FACTORS IMPACTING NEURAL PATHWAYS OF EMOTIONAL PROCESSING

EDITED BY: Yuval Silberman, Nicholas Michael Graziane and Jessica R. Barson
PUBLISHED IN: Frontiers in Behavioral Neuroscience

frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-88971-676-0
DOI 10.3389/978-2-88971-676-0

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FACTORS IMPACTING NEURAL PATHWAYS OF EMOTIONAL PROCESSING

Topic Editors:
Yuval Silberman, The Pennsylvania State University, United States
Nicholas Michael Graziane, The Pennsylvania State University, United States
Jessica R. Barson, Drexel University, United States

Citation: Silberman, Y., Graziane, N. M., Barson, J. R., eds. (2021). Factors Impacting Neural Pathways of Emotional Processing. Lausanne: Frontiers Media SA.
doi: 10.3389/978-2-88971-676-0

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frontiers in Behavioral Neuroscience

EDITORIAL
published: 28 September 2021
doi: 10.3389/fnbeh.2021.755286

Editorial: Factors Impacting Neural Pathways of Emotional Processing

*Yuval Silberman**

Neural and Behavioral Sciences, College of Medicine, The Pennsylvania State University, Hershey, PA, United States

Keywords: editorial, emotional regulation, hippocampus, amygdala, prefrontal cortex, paraventricular thalamus, bed nucleus of the stria terminalis, diet

Editorial on the Research Topic

Factors Impacting Neural Pathways of Emotional Processing

Emotions help guide our everyday choices in complex life situations. We are more likely to continue to perform certain tasks or to prefer certain places/situations if they induce a positive emotional valence, while avoiding those that induce negative valence. This type of emotional processing can also greatly affect memories, with emotionally salient events more likely to be remembered long-term. Such emotional processing is dependent on a widely distributed set of brain regions and neurocircuits that respond to both intrinsic and extrinsic factors. Dysregulation of these emotional processing pathways can lead to a wide variety of serious mental health disorders. Therefore, determining how intrinsic and extrinsic factors can alter emotional processing is likely to provide key understanding of the development of many mental health issues.

The goal of this article collection is to provide an update on how diverse factors, either intrinsic or extrinsic in nature, can alter neural pathways involved in emotional processing. Articles within this collection describe a number of factors that may modulate emotional regulation and result in behavioral changes. Two articles focused on the role of diets in emotional behaviors. Coker et al. reviewed the current literature examining how diets high in fat can alter key neuronal circuitries and neurotransmitter systems involved in emotional processing, how diets can alter insulin and glucose regulation, and how these seemingly disparate systems may interact in the context of alcohol use. In particular, this review focuses on the novel concept that high fat diets may impact neurocircuitry that is often associated with alcohol use disorder. Since high fat diet and alcohol intake often result in activation of overlapping circuits, this article highlights two important views: (1) binge consumption of high fat diets can sensitize increased alcohol intake and thus diet may be a risk factor for development of alcohol use disorders; and (2) high fat diets can be used to mitigate or offset neurocircuit dysregulation during alcohol withdrawal and serve to reduce alcohol intake overall. In addition to neurocircuit changes, diet and alcohol can also alter gut microbiome, which has also been shown to be involved in regulation of emotional processing. In accordance with the hypothesis that the microbiome plays an important role in altering behavioral indices of emotion regulation, Dauge et al. provided novel experimental data showing that treatment with a probiotic formula can decrease anxiety-like and depressive-like behaviors in rodent models of maternal stress and genetic stress sensitivity. Other extrinsic factors described in this collection [i.e., traumatic brain injury (McCorkle et al.), traumatic stress (Piggott et al.), and chronic drug/alcohol exposure (Dao et al.; McKendrick and Graziane)] can alter activity in brain regions known to be involved in emotional processing.

Emotional processing is thought to involve distributed networks of brain regions. In this article collection, Javanbakht et al. utilized a clinical laboratory model of fear conditioning and instructed extinction learning. Their findings indicate that subregions of the prefrontal cortex and parahippocampus gyrus show increased activation during extinction learning. Using the left

Edited and reviewed by:
India Morrison, Linköping University, Sweden

***Correspondence:**
Yuval Silberman
yus72@psu.edu

Specialty section:
This article was submitted to Emotion Regulation and Processing, a section of the journal Frontiers in Behavioral Neuroscience

Received: *08 August 2021*
Accepted: *03 September 2021*
Published: *28 September 2021*

Citation:
Silberman Y (2021) Editorial: Factors Impacting Neural Pathways of Emotional Processing. Front. Behav. Neurosci. 15:755286. doi: 10.3389/fnbeh.2021.755286

ventromedial prefrontal cortex as a seed region, the authors found increased prefrontal cortex activity during extinction learning was associated with significant co-activation of the dorsal medial prefrontal cortex, parahippocampus, insula, and amygdala. Other articles in this collection utilizing preclinical models or analysis of the literature confirm the importance of the prefrontal cortex (Brockway and Crowley) and the amygdala (McCorkle et al.), and further indicate that the bed nucleus of the stria terminalis (Giardino and Pomrenze) and paraventricular nucleus of the thalamus (Barson et al.) are also critically important for regulation of emotional memories and behaviors. Within these brain regions, a number of signaling systems are proposed to be critical for emotional regulation. This is especially important in brain regions which have highly heterogenous cell-types with potential overlapping neurotransmitter and neuropeptide content, like the bed nucleus of the stria terminalis, or in brain regions like the paraventricular nucleus of the thalamus that has multiple cell types across anatomical subregions. Determining how these cell types coordinate emotional memory under typical and dysregulated conditions will be critical as the field continues to develop.

Studies to examine neuronal subtype function on behavior are underway in the prefrontal cortex, as highlighted by two studies within this collection. Both clinical and preclinical evidence suggests that a number of neuropeptides, including neuropeptide Y, corticotropin releasing factor, somatostatin, and endogenous opioids, are critical for emotional memory (Brockway and Crowley). Many of these peptides are co-released from GABAergic neurons in the prefrontal cortex and appear to be responsible for regulating the activity of both local circuits within the prefrontal cortex and the activity of glutamatergic projection neurons. Dao et al. examined how somatostatin neurons in the prefrontal cortex, basolateral and central amygdala, and bed nucleus of the stria terminalis were altered following forced abstinence from alcohol in preclinical models. This work showed that alcohol abstinence increased somatostatin neuron activity in the prefrontal cortex which may dampen overall prefrontal cortex local network activity while somatostatin neuron activity in the bed nucleus of the stria terminalis was also altered. These findings appeared to be more pronounced in female mice compared to male mice, suggesting distinct sex differences within this model. Sex differences were also shown in a study in this collection by Anderson et al. examining a subset of GABAergic neurons in the prefrontal cortex containing parvalbumin. The authors found that genetic deletion of g-protein coupled inwardly rectifying potassium (GIRK) channels from parvalbumin neurons throughout the brain resulted in increased time in the open arms of the elevated plus maze in both males and females, but an increase in immobility episodes in the forced swim test only in males. While the authors found sex differences in reversal learning models, modulation of GIRK expression in parvalbumin neurons did not seem to alter these behaviors. Modulation of GIRK expression resulted in larger afterhyperpolarization of prefrontal cortex parvalbumin containing GABAergic neurons in both sexes but this resulted in increased action potential firing only in male mice. These findings further highlight the importance of examining sex differences as the field continues to develop.

In summary, the articles of this collection highlight the complexities of understanding emotional processing. Such complexities are due to numerous factors, such as distributed neural networks, various levels of neuronal processing, and multiple neurotransmitter and neuropeptide systems. Studies in this collection describe how these factors are altered by intrinsic and extrinsic influences, such as stress, diet, or drug exposure, that can all lead to altered signaling within the distributed network of brain regions involved in emotional regulation. While future studies will be needed to further delineate such mechanisms, the articles in this collection provide novel avenues for these critically important studies to explore.

AUTHOR CONTRIBUTIONS

YS wrote and edited the manuscript.

FUNDING

This work was supported by NIH grants AA026865 and AA022937.

ACKNOWLEDGMENTS

The author specially thanks the co-editors Jessica Barson and Nicholas Graziane for their hard work during the article screening and peer-review process. The author thanks all the contributors and peer-reviewers for making this Special Topics collection extra special.

Conflict of Interest: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Behavioral Neuroscience

ORIGINAL RESEARCH
published: 27 May 2020
doi: 10.3389/fnbeh.2020.00086

Forced Abstinence From Alcohol Induces Sex-Specific Depression-Like Behavioral and Neural Adaptations in Somatostatin Neurons in Cortical and Amygdalar Regions

*Nigel C. Dao[1], Malini Suresh Nair[1,2], Sarah N. Magee[2†], J. Brody Moyer[2], Veronica Sendao[2], Dakota F. Brockway[1,3] and Nicole A. Crowley[1,2,3]**

[1] Department of Biobehavioral Health, Pennsylvania State University, University Park, PA, United States, [2] Department of Biology, Pennsylvania State University, University Park, PA, United States, [3] Neuroscience Curriculum, Pennsylvania State University, University Park, PA, United States

Forced abstinence (FA) from alcohol has been shown to produce a variety of anxiety- and depression-like symptoms in animal models. Somatostatin (SST) neurons, a subtype of GABAergic neurons found throughout the brain, are a novel neural target with potential treatment implications in affective disorders, yet their role in alcohol use disorders (AUD) remains to be explored. Here, we examined the neuroadaptations of SST neurons during forced abstinence from voluntary alcohol consumption. Following 6 weeks of two-bottle choice alcohol consumption and protracted forced abstinence, male and female C57BL/6J mice exhibited a heightened, but sex-specific, depressive-like behavioral profile in the sucrose preference test (SPT) and forced swim test (FST), without changes in anxiety-like behaviors in the elevated plus maze (EPM) and open field test (OFT). FST-induced cFos expressions in the prefrontal cortex (PFC) and ventral bed nucleus of the stria terminalis (vBNST) were altered in FA-exposed female mice only, suggesting a sex-specific effect of forced abstinence on the neural response to acute stress. SST immunoreactivity in these regions was unaffected by forced abstinence, while differences were seen in SST/cFos co-expression in the vBNST. No differences in cFos or SST immunoreactivity were seen in the lateral central nucleus of the amygdala (CEA) and the basolateral amygdala (BLA). Additionally, SST neurons in female mice displayed opposing alterations in the PFC and vBNST, with heightened intrinsic excitability in the PFC and diminished intrinsic excitability in the vBNST. These findings provide an overall framework of forced abstinence-induced neuroadaptations in these key brain regions involved in emotional regulation and processing.

Keywords: forced abstinence, alcohol, sex, stress, somatostatin, prefrontal cortex, extended amygdala

OPEN ACCESS

Edited by:
Jessica R. Barson, Drexel University College of Medicine, United States

Reviewed by:
Sachin Patel, Vanderbilt University Medical Center, United States
Charlis Raineki, The University of British Columbia, Canada
Katherine Mercedes Holleran, Wake Forest Baptist Medical Center, United States

****Correspondence:***
Nicole A. Crowley
nzc27@psu.edu

†Present address:
Sarah N. Magee, Department of Pharmacology, The University of North Carolina at Chapel Hill, Chapel Hill, NC, United States

Specialty section:
This article was submitted to Emotion Regulation and Processing, a section of the journal Frontiers in Behavioral Neuroscience

Received: *24 March 2020*
Accepted: *07 May 2020*
Published: *27 May 2020*

Citation:
Dao NC, Suresh Nair M, Magee SN, Moyer JB, Sendao V, Brockway DF and Crowley NA (2020) Forced Abstinence From Alcohol Induces Sex-Specific Depression-Like Behavioral and Neural Adaptations in Somatostatin Neurons in Cortical and Amygdalar Regions. Front. Behav. Neurosci. 14:86. doi: 10.3389/fnbeh.2020.00086

INTRODUCTION

Alcohol use disorder (AUD) represents one of the most prevalent and costly neuropsychiatric disorders globally and domestically, costing the United States economy an estimated $249 billion due to losses in workplace productivity, and health care and criminal justice expenses (Sacks et al., 2015). Withdrawal from alcohol (both acute and protracted withdrawal) produces a host of negative emotional conditions, such as depression and anxiety (Hershon, 1977; Becker, 2014; Smith et al., 2019). These emotional states can increase the risk for relapse and further hamper an individual's ability to abstain from alcohol. Importantly, the literature suggests that risk of relapse may be different across the sexes, with women more likely to relapse following negative affect-related situations, and men more likely to relapse when in the presence of other alcohol consumers (White et al., 2015; Peltier et al., 2019). Complementary research suggests that alleviating depressive symptoms following abstinence from alcohol may improve treatment outcomes for women (Annis et al., 1998; Holzhauer and Gamble, 2017). Taken together, these data point to a complex relationship between AUD, depression, and treatment outcome, likely moderated by sex.

Previous research has shown that forced abstinence (FA) from alcohol produces depressive-like behavior and a variety of neurobiological changes in rodent models. For instance, Vranjkovic et al. (2018) demonstrated that 24-h immediately following 6 weeks of alcohol drinking, female C57BL/6J mice showed decreased time spent in the open arm of the elevated plus maze (EPM), and increased latency to feed in the novelty suppression of feeding test (NSFT), two commonly used models of anxiety-like behavior. In addition, this model produced increased immobility in the forced swim test (FST) during protracted withdrawal (21 days of abstinence), though it should be noted males were not investigated in these studies (Holleran et al., 2016; Vranjkovic et al., 2018). Similarly, Valdez and Harshberger (2012) demonstrated that male Wistar rats exposed to chronic alcohol show increased immobility in the FST, and that this effect is further enhanced during protracted withdrawal.

Alcohol use disorder and major depressive disorder (MDD) are highly comorbid disorders with overlapping etiology. Novel fast acting antidepressants are able to reduce both binge drinking (Crowley et al., 2019a) and depressive-like behavior following alcohol exposure (Holleran et al., 2016; Vranjkovic et al., 2018) further highlighting the potential overlapping neural circuitry involved in AUD and MDD. Importantly, both the clinical and preclinical depression literature has continuously pointed to a novel subpopulation of gamma aminobutyric acidergic (GABAergic) neurons as a protective, resiliency-conferring population. Somatostatin (SST) neurons are a group of GABAergic neurons that express and release the neuropeptide SST in the cortex, hippocampus, thalamus and amygdala (Fuchs et al., 2017; Abbas et al., 2018; Ahrens et al., 2018). Recent reports have implicated these neurons in a host of neuropsychiatric disorders in addition to MDD, such as bipolar disorder, anxiety disorder, and schizophrenia (Fee et al., 2017). Global genetic upregulation of SST neuronal function reduces depression- and anxiety- like behaviors in male and female mice (Fuchs et al., 2017), and deficits in SST expression are seen in the amygdala of postmortem samples of MDD patients (Douillard-Guilloux et al., 2017). Ketamine reverses the effects of chronic stress on GABAergic transmission in PFC pyramidal neurons by restoring the excitation/inhibition balance in SST neurons (Ghosal et al., 2020). Despite the clear correlation between SST neuronal markers and their function in MDD, both in the human and preclinical animal literature, thus far SST neurons have been poorly investigated in the context of AUD and depression-like phenotypes seen during withdrawal from alcohol.

The current study had two aims: first, to replicate previous behavioral models of forced abstinence from alcohol with both male and female mice. The second aim was to bridge the resiliency-like effect of SST neuronal function seen in the animal depression literature with the alcohol literature, in order to establish whether SST neurons within the prefrontal cortex (PFC), bed nucleus of the stria terminalis (BNST), basolateral amygdala (BLA) and lateral central amygdala (CEA), brain regions known for their role in MDD (Fee et al., 2017) and chronic alcohol exposure (Pleil et al., 2015a) are altered following forced abstinence from alcohol. These findings may shed new insights into the overlapping neural etiology of these highly comorbid disorders, particularly the SST neurons, and provide new directions to the discovery of more effective treatments.

MATERIALS AND METHODS

Animals

Male and female C57BL/6J (stock #000664) were purchased from the Jackson Laboratory. Hemizygous female SST-IRES-Cre::Ai9 were generated from homozygous SST-IRES-Cre (stock #013044, Jackson Laboratory) and homozygous Ai9 (stock #007909, Jackson Laboratory) parents. All mice were at least 8-weeks old at the beginning of alcohol drinking. Mice were individually housed with *ad lib* access to food and water, and were maintained on a 12 h reverse light/dark cycle (lights off at 7am) in temperature- and humidity-controlled vivarium for at least 1 week prior to alcohol exposure. Mouse weights were monitored weekly. All procedures were approved by the Pennsylvania State University's Institutional Animal Care and Use Committee.

Two-Bottle Choice (2BC) Alcohol Drinking

A schematic of the experimental timeline is displayed in **Figure 1A**. After 1 week of acclimatization to single housing, mice were randomly assigned to either drinking or non-drinking groups. Alcohol-drinking mice received continuous access to one sipper bottle of tap water and another of unsweetened, diluted alcohol. Alcohol concentrations increased from 3% in day 1 to 3, to 7% in day 3 to 9, to 10% in day 9 to 42. Bottles were weighed and refilled every 48-h. The positions of the bottles were randomized across mice, and within individual mice were switched weekly to avoid position bias. Following alcohol drinking, mice underwent forced abstinence where they had access to water only. Non-drinking control mice received

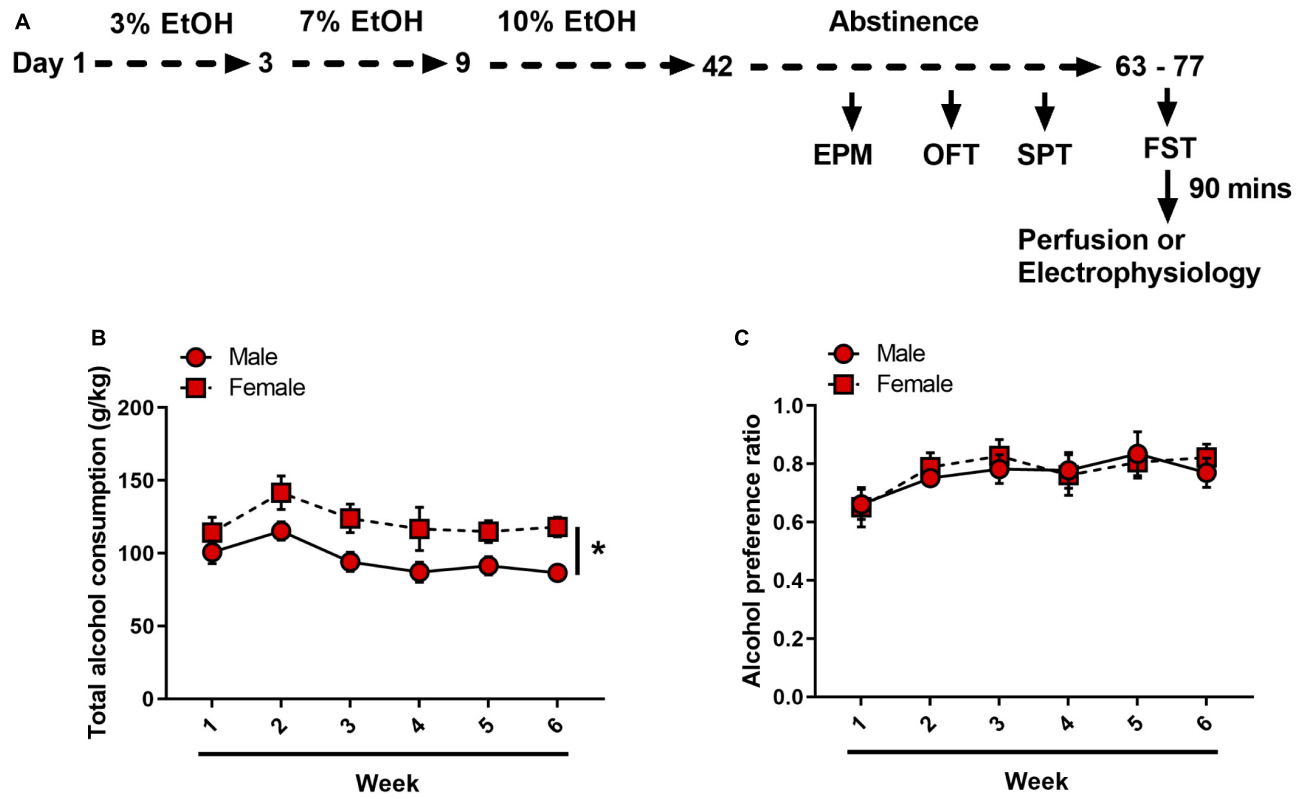

FIGURE 1 | Forced abstinence from alcohol in alcohol ramp two-bottle choice paradigm. **(A)** Experimental timeline. Following 6 weeks of an alcohol ramp procedure, mice underwent a behavior battery (each test 1 week apart). Mice that were used in electrophysiology experiments did not undergo EPM, OPF or SPT. **(B)** Female mice consistently drank more than male mice. **(C)** Both sexes equally preferred alcohol over water. $^{*}p < 0.05$.

continuous access to two water bottles only throughout the experiment. Escalation of alcohol drinking and forced abstinence were modeled off of Holleran et al. (2016).

Behavioral Testing

Mice were tested for anxiety- and depression- like behaviors during abstinence, starting with elevated plus maze (EPM), open field test (OFT), sucrose preference test (SPT) and lastly forced swim test (FST). Prior to behavior, mice were brought to the testing room and allowed to rest for at least 30 min. All tests were done 3-h into the dark cycle, under red light (6 lux). Female SST-Ai9 mice that were used in electrophysiology experiments did not undergo EPM, OFT, and SPT, and were tested in the FST under normal light.

Elevated Plus Maze

One week after the onset of abstinence, mice underwent EPM where they were placed in the center square of the maze (35 × 5 × 40 cm), facing a closed arm (20 cm arm wall height, transparent Plexiglass and gray floor). Mice were allowed to explore the maze for 5 min. Sessions were recorded with EthoVision XT video tracking system (Noldus, Leesburg, VA, United States). Total time spent in open arms and number of entries to open arms were automatically analyzed by EthoVision XT.

Open Field Test

Mice were placed in a corner of a black Plexiglass arena (50 × 50 × 20 cm) and allowed to explore for 20 min. Sessions were recorded with EthoVision XT, and total time spent in center zone and number of entries to center zones were automatically analyzed by EthoVison XT. Center zone was defined as a 12.5 × 12.5 cm area at the center of the arena.

Sucrose Preference Test

Mice received access to one bottle of tap water and another of 1% (w/v) sucrose solution for 12-h, starting 3-h into the dark cycle. Bottles were weighed before and after testing. Sucrose preference ratio was calculated as the volume of sucrose solution consumed divided by total volume of fluid consumed.

Forced Swim Test

Mice were placed in a transparent 5-liter glass beaker filled with approximately 3-liters of water (20 ± 1°C) for 6 min. Total time spent immobile during the last 4 min was recorded and manually scored by a researcher blind to experimental conditions.

Fluorescence Immunohistochemistry

Ninety minutes after FST, mice were deeply anesthetized with Avertin (250 mg/kg) and perfused transcardially with

ice-cold phosphate buffered saline (PBS, pH 7.4) and 4% paraformaldehyde (PFA, pH 7.4). Brains were removed, post-fixed in PFA overnight and placed in 30% (w/v) sucrose solution until sunk. 50-μm free floating sections were sliced with a Compresstome vibrating microtome VF-300-0Z (Precisionary Instruments LLC, Greenville, NC, United States) in a 1:3 series and stored in 30% sucrose/30% ethylene glycol cryoprotectant at −20°C until processed.

Prior to immunostaining, slices underwent antigen retrieval in 10 mM sodium citrate buffer (pH 6.0) at 80°C for 30 min. Slices were washed three times in PBS for 10 min each, and permeabilized in 0.5% Triton X-100 in PBS for 60 min. Nonspecific binding was blocked with 5% normal goat serum (NGS) in 0.1% Triton X-100 in PBS for 60 min. Slices were then incubated in a primary antibody cocktail, including rabbit anti-cFos (1:4000, Cell Signaling Technology, Danvers, MA, United States) and rat anti-somatostatin (1:500, Millipore, Burlington, MA, United States) in 2.5% NGS in 0.1% Triton X-100 in PBS for 48-h at 4°C. Slices were rinsed three times with PBS for 10 min each, and incubated in a fluorophore-tagged secondary antibody cocktail, including goat anti-rabbit Alexa Fluor 488 (1:500, Cell Signaling Technology, Danvers, MA, United States) and goat anti-rat Cy3 (1:500, Millipore, Burlington, MA, United States) for 4-h at room temperature. Slices were rinsed again three times with PBS, with the last step including DAPI (1:10,000), mounted on glass slides, air-dried and coverslipped with Immunomount (Thermo Fisher Scientific, Waltham, MA, United States). Images were obtained with an Olympus BX63 upright microscope (Center Valley, PA, United States) under matched exposure settings. Four to eight images from both hemispheres were taken per region.

Total cFos counts, SST immunoreactivity (IR), and cFos+/SST+ counts were quantified by researchers blind to experimental conditions using ImageJ (National Institutes of Health, Bethesda, MD, United States). For total cFos counts, region of interest (ROI) was delineated, and cFos+ nuclei were automatically quantified under matched criteria for size, circularity and intensity. Each ROI's total Fos count was divided by the ROI's area to give a cFos total density value (Smith et al., 2019). cFos+/SST+ nuclei count was manually quantified, and divided by the ROI's area to give a cFos+/SST+ density value. SST IR was quantified as mean fluorescence intensity of the ROI (Pleil et al., 2015b). At least three sections per region (PFC, dBNST, vBNST, lateral CeA, BLA) were quantified and averaged to obtain one value per mouse.

Electrophysiology

Whole-cell current clamp recordings were conducted similarly to those previously published (Crowley et al., 2016, 2019b). Regions for electrophysiology were determined by cFos and SST results. Based on these results and behavioral results, only female mice were explored. The regions of interest (PFC and vBNST) were identified according to the Allen Mouse Brain Atlas. Following alcohol exposure and abstinence, mice underwent FST. Ninety minutes following FST, female SST-IRES-Cre::Ai9 mice were deeply anesthetized via inhaled isoflurane and rapidly decapitated. Brains were rapidly removed and processed according to the NMDG protective recovery method (Ting et al., 2018). Briefly, brains were immediately placed in ice-cold, oxygenated *N*-methyl-D-glucamine (NMDG)-HEPES aCSF containing the following, in mM: 92 NMDG, 2.5 KCl, 1.25 NaH2PO4, 30 NaHCO3, 20 HEPES, 25 glucose, 2 thiourea, 5 Na-ascorbate, 3 Na-pyruvate, 0.5 CaCl2·2H2O, and 10 MgSO4·7H2O (pH to 7.3–7.4). 300 μM coronal slices containing the PFC and the vBNST were prepared on a Compresstome vibrating microtome VF-300-0Z (Precisionary Instruments, Greenville, NC, United States), and transferred to heated (31°C) NMDG-HEPES aCSF for a maximum of 10 min. Slices were then transferred to heated (31°C), oxygenated normal aCSF (in mM: 124 NaCl, 4.4 KCl, 2 CaCl2, 1.2 MgSO4, 1 NaH2PO4, 10.0 glucose, and 26.0 NaHCO3, pH 7.4, mOsm 300–310), where they were allowed to rest for at least 1-h before use. Finally, slices were moved to a submerged recording chamber (Warner Instruments, Hamden, CT, United States) where they were continuously perfused with heated recording aCSF at a rate of 2 ml/min. Recording electrodes (3–6 MΩ) were pulled from thin-walled borosilicate glass capillaries with a Narishige P-100 Puller (Amityville, NY, United States).

SST-expressing neurons were identified in SST-IRES-Cre::Ai9 mice via presence of tdTomato fluorescence under a 40× immersed objective with 565 nm LED excitation. Measurements of intrinsic excitability included resting membrane potential (RMP), rheobase (the minimum amount of current needed to elicit an action potential during a current ramp protocol), action potential threshold (the membrane potential at which the first action potential fired), and the number of action potentials fired during a voltage-current plot protocol (V-I plot) with increasing steps of depolarizing currents (0–200 pA, 10 pA per step). Hyperpolarizing currents (not shown) were included as a control. Experiments were performed at both RMP and at the standard holding potential of −70 mV. Electrodes were filled with a potassium gluconate-based (KGluc) intracellular recording solution (in mM: 135 K-Gluc, 5 NaCl, 2 MgCl2, 10 HEPES, 0.6 EGTA, 4 Na2ATP, and 0.4 Na2GTP, 287–290 mOsm, pH 7.35).

Signals were digitized at 10 kHz and filtered at 3 kHz using a Multiclamp 700B amplifier, and analyzed using Clampfit 10.7 software (Molecular Devices, Sunnyvale, CA, United States). For all measures, recordings were performed in a maximum of two neurons per subregion, per mouse, and *n* values reported reflect the total number of neurons.

Exclusion Criteria

One male mouse did not consume alcohol (average of 14.75 g/kg per week vs. group average of 108.12 g/kg per week). Since this was the first behavioral assay, this mouse was excluded from all analyses. For immunofluorescence quantification, problematic images (e.g. tears, out of focus, debris) were excluded prior to de-blinding. For electrophysiology recordings, cells that did not fire during the current ramp or V-I plot protocol were excluded. Statistical outliers, identified by Grubb's test, were excluded from the data set.

Statistical Analysis

Data was analyzed in Graphpad Prism 7.0 (San Diego, CA, United States). All datasets were checked for normality (D'Agostino-Pearson's test) and homogeneity (Barlett's test). If found violated, nonparametric Kruskall–Wallis test was performed and Dunn's multiple comparison was used as a posthoc test. Ordinary two-way ANOVA was used for all behavior and fluorescence immunohistochemistry data, followed by Tukey's multiple comparison posthoc test. One sample *t*-test was used to examine alcohol preference over water (theoretical mean 0.5). Student's *t*-test was used to analyze RMP, rheobase and action potential thresholds, while mixed-model two-way ANOVA was used to analyze SST V-I protocol. Statistical significance threshold was set at $\alpha = 0.05$. Data presented show means and standard error of the mean (SEM).

