more responsive to glutamatergic activation, and the different functional properties of these variants

have already been linked to changes in excitability directly in response to glutamate in the hippocampus (Sommer et al., 1990). The effects of alcohol on this circuitry may not be limited to induction of changes in receptor expression and trafficking. Ethanol itself increases membrane permeability and promotes passive depolarization of neurons, and its chemical interactions with exposed site or posttranslational modifications can alter or remove them (Babu et al., 1994).

Conclusions

Electrophysiological data reveal sex differences in glutamatergic circuitry within the amygdala that has direct implications for amygdala output to other brain regions. The data indicate that CeM responsivity to glutamatergic BLA input differs between males and females, but that this difference is erased by an exposure to chronic stress. This may have implications for why the human literature shows no strong correlation in chronicity for men with anxiety disorders and alcoholism, but an apparent vulnerability toward alcohol abuse in women following trauma. One potential explanation is that differences in amygdala circuitry could serve

as protective factors for women against development of alcoholism, and that those differences can be lost as a result of traumatic stress.

Protein analyses in these experiments did not implicate a source for these differences in connectivity, and future work could benefit from identifying subpopulations of BLA and CeM neurons, similar to research into subpopulations of CeL neurons responsible for inhibiting or disinhibiting CeM activity. These data illustrate specific chronic stress-dependent differences in amygdala circuitry and its sensitivity to alcohol related to chronic stress and indicate that sex differences in chronicity of anxiety and alcohol-related disorders may be mediated in part by differences in BLA-CeM connectivity.

REFERENCES

Amano, T., Amir, A., Goswami, S., & Paré, D. (2012). Morphology, PKCδ expression, and synaptic responsiveness of different types of rat central lateral amygdala neurons. Journal of Neurophysiology, 108(12), 3196–3205. https://doi.org/10.1152/jn.00514.2012

Babu, P. P., Kumari, L. R., & Vemuri, M. C. (1994). Ethanol induced alterations in plasma membrane protein phosphorylation of neurons and astrocytes. Molecular and Cellular Biochemistry, 130(1), 41–48. https://doi.org/10.1007/BF01084266

Bali, A., & Jaggi, A. S. (2015). Electric foot shock stress: A useful tool in neuropsychiatric studies. Reviews in the Neurosciences, 26(6), 655–677. https://doi.org/10.1515/revneuro-2015-0015

Burnashev, N., Monyer, H., Seeburg, P. H., & Sakmann, B. (1992). Divalent ion permeability of AMPA receptor channels is dominated by the edited form of a single subunit. Neuron, 8(1), 189–198. https://doi.org/10.1016/0896-6273(92)90120-3

Calhoon, G. G., & Tye, K. M. (2015). Resolving the neural circuits of anxiety. Nature Neuroscience, 18(10), 1394–1404. https://doi.org/10.1038/nn.4101

Ciocchi, S., Herry, C., Grenier, F., Wolff, S. B. E., Letzkus, J. J., Vlachos, I., … Lüthi, A. (2010). Encoding of conditioned fear in central amygdala inhibitory circuits. Nature, 468(7321), 277–282. https://doi.org/10.1038/nature09559

Davis, M. (1992). The Role of the Amygdala in Fear and Anxiety. Annual Review of Neuroscience, 15(1), 353–375. https://doi.org/10.1146/annurev.ne.15.030192.002033

Duvarci, S., & Pare, D. (2014). AMYGDALA MICROCIRCUITS CONTROLLING LEARNED FEAR. Neuron, 82(5), 966–980. https://doi.org/10.1016/j.neuron.2014.04.042

Gilpin, N. W., Herman, M. A., & Roberto, M. (2015). The Central Amygdala as an Integrative Hub for Anxiety and Alcohol Use Disorders. Biological Psychiatry, 77(10), 859–869. https://doi.org/10.1016/j.biopsych.2014.09.008

Grant, B. F., Goldstein, R. B., Saha, T. D., Chou, S. P., Jung, J., Zhang, H., … Hasin, D. S. (2015). Epidemiology of DSM-5 Alcohol Use Disorder: Results From the National Epidemiologic Survey on Alcohol and Related Conditions III. JAMA Psychiatry, 72(8), 757–766. https://doi.org/10.1001/jamapsychiatry.2015.0584

Greger, I. H., Khatri, L., Kong, X., & Ziff, E. B. (2003). AMPA Receptor Tetramerization Is Mediated by Q/R Editing. Neuron, 40(4), 763–774. https://doi.org/10.1016/S0896-6273(03)00668-8