RESULTS

Male and female C57BL/6J mice underwent either an alcohol ramp two-bottle choice procedure and forced abstinence ($N = 11$ males and 10 females) or control condition ($N = 12$ males and 10 females) (**Figure 1A**). On average, over the 6 weeks of alcohol exposure, male mice consumed significantly less alcohol per body weight as compared to female mice [$F_{time}(5, 95) = 6.314$, $p < 0.001$, $F_{sex}(1, 19) = 7.060$, $p = 0.015$, $F_{sex \times time}(5, 95) = 0.685$, $p = 0.635$] (**Figure 1B**). Alcohol preference over water did not differ between male and female mice [$F_{time}(5, 95) = 5.839$, $p < 0.001$, $F_{sex}(1, 19) = 0.041$, $p = 0.84$, $F_{sex \times time}(5, 95) = 0.535$, $p = 0.749$], and both sexes displayed a strong preference for alcohol over water [male: one-sample $t(10) = 5.391$, $p < 0.001$, female: one-sample $t(9) = 7.102$, $p < 0.001$] (**Figure 1C**).

Depressive-Like Behavioral Phenotypes Following Forced Abstinence From Alcohol

Following the first week of abstinence, mice underwent multiple assays (one per week) to assess anxiety- and depression- like behavioral states. In the EPM, open arm duration was comparable between sexes and alcohol conditions [$F_{sex}(1, 39) = 0.379$, $p = 0.531$, $F_{FA}(1, 39) = 0.991$, $p = 0.325$, $F_{sex \times FA}(1, 39) = 0.4$, $p = 0.530$; **Figure 2A**]. Females had higher frequency of entries into the open arms than males, [$F_{sex}(1, 39) = 5.275$, $p = 0.027$] with no effects of FA conditions [$F_{FA}(1, 39) = 0.485$, $p = 0.489$, $F_{sex \times FA}(1, 39) = 0.044$, $p = 0.833$; **Figure 2B**]. In the OFT, no differences were seen in either open field center duration [$F_{sex}(1, 39) = 0.223$, $p = 0.639$, $F_{FA}(1, 39) = 0.878$, $p = 0.354$, $F_{sex \times FA}(1, 39) = 0.066$, $p = 0.797$; **Figure 2C**] or total distance traveled [$F_{sex}(1, 39) = 2.883$, $p = 0.097$, $F_{FA}(1, 39) = 2.502$, $p = 0.121$, $F_{sex \times FA}(1, 39) = 1.575$, $p = 0.217$; **Figure 2D**].

When probing depression-like behavior using the SPT and FST, key differences in both FA exposure and sex emerged. There was a significant main effect of FA history on sucrose preference [$F_{FA}(1, 37) = 9.388$, $p = 0.004$], but no effect of sex [$F_{sex}(1, 37) = 2.983$, $p = 0.092$] and no sex by FA interaction [$F_{sex \times FA}(1, 37) = 0.049$, $p = 0.825$] (**Figure 2E**). Both male and female mice that underwent forced abstinence from FA showed lower sucrose preference, classically interpreted as an anhedonia phenotype. Interestingly, sex differences emerged in the FST. Though male mice exposed to FA showed a significant increase in immobility time, female mice did not (Kruskall–Wallis $\chi = 7.783$, $p = 0.05$, male control vs. male EtOH: $p = 0.02$, female control vs. female EtOH: $p > 0.99$, Dunn's posthoc test; **Figure 2F**). In sum, these data suggest that following forced abstinence from alcohol, male and female mice display a depressive-like behavioral profile without changes in anxiety-like behaviors.

Forced Swim Stress-Induced Neuronal Activation in the PFC Is Modulated by Sex and Forced Abstinence From Alcohol

Next, we probe the neural substrates that may underlie the changes in depressive-like behaviors in cortical and amygdalar areas, including the PFC, BNST, BLA and lateral CeA. In the PFC (**Figure 3A** for representative images), FST-induced neuronal activation, identified by expression of the immediately early gene marker cFos, showed significant main effect of sex [$F_{sex}(1, 33) = 22.44$, $p < 0.001$] and significant interaction between sex and FA conditions [$F_{sex \times FA}(1, 33) = 5.910$, $p = 0.020$], without a main effect of FA [$F_{FA}(1, 33) = 1.198$, $p = 0.281$; **Figure 3B**]. Tukey's posthoc test revealed that FA-exposed female mice showed significantly lower number of cFos nuclei in the PFC than control female mice ($p < 0.05$), control male mice ($p < 0.001$) and FA-exposed male mice ($p < 0.001$). Control female mice also had less cFos nuclei than FA-exposed male mice ($p < 0.05$). SST neuron-specific cFos expression (cFos+/SST+ nuclei) was not altered by sex and FA conditions [$F_{sex}(1, 36) = 2.166$, $p = 0.149$, $F_{FA}(1, 36) = 2.140$, $p = 0.152$, $F_{sex \times FA}(1, 36) = 0.202$, $p = 0.656$; **Figure 3C**]. There were also no changes in SST immunoreactivity [$F_{sex}(1, 35) = 1.889$, $p = 0.178$, $F_{FA}(1, 35) = 0.082$, $p = 0.775$, $F_{sex \times FA}(1, 35) = 0.026$, $p = 0.872$; **Table 1**].

Given that only female mice displayed a change in FST-induced cFos expression in the PFC following forced abstinence from alcohol, we next performed whole-cell patch clamp electrophysiology in SST neurons in the PFC of female SST-Ai9 reporter (**Figure 3D** for representative recording traces). Current clamp experiments revealed that SST neurons had similar RMP [$t(16) = 0.955$, $p = 0.353$, **Figure 3E**]. When cells were held at −70 mV, action potential threshold [$t(16) = 0.223$, $p = 0.825$, **Figure 3F**] and rheobase [$t(16) = 1.096$, $p = 0.290$, **Figure 3G**] were unaltered between FA-exposed mice ($n = 10$ cells, $N = 4$ mice) and control mice ($n = 7$ cells, $N = 3$ mice). V-I plot at the holding potential of −70 mV indicated a significant main effect of current amplitude [$F_{current}(20, 300) = 15.257$, $p < 0.001$[and a significant FA by current amplitude interaction [$F_{FA \times current}(20, 300) = 2.970$, $p < 0.001$), with no main effect of FA [$F_{FA}(1, 15) = 1.539$, $p = 0.234$; **Figure 3H**]. SST neurons in the PFC of FA-exposed female mice fired significantly more action potentials in response to increasing steps of depolarizing currents than those in control female mice.

Recordings at RMP did not reveal any significant difference in rheobase [$t(16) = 0.225$, $p = 0.824$], action potential threshold [$t(16) = 0.267$, $p = 0.792$] and V-I plot [$F_{current}(20, 300) = 42.523$,

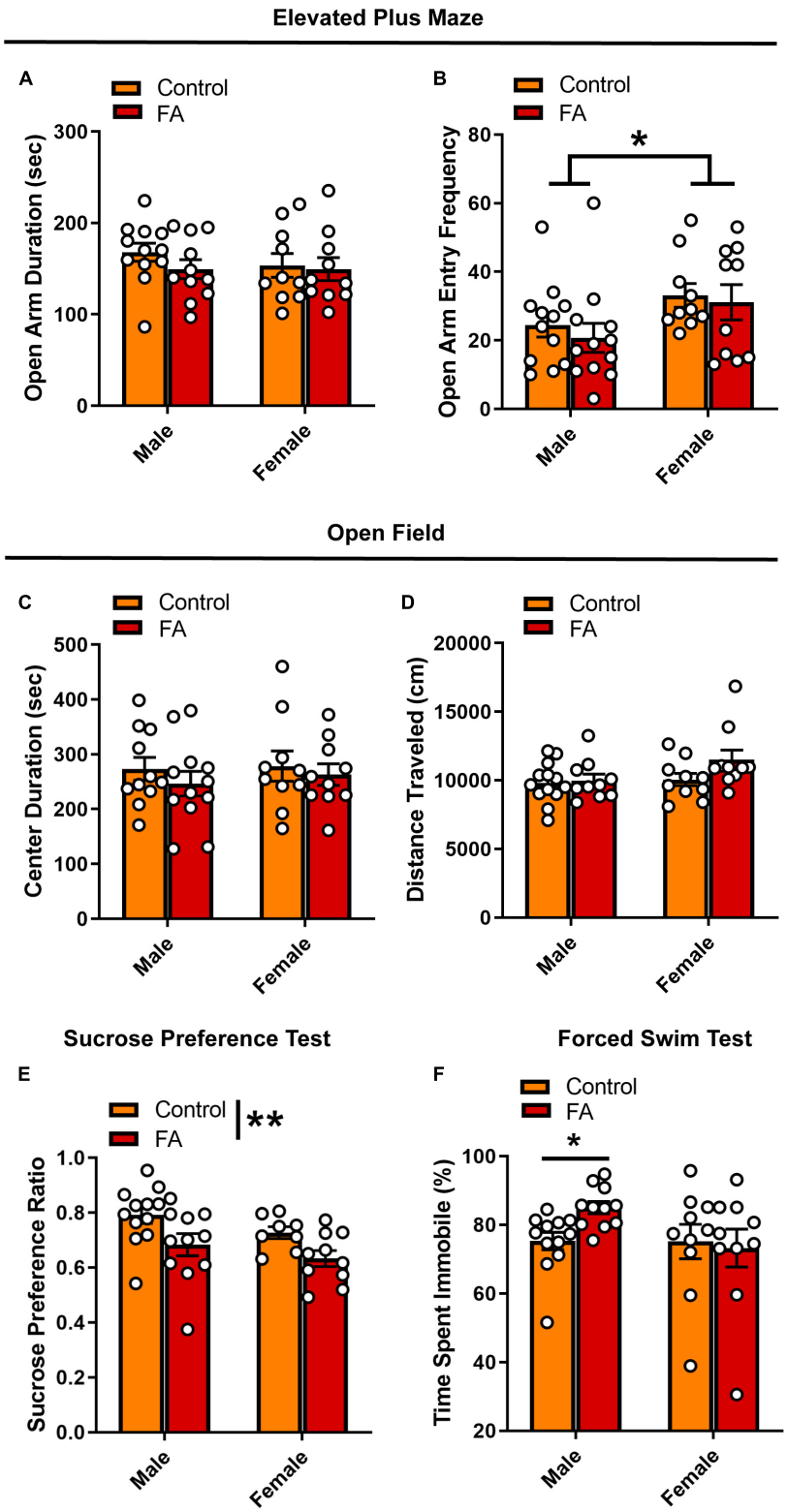

FIGURE 2 | Forced abstinence from alcohol induces a depressive-like behavioral phenotype. **(A)** No significant difference in time spent in the open arm of the EPM was observed across sexes and FA conditions. **(B)** Female mice made more entries to the open arms than male mice, and this effect was not modulated by alcohol conditions. **(C)** No significant difference in time spent in the center zone of the OPT and **(D)** total distance traveled was observed across sexes and FA condition. **(E)** FA-exposed male and female mice displayed lower sucrose preference in the SPT than their control counterparts. **(F)** FA-exposed male mice spent more time immobile in the FST than control male mice. This effect was not observed in female mice. $^{*}p < 0.05$, $^{**}p < 0.01$.

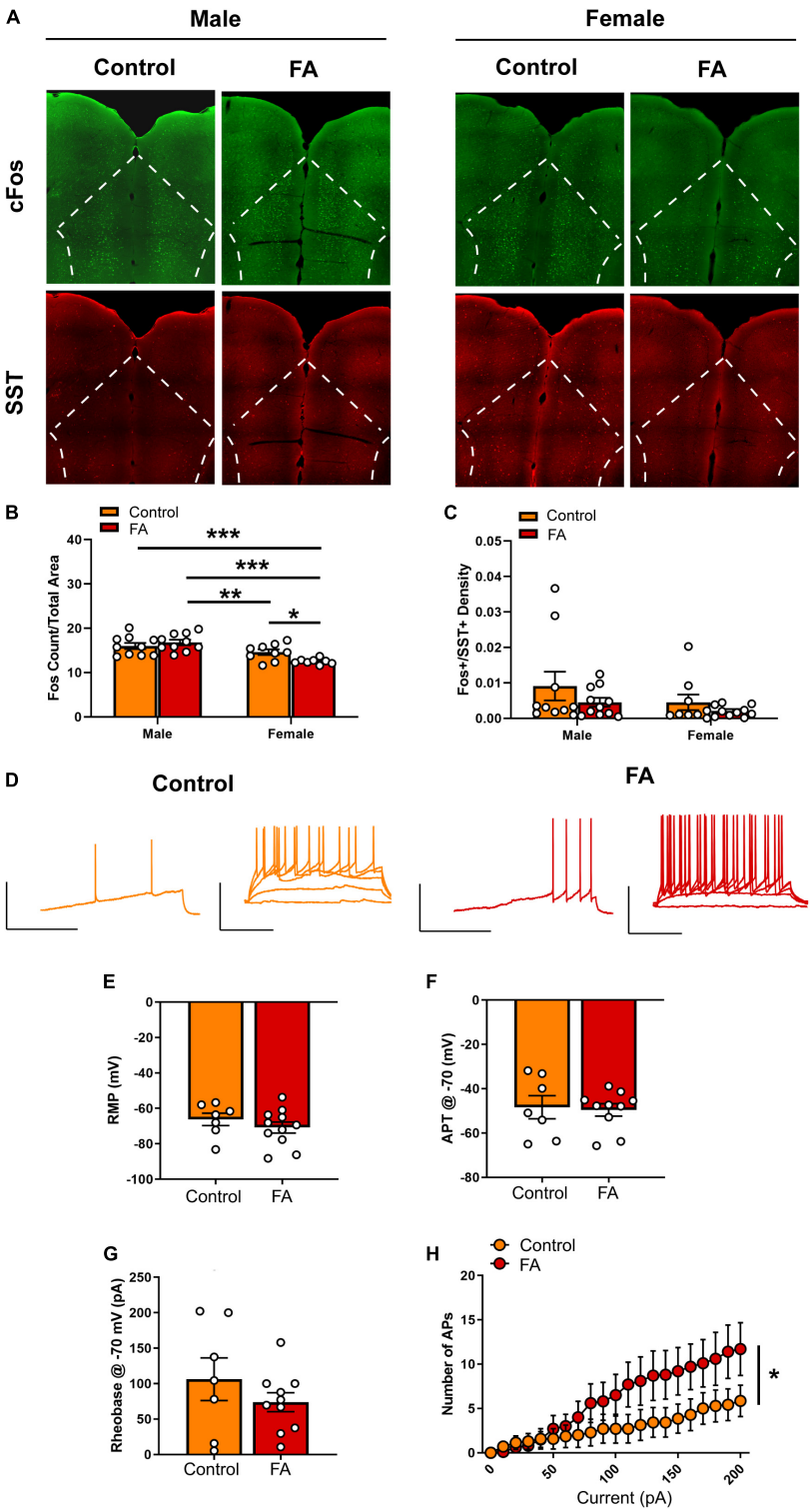

FIGURE 3 | Forced abstinence from alcohol decreases forced swim stress-induced cFos expression and augments intrinsic excitability of SST neurons in the PFC of female mice. **(A)** Representative images of cFos (green) and SST (red) immunofluorescence with the PFC delineated for quantification. **(B)** Male mice displayed more cFos nuclei than female mice following forced swim, and FA-exposed female mice had less cFos nuclei than control female mice. **(C)** SST neuron-specific cFos expression was similar in both sexes and FA conditions. **(D)** Representative traces of current-injected firing from SST neurons in the PL during a ramp protocol (left) and V-I plot protocol (right) at –70 mV. Scale bars 50 mV × 200 ms. **(E)** RMP, **(F)** action potential threshold, and **(G)** rheobase were unaltered in female PL SST neurons. **(H)** FA-exposed female SST neurons fired significantly more action potentials in the V-I plot protocol than control female SST neurons. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

TABLE 1 | Somatostatin Immunoreactivity was not modulated by sex or forced abstinence from alcohol drinking.

	Male		Female	
	Control	FA	Control	FA
PFC	7763 ± 393.8	7827 ± 552.9	6975 ± 561.6	7205 ± 508.1
dBNST	8582 ± 594.4	9136 ± 716.2	8333 ± 780.6	8256 ± 860.7
vBNST	9269 ± 598.1	9162 ± 690.2	9351 ± 1036	8808 ± 783.6
CeA	11372 ± 766.7	11564 ± 1186	11450 ± 1237	11830 ± 1371
BLA	8541 ± 682.1	8805 ± 702.4	7798 ± 655.4	8198 ± 840.8

$p < 0.001$, $F_{FA}(1, 15) = 0.118$, $p = 0.735$, $F_{FA \times current}(20, 300) = 0.421$, $p = 0.987$; data not shown] between FA-exposed mice and control mice.

Together, these data suggest that forced abstinence from alcohol dampened forced swim stress-induced neuronal activation in the female PFC, as indicated by reductions in cFos, likely via an increase in excitability of the GABAergic SST neurons.

Forced Swim Stress-Induced Neuronal Activation in the vBNST Is Modulated by Sex and Forced Abstinence From Alcohol

The dorsal BNST (**Figure 4A** for representative images) showed a significant main sex effect [$F_{sex}(1, 37) = 13.88$, $p < 0.001$], but no main effect of FA [$F_{FA}(1, 37) = 1.646$, $p = 0.207$] or sex by FA interaction [$F_{sex \times FA}(1, 37) = 0.354$, $p = 0.149$] in FST-induced cFos expression (**Figure 4B**). SST neuron-specific cFos expression (Fos+/SST+ nuclei) in the dorsal BNST was unaltered by sex and FA conditions [$F_{sex}(1, 38) = 0.178$, $p = 0.675$, $F_{FA}(1, 38) = 0.134$, $p = 0.715$, $F_{sex \times FA}(1, 38) = 0.0$, $p = 0.988$; **Figure 4C**]. SST immunoreactivity was intact across sexes and FA conditions [$F_{sex}(1, 37) = 0.106$, $p = 0.446$, $F_{FA}(1, 37) = 0.106$, $p = 0.746$, $F_{sex \times FA}(1, 37) = 0.184$, $p = 0.669$; **Table 1**].

The vBNST (**Figure 4A** for representative images) showed a significant main sex effect [$F_{sex}(1, 38) = 13.59$, $p < 0.001$], a significant main FA effect [$F_{FA}(1, 38) = 7.739$, $p = 0.008$], without a sex by FA interaction [$F_{sex \times FA}(1, 38) = 0.815$, $p = 0.372$] in FST-induced cFos expression (**Figure 4D**). Additionally, cFos+/SST+ nuclei in the vBNST revealed a significant main sex effect [$F_{sex}(1, 36) = 7.905$, $p = 0.008$], a significant main effect of FA [$F_{FA}(1, 36) = 4.944$, $p = 0.032$] and a significant sex by FA interaction [$F_{sex \times FA}(1, 36) = 5.918$, $p = 0.020$; **Figure 4E**]. Tukey's posthoc test indicated that FA-exposed female mice showed higher number of Fos+/SST+ nuclei than control female mice ($p < 0.05$), control male mice ($p < 0.01$) and FA-exposed male mice ($p < 0.01$). SST immunoreactivity was similar across sexes and FA conditions [$F_{sex}(1, 37) = 0.030$, $p = 0.862$, $F_{FA}(1, 37) = 0.174$, $p = 0.678$, $F_{sex \times FA}(1, 37) = 0.078$, $p = 0.780$; **Table 1**].

Current clamp recordings from SST neurons in female vBNST (**Figure 4F** for representative recording traces) revealed that the RMP [$t(15) = 1.371$, $p = 0.190$; **Figure 4G**], action potential threshold at RM*P* [$t(15) = 1.387$, $p = 0.185$; **Figure 4H**], and rheobase at RMP [$t(15) = 1.034$, $p = 0.317$; **Figure 4I**] were unaltered in FA-exposed mice ($n = 11$ cells, $N = 4$ mice), compared to control mice ($n = 6$ cells, $N = 3$ mice). These neurons also fired significantly less action potentials in response to increasing steps of depolarizing currents at RMP, as revealed by a significant main effect of current amplitude [$F_{current}(20, 300) = 26.770$, $p < 0.001$], a significant main effect of FA [$F_{FA}(1, 15) = 6.688$, $p = 0.021$] and a significant current by FA interaction [$F_{current \times FA}(20, 300) = 2.737$, $p < 0.001$; **Figure 4J**]. Similar effects were seen when cells were held at −70 mV, including rheobase [$t(15) = 0.851$, $p = 0.408$], action potential threshold [$t(15) = 0.508$, $p = 0.618$], and V-I plot [$F_{current}(20, 300) = 23.391$, $p < 0.001$, $F_{FA}(1, 15) = 4.329$, $p = 0.05$, $F_{current \times FA}(20, 300) = 3.426$, $p < 0.001$; data not shown].

In sum, these data suggest forced abstinence from alcohol induced robust neuroadaptations in the vBNST of female mice, with an increase in overall vBNST neuronal activation, as indicated by cFos, following forced swim stress and a decrease in excitability of the SST subpopulation of vBNST GABAergic neurons.

Forced Swim Stress-Induced Neuronal Activation in the BLA and Lateral CeA Is Not Modulated by Sex or Forced Abstinence From Alcohol

Contrary to the PFC and the vBNST, both the BLA and lateral CeA did not display any appreciable alteration in forced swim stress-induced cFos expression across sexes and FA conditions. In the BLA (**Figure 5A** for representative images), there was a significant main sex effect [$F_{sex}(1, 39) = 5.715$, $p = 0.021$], but not main FA effect [$F_{FA}(1, 39) = 0.041$, $p = 0.839$] or a sex by FA interaction [$F_{sex \times FA}(1, 39) = 0.2850$, $p = 0.596$; **Figure 5B**]. SST immunoreactivity was similar across sexes and FA conditions in the BLA [$F_{FA}(1, 39) = 0.870$, $p = 0.356$, $F_{sex}(1, 39) = 0.211$, $p = 0.648$, $F_{sex \times FA}(1, 39) = 0.008$, $p = 0.925$, **Table 1**].

In the CeA (**Figure 5A**), there was no main sex effect [$F_{sex}(1, 39) = 2.836$, $p = 0.100$], no main FA effect [$F_{FA}(1, 39) = 0.650$, $p = 0.424$], and no sex by FA interaction [$F_{sex \times FA}(1, 39) = 0.2147$, $p = 0.645$; **Figure 5C**] in FST-induced cFos expression. SST immunoreactivity in the CeA was unaltered by FA or sex [$F_{FA}(1, 39) = 0.063$, $p = 0.802$, $F_{sex}(1, 39) = 0.022$, $p = 0.881$, $F_{sex \times FA}(1, 39) = 0.006$, $p = 0.934$, **Table 1**]. Because of this, electrophysiology was not conducted in the BLA and lateral CeA.

DISCUSSION

The current study aimed to replicate previously published findings of abstinence-induced depression in both male and female rodents, and to understand the role SST neurons throughout the brain may play in this phenotype. Both male and female C57BL/6J mice showed an increased depressive-like behavioral profile following forced abstinence from alcohol, without aberrations in anxiety-like behaviors. Though both sexes showed a decrease in sucrose preference (a behavioral phenotype classically interpreted as anhedonia), only male mice showed an increased time spent immobile in the FST. Additionally, we observed plasticity in cortical and amygdalar areas of FA-exposed

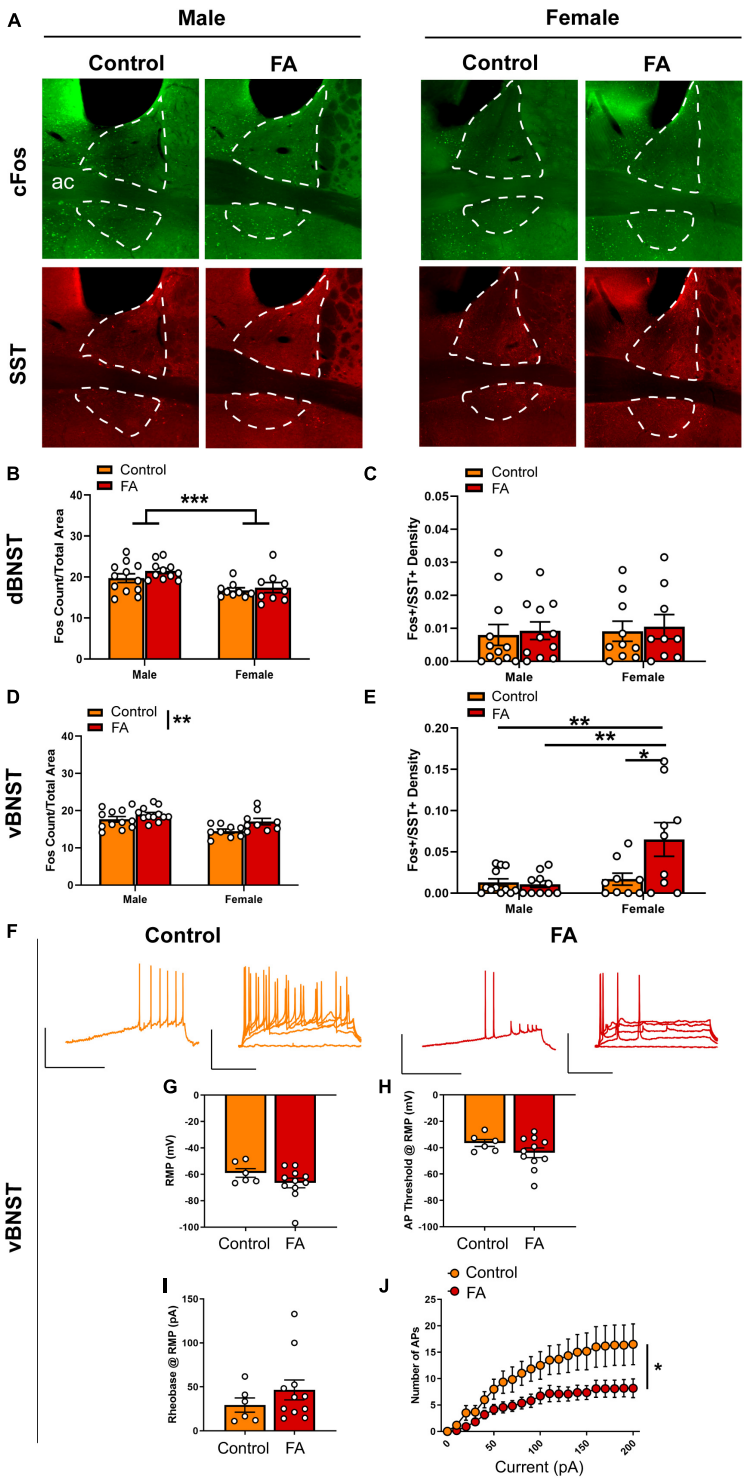

FIGURE 4 | Forced abstinence from alcohol augments forced swim stress-induced neuronal activation and decreases intrinsic excitability of SST neurons in the vBNST. **(A)** Representative images of cFos (green) and SST (red) immunofluorescence in the BNST with the dorsal and ventral subregions delineated for quantification. **(B)** FA-exposed male mice had a higher number of cFos nuclei in the dorsal BNST than control female mice and FA-exposed female mice. **(C)** SST neuron-specific cFos expression in the dorsal BNST was comparable across sexes and FA conditions. **(D)** Control mice had a lower number of cFos nuclei in the vBNST than FA-exposed mice across sexes. **(E)** FA-exposed female mice had a higher number of SST neuron-specific cFos nuclei than control female mice, control male mice and FA-exposed male mice. **(F)** Representative traces of current-injected firing from SST neurons in the vBNST during a ramp protocol (left) and V-I plot protocol (right) at RMP. **(G)** RMP, **(H)** action potential threshold, and **(I)** rheobase were unaltered in female vBNST SST neurons. **(J)** FA-exposed female SST neurons fired significantly less action potentials in the V-I plot protocol than control female SST neurons. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

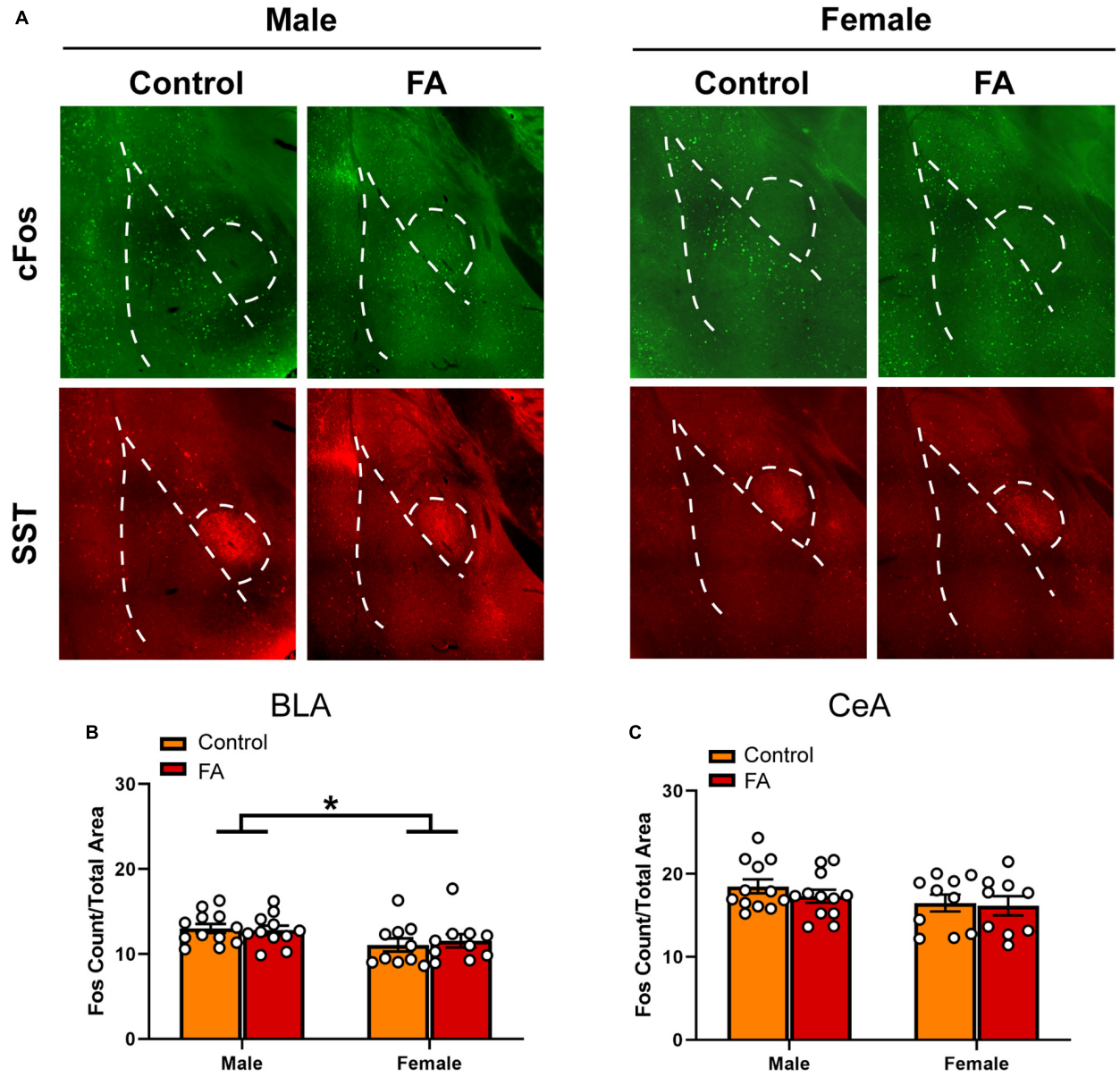

FIGURE 5 | Forced abstinence from alcohol did not influence neuronal activation following forced swim stress in the BLA and CeA. **(A)** Representative images of cFos (green) and SST (red) immunofluorescence in the amygdala with the BLA and CeA delineated for quantification. **(B)** Male mice have a higher number of cfos nuclei in the BLA than female mice, and there was no significant difference in the number of cFos nuclei between FA conditions. **(C)** There was no significant difference in the number of cFos nuclei in the CeA between sexes and FA conditions.

female mice in response to forced swim stress, including alterations in neuronal activation and intrinsic excitability of the GABAergic SST neurons in the PFC and vBNST. These findings highlight the efficacy of this alcohol exposure procedure to model protracted, forced abstinence-induced affective disturbances in both sexes, as well as shed insight into the circuit and cell type-specific adaptations.

Our results corroborate previous preclinical animal studies on sex differences in voluntary alcohol drinking (Almeida et al., 1998; Priddy et al., 2017; Crowley et al., 2019a; Peltier et al., 2019) in which female mice consistently consumed more alcohol than male mice across 6 weeks of access, despite both sexes showing similar alcohol preference over water (**Figures 1B,C**). In humans, while women historically tend to consume less alcohol than men (Rehm et al., 2010), this gap is rapidly closing with substantial increases in the prevalence of alcohol use and binge drinking among women and not men (White et al., 2015; Grucza et al., 2018). Women are also more susceptible to development of alcohol-associated neuropsychiatric disorders, including MDD, anxiety, post-traumatic stress disorder (PTSD) and stress-mediated relapse in alcohol use (Grant et al., 2017; Peltier et al., 2019). A lack of observable differences in anxiety-like

behaviors in the EPM and OFT between control and FA-exposed mice in our study could indicate a transition from the heightened anxious state associated with acute withdrawal and early abstinence (Lee et al., 2015; Pleil et al., 2015a) to a more depressive-like state during protracted abstinence (Driessen, 2001; Stevenson et al., 2009; Heilig et al., 2010).

Previous studies using the same two-bottle choice paradigm demonstrated that female mice develop a heightened depressive-like behavioral phenotype during protracted abstinence from alcohol, as indicated by longer time spent immobile in the FST and longer latency to feed in the novelty-suppressed feeding test (NSFT), that can be alleviated by physical activity (Pang et al., 2013) or administration of the novel fast-acting antidepressant ketamine (Holleran et al., 2016; Vranjkovic et al., 2018). Interestingly, here we observed a more robust depressive-like behavioral phenotype in the FST in FA-exposed male mice, and not FA-exposed female mice. One hypothesis for these differences is that females transition to the abstinence-induced depressive-like state at a more rapid rate than males, such that heightened depressive-like behaviors in females may be observable at an earlier timepoint during abstinence. Alcohol-dependent men and women progress through courses of mood states at different rates during alcohol withdrawal (Bokström et al., 1991). Future experiments should assess the same depressive-like behavioral assays across multiple timepoints during protracted withdrawal. Another possibility is that male and female mice cope with acute stress and manifest depressive-like behaviors differently. Multiple studies in the literature (Kokras et al., 2015; Colom-Lapetina et al., 2017, 2019; Molendijk and de Kloet, 2019) have cautioned against over-interpretation of immobility duration in the FST, which classically has been interpreted as 'behavioral despair' or diminished motivation to escape a stressful environment (Porsolt et al., 1977). Recent work from Colom-Lapetina et al. (2017, 2019) suggests that female rats employ more active strategies to cope with the FST, including climbing and headshaking, while males employ the more classic strategy of immobility. Similar sex differences in coping strategy have also been found in conditioned fear response (Gruene et al., 2015a). Furthermore, we observed a sex difference in the frequency of open arm entries in the EPM (**Figure 2B**), where more entries into the open arm is classically interpreted as lower level of anxiety. Overall, our results suggest that the behavioral adaptations in response to acute swim stress following forced abstinence may be more nuanced in female mice, and future studies should employ a more fine-tuned behavioral analysis to fully elucidate the affective perturbations associated with alcohol exposure and abstinence.

The corticolimbic circuit comprises of highly interconnected regions, including the medial prefrontal cortex (mPFC) and the amygdala and its subregions, that are critically involved in the regulation of emotional behaviors, stress response and reward seeking (Koob, 2009; Koob and Volkow, 2016). The mPFC, including the prelimbic (PL) and infralimbic (IL) subregions, receives glutamatergic inputs from the BLA that can promote anxiety-like behaviors (Felix-Ortiz et al., 2016) and are modulated by chronic stress (Lowery-Gionta et al., 2018; Marcus et al., 2019). Acute withdrawal from alcohol exposure has been found to disrupt synaptic transmission and intrinsic excitability of pyramidal neurons of the mPFC at different stages of alcohol dependence; particularly, the dependence-inducing chronic intermittent ethanol (CIE) model enhances excitatory drive and intrinsic excitability in pyramidal neurons (Pleil et al., 2015b; Cannady et al., 2018), whereas the drinking-in-the-dark (DID) model of pre-dependence binge-like drinking impaired excitatory transmission (Crowley et al., 2019b). Recent evidence further demonstrates an interaction between sex and alcohol exposure on the PFC neurophysiology. Chronic gavage administration of high-dose ethanol enhances excitability of Martinotti neurons (presumably SST neurons) in male PL but decreases excitability of female Martinotti neurons via a reduction in hyperpolarization-induced cation channel (HCN) current (Hughes et al., 2020). Interestingly, this model of alcohol exposure disrupts inhibitory transmission in layer 5/6 pyramidal neurons in both sexes (Hughes et al., 2019), likely via an uncharacterized microcircuit mechanism. Excitatory transmission in PL layer 2/3 pyramidal neurons is decreased following four cycles of DID via distinct cellular mechanisms between the sexes (Crowley et al., 2019a), in which DID targeted cell-surface expression of NMDA and AMPA receptors alterations occur in female PL only. Sex also modulates the effects of stress on the cortical circuitry. Male rats with high level of freezing in fear conditioning have higher spine density in IL neurons that project to the BLA than male rats with low level of freezing (Gruene et al., 2015b), but the effect is not observable in females. Reciprocal projections from the BLA to the PL and IL are more severely affected by early life adversity in females than in males (Honeycutt et al., 2020). Chronic unpredictable stress induces sex-specific alterations in the transcriptomic profiles of SST neurons in the PFC (Girgenti et al., 2019). Here our FST-induced cFos expression data revealed that forced abstinence from alcohol dampened neuronal activation in the female PFC (**Figure 3B**). Hypoactivation of the mPFC in response to stress and subsequent dysregulation of amygdala activity have indeed been observed in humans with MDD and alcoholism (Johnstone et al., 2007; Covington et al., 2010; Seo et al., 2013), as well as rodents in chronic social defeat (Covington et al., 2010) and chronic unpredictable stress (Lam et al., 2018). Withdrawal from CIE exposure similarly reduces cFos expression in the PL and IL of male mice (Smith et al., 2019). Our model, which combines both stress (potential hypoactivation of PFC) and alcohol (potential hyperactivation) may result in more nuanced alterations in this region.

The downregulation of neuronal activation in the PFC may have resulted from augmented inhibitory tone from the GABAergic SST neurons, as we identified an increase in intrinsic excitability of these neurons (**Figures 3H**, **6**). In the cortex, SST neurons are often postulated to be the gatekeeper of thalamo- and cortico-cortical excitatory inputs to pyramidal neurons, via their soma- and dendrite-targeting synapses (Urban-Ciecko and Barth, 2016). These neurons recently gained substantial interest for their role in a host of neuropsychiatric disorders, including MDD, bipolar disorder, and schizophrenia (Fee et al., 2017; Pantazopoulos et al., 2017; Abbas et al., 2018). Global disinhibition of SST neurons via deletion of $GABA_A$ receptors expressing the Υ_2 subunit produces an antidepressant-like

behavioral and neural profile (Fuchs et al., 2017). Acute, chemogenetically induced inhibition of SST neurons in the mPFC is anxiogenic (Soumier and Sibille, 2014), pointing to an overall resiliency-conferring role of SST neurons in the cortex. In our study, it is unclear whether the hyperexcitability of SST neurons following forced abstinence from alcohol directly contributes to the depressive-like states, or is in fact a compensating mechanism to counteract the alcohol-induced adaptations. The latter interpretation, that SST hyperexcitability may be in response to alcohol-induced adaptations, is further supported by the opposing results seen in the vBNST. Future studies should further examine whether SST neurons in the PFC has a causal role in alcohol-related pathological behavioral adaptations.

The extended amygdala, including the BNST and the CeA, is critically involved in regulation of alcohol consumption and withdrawal-associated negative affect (Koob, 2009; Koob and Volkow, 2016; Vranjkovic et al., 2017; Torruella-Suárez et al., 2020). As in the mPFC, withdrawal from both the CIE and DID models modifies synaptic transmission and excitability in the extended amygdala, with heavy emphasis on hyperexcitability in the vBNST and enhanced inhibition of the CeA (Roberto et al., 2010; Lee et al., 2015; Pleil et al., 2015a; Smith et al., 2019). In accordance with these results, our FST-induced cFos expression data demonstrated elevated neuronal activation in the vBNST of FA-exposed female mice (**Figure 4D**). SST neurons in the vBNST were also highly activated in response to FST in FA-exposed female, whereas this activation was very minimal in all other groups (**Figure 4E**). Contrastingly, FA dampens intrinsic excitability in SST neurons of female mice, with fewer numbers of action potentials in response to depolarizing step currents (**Figure 4J**), which could reflect a compensating mechanism to counteract the heightened engagement of vBNST SST neurons following FST. These data suggest that forced abstinence from alcohol may remodel the engagement of the stress-responsive networks in the vBNST, including SST neurons (**Figure 6**).

The circuit organization and functionality of these neuronal populations in the extended amygdala are not fully characterized. There is evidence suggesting that SST neurons in the lateral CeA directly synapse onto SST neurons in the oval nucleus of the BNST (Ahrens et al., 2018). Enhancing excitatory drive on the CeA SST neurons paradoxically disinhibits SST neurons in the oval nucleus in a kappa opioid receptor dependent manner to promote anxiety (Ahrens et al., 2018). As we did not observe any changes in anxiety-like behaviors, or any SST neuron-specific plasticity in the lateral CeA and the dorsal BNST, it is possible that the neuroadaptations in these anxiety-promoting populations

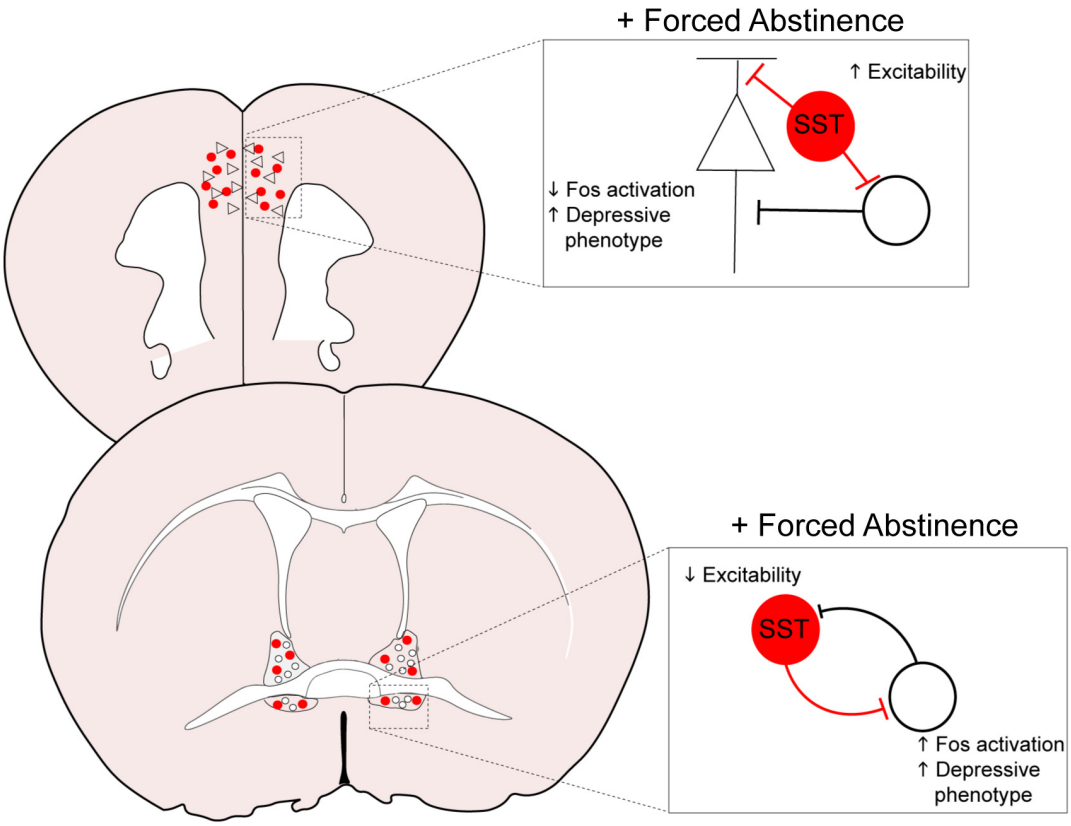

FIGURE 6 | Overall model of neuroadaptations in cortical and amygdalar areas following forced abstinence from alcohol. In the PFC, forced abstinence-induced hyperexcitability of the GABAergic SST neurons may enhance inhibitory tone onto pyramidal neurons and other neighboring populations to dampen neuronal activation in response to forced swim stress. SST neurons in the vBNST otherwise showed diminished intrinsic excitability and consequently disinhibited the local network to enhance neuronal activation in response to forced swim stress.

may be transient and occur during acute withdrawal, subsiding before transition to a depressive-like state. The otherwise robust plasticity in the vBNST SST neurons may implicate a functional distinction between the dorsal and ventral subregions of the BNST. In addition to a dense population of corticotropin-releasing factor (CRF) neurons that are anxiogenic and alcohol drinking-promoting (Pleil et al., 2015b), the vBNST includes an anxiolytic population of GABAergic neurons that project to the ventral tegmental area and the lateral hypothalamus (Marcinkiewcz et al., 2016; Pati et al., 2019). Withdrawal from CIE exposure augments excitability of CRF neurons and increases inhibition onto the anxiolytic midbrain-projecting neurons (Pati et al., 2019). Forced abstinence-induced decrease in intrinsic excitability of SST neurons in the vBNST may result in disinhibition of the CRF neurons to exacerbate negative affect and heighten the risk of stress-induced relapse. Nevertheless, there is no current evidence of direct synaptic input from SST neurons to CRF neurons or midbrain-projecting neurons in the vBNST. Further complicating the matter is the fact that the BNST organization and functionality in females have not been examined to the same degree as in males. In adult men, somatostatin and vasoactive intestinal peptide (VIP) immunostaining reveal larger BNST volume than in adult women (Chung et al., 2002). In rats, female BNST SST neurons do not express estrogen receptors and androgen receptors that are prevalent in male SST neurons (Herbison and Theodosis, 1993; Fernández-Guasti et al., 2000), suggesting that the FA-induced behavioral and neuronal adaptations in females might not be modulated by gonadal hormones. Studies examining the sex-specific properties of the BNST in regulation of emotions and reward seeking, and sex differences in AUD-related neuroadaptations in the BNST, are lacking. Future studies should examine the input and output pathways of these SST populations, as well as their functional role in regulation of alcohol drinking and emotional behaviors with an eye toward sex-specific properties.

Notably, we observed a consistent sex effect in total cFos expression the PFC and the dorsal and ventral subregions of the BNST, in which male mice showed markedly higher level of FST-induced cFos expression (**Figures 3B**, **4B,D**). Given the fact that the majority of the previously cited studies on alcohol-induced neuroplasticity were performed in males, it is interesting to see that FA-exposed male mice seem to be relatively resilient against protracted forced abstinence-induced alterations. One possibility is that, since male mice consumed less alcohol than female mice in the two-bottle choice paradigm when normalized to body weight, the effect of forced abstinence from alcohol in male mice may be too subtle to be detected by the methods employed here. Additionally, as discussed with the sex differences in coping strategies in response to forced swim stress, passive coping in male mice may engage a different circuit and cell-type specificity that could be overlooked. These observations further highlight the importance of sex as a biological variate in neurobiological studies in order to have a better understanding of the pathophysiology underlying neuropsychiatric disorders.

We did not observe any changes in SST protein expression in any cortical or amygdalar regions. We have previously reported that SST neurons in the PL cortex release SST peptide tonically and phasically in a frequency-dependent manner (Dao et al., 2019), hence altered excitability of these neurons in the PFC and the BNST may affect their capacity for neuropeptide release. The SST peptide acts on a family of G protein-coupled receptors, that are mainly inhibitory through Gi/o-dependent signaling (Barnett, 2003). However, SST receptor signaling in these regions has not been characterized. Future studies may merit from examination of the modulatory action of SST peptide, in healthy states and alcohol abuse-associated conditions.

CONCLUSION

The current study identified a host of sex-dependent behavioral adaptations and neuroplasticity in corticolimbic regions that may underlie forced abstinence-induced affective perturbations. SST neurons in the PFC and vBNST emerge as a strong neural candidate that undergo robust plasticity following alcohol exposure and protracted abstinence. These results shed new insight into the neurobiological bases of highly comorbid neuropsychiatric diseases, including MDD and AUDs, and may aid in the development of new and effective treatments.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Pennsylvania State University Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

ND and NC conceived and designed the experiments, analyzed and interpreted the experiments, and wrote the manuscript with feedback from all authors. ND, MS, SM, JM, VS, and DB conducted the experiments.

FUNDING

The experiments were funded by the Brain and Behavior Research Foundation (NARSAD Young Investigator Award; NC), The National Institute on Alcohol Abuse and Alcoholism (R21AA028008; NC), and the Penn State's Social Science Research Institute (Level 2 Award; NC).

ACKNOWLEDGMENTS

The authors would like to thank Dr. Janine Kwapis (Department of Biology, Pennsylvania State University) for assistance in imaging.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
published: 30 June 2020
doi: 10.3389/fnbeh.2020.00114

Chronic Intermittent Ethanol Exposure Increases Ethanol Consumption Following Traumatic Stress Exposure in Mice

Veronica M. Piggott[1,2,3], *Scott C. Lloyd*[1,2,3], *Shane A. Perrine*[1,3] *and Alana C. Conti*[1,2,3]*

[1]*Research & Development Service, John D. Dingell VA Medical Center, Detroit, MI, United States,* [2]*Department of Neurosurgery, Wayne State University School of Medicine, Detroit, MI, United States,* [3]*Department of Psychiatry and Behavioral Neurosciences, Wayne State University School of Medicine, Detroit, MI, United States*

Individuals with post-traumatic stress disorder (PTSD) often use alcohol to cope with their distress. This aberrant use of alcohol often develops into alcohol use disorder (AUD) leading to high rates of PTSD-AUD co-occurrence. Individuals with comorbid PTSD-AUD have more intense alcohol cravings and increased relapse rates during withdrawal than those with AUD alone. Also, individuals with PTSD or AUD alone often show similar psychological behaviors, such as impulsivity and anhedonia. Extensive clinical studies on the behavioral effects of PTSD-AUD comorbidity, namely alcohol use, have been performed. However, these effects have not been well studied or mechanistically explored in animal models. Therefore, the present study evaluated the effects of traumatic stress comorbid with alcohol exposures on ethanol intake, impulsivity, and anhedonia in mice. Adult male C57Bl/6 mice were first exposed to either mouse single-prolonged stress (mSPS), an animal model that has been validated for characteristics akin to PTSD symptoms, or control conditions. Baseline two-bottle choice ethanol consumption and preference tests were conducted after a 7-day isolation period, as part of the mSPS exposure. Next, mice were exposed to air or chronic intermittent ethanol (CIE), a vapor-induced ethanol dependence and withdrawal model, for 4 weeks. Two-bottle choice ethanol drinking was used to measure dependence-induced ethanol consumption and preference during periods intervening CIE cycles. The novelty suppressed feeding (NSF) test was used to evaluate impulsivity and anhedonia behaviors 48 h after mSPS and/or repeated CIE exposure. Results showed that, compared to control conditions, mSPS did not affect baseline ethanol consumption and preference. However, mSPS-CIE mice increased Post-CIE ethanol consumption compared to Control-Air mice. Mice exposed to mSPS had a shorter latency to feed during the NSF, whereas CIE-exposed mice consumed less palatable food reward in their home cage after the NSF. These results demonstrate that mice exposed to both

OPEN ACCESS

Edited by:
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***Correspondence:**
Alana C. Conti
aconti@med.wayne.edu

Specialty section:
This article was submitted to Emotion Regulation and Processing, a section of the journal Frontiers in Behavioral Neuroscience

Received: *07 April 2020*
Accepted: *08 June 2020*
Published: *30 June 2020*

Citation:
Piggott VM, Lloyd SC, Perrine SA and Conti AC (2020) Chronic Intermittent Ethanol Exposure Increases Ethanol Consumption Following Traumatic Stress Exposure in Mice. Front. Behav. Neurosci. 14:114. doi: 10.3389/fnbeh.2020.00114

Abbreviations: AUD, alcohol use disorder; BEC, blood ethanol concentration; CB, cannabinoid; CIE, chronic intermittent ethanol; mSPS, mouse single-prolonged stress; NSF, novelty suppressed feeding; PTSD, post-traumatic stress disorder; WSU, Wayne State University.

mSPS and CIE are more vulnerable to ethanol withdrawal effects, and those exposed to mSPS have increased impulsivity, while CIE exposure increases anhedonia. Future studies to examine the relationship between behavioral outcomes and the molecular mechanisms in the brain after PTSD-AUD are warranted.

Keywords: post-traumatic stress disorder, mouse single-prolonged stress, chronic intermittent ethanol, ethanol consumption, alcohol use disorder, impulsivity, anhedonia

INTRODUCTION

Post-traumatic stress disorder (PTSD) is a serious mental health disorder that people may develop directly or indirectly after experiencing a traumatic event(s). According to the DSM-5, PTSD symptoms include flashbacks, avoidance behavior of the traumatic event(s), negative mood, and hyperarousal (American Psychiatric Association, 2013). The lifetime prevalence of PTSD in the United States population is estimated to be 6.8% (Kessler et al., 2005). Combat-exposed military personnel and Veterans have a higher risk of developing PTSD than civilians (Hoge et al., 2006; Petrakis et al., 2016). Individuals who are diagnosed with PTSD have an increased propensity (28–85%) to develop alcohol use disorder (AUD; Kessler et al., 1995; Baker et al., 2009; Ralevski et al., 2014).

Characteristics of AUD include high tolerance to short-term effects of ethanol and vulnerability to withdrawal symptoms, such as anhedonia, during alcohol abstinence (Becker, 2008; Martinotti et al., 2008; Hatzigiakoumis et al., 2011; Pava and Woodward, 2012). Since alcohol is an effective anxiolytic, individuals with PTSD often self-medicate with alcohol to alleviate PTSD symptoms and negative emotions, which can also contribute to the development of AUD (Carter et al., 2011). Individuals with comorbid PTSD and AUD have more intense alcohol cravings and relapse more frequently during withdrawal than those with AUD only (Brown et al., 1999; Ouimette et al., 1999; Berenz et al., 2017), suggesting that the comorbid disorder is unique from either condition alone.

Clinical research has shown that PTSD symptoms such as hyperarousal and negative mood are strongly related to impulsivity (Armour et al., 2016; Contractor et al., 2016; Roley et al., 2017). Individuals with PTSD symptoms had significant disinhibition behavior compared to traumatized control individuals without PTSD symptoms (Casada and Roache, 2005). Those with PTSD also had impaired judgment in dangerous situations, especially when rewarding stimuli were involved (Casada and Roache, 2005; Roley et al., 2017).

The behavioral outcomes of PTSD-AUD comorbidity such as hyperarousal (Saladin et al., 1995) and increased alcohol relapse (Drapkin et al., 2011; Petrakis and Simpson, 2017) have been shown in clinical studies. However, drinking behavior and other psychological behaviors such as impulsivity and negative affect after traumatic stress and prolonged ethanol exposure and withdrawal have not been fully evaluated in animal models (Gilpin and Weiner, 2017). Mouse single-prolonged stress (mSPS) is based on a rodent animal model that has phenotypes akin to most behavioral and physiological symptoms in clinical PTSD (Liberzon et al., 1997, 1999; Yamamoto et al., 2009; Pitman et al., 2012; Perrine et al., 2016; Flandreau and Toth, 2018). For example, mSPS-exposed mice showed increased freezing behavior when re-exposed to an SPS-associated cue (tone) presented 7 days after mSPS (Perrine et al., 2016), which parallels the augmentation of fear responses when individuals with PTSD encounter trauma-related cues (Garfinkel et al., 2014; Gonzalez and Martinez, 2014). Also, mSPS exposure enhanced dexamethasone suppression of corticosterone levels compared to controls, demonstrating increased negative feedback sensitivity of the hypothalamus-pituitary-adrenal axis, which has been reported in humans with PTSD (Yehuda, 2009; Morris et al., 2012; Perrine et al., 2016). Using this model of traumatic stress exposure, we examined ethanol dependence behaviors using Chronic Intermittent Ethanol (CIE) vapor exposure. CIE is an animal model that has been widely used in alcohol dependence and withdrawal studies (Becker and Lopez, 2004; Becker and Ron, 2014; Anderson et al., 2016a,b; Rose et al., 2016). Repeated CIE involves an extended period of ethanol vapor exposure followed by a brief period of ethanol abstinence. This animal model has shown that mice develop tolerance to the aversive effects of ethanol during the conditioned taste aversion test, which causes escalated ethanol consumption once alcohol dependence has developed (Lopez et al., 2012). Mice exposed to CIE have high blood ethanol concentrations (BECs) that are considered to be intoxicating (Becker and Lopez, 2004). Repeated CIE exposure and ethanol abstinence also induce withdrawal symptoms such as anxiety-like behavior and anhedonia. For example, mice exposed to repeated CIE buried more marbles during the marble burying test and had a longer latency to feed during the novelty suppressed feeding (NSF) test (Rose et al., 2016; Jury et al., 2017).

Using the mSPS and CIE to model PTSD-AUD comorbidity, this study examined the effects of traumatic stress on PTSD-associated behavioral outcomes, namely ethanol intake, impulsivity, and anhedonic responses. We hypothesized that mice exposed to mSPS would demonstrate increased and sustained ethanol consumption and preference after prolonged exposure to ethanol vapor and withdrawal, which would be accompanied by increased impulsivity and anhedonia.

MATERIALS AND METHODS

Animals

Male C57Bl/6 mice (n = 45) were bred in-house at Wayne State University (WSU) facilities. Mice were housed on a 12-h light/dark cycle (lights on at 6 am) in groups of 2–5 in standard microisolator polycarbonate cages under controlled temperature (21–24°C) and humidity (30–40%) with *ad libitum* access to

food and water, except during the mSPS procedure and NSF testing, when food, but not water, was restricted. All procedures were approved by the WSU Institutional Animal Care and Use Committee. All experimental procedures were conducted according to the National Institute of Health Office of Laboratory Animal Welfare for the Care and Use of Laboratory Animals at the WSU Division of Laboratory Animal Resources facilities, which are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. Data from the first cohort of mice (n = 28) were used for the baseline limited access two-bottle choice ethanol drinking test. Data from the second cohort of mice (n = 18) were used for the Post-CIE limited access two-bottle choice ethanol drinking test. Data from the third cohort of mice (n = 27) were used for the NSF test. All cohorts of mice were exposed to mSPS, CIE, and two-bottle choice ethanol drinking.