Gutman, D. A., & Nemeroff, C. B. (2003). Persistent central nervous system effects of an adverse early environment: Clinical and preclinical studies. Physiology & Behavior, 79(3), 471–478. https://doi.org/10.1016/S0031-9384(03)00166-5

Hallett, P. J., Collins, T. L., Standaert, D. G., & Dunah, A. W. (2008). Biochemical fractionation of brain tissue for studies of receptor distribution and trafficking. Current Protocols in Neuroscience, Chapter 1, Unit 1.16. https://doi.org/10.1002/0471142301.ns0116s42

Hasin, D. S., Stinson, F. S., Ogburn, E., & Grant, B. F. (2007). Prevalence, Correlates, Disability, and Comorbidity of DSM-IV Alcohol Abuse and Dependence in the United States: Results From the National Epidemiologic Survey on Alcohol and Related Conditions. Archives of General Psychiatry, 64(7), 830. https://doi.org/10.1001/archpsyc.64.7.830

Jacobson, I. G., Ryan, M. A. K., Hooper, T. I., Smith, T. C., Amoroso, P. J., Boyko, E. J., … Bell, N. S. (2008). Alcohol use and alcohol-related problems before and after military combat deployment. JAMA, 300(6), 663–675. https://doi.org/10.1001/jama.300.6.663

Janak, P. H., & Tye, K. M. (2015). From circuits to behaviour in the amygdala. Nature, 517(7534), 284–292. https://doi.org/10.1038/nature14188

Karst, H., Berger, S., Erdmann, G., Schütz, G., & Joëls, M. (2010). Metaplasticity of amygdalar responses to the stress hormone corticosterone. Proceedings of the National Academy of Sciences of the United States of America, 107(32), 14449–14454. https://doi.org/10.1073/pnas.0914381107

Kessler, R. C. (1995). Posttraumatic Stress Disorder in the National Comorbidity Survey. Archives of General Psychiatry, 52(12), 1048. https://doi.org/10.1001/archpsyc.1995.03950240066012

Kessler, R. C., Chiu, W. T., Demler, O., & Walters, E. E. (2005). Prevalence, Severity, and Comorbidity of 12-Month DSM-IV Disorders in the National Comorbidity Survey Replication. Archives of General Psychiatry, 62(6), 617–627. https://doi.org/10.1001/archpsyc.62.6.617

KESSLER, R. C., PETUKHOVA, M., SAMPSON, N. A., ZASLAVSKY, A. M., & WITTCHEN, H.-U. (2012). Twelve-month and lifetime prevalence and lifetime morbid risk of anxiety and mood disorders in the United States. International Journal of Methods in Psychiatric Research, 21(3), 169–184. https://doi.org/10.1002/mpr.1359

Kim, J., Zhang, X., Muralidhar, S., LeBlanc, S. A., & Tonegawa, S. (2017). Basolateral to central amygdala neural circuits for appetitive behaviors. Neuron, 93(6), 1464-1479.e5. https://doi.org/10.1016/j.neuron.2017.02.034

Krugers, H. J., Hoogenraad, C. C., & Groc, L. (2010). Stress hormones and AMPA receptor trafficking in synaptic plasticity and memory. Nature Reviews Neuroscience, 11(10), 675–681. https://doi.org/10.1038/nrn2913

Läck, A. K., Diaz, M. R., Chappell, A., DuBois, D. W., & McCool, B. A. (2007). Chronic Ethanol and Withdrawal Differentially Modulate Pre- and Post-synaptic Function at Glutamatergic Synapses in Rat Basolateral Amygdala. Journal of Neurophysiology, 98(6), 3185–3196. https://doi.org/10.1152/jn.00189.2007

LeDoux, J. E., Iwata, J., Cicchetti, P., & Reis, D. J. (1988). Different projections of the central amygdaloid nucleus mediate autonomic and behavioral correlates of conditioned fear. Journal of Neuroscience, 8(7), 2517–2529. https://doi.org/10.1523/JNEUROSCI.08-07-02517.1988

Lee, S., Song, B., Kim, J., Park, K., Hong, I., An, B., … Choi, S. (2013). GluA1 phosphorylation at serine 831 in the lateral amygdala is required for fear renewal. Nature Neuroscience, 16(10), 1436–1444. https://doi.org/10.1038/nn.3491