Mouse Single-Prolonged Stress (mSPS)

Adult male mice (10–12 weeks old) were exposed to mSPS which consisted of four consecutive stressors preceding a 7-day incubation period (see **Figure 1A**), as described previously (Matchynski-Franks et al., 2016; Perrine et al., 2016). Mice were restrained for 2 h in 50 ml conical tubes, with a screw top and holes along the tube for adequate air exchange. After 2 h of restraint, mice were immediately exposed to a 10-min group (n = 4–5 mice/group) forced to swim in 26–28°C water in a 4-L glass beaker. Mice were then towel-dried and returned to their home cages where they were exposed to a beaker filled with soiled rat bedding, a predator scent, for 15 min. Immediately after soiled rat bedding exposure, mice were placed in a clean microisolator polycarbonate cage with a cage lid. Cotton balls saturated with diethyl ether anhydrous were gradually placed in the cage at 1-min intervals until mice lost consciousness, which was verified by the toe-pinch method. Control mice (non-mSPS) were handled, weighed, and housed in another room during the mSPS procedure, experiencing no mSPS exposure. After mSPS exposure, both mSPS and control animals were housed individually in clean cages with fresh bedding and left undisturbed for 7 days with *ad libitum* access to food and water and daily health monitoring.

Limited Access Two-Bottle Choice and Chronic Intermittent Ethanol (CIE) Vapor Exposure

Figure 1B shows an overview of the experimental timeline. Stable baseline (Pre-CIE) ethanol intake was achieved beginning on day 8, after a 7-day undisturbed post-mSPS period, using a limited access two-bottle choice paradigm for five consecutive nights. Thirty minutes before the beginning of the dark cycle, mice were introduced to two 150-ml drinking bottles containing 15% v/v ethanol or tap water for 1 h with free access to food. The ethanol solution was prepared fresh daily with 100% ethanol solution and tap water. The positions of the two bottles were altered daily to avoid side preferences. The amount of ethanol consumed was recorded daily by subtracting the weight of the ethanol and water bottles before and after the 1-h ethanol and water consumption period. Ethanol consumption was calculated by converting the ethanol intake in ml (±0.1 ml) and body weight (±0.1 g) to g of ethanol intake/kg of body weight. Ethanol preference was calculated as a ratio of ethanol intake to total fluid consumed. Average ethanol intake and preference per animal were calculated based on average ethanol consumption and preference over the 5-night pre-CIE period.

Approximately 72 h after the last baseline ethanol intake session, mice were counterbalanced into Control-Air, Control-CIE, mSPS-Air, and mSPS-CIE groups according to their baseline ethanol intake levels. Mice, within their home cages, were placed in Plexiglas inhalation chambers (Plas Labs, Lansing, MI, USA) and exposed to ethanol vapor or air for 16 h followed by 8 h room air exposure (ethanol abstinence period) for four consecutive days, which was considered to be one cycle, based on published methods (Becker and Lopez, 2004; Anderson et al., 2016b). Ninety-five percent ethanol was mixed with air and vaporized at a rate of 10 l/min, and the ethanol concentrations in the chambers were monitored daily with a breathalyzer (BAC Track Select S80, San Francisco, CA, USA). Before placement into the ethanol or air chambers, mice were co-administered the alcohol dehydrogenase inhibitor pyrazole (Chem-Impex International, Wood Dale, IL, USA; 1 mM/kg) and ethanol (CIE exposure mice; 1.6 g/kg; 20% w/v) or saline (air exposure mice; 10 ml/kg) intraperitoneally to stabilize BECs and initiate ethanol intoxication, according to published methods (Becker and Lopez, 2004; Anderson et al., 2016b). BECs were measured once per cycle from tail blood samples. Blood samples were collected and centrifuged for 5 min at 2,000 *g*. Plasma was collected and measured using alcohol diagnostic reagents (Pointe Scientific, Canton, MI, USA Cat #: A7504). BEC levels above 175 mg/dl during each cycle indicated intoxication in C57Bl/6 mice (Becker and Lopez, 2004). Average BEC levels across four cycles were recorded. The CIE vapor or air exposure was repeated for 4 weekly cycles with 5-night two-bottle choice Post-CIE drinking sessions during intervening weeks.

Novelty Suppressed Feeding (NSF)

Forty-eight hours after the last day of the 4th CIE cycle, mice were exposed to the NSF test (see **Figure 1B**). Sixty-four hours before the test, mice were given sweetened fruit cereal to be used in the NSF test in their home cages. Mice were then food, but not water, deprived for 48 h with 1 h free access to food every 24 h. On the testing day, a piece of sweetened fruit cereal was placed on a piece of filter paper in the center of an arena (62 cm × 62 cm × 36 cm). Mice were then placed in a random corner of the arena and latency to enter the arena center and feed was recorded in seconds. Mice that took longer than 600 s to take their first bite were eliminated from the test. After the first bite of the sweetened fruit cereal was consumed, mice were immediately removed from the arena and returned to their home cage, where they were offered a pre-weighed piece of sweetened fruit cereal for 5 min to determine home cage consumption. Percent of home cage sweetened fruit cereal consumption was calculated by subtracting the weight of sweetened fruit cereal left in the home cage from the original weight of the sweetened fruit cereal divided by the original weight of the sweetened fruit cereal, multiplied by 100%.

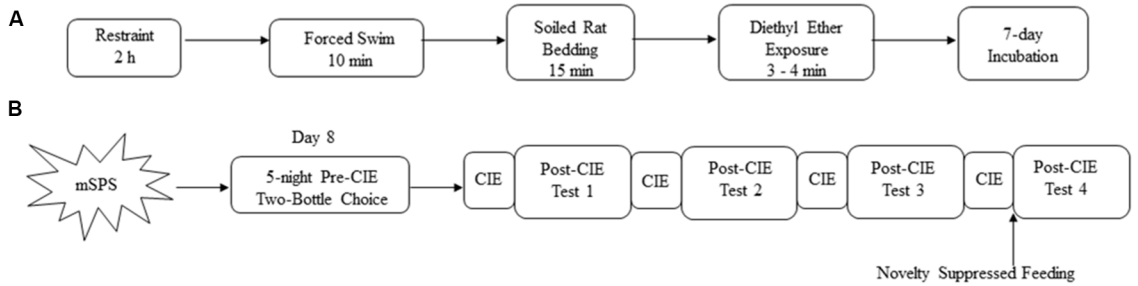

FIGURE 1 | Schematic overview of **(A)** the mouse single-prolonged stress (mSPS) paradigm, **(B)** mSPS, and chronic intermittent ethanol (CIE) exposure with two-bottle choice drinking test. After the mSPS paradigm, mice were exposed to two-bottle choice for five nights. Mice were then exposed to CIE exposure every other week, with a Post-CIE drinking test in between the weeks of CIE exposure.

Data Analysis and Statistics

Data calculations and statistical analyses were performed using MS Excel, GraphPad Prism 6 (San Diego, CA, USA), and Statistica 6.0 (Tulsa, OK, USA). Two mice were removed from the NSF analysis because one mouse's latency to feed did not meet the 600 s or less criterion, and the other mouse datum was a statistical outlier, being more than two standard deviations away from the group mean. A student's two-tailed *t*-test was used to analyze baseline ethanol consumption, baseline ethanol preference, and BEC values. For the Post-CIE ethanol consumption and ethanol preference results under various test weeks, a repeated measure of three-way ANOVA was used with mSPS, vapor exposure, and test week as factors, followed by Fisher's LSD *post hoc* multiple comparisons tests, when appropriate. A two-way ANOVA was used to analyze the latency to feed values and the percentage of in-cage food consumption in the NSF test. All data are reported as mean ± SEM with $p < 0.05$ as the criterion for statistical significance.

RESULTS

mSPS exposure did not increase baseline Pre-CIE ethanol intake (student's two-tailed *t*-test $t_{(26)} = 1.04$; $p = 0.31$; **Figure 2A**) or ethanol preference ($t_{(26)} = 1.56$; $p = 0.13$; **Figure 2B**). However, mSPS-CIE mice escalated their average ethanol consumption after their 1st (6.01 ± 0.27 g/kg) CIE cycle and sustained their average ethanol consumption at the 4th (6.26 ± 0.20 g/kg) CIE cycle. A three-way ANOVA with repeated measures revealed an mSPS effect ($F_{(1,14)} = 17.9$, $p < 0.05$), a CIE effect ($F_{(1,14)} = 5.9$; $p < 0.05$), and a week × mSPS interaction ($F_{(1,14)} = 5.01$; $p < 0.05$) on the average ethanol consumption (**Figure 2C**) after the 1st and 4th CIE cycle. A Fisher's LSD *post hoc* comparison test revealed that the mSPS-CIE ($n = 5$) group significantly increased their vapor-induced ethanol consumption in Test 1 and continued to consume a similar amount of ethanol in Test 4 compared to the Control-Air ($n = 5$; $p < 0.05$), and the Control-CIE groups ($n = 4$; $p < 0.05$) in Test 1. mSPS-Air mice ($n = 4$; $p < 0.05$) also significantly increased their average ethanol consumption compared to the Control-Air group after the first cycle of air. Even though mSPS increased average ethanol consumption, it did not increase ethanol preference, which was approximately 92% (**Figure 2D**) after four cycles of CIE. For the average water consumption, a three-way ANOVA with repeated measures showed an mSPS-effect ($F_{(1,12)} = 18.4$; $p < 0.05$). A Fisher's LSD *post hoc* comparison test showed that the mSPS-Air mice (2.32 ± 0.29 g/kg) and mSPS-CIE (2.08 ± 0.2 g/kg) consumed less water than the Control-CIE mice (3.29 ± 0.35 g/kg) in Test 1. In Test 4, only the mSPS-CIE mice (2.18 ± 0.20 g/kg) consumed less water than both Control-Air (3.00 ± 0.16 g/kg) and Control-CIE (3.16 ± 0.30 g/kg). For the average total fluid consumption, a three-way ANOVA with repeated measures ($F_{(1,12)} = 0.041$; $p = 0.84$) showed no significant differences among groups (**Figure 2E**). In addition, a three-way ANOVA with repeated measures ($F_{(1,14)} = 2.02$; $p = 0.18$) revealed no differences in body weights among groups (**Figure 2F**).

During each CIE cycle, BECs were evaluated in both the Control-CIE and mSPS-CIE groups. **Figure 3** shows the average BEC levels across four cycles between the Control-CIE and mSPS-CIE groups. A student *t*-test revealed that there was no significant difference in BEC levels between Control-CIE ($n = 4$; 258 ± 17 mg/dl) and mSPS-CIE ($n = 5$; 262 ± 18 mg/dl) mice. BEC levels were undetectable in Control-Air and mSPS-Air mice (data not shown).

NSF was performed 48 h after the last day of CIE exposure, before Test 4. **Figure 4** shows the (**Figure 4A**) latency to feed and (**Figure 4B**) % consumption of sweetened cereal in the home cage during the NSF test. One mouse was eliminated because his latency to feed was over 600 s, and an outlier datum was also eliminated from the analysis. Results of the latency to feed showed a mSPS effect (two-way ANOVA; $n = 12$; $F_{(1,21)} = 5.304$; $p < 0.05$). mSPS-exposed mice (108 ± 14 s), either exposed to Air ($n = 7$) or CIE ($n = 5$), had a shorter latency to consume the first bite of sweetened fruit cereal in the arena compared to the Control (non-SPS) mice (189 ± 31 s). Results of the percent home cage cereal consumption revealed an ethanol vapor effect (two-way ANOVA; $n = 11$; $F_{(1,21)} = 19.39$; $p < 0.05$). Mice exposed to CIE (56 ± 4%), either Control ($n = 6$) or previously exposed to mSPS ($n = 5$), consumed less cereal in the home cage compared to mice exposed to Air (83 ± 4%).

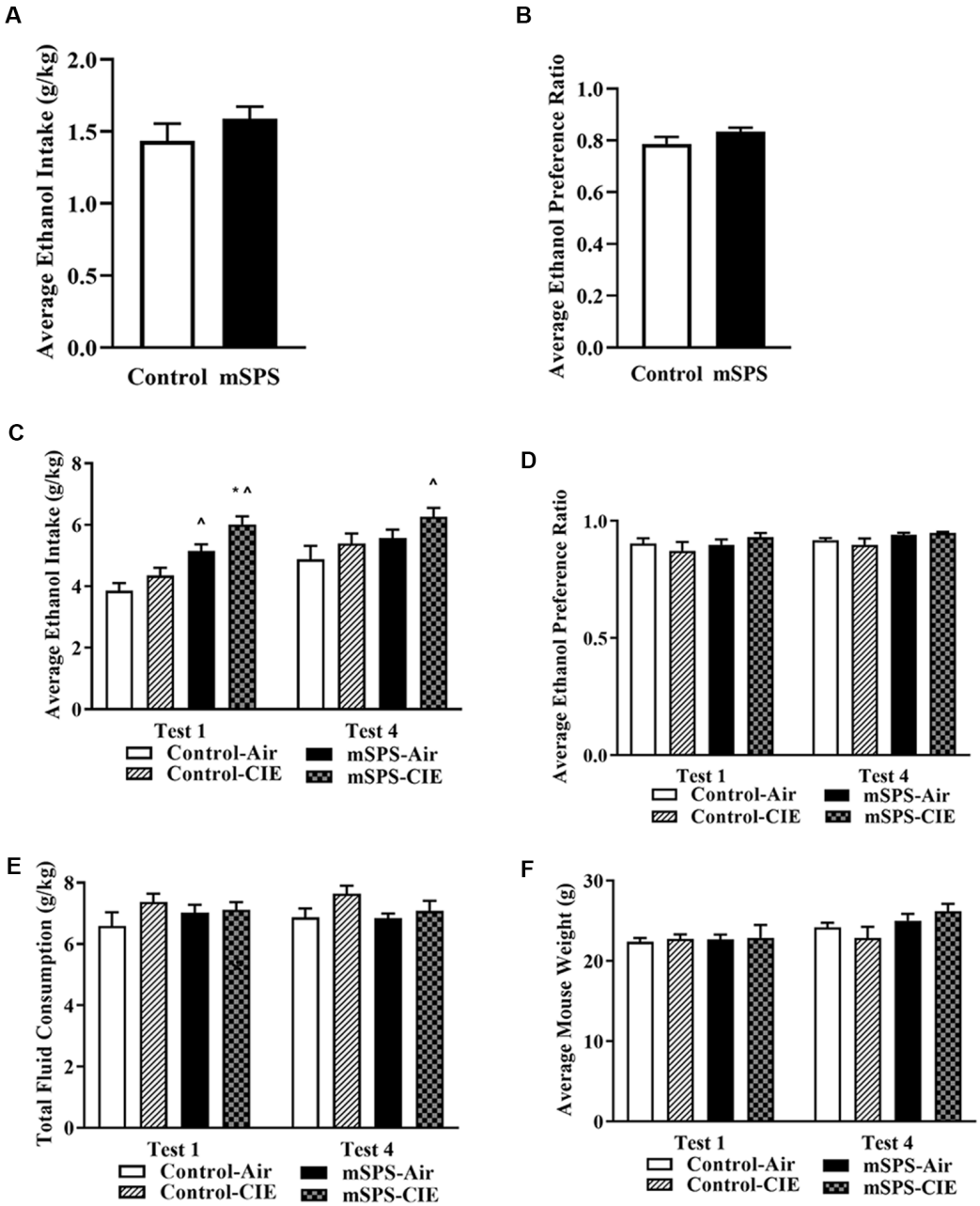

FIGURE 2 | Average of Pre-CIE **(A)** ethanol intake and **(B)** ethanol preference on the 8th day after 7-day of incubation. A student's two-tailed *t*-test revealed no significant impact on ethanol consumption and preference after mSPS ($n = 14$). However, a three-way ANOVA with repeated measures and *post hoc* Fisher-LSD test showed the results in **(C)** that vapor-induced ethanol intake was significantly increased in mSPS-CIE ($n = 5$) compared to Control-Air ($n = 5$; $^p < 0.05$) and Control-CIE mice ($n = 4$; $^*p < 0.05$) in the 1st and 4th Post-CIE ethanol intake test sessions (Test 1 and 4). Mice exposed to mSPS and air control (mSPS-Air: $n = 4$) showed increased ethanol consumption in the 1st post-CIE ethanol intake session (Test 1; $^p < 0.05$) compared to Control-Air mice, but not in subsequent Post-CIE ethanol intake test sessions. The results in **(D)** showed no significant impact on ethanol preference among groups in either test session. A three-way ANOVA with repeated measures showed that neither the **(E)** average total fluid consumption among groups nor the **(F)** mouse body weights were affected in Test 1 and 4. Data are mean ± SEM.

DISCUSSION

The current study evaluated the effects of traumatic stress exposure, chronic alcohol exposure, and co-occurring of both exposures on behavioral outcomes including ethanol intake and preference, impulsivity, and anhedonia. This study hypothesized that mice exposed to mSPS would escalate their ethanol consumption and preference earlier after CIE and withdrawal and sustain their ethanol intake until the end of the study compared to controls. These results were predicted to be

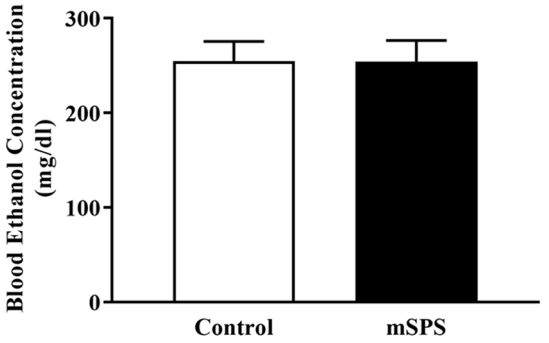

FIGURE 3 | Average blood ethanol concentrations (BECs; mg/dl) across four cycles of CIE vapor exposure. Average BECs in mSPS-CIE mice (n = 5) over four cycles of CIE vapor exposure did not differ from the Control-CIE mice (n = 4). Data are mean ± SEM.

associated with increased impulsivity and anhedonia compared to controls. Results showed that mice exposed to mSPS did not escalate their Pre-CIE two-bottle choice ethanol intake. However, once mSPS-exposed mice were exposed to CIE, which included 8 h/day ethanol abstinence and another 81-h ethanol abstinence period between the CIE cycle and Post-CIE drinking session, their ethanol intake was significantly increased in Test 1 and sustained at Test 4 while their ethanol preference remained unchanged. Even though the ethanol intake was increased in mSPS-CIE mice, their BEC levels did not differ from the Control-CIE mice, indicating both groups were intoxicated during CIE exposure. Finally, mice exposed to mSPS had a shorter latency to feed on a palatable reward, sweetened fruit cereal, during the NSF test, and mice exposed to CIE consumed less sweetened fruit cereal in their home cage after the NSF test.

Previous studies from other laboratories have exposed mice to Pre-CIE drinking (two-bottle choice limited access) for 3–6 weeks to get a stable baseline of ethanol intake before stress and CIE (Anderson et al., 2016a,b; Lopez et al., 2016; Rodberg et al., 2017). Also, these studies showed that non-dependent mice exposed to different stressors did not escalate ethanol consumption if mice were exposed to a limited access drinking paradigm only (Anderson et al., 2016a; Lopez et al., 2016). Following this, our current study also showed that mice exposed to traumatic stress did not increase their baseline ethanol consumption, indicating that ethanol dependence does not develop during the baseline drinking session.

Effects of stress on ethanol consumption behaviors have provided variable results in animal models (Pohorecky, 1990; Sillaber and Henniger, 2004; Yang et al., 2008; Becker et al., 2011; Cozzoli et al., 2014; Lopez et al., 2016). Rodents' drinking behaviors can be affected by different types of stress or durations of ethanol access. One study showed that restraint stress did not change ethanol consumption when mice had 24-h ethanol access (Yang et al., 2008), while another study found ethanol consumption to be decreased after restraint stress when mice had 2-h limited ethanol access (Cozzoli et al., 2014). Also, mice exposed to predator odor stress escalated their alcohol consumption 2 days after stress (Cozzoli et al., 2014). In a modified SPS study, rats that were exposed to traumatic stress had a higher alcohol preference score during a conditioned place preference (CPP) test compared to controls (Yu et al., 2016), which indicated that traumatic stress could cause an escalation of ethanol intake.

In comorbid stress and AUD study, mice escalated their ethanol consumption after exposure to CIE with forced swim stress but had no change in their ethanol consumption after CIE with social defeat stress exposure (Lopez et al., 2016). This study further demonstrated that mice exposed to forced swim stress once during the last cycle of CIE did not increase their ethanol consumption (Anderson et al., 2016a; Lopez et al., 2016). These studies underscored the importance of the experimental

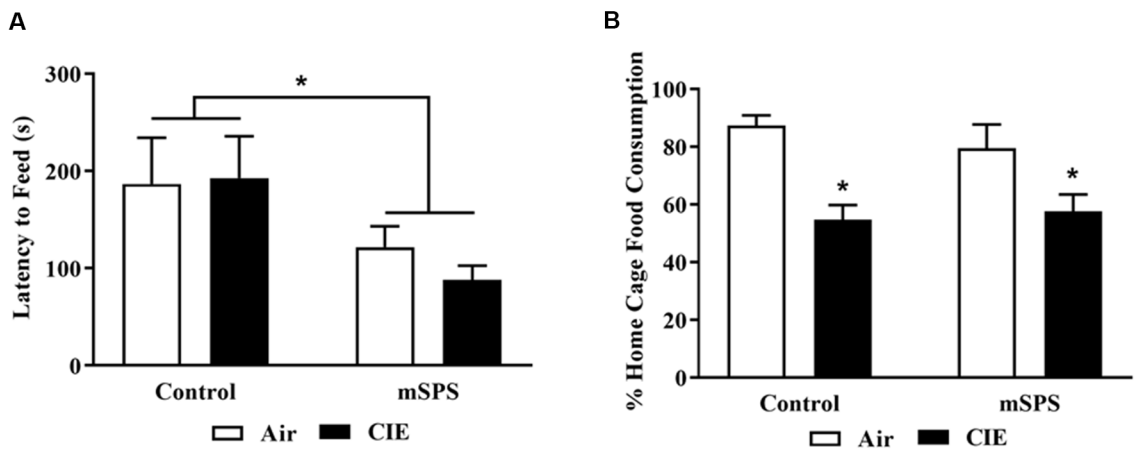

FIGURE 4 | Average of **(A)** latency to feed during the novelty suppressed feeding (NSF) test and **(B)** percent sweetened fruit cereal consumed in the home cage, after the 4th cycle of CIE (before Test 4). mSPS increased impulsivity behavior **(A)**, and CIE exposure decreased home cage sweetened cereal intake in mice (n = 5–6; **B)**. A two-way ANOVA revealed that mSPS-exposed mice, either exposed to CIE or Air, had a shorter latency to take the first bite of a sweetened fruit cereal (*$p < 0.05$) compared to mSPS controls. A two-way ANOVA also revealed that CIE-exposed mice, either exposed to mSPS or Control (non-mSPS) conditions previously, consumed less sweetened cereal in their home cages compared to Air-exposed mice. Data are mean ± SEM.

design of stress and alcohol exposure comorbidity (types and durations of stress, duration, and route of ethanol exposure). Our Post-CIE two-bottle choice limited access had comparable results. mSPS-CIE and mSPS-Air mice consumed a high amount of ethanol during the Post-CIE two-bottle choice session after the 1st CIE cycle, mainly due to stress-induced ethanol consumption. A ceiling effect likely prevented the mSPS-exposed group from further escalating their ethanol intake by Test 4 compared to Test 1, as their ethanol consumption was elevated earlier (Test 1) than that of the Control-Air and Control-CIE groups. At Test 4 (after the 4th cycle), only mSPS-CIE mice showed exacerbated ethanol intake with no sustained changes in any other testing group, which is consistent with the results from published studies (Anderson et al., 2016a; Lopez et al., 2016). The Control-CIE group demonstrated a gradual increase in ethanol intake, as it has been shown in published studies (Lopez et al., 2012; Anderson et al., 2016a). Use of a 5-days pre-CIE baseline drinking session in the present study, instead of a 6-week pre-CIE baseline drinking session used in the original paradigm (Becker and Lopez, 2004), could explain this gradual increase of ethanol intake observed in the Control-CIE group. Yet, a novel finding for the present Post-CIE drinking study is that mSPS, which consists of a variety of stressors, had a rapid and protracted effect on ethanol consumption behavior, as seen in Test 1 and 4. Mice were exposed to mSPS 8 weeks before the Test 4 drinking session, and mice were not exposed to another mSPS during this period of eight-weeks. Therefore, mSPS-CIE comorbidity has a unique effect that caused mice to escalate their ethanol intake, similar to the increased ethanol intake phenomenon reported in humans with PTSD and AUD (Brown et al., 1999; Ouimette et al., 1999; Berenz et al., 2017). Even though mSPS-CIE mice escalated their ethanol intake in Test 4, mice exposed to either Control (non-mSPS) or mSPS had similar BEC levels. This result indicates that both Control-CIE and mSPS-CIE groups reached the critical threshold of intoxication ($\geq$175 mg/dl) that is a criterion for the CIE animal model (Becker and Lopez, 2004). Both Pre-CIE and Post-CIE ethanol preference showed no difference among treatment groups, suggesting that ethanol intake may have produced a ceiling effect in our mice, which could obscure changes in mSPS-induced ethanol preference.

Clinical studies have shown that impulsive behavior is highly correlated with PTSD (Garfinkel et al., 2014; Armour et al., 2016; Contractor et al., 2016; Roley et al., 2017) and AUD (Dick et al., 2010) alone, as well as with the combination of PTSD and substance use disorder (Weiss et al., 2017). The NSF test is commonly used to measure novelty-induced hyponeophagia (Samuels and Hen, 2011). This test has been used to examine anxiety-like behavior after alcohol withdrawal (Pang et al., 2013; Holleran et al., 2016; Jury et al., 2017; Sidhu et al., 2018), as well as impulsive behavior (Bevilacqua et al., 2010; Angoa-Pérez et al., 2012) in mice. In impulsivity studies, mice had a shorter latency to feed in a novel environment (Bevilacqua et al., 2010; Angoa-Pérez et al., 2012), whereas, in ethanol-induced anxiety studies, mice had a longer latency to feed in a novel environment (Pang et al., 2013; Holleran et al., 2016; Jury et al., 2017; Sidhu et al., 2018). Also, anxiety-like behavior has been observed in CIE-exposed mice using both the marble-burying and the NSF tests (Rose et al., 2016; Jury et al., 2017; Sidhu et al., 2018). However, unlike the previous studies from others (Jury et al., 2017; Sidhu et al., 2018), our CIE-exposed mice did not show anxiety-like behavior, as they did not have a longer latency to feed in a novel environment. Our contradicting results could be due to differences in our CIE paradigm before the NSF test. Our CIE paradigm before the NSF test consisted of four cycles of CIE with a two-bottle choice limited drinking test during intervening weeks, whereas other studies used repeated CIE with no intervening drinking sessions. An extra two-bottle choice limited access drinking test in between CIE exposure could account for our NSF results differing from those of others. In our study, mSPS-exposed mice had a shorter latency to feed in a novel environment after both Air and CIE vapor exposures, which could be interpreted as impulsivity-like behavior, based on the findings from published studies (Bevilacqua et al., 2010; Angoa-Pérez et al., 2012). Additional studies have reported that C57/Bl6 mice show resilience to anxiety-like behaviors, with results being dependent on the ethanol intake regimen (Cox et al., 2013), which could explain why we did not see anxiety-like behavior on CIE-exposed mice.

Another behavior related to negative affect that is associated with AUD and PTSD is anhedonia, which can be reflected in the NSF test. According to the DSM-5, anhedonia is one of the symptoms associated with PTSD, as well as ethanol withdrawal (Becker, 2008; American Psychiatric Association, 2013). Mice exposed to a 6-week two-bottle choice drinking session followed by 2-week ethanol abstinence showed a decrease in saccharin consumption during a saccharin preference test, which suggests that mice showed anhedonia-like behavior after ethanol withdrawal (Pang et al., 2013). Furthermore, rats exposed to SPS consumed less saccharin during a saccharin preference test, consumed less cocaine during cocaine self-administration, and had a lower cocaine preference score during a CPP test, which indicates anhedonia-like behavior (Enman et al., 2015). In the current study, mice exposed to repeated CIE vapor exposure, with prior exposure to either Control or mSPS conditions, consumed less sweetened fruit cereal in their home cage after the NSF test, indicating anhedonia-like behavior. These results parallel the results of the saccharin preference test study (Enman et al., 2015). One would expect that mSPS-Air mice would have anhedonia-like behavior, as was seen in the SPS-cocaine rat study (Enman et al., 2015). However, the SPS effect may resolve 2 weeks after the exposure in the absence of additional insults, such as cocaine or ethanol exposure, within the 2 weeks (Liberzon et al., 1997, 1999; Feng et al., 2015).

In conclusion, this study examined the effects of traumatic stress and ethanol exposure on ethanol intake and negative affect behaviors such as impulsivity and anhedonia. The combined exposure produced a protracted increase in dependence-induced ethanol intake. Also, traumatic stress exposure in mice caused impulsivity-like behavior, and repeated CIE vapor exposure resulted in anhedonia-like behavior. Future studies will examine the connections between these behavioral outcomes and the molecular mechanisms in the brain after PTSD-AUD exposure.

The cannabinoid (CB) system is one of the systems that could be affected by the comorbidity of PTSD and AUD, as previously reported by our lab (Matchynski-Franks et al., 2016). CB signaling is known to modulate the activation of stress responses (Crowe et al., 2014). Besides, cannabinoid 1 (CB1) receptors and the endocannabinoids play an important role in the motivation and reinforcement of ethanol and ethanol withdrawal (Varodayan et al., 2017). For example, CB1 receptor expression and function were downregulated while the ethanol consumption was increased after mice were exposed to CIE for two cycles (DePoy et al., 2013). However, the mechanisms by which the comorbidity of PTSD and AUD affects CB function are not yet known. Therefore, it is important to examine the disruption of CB regulation of PTSD and AUD comorbidity which could cause negative behavioral outcomes such as those found in this study.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Wayne State University Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

VP designed the experiments, performed the experiments, analyzed the data, and drafted the manuscript. SL performed the experiments. SP reviewed the data and interpreted the results. AC designed the experiments, reviewed the data, and interpreted the results. All authors provided critical inputs and revisions on the manuscript.

FUNDING

These studies were supported by resources from the John D. Dingell VA Medical Center in Detroit, Michigan, and were funded by U.S. Department of Veterans Affairs (VA) CDA-2 5-IK2-RX002686-02 (VP), CURES NIEHS P30 ES020957 (AC, SP), and in part by VA Merit 5-I01-RX002252-02 (SP, AC).

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Behavioral Neuroscience

REVIEW
published: 29 September 2020
doi: 10.3389/fnbeh.2020.582147

Drug-Induced Conditioned Place Preference and Its Practical Use in Substance Use Disorder Research

Greer McKendrick[1,2] *and Nicholas M. Graziane*[3]*

[1]*Neuroscience Graduate Program, Penn State College of Medicine, Hershey, PA, United States,*
[2]*Department of Anesthesiology and Perioperative Medicine, Penn State College of Medicine, Hershey, PA, United States,*
[3]*Departments of Anesthesiology and Perioperative Medicine and Pharmacology, Penn State College of Medicine, Hershey, PA, United States*

The conditioned place preference (CPP) paradigm is a well-established model utilized to study the role of context associations in reward-related behaviors, including both natural rewards and drugs of abuse. In this review article, we discuss the basic history, various uses, and considerations that are tied to this technique. There are many potential takeaway implications of this model, including negative affective states, conditioned drug effects, memory, and motivation, which are all considered here. We also discuss the neurobiology of CPP including relevant brain regions, molecular signaling cascades, and neuromodulatory systems. We further examine some of our prior findings and how they integrate CPP with self-administration paradigms. Overall, by describing the fundamentals of CPP, findings from the past few decades, and implications of using CPP as a research paradigm, we have endeavored to support the case that the CPP method is specifically advantageous for studying the role of a form of Pavlovian learning that associates drug use with the surrounding environment.

Keywords: conditioned place preference, CPP, drug reward, addiction-like behavior, drugs of abuse, substance use disorder, addiction, rodent model

OPEN ACCESS

Edited by:
Gregg Stanwood,
Florida State University, United States

Reviewed by:
Katherine Mercedes Holleran,
Wake Forest Baptist Medical Center, United States
Ellen M. Unterwald,
Temple University, United States

***Correspondence:**
Nicholas M. Graziane
ngraziane@pennstatehealth.psu.edu

Specialty section:
This article was submitted to Emotion Regulation and Processing, a section of the journal Frontiers in Behavioral Neuroscience

Received: *10 July 2020*
Accepted: *02 September 2020*
Published: *29 September 2020*

Citation:
McKendrick G and Graziane NM (2020) Drug-Induced Conditioned Place Preference and Its Practical Use in Substance Use Disorder Research. Front. Behav. Neurosci. 14:582147. doi: 10.3389/fnbeh.2020.582147

INTRODUCTION

Conditioned place preference (CPP) was developed as a technique to assess the reinforcing properties of opioid drugs (Rossi and Reid, 1976; Katz and Gormezano, 1979; Mucha and Iversen, 1984). Now, CPP is widely used to test context associations based on the rewarding properties of an unconditioned stimulus in many organisms including, rodents (Lu et al., 2005; Akbarabadi et al., 2018; Cunningham, 2019), flies (Kaun et al., 2011), *C. elegans* (Musselman et al., 2012; Engleman et al., 2018), planaria (Hutchinson et al., 2015; Mohammed Jawad et al., 2018; Adams and Byrne, 2019; Phelps et al., 2019), primates (Wang et al., 2012; Borges et al., 2015; Yan et al., 2015; Wu et al., 2016), and humans (Thewissen et al., 2006; Childs and De Wit, 2009, 2013, 2016). Although a widely used behavioral model, CPP is a complex behavior that incorporates Pavlovian learning, memory, and motivated behaviors. Due to the complexity, CPP findings are often difficult to understand and interpret. The purpose of this review article is to define common terms used throughout the CPP literature, as well as to discuss factors that are likely to contribute to CPP behaviors in mammals. We include a section related to the neurobiology of opioid-induced conditioned place preference and we conclude by discussing how CPP and addiction-like

behaviors can be combined experimentally to assess spatial memory involved in affective states, and to provide a quantifiable readout of context/environment-specific drug-seeking.

THE BIOLOGICAL PURPOSE OF PAVLOVIAN LEARNING AND HOW IT RELATES TO DRUG-INDUCED CPP

CPP is posited to be based on Pavlovian learning which refers to our ability to form relationships between temporally-associated stimuli. This form of learning as stated elegantly by Fanselow and Wassum (2015), has an evolutionary function that enables us to anticipate events and alter our behavior accordingly to promote survival (Fanselow and Wassum, 2015). Pavlovian learning is advantageous to reproduction (Domjan and Gutiérrez, 2019) as it influences hormonal responses (Graham and Desjardins, 1980), sexual performance (Zamble et al., 1985), and attraction (Domjan, 1994). For example, fish or quail exposed to a paired cue while seeing, but not interacting with a female, will have an increased number of offspring (fish) or increased number of sperm production and fertilized eggs (quail) when the cue is presented and the barrier between the male and female is removed (Hollis et al., 1989; Matthews et al., 2007). Additionally, Pavlovian learning prepares us for food consumption such that eating and digestion occur simultaneously. Pavlov showed that our physiological response to a cue associated with food will elicit salivary secretion and this salivary section is food-dependent (Pavlov, 2010). For example, the meat will evoke thick and viscous saliva containing high levels of mucus, while different substances like salt, acid, and mustard will evoke the release of "watery" saliva (Pavlov, 2010). Furthermore, Pavlovian learning prepares us for danger as well as rewards. In fear conditioning, neutral stimuli that become associated with an aversive event will evoke freezing behaviors in rodents (Rescorla, 1968; Fanselow, 1986; Iwata and Ledoux, 1988; Maren, 1999), while stimuli associated with drugs of abuse will evoke homeostatic alterations to counter previously experienced drug-induced changes (see "Opponent Process Theory in the Factors to Consider" Section in Siegel et al., 1982). In terms of substance use disorders, Pavlovian learning is critically important for context-induced relapse where re-exposure to drug-associated contexts evokes strong drug-craving (O'Brien et al., 1986, 1992). To study this form of relapse, one must understand how the brain forms and retains drug-context associations, which can be preclinically modeled using the CPP paradigm.

CONDITIONED PLACE PREFERENCE AS A MEASURE OF DRUG REWARD

CPP is used to measure associations formed between a rewarding stimulus (e.g., drug) and a contextual environment (Tzschentke, 2007). The paradigm uses a two or three-compartment apparatus with each compartment displaying distinct contextual characteristics (e.g., wall colors/patterns and floor texture). The CPP model consists of three phases: habituation, conditioning, and post-conditioning (i.e., CPP test). During habituation, animals are given free access to all compartments before they are returned to their home cage. The habituation sessions serve two purposes. First, they expose the animal to the apparatus, which is intended to habituate the animal to the environment, and second, they provide a measure of an animal's baseline preference for each compartment. Measuring the baseline preference allows the experimenter to perform a biased design [pairing a drug with the least preferred side to avoid ceiling effects when the drug is assigned to an already preferred environment (Cunningham et al., 2003)] or an unbiased design [randomly pairing the drug with a context; advantages and disadvantages of both designs can be found here (Cunningham et al., 2006)] as well as exclude animals based on predefined exclusion criteria (e.g., spending >80% of the time in one compartment). However, implementing a biased or unbiased design is up to the experimenter as evidence suggests, at least with morphine, that there are no differences in the outcome of CPP when employing a biased or unbiased approach (Blander et al., 1984). Conditioning sessions consist of a non-contingent (experimenter administered) injection of vehicle (control) or drug given before placing and confining the animal in a distinct context. Control and drug conditioning sessions occur on the same day (separated by 4–6 h) or on alternating days. These pairings take place one time or over multiple days. During the conditioning session, the drug-context associations become acquired (often referred to as the acquisition phase). Lastly, following conditioning sessions, animals undergo a CPP test where they are again given free access to all compartments and the time spent in the drug-paired side is measured, which provides a measure of CPP expression. Selective administration of test compounds can be used to assess effects on different phases of CPP. Administration prior to the drug-context pairing (i.e., conditioning phase) assesses the test compound's effects on the acquisition of CPP, while administration prior to the CPP test measures effects of the test compound on the CPP expression. CPP is measured as the total time spent in each context on test day, or as a CPP score. CPP scores are calculated as either: (i) time in the drug-paired context on test day minus time in the drug-paired context during habituation; or (ii) time in the drug-paired context on test day minus time in the vehicle-paired context on test day. Significant increases in time spent in the drug-paired side is associated with the rewarding properties of the drug.

For CPP, in the context of Pavlovian learning, the drug (i.e., the unconditioned stimulus) is expected to elicit a hedonic feeling of pleasure (i.e., an unconditioned response; **Figure 1**). The drug is paired with a distinct context in the CPP chamber (i.e., a neutral stimulus), which, following conditioning, becomes a conditional stimulus. After conditioning, in the absence of the drug (i.e., the unconditioned stimulus), the drug-paired chamber (i.e., conditional stimulus) is expected to evoke hedonic feelings of pleasure (i.e., conditioned response) leading to approach behaviors toward, and increased time spent in the drug-paired chamber. This approach behavior toward the drug-paired context is similar to sign-tracking behaviors (Huston et al., 2013) which refer to behaviors that are directed toward a stimulus as a result of that

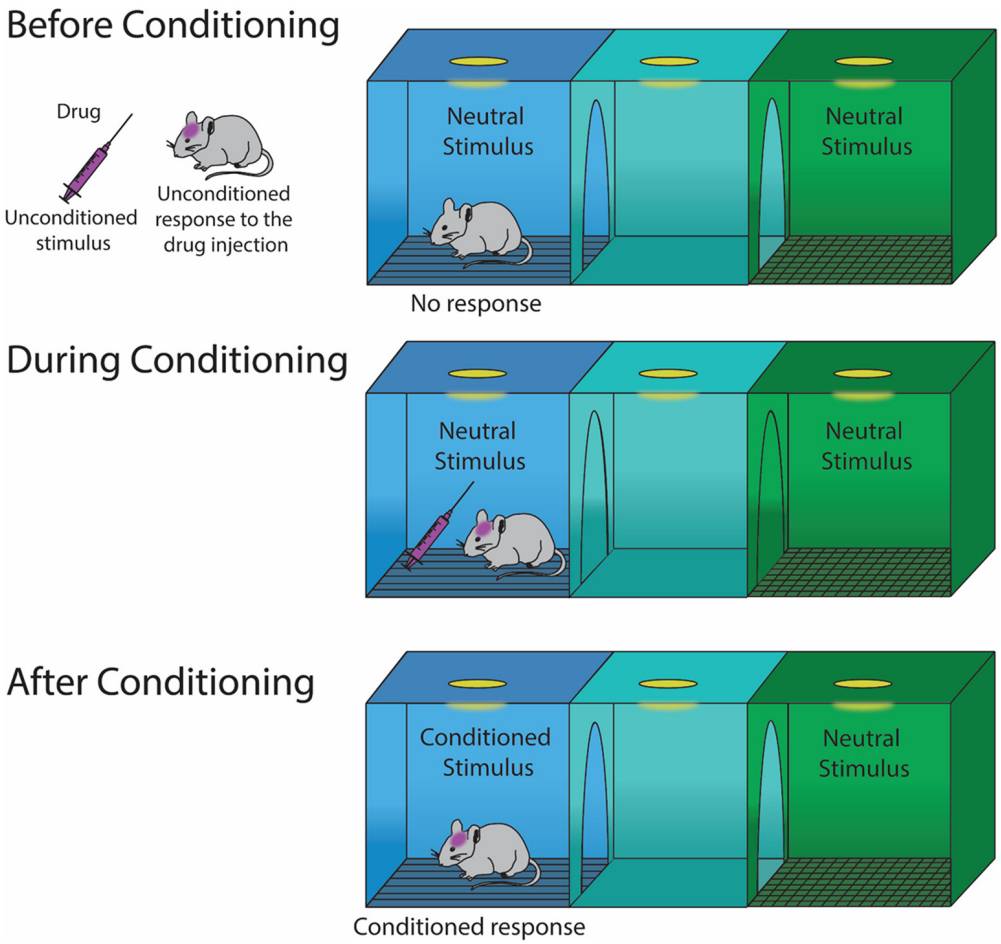

FIGURE 1 | Illustration of Pavlovian learning during conditioning with a drug that elicits hedonic feelings of pleasure. Before conditioning, a drug injection elicits an unconditioned response of pleasure. During conditioning, the neutral stimulus becomes a conditioned stimulus, which results in a conditioned response.

stimulus becoming associated with a reward (Huys et al., 2014). Despite this seemingly straightforward behavioral response, there may be many additional underlying factors that contribute to drug-induced CPP. The next section discusses factors that independently and/or synergistically may regulate this complex behavior.

FACTORS TO CONSIDER WHEN INTERPRETING CONDITIONED PLACE PREFERENCE

As stated above, conditioning in the CPP paradigm refers to pairing a drug with a context. Evidence suggests that a single drug-context pairing (Bardo and Neisewander, 1986; Fenu et al., 2006; Grisel et al., 2014; Nentwig et al., 2017) or repeated drug-context pairings (Cunningham et al., 2006; Dickinson et al., 2009; Ma et al., 2011; Otis and Mueller, 2011; Koo et al., 2014) induces CPP, but these varied exposure protocols may be influenced by different underlying factors including the rewarding properties of the drug, removal of an aversive state, conditioned behaviors, memory, and/or motivated states (**Figure 2**).

First, a logical interpretation of CPP is that it is mediated by the rewarding properties of the drug. Therefore, the animal seeks out or prefers the drug-paired context during the CPP test because this behavioral response has produced a beneficial, rewarding outcome. This is a valid interpretation and supported by neurobiological responses related to reward encoding that occurs during the conditioning sessions (Tzschentke, 1998, 2007). Additional support comes from human data which not only demonstrate that drug "liking" predicts room liking scores, but also shows the validity of CPP as a translational procedure. In one study, human male and female subjects received either *d*-amphetamine (20 mg) or placebo using a biased design (paired group). Treatments were alternated across successive sessions. A second group received *d*-amphetamine (20 mg) and a placebo in both rooms (unpaired group). The subjective mood was assessed using the Profile of Mood States and participants rated their liking and preference for the testing rooms once before the conditioning sessions and once during re-exposure to the test session after conditioning (Childs and De Wit, 2013). Using

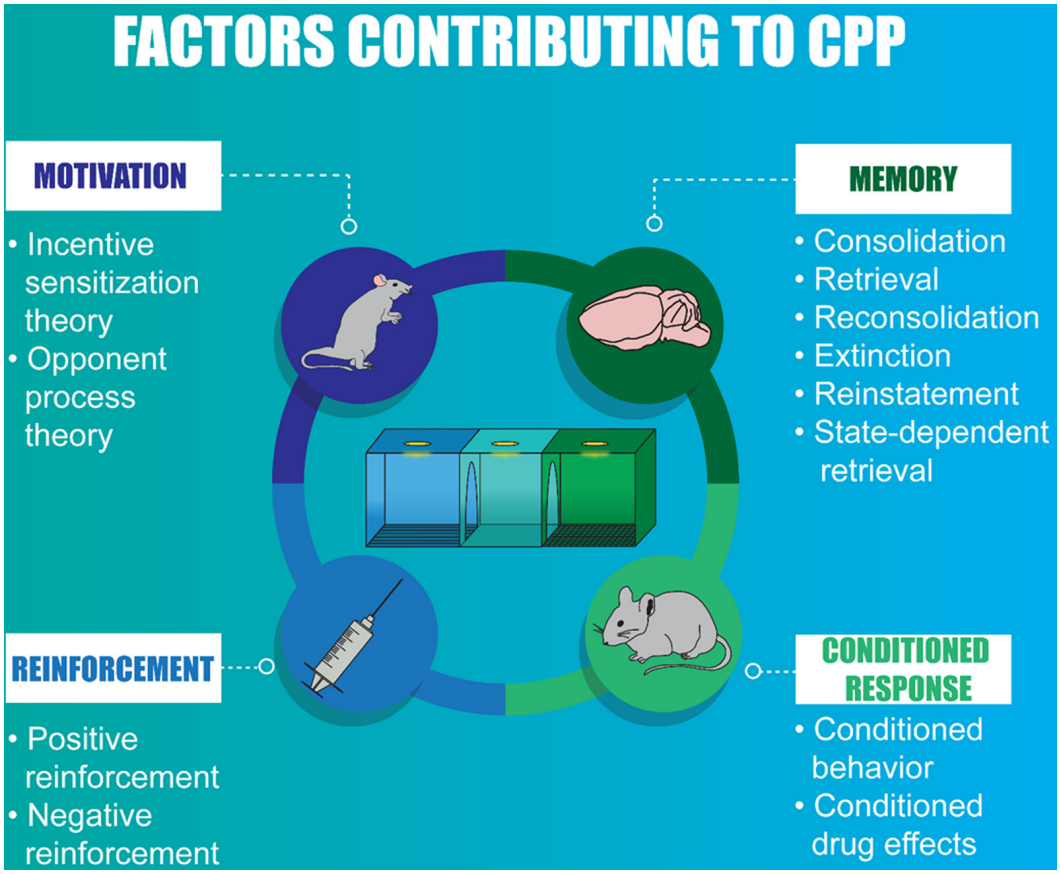

FIGURE 2 | Infographic summarizing the factors that contribute to conditioned place preference (CPP).

this model, Childs and De Wit (2013) showed that the acute positive and negative subjective responses to *d*-amphetamine significantly predicted changes in room liking after conditioning. Additionally, the authors also showed that the context of drug administration can influence acute drug effects on re-administration. This is supported by subjects in the paired group experiencing greater subjective stimulation and drug craving after *d*-amphetamine on the second administration relative to the first (Childs and De Wit, 2013). However, this context-dependent change in subjective drug response is likely drug class-specific as the same authors showed that alcohol effects were consistent across repeated administrations in the same vs. different contexts (Childs and De Wit, 2016). In this latter study, the authors showed that social drinkers developed a place preference for locations paired with alcohol, which was enhanced in subjects experiencing sedative effects from alcohol in those locations (Childs and De Wit, 2016). Therefore, interpreting CPP in rodents as drug reward is validated by human research and is dependent upon the drug, the drug-dose used, and/or the drug-exposure paradigm.

Removal of an aversive state to evoke CPP has been observed in animals expressing chronic pain, in which pairing a pain-relieving drug with a context during conditioning elicits CPP for the drug-paired context (King et al., 2009; Cahill et al., 2013; Navratilova et al., 2013). Additionally, in animals expressing chronic pain, inhibiting the anterior cingulate cortex (ACC), a brain region involved in pain expression, during context pairing elicits CPP for the context paired with ACC inhibition (Gao et al., 2020). These findings suggest that the removal of an aversive state, such as pain, reinforces the animal's behavioral response to prefer the drug-paired context. Decades of pre-clinical and clinical research have illustrated that, following repeated administration of many drugs of abuse (e.g., opioids, psychostimulants, nicotine, and alcohol), behaviors associated with negative affective states are observed during drug abstinence. These negative states can be physiological, including withdrawal and craving, but also psychological, such as anxiety and depression, and both separately or combined, may be relieved by drug exposure during conditioning, thus facilitating CPP. For example, we found that 5 days of repeated morphine exposure elicits anxiety-like behaviors as well as CPP and that removing the morphine-induced anxiety-like behavior using ketamine is sufficient to block morphine CPP (McKendrick et al., 2020a). Since we did not observe any physiological signs of withdrawal, such as jumping, wet dog shakes, teeth chattering, rearing,

tremor, and diarrhea [which coincides with the lack of observed somatic withdrawal symptoms following a more prolonged injection regimen of five daily morphine (10 mg/kg, i.p.) injections over 4 weeks (Robinson and Kolb, 1999)], our results suggest that CPP may be elicited, not only by the rewarding properties of morphine but also by the ability of morphine to relieve "psychological" rather than physiological withdrawal symptoms. There is also evidence that anxiety-like behaviors are linked to somatic withdrawal. Escalating doses of morphine (20–100 mg/kg, i.p.), over 6 days, induce anxiety-like behaviors in the marble burying task, as well as resulting in significant increases in piloerection, jumps, and ptosis (Becker et al., 2017). These studies demonstrate how altering the morphine exposure and concentration paradigm allows the researcher to distinguish morphine-induced negative-affective states from negative affect confounded by somatic signs of withdrawal. In line with this, fewer days of morphine exposure [three morphine injections (10 mg/kg) every other day] does not elicit anxiety-like behaviors (Benturquia et al., 2007), which highlights how the dosing regimen impacts the behavioral paradigm.

Other classes of drug of abuse may also evoke aversive states during conditioning such that the potential "rewarding" effects are mediated by the removal of aversive states. In line with this, pairing a distinct context with an intravenous injection of cocaine during conditioning elicits CPP when the pairing occurs immediately or 5 min after the injection (Ettenberg et al., 1999), however, cocaine-context pairings that occur 15 min after the cocaine injection elicit conditioned place aversion (Ettenberg et al., 1999). Likewise, ethanol, nicotine, and amphetamine exposure show that the immediate effects are rewarding, but that the delayed effects are aversive (Fudala and Iwamoto, 1986, 1987, 1990; Cunningham et al., 1997). Others have shown that, following chronic (14–28 days) non-contingent cocaine administration, rodents displayed less open arm exploration in the elevated plus-maze (Fung and Richard, 1994; Sarnyai et al., 1995; Basso et al., 1999; Rudoy and Van Bockstaele, 2007), which is an indication of the rodent expressing anxiety-like behavior. Furthermore, evidence suggests that repeated, non-contingent cocaine injections (i.e., daily cocaine injections that occur over 5 or 8 days) elicit cocaine-induced anxiety-like behavior when tested on abstinence day 9 or 15 (Valzachi et al., 2013; Hu et al., 2016). Therefore, it is possible that conditioning sessions that occur over many days result in drug-context pairings that alleviate drug-induced negative affective states, subsequently leading to preference for the drug-paired chamber.

Conditioning in the CPP paradigm may also elicit conditioned behavior and conditioned drug effects which, theoretically, may lead to increased or decreased time spent in the drug-paired side during CPP tests (for review, see Huston et al., 2013). Conditioned behaviors, which may be simple or complex, occur spontaneously during conditioning and are inadvertently reinforced during drug exposure, resulting in an increased frequency of the behavior (Skinner, 1948; Staddon and Simmelhag, 1971; Huston et al., 2013). During the test, the drug-paired context may elicit spontaneous behavior (e.g., grooming, rearing, and repetitious movements) and prevent the animal from leaving the conditioned compartment (Huston et al., 2013). Conditioned drug effects refer to drug-induced behavioral responses that become associated with a drug-paired context. After conditioning, re-exposure to the drug-paired context may elicit the reinforced behavior, which may prevent the animal from leaving the drug-paired context, or mask drug-induced CPP (Huston et al., 2013). An example of masked drug-induced CPP is evident from hyperactivity in animals following cocaine administration. This cocaine-induced hyperactivity becomes conditioned to the drug-paired context, which results in conditioned hyperactivity during the CPP test (Saunders et al., 2014). This increased locomotion may increase the probability that the animal leaves the conditioned compartment, thus, inadvertently reducing the true cocaine-induced CPP (Huston et al., 2013).

Memory is another factor to consider that may influence drug-induced CPP. Most CPP tests occur during a period of drug abstinence, so the learned associations that occur during conditioning session/s would have to have been consolidated and maintained for the animal to be able to recall the association when re-exposed to the CPP chamber on test day. Upon drug re-exposure, the memory is retrieved and destabilized, which enables the memory to be updated with new information. Subsequently, the memory is restabilized in a process called reconsolidation (Torregrossa and Taylor, 2013, 2016). Therefore, it is plausible that drug-induced CPP relies on three phases of memory: consolidation, retrieval, and reconsolidation (Milton and Everitt, 2010). Each memory phase is vulnerable to interference in a CPP paradigm, depending upon the time point that the memory interference is initiated by the experimenter. Typically, administering a test compound shortly *after* a conditioning session will assess effects on memory consolidation (Cervo et al., 1997; Hsu et al., 2002; Robinson and Franklin, 2007; Yu et al., 2009). After conditioning is completed, exposing an animal to a test compound just before re-exposure to the CPP apparatus will assess the effects on memory retrieval (Miller and Marshall, 2005; Yim et al., 2006; Fan et al., 2013), and exposing an animal to a test compound following re-exposure to the CPP apparatus will assess the effects on memory reconsolidation (Brown et al., 2007; Otis et al., 2013; Sartor and Aston-Jones, 2014). The timing of test compound administration, if pharmacologically mediated, depends upon the pharmacokinetic properties of the compound. Of note, re-exposure to the CPP apparatus is not the only way to retrieve drug-associated contextual memory as the rewarding properties of the drug may establish state-dependent retrieval (Overton, 1972). In state-dependent retrieval, CPP is more strongly expressed in the presence, vs. the absence, of the drug. This occurs as the learned associations are formed in the presence of the drug during conditioning. Therefore, if the animal learns the associations in a drugged state and performs the test in a drug-free state, retrieval deficits may result due to changes in the internal state of the animal (Spear, 1978; Urcelay and Miller, 2008).

Additionally, CPP memory is liable to extinction and reinstatement. Extinguishing CPP occurs over many days and is often performed by confining the animal to the drug-paired compartment in the absence of the drug, then, on the next day, the animal is given free access to all compartments. This procedure is repeated until the animal reaches extinction criteria (Hearing et al., 2016). CPP is then reinstated with a drug-prime injection or stress exposure (Aguilar et al., 2009).

Reinstatement paradigms are frequently compared to the human experience known as "relapse," but an important distinction is that relapse in humans is often characterized by a resumption of drug-taking, whereas in rodent models, these reinstatement models are performed in a drug-free state and/or without the ability to continue drug exposure (Sanchis-Segura and Spanagel, 2006). Therefore, it is more accurate to state that reinstatement in CPP more directly reflects a continuation of CPP behaviors, whether it be triggered by a drug-prime injection or a stressor. Types of stressors that have been utilized to trigger reinstatement include: (1) naturalistic stressors, such as water/food deprivation, physical restraint stress (Ribeiro Do Couto et al., 2006), painful stimuli such as the foot-shock paradigm (Wang et al., 2000; Sanchez and Sorg, 2001), and fear/anxiety-inducing stimuli such as the forced swim stress (Sanchez and Sorg, 2001; Ribeiro Do Couto et al., 2006; Redila and Chavkin, 2008); (2) social disruption/conflict stressors including social isolation and maternal deprivation (Ribeiro Do Couto et al., 2006; Calpe-López et al., 2020); and (3) pharmacological stressors, such as injections of agonists of the kappa opioid system (Redila and Chavkin, 2008), and yohimbine (Mantsch et al., 2010). While comparisons of drug-prime vs. stressor-induced reinstatement models are common concerning operant drug self-administration paradigms, they are rather limited in the field of CPP (Mantsch et al., 2016). The findings of Ribeiro Do Couto et al. (2006) demonstrate that social defeat stress is similar to physical restraint stress at reinstating morphine conditioned place preference. Also, one study by Wang et al. (2000) found that both foot-shock stress and an acute morphine prime injection sufficiently reinstated extinguished morphine conditioned place preference. Therefore, future studies are needed to directly compare CPP reinstatement models.

Motivation may also contribute to increases in time spent in the drug-paired compartment during CPP tests. Evidence for this comes from a study showing that a hungry animal will approach contexts previously associated with food, whereas the same animal, when water-deprived, will approach contexts associated with fluid (Perks and Clifton, 1997). Similarly, with drugs of abuse, morphine-dependent chimpanzees given daily, passive injections of morphine and then trained to choose between a white box hiding a syringe filled with morphine or a black box hiding a banana, will choose the white box when deprived of morphine, and choose the black box when pretreated with their daily dose of morphine (Spragg, 1940). These drug-induced motivated behaviors are potentially explained by the combined incentive sensitization and opponent-process theories of substance use disorders (Koob et al., 1989; Robinson and Berridge, 1993, 2008). Here, the drug of abuse elicits an unnatural, strong hedonic sensation of pleasure resulting in the drug becoming highly salient, attractive, and "wanted" (Robinson and Berridge, 1993). Meanwhile, the brain automatically compensates and dampens drug reward by recruiting opponent processes, which, over time, following repeated drug exposure, become quicker, stronger, and longer-lasting, leading to negative affective states (Solomon and Corbit, 1978; Koob et al., 1989; Koob and Le Moal, 2008; Grisel, 2019). It is possible that, in patients diagnosed with substance use disorders, a reward is required to sufficiently curtail these negative affective states. Given the incentive salience that the nervous system attributes to the act of drug taking, the negative affective state may drive drug craving and the recall of Pavlovian associations related to drug taking, thus directing motivated drug-seeking behaviors (O'Brien, 1975; Perkins and Grobe, 1992; Zinser et al., 1992; Wetter et al., 1994; Cooney et al., 1997; Baker et al., 2004; Conklin and Perkins, 2005; Fox et al., 2007; Wikler, 2013).

When interpreting CPP, considering factors such as the rewarding properties of the drug, alleviation of aversive states, conditioned behavior, conditioned drug effects, memory, and/or motivational states, has the potential to lead to more comprehensive assessments. Additionally, considering how these factors work independently and/or synergistically has the potential to explain drug-specific effects that direct behaviors toward, or away from, a stimulus, and/or the underlying neurobiological mechanisms contributing to the behavior. These factors may be unique to addictive-drug categories (e.g., opioids, psychostimulants, cannabis, dissociative, inhalants, depressants, and hallucinogens), or to addictive drugs vs. natural rewards (Spiteri et al., 2000; Yonghui et al., 2006; Steiner et al., 2013).