Lehavot, K., Stappenbeck, C. A., Luterek, J. A., Kaysen, D., & Simpson, T. L. (2014). Gender differences in relationships among PTSD severity, drinking motives, and alcohol use in a comorbid alcohol dependence and PTSD sample. Psychology of Addictive Behaviors : Journal of the Society of Psychologists in Addictive Behaviors, 28(1), 42–52. https://doi.org/10.1037/a0032266

Logrip, M. L., Oleata, C., & Roberto, M. (2017). Sex differences in responses of the basolateral-central amygdala circuit to alcohol, corticosterone and their interaction. Neuropharmacology, 114, 123–134. https://doi.org/10.1016/j.neuropharm.2016.11.021

Logrip, M. L., & Zorrilla, E. P. (2012). Stress history increases alcohol intake in relapse: Relation to phosphodiesterase 10A: Stress history alters relapse. Addiction Biology, 17(5), 920–933. https://doi.org/10.1111/j.1369-1600.2012.00460.x

Man, H.-Y. (2011). GluA2-lacking, calcium-permeable AMPA receptors – inducers of plasticity? Current Opinion in Neurobiology, 21(2), 291–298. https://doi.org/10.1016/j.conb.2011.01.001

Mao, L.-M., Diaz, J. A., Fibuch, E. E., & Wang, J. Q. (2013). Regulation of phosphorylation of synaptic and extrasynaptic GluA1 AMPA receptors in the rat forebrain by amphetamine. European Journal of Pharmacology, 715(0), 164–171. https://doi.org/10.1016/j.ejphar.2013.05.027

McLean, C. P., Asnaani, A., Litz, B. T., & Hofmann, S. G. (2011). Gender differences in anxiety disorders: Prevalence, course of illness, comorbidity and burden of illness. Journal of Psychiatric Research, 45(8), 1027–1035. https://doi.org/10.1016/j.jpsychires.2011.03.006

Mons, N., & Beracochea, D. (2016). Behavioral Neuroadaptation to Alcohol: From Glucocorticoids to Histone Acetylation. Frontiers in Psychiatry, 7, 165. https://doi.org/10.3389/fpsyt.2016.00165

Murray, J., Ehlers, A., & Mayou, R. A. (2002). Dissociation and post-traumatic stress disorder: Two prospective studies of road traffic accident survivors. The British Journal of Psychiatry, 180(4), 363–368. https://doi.org/10.1192/bjp.180.4.363

Nakanishi, S. (1992). Molecular Diversity of Glutamate Receptors and Implications for Brain-Function. Science, 258(5082), 597–603. https://doi.org/10.1126/science.1329206

Ogilvie, K. M., & Rivier, C. (1997). Effect of alcohol on the proestrous surge of luteinizing hormone (LH) and the activation of LH-releasing hormone (LHRH) neurons in the female rat. The Journal of Neuroscience: The Official Journal of the Society for Neuroscience, 17(7), 2595–2604.

O'Neill, P.-K., Gore, F., & Salzman, C. D. (2018). Basolateral amygdala circuitry in positive and negative valence. Current Opinion in Neurobiology, 49, 175–183. https://doi.org/10.1016/j.conb.2018.02.012

Pachernegg, S., Münster, Y., Muth-Köhne, E., Fuhrmann, G., & Hollmann, M. (2015). GluA2 is rapidly edited at the Q/R site during neural differentiation in vitro. Frontiers in Cellular Neuroscience, 9. https://doi.org/10.3389/fncel.2015.00069

Pin, J., & Duvoisin, R. (1995). The Metabotropic Glutamate Receptors—Structure and Functions. Neuropharmacology, 34(1), 1–26. https://doi.org/10.1016/0028-3908(94)00129-G

Roberto, M., Bajo, M., Crawford, E., Madamba, S. G., & Siggins, G. R. (2006). Chronic Ethanol Exposure and Protracted Abstinence Alter NMDA Receptors in Central Amygdala. Neuropsychopharmacology, 31(5), 988–996. https://doi.org/10.1038/sj.npp.1300840

Roberto, M., Madamba, S. G., Stouffer, D. G., Parsons, L. H., & Siggins, G. R. (2004). Increased GABA release in the central amygdala of ethanol-dependent rats. The Journal of Neuroscience: The Official Journal of the Society for Neuroscience, 24(45), 10159–10166. https://doi.org/10.1523/JNEUROSCI.3004-04.2004