NEUROBIOLOGY OF DRUG-INDUCED CPP: FOCUS ON OPIOID CPP

Ongoing research investigates the neurobiological mechanisms that regulate CPP, with evidence supporting the role of the central nervous system in mediating learned associations. Seminal work by Schultz et al. (1997) showed that, in monkeys, dopamine neuron firing occurred directly after a juice reward, but over time, these neurons began to fire upon exposure to a light cue that preceded the reward. Further support comes from studies showing that neuronal activation in the ventral tegmental area (VTA), a brain region where dopamine neurons are expressed, is necessary for the acquisition of morphine CPP (Harris et al., 2004; Moaddab et al., 2009). Additional studies show that lesions of VTA dopaminergic terminals in the ACC block opioid-induced CPP (Narita et al., 2010), while *in vivo* stimulation of VTA dopaminergic projections to the nucleus accumbens enhances morphine CPP (Koo et al., 2012). Additionally, increases in dopamine and dopamine metabolites in the nucleus accumbens are correlated with morphine CPP (Ma et al., 2009), and blocking dopamine receptors in the nucleus accumbens and basolateral amygdala prevents the acquisition of morphine CPP (Fenu et al., 2006; Lintas et al., 2011, 2012). Although evidence suggests that the nucleus accumbens and potentially the VTA are not necessary and sufficient for

the acquisition of morphine CPP (shown by lesions in the nucleus accumbens or CPP tests in dopamine-deficient mice; Olmstead and Franklin, 1996; Hnasko et al., 2005), more recent reports show that transient inactivation of the VTA or nucleus accumbens inhibits both acquisition and expression of morphine CPP (Moaddab et al., 2009; Esmaeili et al., 2012).

Further support of the nucleus accumbens and VTA in mediating morphine CPP comes from studies focusing on molecular signaling cascades in these brain regions. For example, evidence suggests that the activation of p38 mitogen-activated protein kinase (MAPK) and the transcription factor, nuclear factor-κB (NF-κB) in the nucleus accumbens is critically involved in the acquisition of morphine CPP and that this signaling cascade potentially relies upon the activation of transient receptor potential vanilloid type 1 channel (TRPV1; Zhang et al., 2011, 2012; Hong et al., 2017). Furthermore, antagonizing the transcription factor, ΔFosB in dynorphin-expressing medium spiny neurons, which are putative dopamine D1receptor-expressing medium spiny neurons (McDevitt and Graziane, 2018), or inhibiting the transcription factor cAMP response element (CRE)-binding protein (CREB) in the nucleus accumbens, decreases and increases morphine CPP, respectively (Nestler et al., 2001; Barrot et al., 2002; Zachariou et al., 2006). Lastly, the inhibition of phosphodiesterase (PDE) 10A, which inhibits cAMP- and cGMP-mediated intracellular signaling and is selectively expressed in the nucleus accumbens, inhibits the acquisition of morphine-induced CPP (Mu et al., 2014). In the VTA, inhibition of the mitogen-activated protein kinase kinase (MEK)-extracellular signal-regulated kinase (ERK) pathway blocks the acquisition of morphine CPP (Lin et al., 2010).

Also, there are other brain regions involved in opioid CPP such as the pedunculopontine tegmental nucleus (PPTg), which, when lesioned, blocks morphine-induced CPP (Olmstead and Franklin, 1997; Olmstead et al., 1998). The PPTg sends cholinergic inputs to the VTA, which elicit dopamine-neuron depolarization and increase firing in activated neurons (Floresco et al., 2003). Therefore, it would be expected that the cholinergic neurons of the PPTg are involved in the formation of opioid-context associations *via* activation of dopamine neurons in the VTA. However, evidence suggests that, in heroin-induced CPP, PPTg cholinergic cells that project to the VTA are not involved in opioid CPP (Steidl et al., 2014). Rather, orexin neurons that project from the lateral hypothalamus to the VTA, and the hippocampal dentate gyrus, are critical in the formation of associations between contextual cues and morphine (Harris et al., 2007; Guo et al., 2016) with evidence suggesting that this morphine-induced activation of orexinergic neurons relies on corticotropin-releasing factor 1 receptor (CRF1R) activation in morphine CPP (Lasheras et al., 2015).

Seminal work in the field of fear conditioning supports the role of the hippocampus in mediating contextual encoding (Selden et al., 1991; Kim and Fanselow, 1992; Phillips and Ledoux, 1992) and research in opioid-induced contextual learning suggests that the hippocampus may play a similar role. The activation of cholinergic and dopaminergic systems in the dorsal hippocampus regulates the acquisition of morphine CPP (Rezayof et al., 2003, 2006). Additionally, long-term potentiation (enhanced synaptic transmission) in the CA1 region of the hippocampus, which is associated with learning and memory (Kauer and Malenka, 2007), is disrupted (unknown whether it is blocked or occluded) in rodents expressing morphine CPP (Portugal et al., 2014). *In vivo* electrophysiological studies have shown that long-term potentiation of glutamate transmission at hippocampal ventral subiculum to the nucleus accumbens shell is facilitated in rats following re-exposure to the morphine-paired chamber (Li et al., 2017), with evidence suggesting that this potentiation is involved in spatial learning (Goto and Grace, 2005). Recently, it has been shown that astrocytic μ-opioid receptor activation in the CA1 region of the hippocampus is necessary and sufficient to enhance synaptic transmission at Schaffer collateral to CA1 synapses and that this long-term potentiation leads to the acquisition of contextual memory (Nam et al., 2019).

There are also molecular signaling cascades in the hippocampus involved, in part, in long-term potentiation that are important for the acquisition of morphine CPP. Inhibiting phosphatidylinositol 3-kinase (PI3K) or its downstream target mammalian target of Rapamycin (mTOR) in hippocampal CA3 prevents the acquisition of morphine CPP and inhibits the morphine-induced activation of PI3K-Akt signaling pathway (Cui et al., 2010). Additionally, inhibiting ERK in the ventral hippocampal-medial prefrontal cortical circuit blocks the formation of opiate contextual memory (Wang et al., 2019).

The central amygdala, another brain region involved in fear conditioning (Ciocchi et al., 2010; Goode and Maren, 2019), also influences the acquisition of morphine CPP in this case, through NMDA receptor and dopamine-D1 receptor activation (Zarrindast et al., 2003; Rezayof et al., 2007). Additionally, inhibition of MEK or NMDA receptors in the central amygdala blocks the expression of morphine-induced place preference (Li et al., 2011).

Cortical regions are also involved in the acquisition of morphine-induced Pavlovian learning including viscerosensory regions like the somatosensory cortex and granular insular cortex, which when lesioned, block morphine CPP (Meng et al., 2009; Li et al., 2013). Additionally, blocking NMDA receptors in the prelimbic cortex, a brain region involved in promoting relapse to both fear and drug-seeking (Ma et al., 2014; Goode and Maren, 2019), potentiates the acquisition of morphine CPP, likely mediated by dopamine receptor activation, glutamate release, and basolateral amygdala activation (Bishop et al., 2011). Furthermore, norepinephrine depletion in the medial prefrontal cortex impairs the acquisition of morphine CPP (Ventura et al., 2005).

As evidenced above, morphine CPP is regulated by glutamatergic, cholinergic, and dopaminergic systems. In addition to this, preclinical evidence suggests that morphine-context associations rely on signaling from another neurotransmitter, hormonal, and neuromodulatory systems, including opioid, GABA, norepinephrine, serotonin, cannabinoid, nitric oxide, hypocretin/orexin, neuropeptide S, and cholecystokinin (Tzschentke, 1998, 2007; Le Merrer et al., 2009; Li et al., 2009; Billa et al., 2010; Karimi et al., 2013;

Ghavipanjeh et al., 2015; Loureiro et al., 2016; Zhang et al., 2016; Azizbeigi et al., 2019) as well as systems involved in immune function and inflammation (Ghahremani et al., 2006; Zhang et al., 2012; Chen et al., 2017). Additionally, evidence suggests that morphine-induced suppression of endogenous histamine is important for morphine CPP as bilateral lesions of the tuberomammillary nucleus, a brain region that expresses histamine-releasing neurons, potentiated the development of morphine CPP (Gong et al., 2007). Also, activation of scaffolding proteins such as receptor for activated protein kinase C 1 (RACK1) is necessary for morphine CPP (Wan et al., 2011; Liu et al., 2016). Given the rewarding properties of drugs of abuse, it is reasonable to expect that a drug of abuse that elicits hedonic feelings (i.e., pleasant sensations) will activate neurobiological mechanisms that signal reward during each conditioning trial. However, many of the neurobiological mechanisms described above are potentially involved in negative affective states depending upon the drug exposure paradigm, the induction of dependence or tolerance, and/or the drug class used. The suppression of these negative affective states during conditioning may also contribute to CPP.

Negative affect observed during drug abstinence is timed with neurobiological responses that mediate negative affective states (Koob, 2013, 2020). For example, following repeated exposure to morphine, there are increases in norepinephrine-induced modulation of the extended amygdala (Aston-Jones et al., 1999; Delfs et al., 2000; Smith and Aston-Jones, 2008), activation of the amygdalar corticotrophin-releasing factor (CRF) system (Heinrichs et al., 1995; Maj et al., 2003), norepinephrine release in the extended amygdala (Fuentealba et al., 2000; Aston-Jones and Harris, 2004), and decreases in dopamine transmission (Diana et al., 1995). Also, following repeated exposure to cocaine, the lateral habenula, a brain region whose increased activity is correlated with aversive states (Graziane et al., 2018), has increased activation 15 min after repeated cocaine administration (Jhou et al., 2013), with evidence suggesting that this increase in cocaine-induced lateral habenula activation lasts until abstinence day 2 in rodents with a history of cocaine self-administration (Neumann et al., 2014). Additionally, lateral habenula neuronal firing is increased *in vivo* during ethanol-induced conditioned taste aversion (Tandon et al., 2017). Finally, when activated, the dynorphin-κ opioid system produces aversion and dysphoria in humans and in animals (McLaughlin et al., 2003, 2006; Land et al., 2008, 2009; Sirohi and Walker, 2015), with evidence suggesting that this system is activated during drug abstinence, potentially driving drug-induced negative affective states (Mucha and Herz, 1985; Pfeiffer et al., 1986; Wee and Koob, 2010; Chartoff et al., 2012).

Combined, this section highlights how many different brain regions involved in signaling salient cues (VTA and nucleus accumbens), contributing to affective, emotional, and cognitive control (amygdala, insula, prefrontal cortex, and ACC), signaling sensation (somatosensory cortex), and processing spatial information and memory (hippocampus) work together to acquire and maintain drug-context associations. Interestingly, these same brain regions are implicated in processes related to pain (Bushnell et al., 2013; Navratilova and Porreca, 2014) and fear (Goode and Maren, 2019) demonstrating how pathological processing within and between these brain regions can lead to pathological behaviors that are easily differentiated clinically, but influence similar neurocircuit connections, albeit, likely in different ways.

CONDITIONED PLACE PREFERENCE COMBINED WITH ORAL SELF-ADMINISTRATION

Substance use disorder is a chronic, relapsing condition that is characterized by specific hallmark behaviors including the difficulty to stop drug use, augmented motivation to seek and take drugs, continued use despite adverse consequences, and high susceptibility to relapse. Addiction-like behaviors, therefore, encompass all aspects of behavior that contribute to these criteria and can be observed in both basic and clinical settings. CPP fundamentally tests the incentive value of contexts, and how environmental conditions contribute to the formation of drug-context associations. The long-term nature of drug context-seeking behavior is evident in our CPP paradigm, in which we observe a robust CPP with 28 days of abstinence following conditioning and drug exposure (McKendrick et al., 2020a). In line with this finding, the motivation to seek a context associated with a drug is seen by the induction of approach behaviors, with CPP tests following conditioning (Aguilar et al., 2009), which can persist for 12 weeks without any additional drug exposure (Mueller et al., 2002). Similar to operant self-administration models, reinstatement following extinction is also reliably shown in CPP. Reinstatement of drug-induced CPP can be induced by both stress (Wang et al., 2002; Aguilar et al., 2009) and drug-primes (Mueller et al., 2002; Aguilar et al., 2009), all with the added component of drug-associated contexts. The ability to reinstate CPP is indicative of persistent drug-associated memories, which likely results in the propensity to elicit drug cravings and/or directs drug-seeking long into abstinence (O'Brien et al., 1986, 1992). Furthermore, work by LeCocq et al. (2020) has advocated the ability of drug-associated contexts to serve as a vital trigger for reinstatement and renewal of extinguished addiction-like behaviors.

Understanding how drugs of abuse become associated with contexts is critical in the study of addiction to deconstruct how contexts influence drug-seeking behaviors, relapse propensity, and treatment success. Through repetitive pairings with drug use, contexts that were previously neutral gain incentive salience, and this intense association can serve to reinforce the cyclical nature of drug-seeking behaviors. In animal models of drug abuse, the ABA renewal paradigm [whereby the subject is conditioned in one context (A), extinguished in another (B), and is then re-exposed to the original context (A)] emphasizes how environmental contexts that have been associated with drug use can directly prompt reinstatement (LeCocq et al., 2020). Clinical research has established that drug-associated contexts promote cue reactivity, elevate craving responses, and are sufficient to elicit context-induced relapse (LeCocq et al.,

2020). Recent studies have suggested that a way to improve the discovery of more effective treatments is to accentuate the influence of environmental contexts and their influence on drug-seeking behaviors (Everitt and Robbins, 2005; Aguilar et al., 2009; LeCocq et al., 2020). Therefore, CPP serves as a specialized paradigm that can be exploited just for this purpose.

Seeking the drug-paired context (approach toward and spending more time in the drug-paired context) is not typically considered drug-seeking. This is because drug-seeking behaviors are associated with operant responses for a drug, and occur during an extinction session in a self-administration model [see Marchant et al. (2013) for a description of this model]. Here, an animal that has learned to press a lever or nose poke into an active hole to receive an intravenous drug injection, continues this behavior in the absence of the drug. The amount of lever presses is measured and associated with drug-seeking behavior. In the CPP model, the drug is administered non-contingently in a paired context, so the approach to the context and time spent in the context is not typically considered drug seeking because the drug has not been operantly available during the conditioning session. However, one may argue that drug-seeking requires a motivated response directed toward a context associated with the drug. In this case, drug-context seeking is a critical step in the process of drug-seeking. The problem, thus far, with this argument is that there has not been any direct measure in the CPP paradigm to demonstrate that drug-context seeking is linked to drug-seeking behaviors. Recently, we have developed a novel CPP approach in which mice can consume solutions while confined to either context, thus, enhancing the paradigm by including a voluntary, motivated behavior [see McKendrick et al. (2020b) for details regarding methodology, set-up, and figures illustrating the procedure]. This oral self-administration model can include natural rewards such as sucrose and saccharin solutions, or drug-containing solutions like morphine. When mice are conditioned with a solution of 0.1 mg/ml morphine dissolved in 0.2% saccharin in one chamber and only 0.2% saccharin in the opposing chamber, there is a significant preference for the morphine-paired context on test day. Furthermore, to incorporate instrumental, drug-seeking behaviors with CPP, water bottles were placed in each context on test day. In addition to CPP, morphine-conditioned mice consumed significantly more water on the morphine-paired side, a finding not observed in saccharin controls (McKendrick et al., 2020b). These results suggest that drug-context seeking in the CPP model is not a passive state and is potentially important for directing drug-seeking behavior. As denoted above, this method can expand on traditional CPP paradigms by including a voluntary drug-taking aspect, which allows one to study the importance of learned drug-context associations that are based on distinguishing spatial characteristics represented by distinct environments, similar to human experiences.

CONCLUSION

Drug-induced CPP is a Pavlovian-based behavior, used to model the transition of a neutral stimulus to a conditioned stimulus, which drives a conditioned response (i.e., approach behaviors to a drug-paired context). This complex behavior consists of many overlapping components that may work synergistically or independently to drive place preference. Although not considered a gold standard for modeling addiction-like behaviors, CPP provides a valuable tool that can be used to understand how drugs of abuse become associated with environmental contexts, a process which is implicated in context-induced drug craving and relapse (O'Brien et al., 1986, 1992). Additionally, this approach can provide insight into contingency awareness [knowledge that the conditioned stimuli predict the unconditioned stimulus (Grillon, 2002)], which relies on conscious cognitive operations (Dawson and Furedy, 1976; Lovibond and Shanks, 2002). Despite the limitation of non-contingent drug administration, drug-induced CPP provides a measure of motivated approach behaviors toward a drug-associated environment, which is a critical step in drug-seeking behaviors.

AUTHOR CONTRIBUTIONS

GM and NG developed the focus of this review article and wrote the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the Brain and Behavioral Research NARSAD Young Investigator Award (27364NG), the Pennsylvania State Junior Faculty Scholar Award (NG), the Pennsylvania Department of Health using Tobacco CURE Funds (NG), and the Pennsylvania State Research Allocation Project Grant (NG).

ACKNOWLEDGMENTS

We thank Dr. Diane McCloskey for edits and comments on this project and Dr. Yuval Silberman for his input on the focus of the review article. We would also like to thank Reviewers Katherine Mercedes Holleran and Ellen M. Unterwald who suggested many important additions to the final version of this manuscript, including, but not limited to, elaboration on reinstatement modalities and neurobiology of CPP, the inclusion of human data, and the proper use of terminology.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Behavioral Neuroscience

REVIEW
published: 20 October 2020
doi: 10.3389/fnbeh.2020.588400

Turning the ′Tides on Neuropsychiatric Diseases: The Role of Peptides in the Prefrontal Cortex

Dakota F. Brockway[1] *and Nicole A. Crowley*[1,2]*

[1]Neuroscience Curriculum, Pennsylvania State University, University Park, PA, United States, [2]The Department of Biology, Pennsylvania State University, University Park, PA, United States

Recent advancements in technology have enabled researchers to probe the brain with the greater region, cell, and receptor specificity. These developments have allowed for a more thorough understanding of how regulation of the neurophysiology within a region is essential for maintaining healthy brain function. Stress has been shown to alter the prefrontal cortex (PFC) functioning, and evidence links functional impairments in PFC brain activity with neuropsychiatric disorders. Moreover, a growing body of literature highlights the importance of neuropeptides in the PFC to modulate neural signaling and to influence behavior. The converging evidence outlined in this review indicates that neuropeptides in the PFC are specifically impacted by stress, and are found to be dysregulated in numerous stress-related neuropsychiatric disorders including substance use disorder, major depressive disorder (MDD), posttraumatic stress disorder, and schizophrenia. This review explores how neuropeptides in the PFC function to regulate the neural activity, and how genetic and environmental factors, such as stress, lead to dysregulation in neuropeptide systems, which may ultimately contribute to the pathology of neuropsychiatric diseases.

Keywords: prefrontal cortex, peptides, animal models, circuitry, neurons, animal behavior

OPEN ACCESS

Edited by:
Jessica R. Barson, Drexel University, United States

Reviewed by:
Sofiya Hupalo, National Institutes of Health (NIH), United States
Max Joffe, Vanderbilt University, United States

***Correspondence:**
Nicole A. Crowley
nzc27@psu.edu

Specialty section:
This article was submitted to Emotion Regulation and Processing, a section of the journal Frontiers in Behavioral Neuroscience

Received: *28 July 2020*
Accepted: *09 September 2020*
Published: *20 October 2020*

Citation:
Brockway DF and Crowley NA (2020) Turning the ′Tides on Neuropsychiatric Diseases: The Role of Peptides in the Prefrontal Cortex. Front. Behav. Neurosci. 14:588400. doi: 10.3389/fnbeh.2020.588400

INTRODUCTION

The Prefrontal Cortex in Humans and Rodents: Executive Control Over Neuropsychiatric Disorders

The prefrontal cortex (PFC), located in the anterior portion of the frontal lobe, is responsible for several higher-order behaviors including executive function and response to emotional stimuli (Salzman and Fusi, 2010; Grossmann, 2013). The PFC of humans has been implicated in many stress-related neuropsychiatric disorders, including anxiety (Park and Moghaddam, 2017), major depressive disorder (MDD; Murray et al., 2011), post-traumatic stress disorder (PTSD; Koenigs and Grafman, 2009), and substance use disorders (Goldstein and Volkow, 2011). Importantly, the PFC is one of the brain regions most sensitive to the detrimental effects of stress (Arnsten, 2009; Kolb et al., 2012). Stress has been shown to lead to PFC dysfunction observed in various neuropsychiatric disorders. Moreover, the PFC is known to undergo profound alterations throughout development (Teffer and Semendeferi, 2012), and is one of the last areas of the cortex to develop (Fuster, 2001).

In addition to the extensive human literature, the role of the PFC in behaviors associated with stress and neuropsychiatric disease has been heavily studied using rodent and non-human primate models. Though the role of the PFC in animal models has been heavily debated, recent attempts to standardize the definition and anatomical framework of the PFC have led to increased consistency of research (for review and synthesis see Carlén, 2017; Laubach et al., 2018). The PFC is divided dorsoventrally into various subregions; the human literature often divides the PFC into the lateral PFC (Broadmann areas 9–12 and 25) and the medial PFC (Broadmann Areas 9–12 and 44–46; Grossmann, 2013), whereas the rodent literature often sub-divides the PFC into infralimbic, prelimbic, and anterior cingulate cortex (Laubach et al., 2018). Animal model-based investigations of the PFC are allowing for a greater understanding of prefrontal cortical networks.

The PFC has both complex local circuitry and connections with other brain regions (Kolb et al., 2012). The PFC is heavily connected with other regions such as the brainstem, the thalamus, the basal ganglia, and limbic system (for review and synthesis see Van Eden and Buijs, 2000; Fuster, 2001). Its well-organized reciprocal connections with the mediodorsal nucleus of the thalamus (MD) is used as a criterion for identifying the PFC in a variety of species (Ferguson and Gao, 2015). Connections between the MD and PFC have been linked with cognitive impairment observed in many different neuropsychiatric disorders (for review and synthesis see Ouhaz et al., 2018). Moreover, excitatory afferents to the PFC arise from several other brain regions including limbic areas related to emotion such as the amygdala (Porrino et al., 1981; Lowery-Gionta et al., 2018), hippocampus (Thierry et al., 2000; Dégenètais et al., 2003; Bogart and O'Donnell, 2018), and hypothalamus (Kievit and Kuypers, 1975; Jacobson et al., 1978). Afferent projections from limbic regions carry to the PFC information about internal states and motivational significance and likely play a major role in executive control over emotional behavior (LeDoux, 1993; Fuster, 2001). The PFC also sends glutamatergic projections to multiple brain regions responsible for regulating emotional behaviors (some of them reciprocal) including the amygdala (McGarry and Carter, 2017; Bloodgood et al., 2018), the bed nucleus of the stria terminalis (BNST; Crowley et al., 2016), the striatum (Stuber et al., 2011; Britt et al., 2012; Bloodgood et al., 2018), and the periaqueductal gray (Siciliano et al., 2019).

Peptide Populations Within the Prefrontal Cortex

Neurons within the PFC express a variety of markers and neurotransmitters (Van De Werd et al., 2010). Neuropeptides are strings of amino acids connected by peptide bonds found in the central nervous system (CNS) which play a key role in modulating neural activity (see van den Pol, 2012 for review and synthesis). Unlike classical neurotransmitters (e.g., amino acid neurotransmitters such as GABA and glutamate), neuropeptides are large molecules that are stored in large dense-core vesicles. They are often co-released along with other amino acid neurotransmitters and neuropeptide release is not restricted to the synapse. Neuropeptides diffuse long distances to act on G-protein coupled receptors. Compared to fast-acting amino acid neurotransmitters the response of receptive cells to neuropeptides is slow (often several seconds to minutes), which makes the investigation of neuropeptides complex.

Peptide-expressing gamma-aminobutyric acid (GABA)-ergic neurons (which often co-release neuropeptides) in the PFC have received dense focus throughout the decades due to their strong regulation of both glutamatergic inputs to and outputs from the PFC, as well as their ability to modulate each other. This, combined with the extensive role of the PFC in stress and neuropsychiatric disorders, has led to a keen interest in their function (Northoff and Sibille, 2014; Fogaça and Duman, 2019; McKlveen et al., 2019; Ghosal et al., 2020). These neuronal populations release both GABA and their respective neuropeptides, allowing for complex regulatory control over PFC circuits though the precise dynamics of peptide transmission vs. GABA transmission are still being elucidated throughout the brain. Also, some GABA/peptidergic neurons are thought to express multiple peptides, and it is unclear under what conditions these individual (and sometimes functionally opposing) peptides are released. Despite these challenges in studying neuropeptides, recent technological advances have made it easier to investigate peptidergic transmission in various brain regions (Al-Hasani et al., 2015, 2018; Crowley et al., 2016), including detecting neuropeptide release within the PFC (Dao et al., 2019).

Recent research outlined in this review sheds light on the role of diverse neuropeptides in the PFC in regulating cortical networks and controlling emotional behaviors. The current review focuses on some of the major neuropeptide populations within the PFC—notably neuropeptide Y (NPY), corticotrophin-releasing factor (CRF), somatostatin (SST), dynorphin opioids (DYN), and the endorphin/enkephalin opioid systems. Where possible, each section will explore the peptide expression and known effects, the effects of known receptors, and the role the peptide and receptors play in a variety of neuropsychiatric diseases. Importantly, this review attempts to bridge clinical studies of psychiatric populations with preclinical research investigating the neural circuit actions of PFC neuropeptides and how dysregulation of these systems contributes to specific behaviors associated with diseased states (**Figure 1**).

It is important to note that the profound overlap and complexity in human neuropsychiatric diseases is not well recapitulated in animal models; many animal models represent a single representation of behavior involved in disease but do not fully encompass the actual human manifestation of the disease. Disorders like MDD and substance use disorder each have many criteria for diagnosis, of which an individual only needs to meet a few characteristics. This results in great heterogeneity in the expression of neuropsychiatric disorders, and likely, great heterogeneity in underlying causes. Also, the interpretation of some animal models has evolved (for example Commons et al., 2017), further muddling the literature. While these stipulations for the interpretation of the animal literature exist, preclinical behavioral models allow for greater investigation of the peptides in context: animal models allow for a greater teasing apart of the underlying neurocircuitry, synaptic dynamics, and relationship

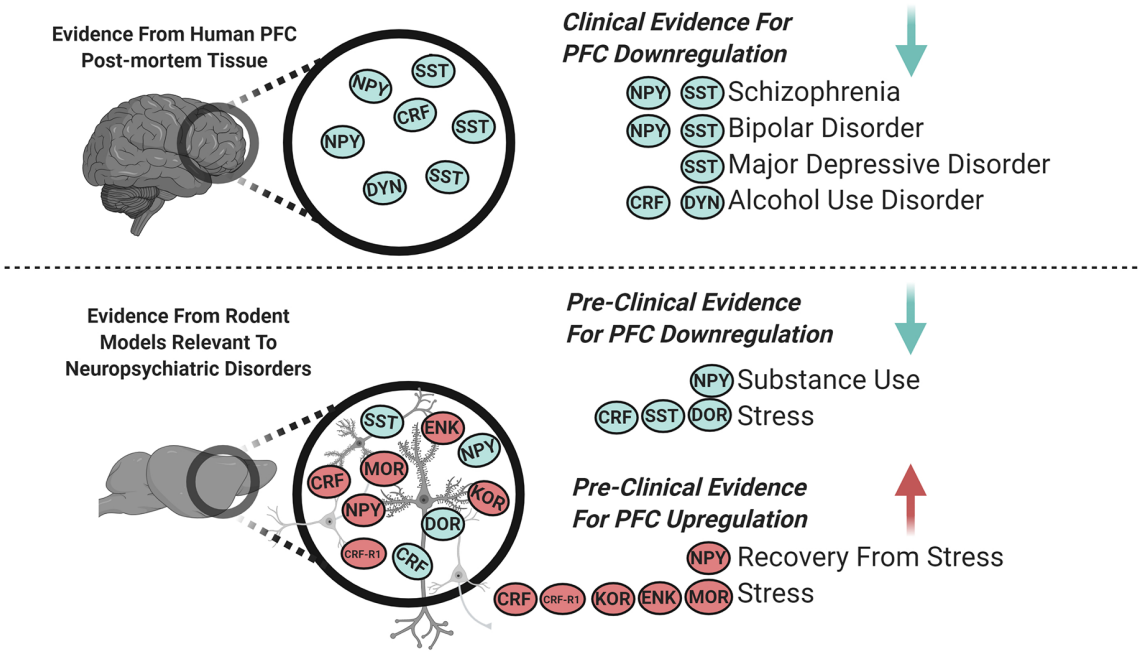

FIGURE 1 | Graphical summary of the major clinical and pre-clinical findings covered in this review demonstrating dysregulated expression of neuropeptides and their receptors in the prefrontal cortex (PFC; blue indicates downregulation and red indicates upregulation). Clinical evidence from PFC of human post-mortem tissue indicates downregulation of somatostatin (SST) and neuropeptide Y (NPY) in schizophrenia and bipolar disorder, SST in major depressive disorder (MDD), and corticotropin-releasing factor (CRF) and dynorphin (DYN) in alcohol use disorder. Pre-clinical evidence from the PFC of rodent models relevant to neuropsychiatric disorders indicates that; NPY is downregulated with substance use, SST, and delta-opioid receptors (DOR) are downregulated following stress. NPY is upregulated during recovery from stress, and CRF receptor 1 (CRF-R1), kappa opioid receptors (KOR), enkephalin (ENK) and, mu-opioid receptors (MOR) are upregulated following stress. CRF is downregulated following acute stress and is increased following chronic stress.

to other peptides and neurotransmitters. These positive and negative aspects of human and animal work may be in part responsible for the lack of cohesion between the preclinical and clinical literature, discussed in greater detail throughout the review.

NEUROPEPTIDE Y (NPY)

Neuropeptide Y Signaling and Overall Peptide Actions

Neuropeptide Y (NPY) is a 36-amino-acid neuropeptide with potent physiological effects and dense expression in the central and peripheral nervous systems (PNS; Tatemoto et al., 1982; Adrian et al., 1983; Allen et al., 1983). It is structurally similar to peptide YY and pancreatic polypeptide and is named for its abundance of tyrosine (Y) residues (Tatemoto et al., 1982). NPY in the PNS is co-localized with noradrenaline in sympathetic nerves (Lundberg et al., 1982), and has been shown to regulate sympathetic nervous system functions such as increasing blood pressure and causing vasoconstriction (Lundberg et al., 1982; Fuxe et al., 1983).

NPY in the CNS has been shown to modulate neural activity and regulates a variety of physiological functions including feeding, mood, and memory (Fuxe et al., 1983; Wahlestedt et al., 1989; Berglund et al., 2003; Sabban et al., 2016). A growing body of evidence indicates that NPY in the CNS plays a role in the stress response (Wahlestedt et al., 1989; Heilig, 2004; Thorsell, 2008), and stress-related neuropsychiatric disorders such as anxiety, depression (Wahlestedt et al., 1989; Zukowska-Grojec, 1995; Heilig, 2004; Hou et al., 2006), and substance use disorder (Ehlers et al., 1998; Pleil et al., 2015; Robinson and Thiele, 2017). NPY has gained attention as an anti-stress peptide, it promotes resilience to stress and reduces behaviors relevant to anxiety *in vivo* (Eaton et al., 2007; Cohen et al., 2012).

NPY is considered one of the most abundant neuropeptides in the CNS (Adrian et al., 1983; Allen et al., 1983). NPY containing neuronal cell bodies and axons are found in the PFC of several different species including humans (Chan-Palay et al., 1985; Eaton et al., 2007; Robinson et al., 2019a). NPY in the cortex is known to be expressed in non-pyramidal neurons, and like other neuropeptides, is co-localized with the inhibitory neurotransmitter GABA (Hendry et al., 1984). It is often co-expressed with other peptides, namely somatostatin. Furthermore, NPY is released following physiological stimulation of NPY expressing (NPY+) GABAergic neurons in other brain regions (Li et al., 2017).

NPY+ neurons in the PFC are thought to synapse locally as well as to potentially project to other regions

(Chan-Palay et al., 1985). Interestingly NPY+ neurons form connections between subregions of the PFC (Saffari et al., 2016). NPY+ neurons in the infralimbic cortex have been found to synapse onto pyramidal cells in the prelimbic cortex. NPY+ neurons in the PFC are activated by the claustrum and mediate local inhibition over pyramidal cells (Jackson et al., 2018). Due to the nature of NPY signaling both locally within the PFC and connections with other regions, NPY in the PFC is positioned as a powerful regulator over cortical networks.

The effects of the NPY peptide in the CNS are mediated primarily by five different G-protein coupled receptors, Y1, Y2, Y4, Y5, and Y6 (Pedragosa-Badia et al., 2013), though like for many peptides, off-target effects have not been completely ruled out. The Y6 receptor is non-functional in humans and rats but is functional in mice (Starbäck et al., 2000). The function of NPY in the CNS has been most well-characterized on $G_{i/o}$-coupled Y1 and Y2 receptors (Kopp et al., 2002; Kash and Winder, 2006; Gilpin et al., 2011; Robinson and Thiele, 2017). The action of NPY is both cell and receptor-specific, for example, activation of Y1 but not Y5 receptors results in a rise in intracellular calcium in smooth muscle cells (Pons et al., 2008). In other regions, Y1 receptors act predominately postsynaptically, and Y2 receptors act predominately presynaptically.

NPY receptors in the PFC are found on both pyramidal neurons and GABAergic neurons and correspondingly, NPY has been shown to alter both inhibitory and excitatory signaling onto pyramidal neurons within the PFC (Vollmer et al., 2016), indicating circuit mediated effects. Vollmer et al. (2016) found that bath application of 1 μM NPY increases $GABA_A$ receptor-mediated inhibitory postsynaptic currents (IPSCs) and a decrease in evoked α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR)-mediated excitatory postsynaptic currents (EPSCs) onto layer 5 pyramidal cells in the infralimbic cortex. Collectively, this suggests that NPY may function to reduce action potential discharge of pyramidal neurons of the PFC. Moreover, NPY has been shown to increase the release of other neurotransmitters in the PFC such as dopamine (Ault and Werling, 1998). The physiological effect of NPY in the PFC likely depends on several factors, such as the postsynaptic cell, receptor subtype, and locus of action. More work is needed to provide a cohesive understanding of NPY's neuromodulatory role in the PFC.

Stress and Post Traumatic Stress Disorder (Pre-clinical Evidence)

Pre-clinical rodent studies indicate that the effect of stress on NPY expression in the PFC is dependent on multiple factors, including the duration and type of stressor, the length of recovery, sex, and genotype. One study in Male Long Evans rats exposed to an acutely stressful event consisting of electric foot shocks found no significant change in NPY peptide after 7 days of recovery (Schmeltzer et al., 2015). Further, Schmeltzer et al. (2015) found that rats subjected to 7 days of chronic variable stress procedures, which also included foot shocks, showed no significant differences in NPY concentration in the PFC after 7 days of recovery. A separate study, also using male Long Evans rats subjected to 7 days of chronic variable stress, demonstrated no change in PFC NPY peptide 16 h following chronic variable stress; however, unlike the previous study, they found significantly increased NPY peptide in the PFC after 7 days of recovery (McGuire et al., 2011), suggesting NPY may be involved in adaptive responses to stress. However, a study using a longer duration of stress found that male Sprague–Dawley rats exposed to 36 days of a chronic unpredictable stress paradigm and 1 day of recovery demonstrated significantly reduced NPY mRNA in the PFC (Banasr et al., 2017). Together these studies suggest that the effect of stress on NPY may be dependent on the duration of the stressor, and specific dynamics of the stress procedure, including post-stress recovery time before data collection. It is possible that NPY is initially downregulated following long durations of stress but becomes upregulated during recovery as an adaptation, and more comprehensive studies are needed to confirm this hypothesis. Importantly, it has yet to be fully explored how dysregulation in NPY expression following stress exposure may be a positive or negative adaptation to further stress.

Sex hormones may also play a role in how stress affects NPY expression. A recent study explored NPY expression in the PFC in male, female, and ovariectomized female C57BL/6 mice exposed to 21 days of a chronic variable stress paradigm and 3 days of recovery (Karisetty et al., 2017). Karisetty et al. (2017) found increased NPY mRNA in the PFC in females but not in males or ovariectomized females—highlighting the increasing need for investigations into the role of sex differences. Taken together, these studies indicate NPY in the PFC is largely reduced during stress, whereas it becomes upregulated during recovery. However, the effect of stress on NPY in the PFC seems to be sex-dependent. Stress may be a contributing factor to the NPY pathology observed in neuropsychiatric disorders, and more studies are needed to understand how stress and recovery interact to regulate NPY expression in both males and females.

PTSD is a neuropsychiatric disorder that often occurs in individuals who have witnessed or experienced a traumatic or stressful event [American Psychological Association (APA) (2013)]. Symptoms of PTSD include intrusive thoughts, avoidance of reminders of a traumatic event, alterations in cognition and mood, alterations in arousal, and reactivity. Given the interaction between stress and NPY, NPY may play a role in behaviors associated with PTSD. McGuire et al. (2011) found that the increase in NPY after 7 days of chronic variable stress and 7 days of recovery in rats was associated with exaggerated fear response and recall. Also, NPY infused into the infralimbic region of the PFC in male Sprague–Dawley rats inhibits the consolidation of extinction, resulting in impaired retrieval of extinction memory *via* the Y1 receptor (Vollmer et al., 2016). In short, these preclinical findings indicate that increased NPY may contribute to fear-related behaviors associated with PTSD.

Human subjects with PTSD demonstrate decreased NPY in the cerebrospinal fluid (CSF; Sah et al., 2009, 2014). It is unknown how NPY regulation is changed in the PFC of PTSD subjects. Pre-clinical findings from Vollmer et al. (2016) would suggest increased NPY expression in the PFC of PTSD patients however clinical studies are needed to confirm this

hypothesis. Importantly, the causes of PTSD in humans is incredibly varied—ranging from life events (Simon et al., 2020), natural disasters (Cénat et al., 2020), domestic violence (Kofman and Garfin, 2020), pandemics (Kaseda and Levine, 2020) to other causes, and it is unlikely that the animal literature models the breadth of these events.

Substance Use Disorder (Pre-clinical Evidence)

To meet the criteria for substance use disorder (including alcohol use disorder) individuals must display at least 2 of 11 symptoms ranging from impaired control over substance use, social impairment, risky behavior, and the development of tolerance and withdrawal [American Psychological Association (APA) (2013)]. Several animal studies that investigated the role of NPY in the CNS on alcohol consumption indicate that lack of NPY can promote alcohol consumption. For instance, NPY knockout mice exhibit increased alcohol consumption (Thiele et al., 1998, 2000; Robinson and Thiele, 2017). Recently, the relationship between NPY in the medial PFC (mPFC) and binge alcohol consumption was explored in male and female C57BL/6J mice using the drinking in the dark paradigm (Robinson et al., 2019a). Robinson et al. (2019a) found that binge drinking reduced NPY immunoreactivity in the mPFC. Also, Robinson et al. (2019a) discovered opposing effects of Y1 and Y2 receptors in the mPFC, consistent with the literature on NPY and drinking elsewhere in the brain, such as the BNST (Kash et al., 2015). Robinson et al. (2019a) found that separate activation of Y1 receptors and inhibition of Y2 receptors both resulted in decreased binge ethanol intake in the mPFC, suggesting that NPY may reduce alcohol consumption through activation of Y1 receptors. Because Y2 receptors are predominantly auto-receptors on NPY neurons, antagonism of the Y2 receptor may promote activation of NPY neurons and subsequent NPY Y1 receptor activation, thus the synaptic location may account for this differential effect. NPY Y1 and Y2 receptors both signal through Gi/o signaling cascades, little evidence thus far suggests differences in NPY affinity for these receptors, or in the intracellular signaling cascades at either receptor (Kash et al., 2015), thereby supporting Robinson et al.'s (2019a) conclusion that synaptic location and local circuitry are at play. Moreover, other drugs of abuse such as cocaine lead to reductions in NPY in the PFC (Wahlestedt et al., 1991). These findings indicate that NPY and its receptors play an important role in alcohol consumption, and the effect of NPY likely depends largely on the specific NPY receptor which is activated. NPY and its corresponding receptors in the PFC are hypothesized to regulate behaviors associated with substance use disorder in humans, but clinical investigations are needed to confirm this hypothesis. Importantly, NPY is downregulated by multiple forms of substance use (both binge alcohol and cocaine) in animal models.

Major Depressive Disorder (Pre-clinical and Clinical Evidence)

MDD is characterized by a combination of symptoms including, depressed mood and loss of interest or pleasure, present during the same 2-week period [American Psychological Association (APA) (2013)]. Clinical studies indicate that NPY concentration in the frontal cortex is largely unchanged in patients with a clinical diagnosis of MDD, however, it may be involved with emotional regulation. One study found NPY was significantly lower in victims of suicide when compared to accidental-death control subjects (Widdowson et al., 1992) suggesting that NPY deficits in this region may be linked with emotional regulation and depression. However, a subsequent study did not support a role for NPY in MDD (Ordway et al., 1995). When comparing victims of suicide with a co-occurring diagnosis of MDD to accidental-death controls with no diagnosed psychiatric disorders, there was no significant difference in NPY concentration (Ordway et al., 1995). Consistent with this result, another study in humans found no change in the levels of PFC NPY in subjects diagnosed with MDD (Kuromitsu et al., 2001). The relationship between MDD and suicide is complex and remains to be fully elucidated, particularly in terms of causality, therefore while NPY may play a role in emotional regulation, it appears largely unaffected in the clinical population diagnosed with MDD. Further, there were no significant differences in prefrontal Y1 or Y2 receptor mRNA between control and MDD subjects (Caberlotto and Hurd, 2001). Together, clinical studies indicate that in humans diagnosed with MDD, expression NPY and NPY Y1 and Y2 receptors in the PFC are largely unchanged.

Genes regulating NPY have been shown to interact with environmental factors such as stress to increase susceptibility to negative emotional symptoms associated with anxiety and depression (Sommer et al., 2010). Sommer et al. (2010) identified a variant allele in the NPY promoter which results in increased NPY mRNA in the anterior cingulate cortex subregion of the PFC. This variation in the NPY gene increases susceptibility to stress and may contribute to the symptoms of depression. This finding in humans links genetic regulation of NPY with emotional symptoms following stress, however, it does not provide direct evidence for the role of NPY in stress or MDD.

Despite these clinical findings, pre-clinical rodent models exhibiting behaviors associated with depression point towards decreased NPY in the PFC. Both types of NPY mRNA were found to be downregulated in the PFC of a rat model of depression (Flinders Sensitive Line, FSL) when compared to controls (Flinders Resistant Line, FRL; Melas et al., 2012). The FSL is a selectively bred rat line that partially resembles the behavior of depressed individuals and exhibits other neurochemical changes associated with depression (Overstreet et al., 2005). Another model using an intraperitoneal injection of lipopolysaccharide (LPS) to induce depressive-like behavior in Sprague–Dawley rats found decreased NPY and Y2 receptor expression in the mPFC (Wang et al., 2019). It is important to note that these models do not fully capitulate the disease pathology observed in humans with MDD, and their construct, face, and predictive validity must be assessed when comparing these models with clinical populations.

Preclinical studies using rodent models are helping to investigate the potential of NPY to modulate behaviors associated with depression. Wang et al. (2019) also found that NPY itself

can reduce depressive-like behavior (as measured by open field test and sucrose preference test) in LPS treated rats when administered in the PFC. Local infusion of NPY into the mPFC reduced LPS-induced depressive-like behaviors in both the open field test and sucrose preference test in Sprague–Dawley rats. This effect was determined to be mediated by the Y2 receptor, as PFC administration of the Y2 receptor antagonist abolished, and administration of Y2 receptor agonist mimicked, antidepressant-like behavioral effects of NPY. However, a different study in male Sprague–Dawley rats found that NPY infusion into the infralimbic cortex subregion of the mPFC did not affect depression-like behavior in the forced swim test (Vollmer et al., 2016). As interpretations of behavior in the forced swim test continue to evolve (Commons et al., 2017), NPY may affect a specific subset of behaviors associated with depression such as the behaviors measured by the open field test and sucrose preference test and not affect other types of behaviors such as those measured by forced swim test, and more studies are needed to determine the specific behavioral effect of NPY. Another possibility is that the effect of NPY in the PFC is region-specific even within the PFC, and its effect may depend largely on the site of injection. The pre-clinical work demonstrates support for the hypothesis that NPY plays a role in regulating a specific subset of behaviors associated with depression, and further pre-clinical and clinical studies are needed to fully examine this hypothesis.

Schizophrenia and Bipolar Disorder (Pre-clinical and Clinical Evidence)

Many neuropsychiatric disorders have been linked with reductions in PFC NPY, suggesting that it plays an essential role in regulating emotional behaviors. Notably, NPY in humans is found to be altered in schizophrenia, and bipolar disorder (Wu et al., 2011). Schizophrenia is characterized by symptoms such as delusions, hallucinations, social and occupational dysfunction [American Psychological Association (APA) (2013)]. Bipolar disorder is characterized by extreme emotional states that occur at distinct times called mood episodes. These mood episodes are often characterized as manic, or depressive. Multiple studies have observed expression deficits in NPY and NPY mRNA in the PFC from post-mortem tissue of subjects with schizophrenia and subjects with bipolar disorder (Gabriel et al., 1996; Kuromitsu et al., 2001; Hashimoto et al., 2008a), although it is important to note that Caberlotto and Hurd (1999) found decreased NPY mRNA only in post mortem tissue of subjects with a clinical diagnosis of bipolar disorder, and not those with schizophrenia or other disorders. Moreover, NPY Y1 and Y2 receptor mRNA in the PFC was unaltered in post mortem tissue of subjects with bipolar disorder, and schizophrenia when compared to healthy controls (Caberlotto and Hurd, 2001). Male rats treated with Lithium exhibited increased NPY-like immunoreactivity in the frontal cortex, suggesting that NPY may be involved in the response to treatments to MDD and bipolar disorder. Together these findings indicate that deficits in NPY expression in the PFC are observed in both schizophrenia and bipolar disorder.

CORTICOTROPIN-RELEASING FACTOR (CRF)

CRF Neuropeptide Signaling and Overall Actions

Corticotropin-releasing factor (CRF) also known as Corticotropin-releasing hormone (CRH; referred to here as CRF) is a 41-amino acid neuropeptide which belongs to a family of neuropeptides including Urocortin 1 (Vaughan et al., 1995), Urocortin 2 (Reyes et al., 2001), and Urocortin 3 (Lewis et al., 2001). CRF was first characterized by hypothalamic extracts for its ability to stimulate the release of corticotropin and beta-endorphin from rat anterior pituitary cells *in vitro* (Vale et al., 1981). CRF is highly expressed in the paraventricular nucleus of the hypothalamus (Swanson et al., 1983) where it acts to activate the primary stress response pathway or hypothalamic pituitary adrenal (HPA) axis by promoting the release of stress hormones such as glucocorticoids and cortisol from the adrenal gland (Turnbull and Rivier, 1997; Bale and Vale, 2004; Dedic et al., 2017).

In addition to its role in the hypothalamus, CRF acts in other regions of the CNS where it functions to robustly modulate circuit function and to regulate behaviors associated with stress and addiction (Koob and Heinrichs, 1999; Kash and Winder, 2006; Orozco-Cabal et al., 2006; Silberman et al., 2013). CRF is widely distributed throughout the mammalian brain and is highly expressed in the PFC of mammals including humans (Pandey et al., 2019), rats (Swanson et al., 1983), and mice (Chen et al., 2020). CRF expressing neurons (CRF+) are found in the PFC and are predominately in layers II and III (Swanson et al., 1983). CRF+ neurons in the PFC are a subclass of inhibitory neurons, a large portion of which also express vasoactive intestinal polypeptide (VIP) or calretinin (Chen et al., 2020). Importantly, CRF+ neurons in the PFC become active during stress (Chen et al., 2020) and withdrawal (George et al., 2012). Activation of PFC CRF+ neurons results in local CRF release to modulate cognition and behavior (Hupalo et al., 2019b). Direct administration of CRF into the PFC results in impaired working memory, and CRF antagonism improves working memory, indicating that CRF acts in the PFC to regulate cognitive behaviors (Hupalo and Berridge, 2016). The dysfunction of CRF in the CNS occurs in stress-related disorders including PTSD, MDD, and anxiety (Hupalo et al., 2019a). Taken together, these findings suggest an important interaction between stress and CRF release which may contribute to neuropsychiatric disease.

The action of CRF is exerted through two major G protein-coupled receptors subtypes: CRF-R1 and CRF-R2 (Lovenberg et al., 1995; Perrin et al., 1995; Grammatopoulos et al., 2001; Dautzenberg and Hauger, 2002; Hauger et al., 2003). CRF-R1 and CRF-R2 are present in the PFC, however, CRF-R2 is expressed at low levels in the PFC of rodents (de Souza et al., 1985; Millan et al., 1986; Sánchez et al., 1999; Van Pett et al., 2000). CRF receptor expression in the cortex shows a high correlation with the distribution of CRF (de Souza et al., 1985) and CRF depresses excitatory synaptic transmission in PFC slices (Zieba et al., 2008).

This supports the importance of CRF as a neuromodulator in the PFC.

In other regions such as the BNST and CEA, CRF-R1 and CRF-R2 have been found to exert opposing roles on physiology and stress-induced behaviors (Liu et al., 2004; Funk and Koob, 2007; Lowery-Gionta et al., 2012; Tran et al., 2014). Given the differences between CRF-R1 and CRF-R2 in the PFC, this effect is likely consistent. For instance, CRF-R1 acts postsynaptically, while CRF-R2 acts presynaptically in the PFC (Orozco-Cabal et al., 2008). CRF-R2 is present on presynaptic terminals in the PFC which originate from CRF+ neurons in the basal lateral amygdala (BLA; Yarur et al., 2020). Activation of presynaptic CRF-R2 limits the excitatory transmission to the PFC from the basolateral amygdala. CRF has a higher affinity for CRF-R1 (Lovenberg et al., 1995) and CRF-R2 is expressed at lower levels as compared to CRF-R1 in the PFC of rodents (Van Pett et al., 2000). The opposing function of CRF receptors is similar to the opposing function of NPY-Y1 and Y2 receptors, and the ratio of these two CRF receptors may be responsible for the net effect of the peptide in that region. Therefore, dysregulation in the expression of one or both of these receptors may contribute to the dysfunction of the CRF system.

This review focuses specifically on CRF and its action in the PFC although CRF+ neurons whose cell bodies are located in the PFC also project to other regions where they release CRF. For instance, CRF+ neurons in the PFC project to the nucleus accumbens (NAc; Itoga et al., 2019) where they act to modulate behavior through activation of CRF receptors in those regions (Kai et al., 2018). It is important to note that males and females have been shown to exhibit different behavioral responses to CRF (Wiersielis et al., 2016). Despite this finding, much of the work on the role of PFC CRF has focused exclusively on male rodents, and sex differences have been understudied. Future work is warranted to examine how dysfunction in the CRF system may contribute to behaviors related to anxiety and neuropsychiatric disorders separately in males and females.

Stress and Post Traumatic Stress Disorder (Pre-clinical Evidence)

Multiple pre-clinical studies indicate that stress impacts the expression of CRF and CRF receptors and CRF may contribute to behaviors such as cognitive deficits associated with neuropsychiatric disease. Few preclinical studies have examined the relationship between stress and CRF-R2, and most work has focused largely on CRF-R1.

The relationship between stress, CRF, and CRF receptors in the PFC depends on the condition and duration of the stressor. Rodent models demonstrate that acute stress increases CRF and CRF-R1 mRNA in the PFC. Acute restraint stress increases both CRF mRNA and CRF-R1 mRNA in the PFC of Sprague–Dawley male rats (Meng et al., 2011). Moreover, mice (C57Bl/6N and CD1) exposed to acute social defeat stress exhibited increased CRF-R1 mRNA in the cingulate, prelimbic, and infralimbic regions of the PFC (Uribe-Mariño et al., 2016). Moreover, two stress-based rodent models of PTSD-like behaviors (though rodent models of PTSD are still evolving and emerging), one which subjected male rats to a single prolonged stressor consisting of 2-h restraint and 20 min forced swim (Wang et al., 2019), and another which subjected adolescent rats to inescapable electric foot shocks (Li et al., 2015) both resulted in increased CRF-R1 in the PFC. These experiments provide pre-clinical evidence that acute stress leads to increased CRF and CRF-R1 expression within the PFC.

Animal models of chronic stress, on the other hand, demonstrate that chronic stress leads to unchanged or decreased CRF mRNA, representing a possible adaptation to repeated stress. One study found Male Sprague–Dawley rats subjected to chronic immobilization stress (3 h a day for 21 days) showed no change in PFC CRF mRNA (Chen et al., 2008). A separate study found chronic social defeat stress (10 days) in Wistar male rats resulted in decreased CRF mRNA and increased CRF-R1 mRNA in the PFC (Boutros et al., 2016). Importantly, both acute and chronic stress lead to increased expression of CRF-R1.

CRF in the PFC has been shown to regulate behaviors associated with PTSD. Infusion of CRF into the vmPFC produces avoidance of stimuli paired with a traumatic stressor (Schreiber et al., 2017). Conversely, blockade of CRF signaling *via* CRF-R1 antagonism in the vmPFC reverses avoidance of stimuli paired with traumatic stress. Furthermore, CRF-R1 activation in the PFC following acute social defeat stress results in cognitive dysfunction (Uribe-Mariño et al., 2016). This suggests that increased CRF and CRF-R1 may contribute to behaviors associated with PTSD, and future clinical studies are needed to test this hypothesis.

Taken together these pre-clinical findings suggest that acute stress results in increased CRF whereas chronic stress results in decreased CRF. Both acute and chronic stress result in increased CRF-R1. Interestingly, CRF itself positively regulates the expression of CRF-R1 in cultured neurons (Meng et al., 2011). This suggests that the release of CRF in the PFC in response stressors may correspondingly regulate expression of CRF-R1. Increased CRF-R1 following acute stress may represent a neuroadaptation to increased CRF expression in response to stress, and this may upregulation may persist despite the downregulation of CRF with chronic stress. Deviation from homeostatic levels of CRF and CRF-R1 may contribute to PFC neurological dysfunction observed in neuropsychiatric disorders. Further pre-clinical studies are needed to compare the effects of acute and chronic stress on the expression of CRF, CRF-R1, and CRF-R2. Moreover, investigations into the expression of CRF and its receptors at multiple time points during a prolonged stressor may provide insight into the role of the CRF system in adaptation to stress.

Anxiety and Depression (Pre-clinical and Clinical Evidence)

Clinical and pre-clinical studies both point towards a role for CRF and its receptors in anxiety and depression (for review and synthesis see Owens and Nemeroff, 1993). It has been well validated that the concentration of CRF is increased in the CSF of depressed patients and suicide victims. In the PFC, pre-clinical evidence indicates increased CRF-R1 receptor expression in response to both acute and chronic stress. Given the connection between stress and depression this effect might suggest that

the clinical literature would show an increase in CRF receptor expression in humans with symptoms of depression, however, this is paradoxically not the case. Clinical evidence indicates that suicide victims exhibit a reduced density of CRF receptors in the frontal cortex as evidenced by a 23% reduction in CRF binding sites in brain tissue compared to healthy controls (Owens and Nemeroff, 1993). There are various possible reasons for these discrepancies. Importantly, the relationship between stress and suicide is not well established (i.e., not all suicide is precipitated by clear stressors, and not all stress leads to suicide). Also, Owens and Nemeroff (1993) did not distinguish between CRF-R1 and CRF-R2, and CRF-R2 is known to be less prominent in rodents than in primates. Further clinical studies are needed to determine the expression of CRF, CRF-R1, and CRF-R2 in the PFC of patients with depression. Most pressing, is that post-mortem evidence from victims of suicide does not encompass the large range of symptomology of depression, and investigation of other categories, such as MDD, is important for comparison to the animal literature.

Pre-clinical animal models also provide support for the role of PFC CRF in behaviors associated with anxiety and depression. Microinjection of CRF (0.02 μg) into the mPFC increased anxiety-like behavior in the elevated plus-maze (EPM) in both acute and chronically stressed rats (Jaferi and Bhatnagar, 2007). Interestingly, in unstressed rats, microinjection of a larger amount of CRF (0.2 μg) into the frontal cortex reduced anxiety-like behavior in the EPM. One study using various dosages of CRF in unstressed male Wistar rats found that CRF exerts opposing effects on anxiety-related behavior in the EPM depending on dose (Ohata and Shibasaki, 2011). CRF microinjected into the mPFC increases anxiety-like behavior in the EPM at lower doses (0.05 μg) and reduces anxiety-like behavior at higher doses (1.0 μg). Together, these studies reveal that CRF acts to modulate behavior associated with anxiety and depression as measured by the EPM in rodents, and the directionality of this effect may be in part, state-dependent and in part, dose-dependent.

The effect of CRF on anxiety-like behavior depends on the activation of CRF-R1. CRF microinjected into the mPFC of male Swiss mice increased anxiety-like behavior in the EPM and importantly, when a CRF-R1 antagonist was microinfused before CRF, the effect of CRF on anxiety-like behavior was blocked (Miguel et al., 2014). This study indicates that the effect of CRF on anxiety-like behavior is dependent on the CRF-R1. A separate study in male CD1 mice exposed to a live predator demonstrated that infusion of a CRF-R1 specific agonist into the mPFC reduced anxiety-like defensive behaviors including avoidance and freezing (Pentkowski et al., 2013). This supports the hypothesis that activation of CRF-R1 within the PFC regulates various behaviors associated with anxiety. CRF *via* activation of CRF-R1 in the PFC has also been shown to regulate anxiety-related behaviors through the sensitization of serotonin 5-hydroxytryptamine receptor subtype 2 (5-HT2R) signaling (Magalhaes et al., 2010), indicating a possible interaction between CRF and neurotransmitters such as serotonin. Collectively, these studies indicate that CRF plays a dual role in modulating anxiety-like behaviors through the activation of CRF-R1. Deviation from homeostatic levels of CRF may contribute to the pathology of anxiety and depression, and future clinical studies are needed to confirm this hypothesis.

Substance Use Disorders (Pre-clinical and Clinical Evidence)

CRF systems in the brain become activated by stressors including excessive drug use, and dysfunction of CRF contributes to negative emotional states associated with withdrawal and addiction (Koob, 2013; McReynolds et al., 2014; Zorrilla et al., 2014). The human literature points towards altered CRF and its receptors in the PFC as playing a role in various substance use disorders. Post-mortem tissue from individuals with alcohol use disorder exhibit significantly decreased CRF, CRF-R1, and increased CRF-R2 mRNA in the PFC (Gatta et al., 2019). Genetic variation in CRF receptors also contributes to increased maladaptive substance use. Human genetic variation in CRF-R1 (rs110402) has been shown to interact with stress to modulate alcohol consumption and PFC activity (Glaser et al., 2014). Overall, the clinical data support a role for CRF, CRFR-1, and CRFR-2 in the PFC in substance use disorders specifically alcohol use disorder, and genetic variation in this system may contribute to the pathology.

Pre-clinical animal models also demonstrate that drug use can alter CRF expression through various mechanisms. Chronic nicotine use decreases CRF mRNA in the PFC of rodents (Carboni et al., 2018). Male Sprague–Dawley rats exhibited decreased CRF mRNA following chronic nicotine exposure (0.4 mg/kg intraperitoneal once daily for 5 days) while CRF was unchanged following acute administration of nicotine (0.4 mg/kg intraperitoneal once daily for 1 day; Carboni et al., 2018). No change in CRF mRNA was observed following 3 cycles of binge drinking in male C57BL/6J mice, however, these mice exhibited decreased CRF binding protein in the PFC (Ketchesin et al., 2016). CRF binding protein (CRF-BP) is expressed in the PFC and binds CRF with a high affinity to regulate the activity of CRF receptors (Ketchesin et al., 2017). On the other hand, heroin self-administration was not associated with alterations in CRF mRNA or CRF-BP mRNA in male Sprague–Dawley rats (McFalls et al., 2016). The variability in these results indicates that the relationship between drug use and altered CRF depends on several factors including the pharmacology of the drug in question, and the duration of drug use or abuse. Also, the difference may emerge based on whether the drug was experimenter-administered (i.e., intraperitoneal) or consumed by choice. Moreover, CRF activity can be regulated independently by other factors such as CRF-BP.