Roberto, M., Schweitzer, P., Madamba, S. G., Stouffer, D. G., Parsons, L. H., & Siggins, G. R. (2004). Acute and Chronic Ethanol Alter Glutamatergic Transmission in Rat Central Amygdala: An In Vitro and In Vivo Analysis. Journal of Neuroscience, 24(7), 1594–1603. https://doi.org/10.1523/JNEUROSCI.5077-03.2004

Sah, P., Faber, E. S. L., Armentia, M. L. D., & Power, J. (2003). The Amygdaloid Complex: Anatomy and Physiology. Physiological Reviews, 83(3), 803–834. https://doi.org/10.1152/physrev.00002.2003

Sanford, C. A., Soden, M. E., Baird, M. A., Miller, S. M., Schulkin, J., Palmiter, R. D., … Zweifel, L. S. (2017). A Central Amygdala CRF Circuit Facilitates Learning about Weak Threats. Neuron, 93(1), 164–178. https://doi.org/10.1016/j.neuron.2016.11.034

Schuckit, M. A. (2009). Alcohol-use disorders. The Lancet, 373(9662), 492–501. https://doi.org/10.1016/S0140-6736(09)60009-X

Shackman, A. J., Tromp, D. P. M., Stockbridge, M. D., Kaplan, C. M., Tillman, R. M., & Fox, A. S. (2016). Dispositional negativity: An integrative psychological and neurobiological perspective. Psychological Bulletin, 142(12), 1275–1314. https://doi.org/10.1037/bul0000073

Shalev, A. (1992). Posttraumatic Stress Disorder among Injured Survivors of a Terrorist Attack. The Journal of Nervous and Mental Disease, 180(8), 505–509.

Shalev, A. Y., Freedman, S., Peri, T., Brandes, D., & Sahar, T. (1997). Predicting PTSD in trauma survivors: Prospective evaluation of self-report and clinician-administered instruments. The British Journal of Psychiatry; London, 170, 558–564.

Sinha, R. (2012). How Does Stress Lead to Risk of Alcohol Relapse? Retrieved March 22, 2019, from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3788822/

Sommer, B., Keinanen, K., Verdoorn, T. A., Wisden, W., Burnashev, N., Herb, A., … Seeburg, P. H. (1990). Flip and flop: A cell-specific functional switch in glutamate-operated channels of the CNS. Science, 249(4976), 1580–1585. https://doi.org/10.1126/science.1699275

Sonne, S. C., Back, S. E., Diaz Zuniga, C., Randall, C. L., & Brady, K. T. (2003). Gender differences in individuals with comorbid alcohol dependence and post-traumatic stress disorder. The American Journal on Addictions, 12(5), 412–423.

Swanson, G. T., Kamboj, S. K., & Cull-Candy, S. G. (1997). Single-channel properties of recombinant AMPA receptors depend on RNA editing, splice variation, and subunit composition. The Journal of Neuroscience: The Official Journal of the Society for Neuroscience, 17(1), 58–69.

Traynelis, S. F., Wollmuth, L. P., McBain, C. J., Menniti, F. S., Vance, K. M., Ogden, K. K., … Dingledine, R. (2010). Glutamate Receptor Ion Channels: Structure, Regulation, and Function. Pharmacological Reviews, 62(3), 405–496. https://doi.org/10.1124/pr.109.002451

Tye, K. M., Prakash, R., Kim, S.-Y., Fenno, L. E., Grosenick, L., Zarabi, H., … Deisseroth, K. (2011). Amygdala circuitry mediating reversible and bidirectional control of anxiety. Nature, 471(7338), 358–362. https://doi.org/10.1038/nature09820

Vesga-López, O., Schneier, F., Wang, S., Heimberg, R., Liu, S.-M., Hasin, D. S., & Blanco, C. (2008). Gender differences in Generalized Anxiety Disorder: Results from the National Epidemiologic Survey on Alcohol and Related Conditions (NESARC). The Journal of Clinical Psychiatry, 69(10), 1606–1616.

Whitaker, A. M., Gilpin, N. W., & Edwards, S. (2015). Animal Models of Post-Traumatic Stress Disorder and Recent Neurobiological Insights. 22.

Yang, Y., Wang, X., Frerking, M., & Zhou, Q. (2008). Delivery of AMPA receptors to perisynaptic sites precedes the full expression of long-term potentiation. Proceedings of the National Academy of Sciences, 105(32), 11388–11393. https://doi.org/10.1073/pnas.0802978105