Alterations to CRF receptors in the PFC observed in humans with alcohol use disorder (Gatta et al., 2019) are also associated with various forms of substance use in rodents. Deficits in CRF are characteristic of high drinking alcohol-preferring male rats—for example, Ehlers et al. (1992) note decreased CRF concentration in the PFC of these rats. Besides these rats have been demonstrated to have increased cortical activity in the frontal cortex following CRF administration, suggesting that CRF receptors in this region may also be dysregulated

(Ehlers et al., 1992). Increased heroin self-administration is associated with increased CRF-R1 in the PFC in male Sprague–Dawley rats (McFalls et al., 2016). CRF-R1 antagonism in the PFC reduced impulsivity and resulted in profound reductions in binge motivated alcohol drinking in male and female rats who had undergone early life maternal separation (Gondré-Lewis et al., 2016). Both chronic nicotine (Carboni et al., 2018) and repeated cocaine exposure (Orozco-Cabal et al., 2008) resulted in increased CRF-R2 expression in the PFC in male Sprague–Dawley rats.

CRF receptors have been shown to differentially regulate ethanol use behavior. In other brain regions such as the central extended amygdala (CEA) CRF-R1 (Lowery-Gionta et al., 2012) and CRF-R2 (Funk and Koob, 2007) play opposing roles in ethanol consumption. Substance use can modulate excitatory BLA inputs to mPFC through activation of presynaptic CRF-R2 (Orozco-Cabal et al., 2008). Orozco-Cabal and colleagues demonstrated that chronic cocaine results in increased functionality of presynaptic CRF-R2 and loss of postsynaptic function of CRF-R1 in the PFC of male rats. Moreover, an interesting, recent study found that inhibition of CRF-R2 and separate activation of CRF-R1 in the PFC both resulted in decreased binge-like ethanol consumption in male and female C57BL/6J mice, confirming that much like in the CEA, these two receptors may play opposite roles in substance use (Robinson et al., 2019b). In this work, Robinson et al. (2019b) demonstrated that co-administration of CRF-R1 and CRF-R2 antagonists attenuated the behavioral effect of CRF-R1 antagonist. This suggests that decreased binge-like ethanol drinking resulting from inhibition of CRF-R1 may result from increased activation of the CRF-R2, providing strong evidence in support of an important role of both CRF-R1 and CRF-R2 in the PFC in regulating substance abuse. However, in separate work, blocking CRF-R2 in the PFC partially inhibited cocaine-primed reinstatement of cocaine conditioned place preference (Guan et al., 2014). Overall, these findings highlight how different substances may differentially affect CRF and its receptors. These pre-clinical finding along with the clinical finding from Gatta et al. (2019) suggest that CRF-R1 and CRF-R2 may play opposing roles in substance use, and more studies are needed to confirm this hypothesis.

SOMATOSTATIN (SST)

SST Neuropeptide Signaling and Overall Actions

Somatostatin (SOM or SST), also known as somatotropin releasing inhibitory factor (SRIF; referred to here as SST), was characterized over 50 years ago as a hypothalamic extract capable of inhibiting the release of growth hormone from the rat anterior pituitary *in vitro* (Krulich et al., 1968). Somatostatin was originally described as a 14 (SST-14) amino acid peptide (Brazeau et al., 1973). Later, a second N-terminally extended bioactive form consisting of 28 amino acids (SST-28) was isolated and characterized (Pradayrol et al., 1980). Both isoforms are generated from the same precursor, prosomatostatin (Benoit et al., 1990). SST exhibits diverse physiological effects such as regulation of visceral functions, and inhibition of a variety of biological processes including anterior pituitary hormone secretion, insulin secretion, glucagon secretion, immune responses, DNA synthesis, and cell division (Brown and Taché, 1981; Kumar and Grant, 2010; Eigler and Ben-Shlomo, 2014; Morisset, 2017). In short, somatostatin is known to inhibit various cellular processes such as the secretion of hormones and other secretory proteins (Benali et al., 2000; Morisset, 2017). Somatostatin has been gaining attention for its role in the CNS as a neuromodulator, and in regulating behaviors linked to stress including substance abuse and affective disorders (Liguz-Lecznar et al., 2016; Robinson and Thiele, 2020).

SST in the CNS is highly evolutionarily conserved, and expression has been observed in several different species including humans, non-human primates, and rodents (Iritani and Satoh, 1991). There is a large amount of SST expression (both SST-14 and SST-28) in the PFC (Hayashi and Oshima, 1986; Lewis et al., 1986). Also, SST+ immunoreactive neurons are present at high densities in the PFC in several non-human species including macaque monkeys (Yamashita et al., 1989). SST expression is often used to classify inhibitory GABAergic neurons which mainly synapse on the dendrites of pyramidal cells within the cortex (Melchitzky and Lewis, 2008), though they have also been shown to project onto other populations including inhibitory neurons in the PFC (Cummings and Clem, 2020). SST+ neurons in the PFC have been shown to release SST under basal or tonic conditions as well as following activation (Dao et al., 2019); therefore, changes in the number or activity of SST cells in the PFC may not only result in altered GABAergic signaling but also altered SST tone. The GABAergic properties of SST cells in the cortex are known to control network activity, and the implications of SST-specific GABAergic dysfunction on neuropsychiatric disorders have been previously reviewed (Liguz-Lecznar et al., 2016; Urban-Ciecko and Barth, 2016; Robinson and Thiele, 2020).

SST exerts its biological function by activating any of five g protein-coupled SST receptors (SST-R1 to SST-R5) which are predominately Gi/Go coupled and result in inhibition of adenylyl cyclase (Patel et al., 1994; Liguz-Lecznar et al., 2016). SST-14 and SST-28 both bind and activate SST receptors with differing affinities (for example, SST-28 exhibits a greater affinity for SST-R5 than SST-14 (Liguz-Lecznar et al., 2016). SST-14 and SST-28 have been shown to exhibit differing biological effects (Hadjidakis et al., 1986). Several SST agonists and antagonists are used clinically for the treatment of diseases such as acromegaly and neuroendocrine tumors (Rai et al., 2015).

All five somatostatin receptors have been observed by immunohistochemistry in the frontal cortex of the human brain (Kumar, 2005). SST-R1 immunoreactivity is observed in the dendrites and soma of both pyramidal and non-pyramidal cells in the frontal cortex (Kumar, 2005) and is mainly presynaptic in other regions (Liguz-Lecznar et al., 2016). SST-R2 immunoreactivity was found to be confined mainly to pyramidal cells and was abundantly expressed in dendrites and processes (Kumar, 2005). SST-R3 immunoreactivity was less predominant and was observed in pyramidal cells as well as other cells such

as immune cells in the frontal cortex (Kumar, 2005). SST-R3 has been shown to exist on neuronal cilia in other regions (Liguz-Lecznar et al., 2016). SST-R4 and SST-R5 expression was observed in the dendrites (Kumar, 2005).

In vitro studies have demonstrated that the response of cortical neurons to SST is dependent upon the concentration and corresponding receptor activation (Delfs and Dichter, 1983). Delfs and Dichter (1983) found that in cultured rat cortical neurons low concentrations of SST-14 (100 pM–1 μM) caused an excitatory response and depolarization in neurons while at higher concentrations (10 μM–1 mM) SST-14 was more likely to have no effect or to produce an inhibitory response. SST-14 and SST-28 have also been demonstrated to exhibit opposing effects on rat cortical neurons in culture (Wang et al., 1989). Wang et al. (1989) found SST-14 increased a delayed rectifier potassium current in cortical neurons, while SST-28 reduced the current. A separate study found somatostatin applied microiontophoretically to neurons in the frontal cortex elicited a dose-dependent increase in activity and caused excitation in pyramidal cells (Olpe et al., 1980). This excitatory response was likely not a result of decreased GABAergic inhibition supporting a role for somatostatin in increasing frontal cortical activity. With the recent development of receptor-specific agonists and antagonists, there is a pressing need for rigorous region and receptor-specific investigations into the neurophysiological effects of SST. Moreover, few studies have investigated both SST-14 and SST-28, and some fail to differentiate between the two. Therefore, future studies investigating the effect of both SST-14 and SST-28 are warranted.

Results concerning the action of SST on neuronal activity are sparse, and very few experiments have been conducted in the PFC. SST+ neurons in the PFC have been shown to release somatostatin in an activity dependent manner (Dao et al., 2019), and dopamine can stimulate cortical SST release (Thal et al., 1986). The release of SST in the PFC indicates that it likely functions as an important neuromodulator in this region. A thorough investigation of SST's pharmacological action on neurotransmission in the PFC would provide the field with a framework for understanding how SST release impacts neurotransmission and behavior, and how deficits in SST observed in neuropsychiatric disorders contributes to PFC dysregulation. Importantly, studies of this nature may bridge the gap between the literature concerning the activity of SST neurons and corresponding neuropsychiatric diseases.

Stress (Pre-clinical Evidence)

Somatostatin mRNA and peptide in the PFC are reduced following stress in rats (Banasr et al., 2017; Li et al., 2018). Male Sprague–Dawley rats exposed to 36 days of chronic unpredictable stress and 1 day of recovery exhibited significantly decreased SST mRNA measured by quantitative polymerase chain reaction (qPCR) in the PFC when compared to home cage control (Banasr et al., 2017). A study using liquid chromatography-mass spectrometry to probe the effect of multiple stressful experiences during adolescence on a broad range of neuropeptides in the PFC and hippocampus in adulthood uncovered deficits in SST-28 following adolescent stress (Li et al., 2018). Li et al. (2018) found male Wistar Han rats that underwent the peripubertal stress protocol (including exposure to fox odor and elevated platform) from postnatal day 28–42 as well as 1 h of restraint stress before sacrifice demonstrated decreased SST-28 in the PFC. In both studies the decrease was not significant in the hippocampus, suggesting that changes in SST following stress are region specific (Banasr et al., 2017; Li et al., 2018).

Stress has also been shown to affect the levels of SST receptors in the PFC (Faron-Górecka et al., 2018). Male Wistar Han rats exposed to 7 weeks of chronic mild stress exhibited increased SST-R2 binding in the PFC following stress (Faron-Górecka et al., 2018). The literature shows conflicting results concerning the effect of stress on SST+ cell number and may depend on the sex and duration of stress. Male and female SST-tdT reporter mice exposed to 14 days of chronic unpredictable stress displayed significantly decreased SST-tdT+ neurons compared to control (Girgenti et al., 2019), while in a similar study, no significant decrease in SST cell number in male Wistar rats subjected to 9 weeks of chronic mild stress was observed (Czéh et al., 2018). The longer duration of stress and the use of only males in Czéh et al. (2018) may partially account for the different results. Studies investigating the effects of both acute and chronic stress of different durations may help to uncover how the concentration of SST changes throughout a stressor.

Major Depressive Disorder and Bipolar Disorder (Pre-clinical and Clinical Evidence)

Ample clinical evidence points towards decreased SST in human subjects with MDD and bipolar disorder. Subjects with bipolar disorder exhibit decreased SST mRNA in the PFC (Fung et al., 2014). Post mortem tissue from subjects with MDD exhibit a significant reduction in the expression of SST mRNA (measured by qPCR) and SST precursor protein (measured by western blot for prepro-SST) in the dorsolateral PFC (Sibille et al., 2011) and subgenual anterior cingulate cortex (Tripp et al., 2011). Recent studies have linked brain-derived neurotrophic factor (BDNF) expression with altered SST. Deficits in BDNF lead to decreased SST (Du et al., 2018), and BDNF itself may be required to maintain SST gene expression (Glorioso et al., 2006). This is consistent with the similar developmental expression profile of BDNF and SST mRNA which both increases during early adolescence (Du et al., 2018) and subsequently decrease with aging (Hayashi et al., 1997; McKinney et al., 2015). Decreased SST mRNA in the PFC in human subjects with MDD is correlated with reduced BDNF mRNA (Oh et al., 2019). Oh et al. (2019) found that C57BL/6J mice exposed to 7 weeks of chronic stress (an unpredictable chronic mild stress protocol) exhibited deficits in dendritic BDNF in the PFC and this decrease in dendritic BDNF may lead to a low neurotropic supply to SST neurons. Therefore, decreased BDNF may contribute to reduced SST expression and behavioral symptoms of depression (Oh et al., 2019).

Antidepressants have also been shown to modulate SST and SST receptors in the PFC in rodents. Male Sprague–Dawley rats chronically administered the antidepressant citalopram

exhibited increased SST and SST-R2 density (measured by autoradiography) in the PFC and frontal cortex respectively (Pallis et al., 2009). No change in SST in the PFC was observed after treatment with the antidepressant desmethylimipramine indicating that the effect may be dependent on the pharmacology of the antidepressant. Male Sprague–Dawley rats also showed no differences in somatostatin receptors [measured by (125i)Tyrl 1-somatostatin binding] in the PFC following acute and chronic desipramine treatment (Gheorvassaki et al., 1992). These results indicate that the pharmacology of the antidepressant may determine the effect on SST and SST receptors. Understanding the effects of antidepressants on SST may help to uncover whether changes in SST contribute to the pharmaceutical efficacy of antidepressants.

Schizophrenia (Pre-clinical and Clinical Evidence)

SST has been well studied for its role in schizophrenia and behavior related to schizophrenia in clinical and pre-clinical studies. Multiple studies have investigated the expression of SST in schizophrenic subjects (post-mortem tissue). DNA microarray for expression of GABA-related transcripts in the dorsolateral PFC of schizophrenic subjects (post mortem tissue) and matched controls revealed a robust decrease in SST mRNA in subjects with schizophrenia (Hashimoto et al., 2008a). The difference in expression of SST from healthy controls was greater than all other transcripts analyzed including NPY, GAD67, and GABA receptor subunits. This reduction in SST was further validated using Real-time qPCR and *in situ* hybridization and was replicated in multiple different studies in human subjects using qPCR (Hashimoto et al., 2008b; Fung et al., 2014; Tsubomoto et al., 2019). SST mRNA was found to be reduced (through *in situ* hybridization histochemistry and qPCR) in the orbitofrontal cortex in subjects with schizophrenia (also post mortem tissue; Joshi et al., 2015). A subsequent study in schizophrenic subjects (post mortem tissue) found that decreased SST expression in the dorsolateral PFC in schizophrenia is confined to layers 2 through 6, and both the density of SST+ neurons and the expression of SST mRNA per neuron were reduced (Morris et al., 2008). Collectively, these studies demonstrate substantial evidence in support of decreased SST in the PFC in patients with diagnosed schizophrenia.

The levels of SST mRNA were not altered in the dorsolateral PFC of monkeys chronically exposed to antipsychotic medications (Hashimoto et al., 2008b), suggesting that administration of antipsychotics as a treatment itself is not the cause of the reduced SST mRNA in schizophrenia. Moreover Rats given a single administration of haloperidol resulted in either unchanged or increased SST mRNA in the PFC further supporting the hypothesis that the decrease in SST mRNA observed in the PFC of human schizophrenic subjects reflects the disease process and is not a byproduct of antipsychotic treatment (Sakai et al., 1995).

Differential expression of SST receptors is also seen in individuals with schizophrenia. Subjects (post mortem tissue) with schizophrenia exhibited unchanged SST-R1 but significantly decreased SST-R2 mRNA in the dorsolateral PFC, and this reduction was localized to pyramidal cells in layers 5–6 (Beneyto et al., 2012). SST-R2 expression was not affected in macaque monkeys exposed to chronically high doses of antipsychotics, or in patients on or off antipsychotics at the time of death; however, macaque monkeys exposed to low doses of the antipsychotic haloperidol (a common antipsychotic for the treatment of schizophrenia) demonstrated reduced SST-R2, suggesting the results should be interpreted cautiously (Beneyto et al., 2012).

Animal studies are providing insight into the relationship between decreased PFC SST transmission and behavioral processes disrupted in schizophrenia. A recent study used viral gene knockdown to determine the behavioral effects of SST in the PFC (Perez et al., 2019). Perez et al. (2019) found that male and female Sprague–Dawley rats which underwent viral-mediated gene knockdown of SST in the PFC exhibit behavioral deficits in the negative (social interaction test) and cognitive (reversal learning test) domains consistent with those observed in schizophrenia. In two different rodent models of schizophrenia, the MK-801 model in Long Evans rats (Murueta-Goyena et al., 2020) and the BRINP1-KO model in mice (Kobayashi et al., 2018), the number of SST+ immunoreactive neurons is decreased, although this does not necessarily represent a change in the SST peptide.

The deficits in SST and SST+ neurons observed in subjects with schizophrenia and animal models of schizophrenia may be a downstream consequence of impaired BDNF signaling. Consistent with this hypothesis, strong positive correlations between BDNF protein levels and SST mRNA levels were observed in the PFC of human subjects (post mortem tissue) with schizophrenia, suggesting that BDNF may function to regulate SST expression in the PFC (Mellios et al., 2009). This parallels the findings suggesting an interaction between BDNF and SST in MDD. SST has also been shown to be regulated by BDNF through the tyrosine receptor kinase B (trkB) receptor as evidenced by reduced expression of SST in the PFC of trkB hypomorphic mice, which express significantly lower levels of trkB (Morris et al., 2008). These studies support the hypothesis that BDNF underlies changes in SST in the PFC, and may precede changes in SST, though more work is needed to understand this relationship.

DYNORPHIN

Dynorphin Signaling and Overall Peptide Actions

Dynorphin, an endogenous member of the opioid neuropeptide family (Goldstein et al., 1979), is thought to mediate negative emotional states associated with stress, depression, and drug use withdrawal (Koob and Le Moal, 2008; Bruchas et al., 2010; Knoll and Carlezon, 2010; Hang et al., 2015). Dynorphin refers to a group of neuropeptides derived from the preprodynorphin gene including Dynorphin-A 32 amino acids (Fischli et al., 1982), which binds with high affinity to kappa opioid receptors (KORs; Chavkin et al., 1982; James et al., 1982; Kakidani et al., 1982; Hauser et al., 2005). Dynorphin and KORs are present throughout the brain and activation of this

system generally promotes dysphoria, anxiety-like behavior, and behaviors associated with substance use disorders (Wee and Koob, 2010; Crowley and Kash, 2015).

KORs are G protein-coupled receptors, encoded by the oprk1 gene, which are selectively activated by dynorphin (Karkhanis et al., 2017). KORs can signal *via* multiple signaling pathways including G-i/o protein-coupled inhibition of adenylyl cyclase (Konkoy and Childers, 1993; Lawrence et al., 1995; Dhawan et al., 1996; Karkhanis et al., 2017), stimulation of inwardly rectifying potassium channels (Henry et al., 1995), activation of p38 MAPK (Bruchas et al., 2006), and activation of ERK 1/2 (McLennan et al., 2008). The effects of dynorphin/KORs on neurotransmission are variable and depend on the brain region, neuron the receptor is on, and whether the receptor is expressed pre- or post- synaptically (Karkhanis et al., 2017). In other regions of the cortex, dynorphin has been shown to act presynaptically to inhibit the release of both GABA and glutamate in the same brain region (Li et al., 2012; Crowley et al., 2016). The dynorphin/KOR system is a critical mediator of both stress response and stress-induced relapse and has been linked with the CRF system (Bruchas et al., 2010). Stress-induced CRF activation leads to dynorphin release and subsequent modulation of mood by KOR activation.

Both Dynorphin and KORs are highly evolutionarily conserved and are present in the PFC in both humans and rodents (Zamir et al., 1984a,b; Dawbarn et al., 1986; McIntosh et al., 1987; Wevers et al., 1995; Hurd, 1996; Mansour et al., 1996; Svingos and Colago, 2002). KORs predominate over other types of opioid receptors such as mu-opioid receptors (MORs) in the PFC (Lahti et al., 1989). Dynorphin neurons comprise a subset of neurons in the PFC which express pre-prodynorphin and are GABAergic (Sohn et al., 2014). Approximately one-quarter of dynorphin neurons also express the neuropeptide somatostatin (Sohn et al., 2014).

Recent studies have revealed an important neuromodulatory role for the dynorphin/KOR system in the PFC. KORs in the mPFC is thought to be largely presynaptic and are localized on axons and axon terminals (Svingos and Colago, 2002). Presynaptic KORs can regulate synaptic input from other regions onto the PFC, and activation of KORs in the PFC has been shown to negatively regulate glutamatergic synaptic transmission from the BLA (Tejeda et al., 2015). Also, Tejeda et al. (2015) demonstrated that activation of KORs in the PFC decreases the frequency of miniature EPSCs onto layer 5 pyramidal cells. This work also demonstrated that activation of KORs in the PFC also directly inhibits dopamine terminals to reduce dopamine release in the PFC. Currently, Dynorphin/KOR activation is known to reduce dopamine release and dampen glutamatergic input onto pyramidal cells in the PFC, though other effects in the PFC (both on other cell populations and other neurotransmitters and peptides) have yet to be fully elucidated.

Stress and Anxiety (Pre-clinical Evidence)

KOR mRNA and protein are affected by stress. Repeated forced swim stress in male C57BL/6 resulted in increased expression of KOR mRNA (Flaisher-Grinberg et al., 2012) and a separate study found an increase in KOR protein in male Swiss mice exposed to repeated forced swim stress (Rosa et al., 2018a). Together these results suggest that stress, specifically forced swim stress, leads to increased KOR expression. It is unknown how stress affects dynorphin expression in the PFC and future studies are needed to test this.

Dynorphin is thought to mediate dysphoria, and broadly, promote behaviors associated with anxiety. Pre-clinical rodent models indicate that the behavioral effect of dynorphin in the PFC may depend on the region of activation. Mice subjected to chronic constriction injury of the right sciatic nerve exhibit significantly increased pro-dynorphin and KOR mRNA expression in the PFC (Palmisano et al., 2019). Studies investigating the effects of dynorphin agonists and antagonists on behaviors associated with anxiety reveal a subregion specific effect of KOR activation. Microinjections of a KOR agonist (U50, 488H) or a dynorphin derivative (E-2078) into the mPFC in rats led to place aversion in the conditioned place preference paradigm suggesting that activation of KOR is associated with aversive effects in the mPFC (Bals-Kubik et al., 1993). Consistent with this, another study using the selective KOR antagonist nor-binaltorphamine (norBNI) in male Long-Evans rats found intra-mPFC injection increased center time in the open field test, suggesting decreased defense/withdraw anxiety (Tejeda et al., 2015). Together, these findings point towards a role for dynorphin and KOR activation in the mPFC in mediating aversive states and behaviors associated with anxiety.

However, these studies did not investigate the effect in specific subregions of the mPFC. Male CD-1 mice injected with the selective KOR agonist U-69, 593 in the infralimbic cortex exhibited dose-dependent decreases in avoidance behaviors in the EPM, and defensive/withdrawal anxiety in the open field (Wall and Messier, 2000). They also exhibited evidence for enhanced memory in two separate memory tests: the EPM transfer-latency test and the Y-maze test. A subsequent study by Wall and Messier (2000) investigated the effect of blocking KOR activation selectively in the infralimbic cortex using the KOR antagonist norBNI. They found that pretreatment with one injection of norBNI in the infralimbic cortex dose-dependently increased anxiety-like behavior as well as disrupted working memory in the same behavioral tasks. Importantly, the effect on anxiety-like behavior after infusion of either a KOR agonist or antagonist in the infralimbic cortex was long-lasting, and differences in EPM behavior were observed in both studies after a 24-h delay (Wall and Messier, 2000). This finding is inconsistent with the effect of KOR observed by other researchers in the mPFC, but this discrepancy may be due to the region of injection, and the effect observed by Wall and Messier may be specific to the infralimbic subregion of the PFC, which has been shown to play opposing roles to more dorsal regions of the PFC such as the prelimbic cortex in other neuropeptides. More work is needed to understand the region-specific behavioral effect of KOR activation/inhibition in different subregions of the PFC.

Substance Use Disorder (Pre-clinical and Clinical Evidence)

Human studies point toward a dysregulation of dynorphin/KOR systems in subjects with alcohol or substance use disorders.

Prodynorphin CpG dinucleotides that overlap with SNPs were differentially methylated in the dlPFC of postmortem brains from alcohol-dependent individuals (Taqi et al., 2011) suggesting a possible role for dynorphin in behaviors associated with substance dependency. One study found prodynorphin and dynorphin (both A and B) mRNA were upregulated in the dlPFC of alcoholics (Bazov et al., 2013). A second study by Bazov et al. (2018) found prodynorphin is downregulated in the PFC of alcoholics while KOR expression itself was unchanged. The authors of both studies hypothesize that the different findings are likely because the first study was underpowered, while the second study from 55 control and 53 alcoholic subjects provided a more sufficient dataset (Bazov et al., 2018). Post mortem brains from individuals with a history of marijuana use or stimulant use had increased prodynorphin mRNA in the anterior cingulate and dorsolateral prefrontal cortices respectively, but no change was found in the brains of individuals with a history of alcohol use (Peckys and Hurd, 2001). The human findings point towards dysregulation of prodynorphin with substance use, but the directionality of this effect may depend on the specific pharmacology and consumption pattern of the drug of abuse, as well as severity of abuse.

Alcohol has been shown to regulate dynorphin in rodents. Alcohol increases the density of dynorphin expressing-cells in the mPFC in rats consuming ethanol chronically compared to water controls, as assessed by digoxigenin-labeled *in situ* hybridization histochemistry (Chang et al., 2010). Male Sprague–Dawley rats treated with alcohol at a dose of 1.5 g/kg three times for 1 day exhibited increased prodynorphin in the PFC, however, no change was detected after 5 days (D'Addario et al., 2013). Prenatal alcohol exposure can lead to increased prodynorphin mRNA in the PFC in infant rats (age not further specified; Wille-Bille et al., 2018). Like other drug use, alcohol use also causes increased expression of prodynorphin. Also, directly modulating KORs has been shown to regulate drug reward. In Male Sprague–Dawley rats which received once-daily injections of cocaine (20.0 mg/kg, intraperitoneal) for 5 days, site-specific activation of mPFC KORs exacerbated the development of behavioral sensitization and increased cocaine-evoked dopamine levels (Chefer et al., 1999). More work is needed to determine how KOR activation in the PFC influences drug reward and behaviors associated with substance use disorders.

Consistent with the human literature, pre-clinical animal studies also indicate altered dynorphin/KOR following the administration of substances of abuse other than alcohol. Acute (8 mg/kg, intraperitoneal) administration of 3,4-methylenedioxy-N-methylamphetamine (ecstasy) in male Sprague–Dawley rats raised levels of prodynorphin mRNA in the PFC and decreased levels of Dynorphin-A (Di Benedetto et al., 2006). Interestingly, Di Benedetto et al. (2006) observed no change after chronic treatment. Male Wistar rats treated with both acute and chronic morphine (8.0 mg/kg intraperitoneal, once daily for one or five consecutive days) exhibited increased KOR mRNA in the PFC (Yu et al., 2012). However, at the protein level, acute morphine treatment did not affect KORs in the PFC, while chronic morphine caused downregulated KOR protein (Yu et al., 2014). Nicotine has also been shown to lead to changes in prodynorphin expression (Carboni et al., 2016). Chronic and sub-chronic administration of nicotine led to increased expression of prodynorphin mRNA in the PFC of Sprague–Dawley rats. However, this was not observed after acute administration, and no change was found in KOR mRNA with either administration paradigm. In summary, acute ecstasy, acute and chronic morphine, and sub-chronic and chronic administration of nicotine all led to increased expression of prodynorphin. More research is needed to uncover how KOR's and dynorphin itself is altered with drug use.

Importantly, prodynorphin and KOR mRNA were found to be unchanged in the anterior cingulate and dorsolateral prefrontal cortices of post mortem brains of subjects diagnosed with schizophrenia, bipolar disorder, or major depression, and was not associated with antipsychotic treatment or suicide (Peckys and Hurd, 2001). This points towards dynorphin having a particular sensitivity to stress and/or drugs of abuse and maybe a central part of the addiction process. Based on the postulated role of the dynorphin/KOR system in mediating negative affect (such as withdrawal from drugs of abuse), more work is needed to further characterize the role of this system in the PFC concerning substance use disorders.

ENDORPHIN AND ENKEPHALIN

Endorphin and Enkephalin Signaling and Overall Peptide Actions

Endorphin and enkephalin, like dynorphin, are endogenous opioid neuropeptides that are present in the CNS (Rossier, 1988). Despite their similar behavioral effects, and pharmacological action, they are derived from distinct precursors. Endorphins (canonically β-endorphins; Bruijnzeel, 2009) are derived from pro-opiomelanocortin, and enkephalins (met-enkephalin and leu-enkephalin) are derived from proenkephalin (Rossier, 1988). Endorphins and enkephalins play a role in motivational and stress circuits and have been implicated in neuroadaptations to drug abuse (Koob and Volkow, 2016). Both endorphin and enkephalin have profound pain-relieving effects and promote euphoria (Shenoy and Lui, 2018; Hicks et al., 2019).

Endorphins, enkephalins, and their precursors are highly evolutionarily conserved and are expressed in the PFC in several species including humans and rodents (Matthews et al., 1992; Hurd, 1996; Leriche et al., 2007). To date, few studies have directly examined the behavioral or physiological function of these peptides in the PFC and instead have focused on their corresponding receptors. Endorphins and enkephalins modulate neural activity through activation of G protein-coupled (GPCR) opioid receptors (Corder et al., 2018) which are also present in the PFC (Lahti et al., 1989). Endorphins preferentially bind and activate mu–opioid GPCRs (MORs), while enkephalins are non-selective agonists with an affinity for both MORs and delta-opioid GPCRs (DORs; Corder et al., 2018). DORs have been shown to regulate anxiety-like behavior, and site-specific activation of DORs in the PFC in mice reduced anxiety-like behavior (Lahti et al., 1989). Despite this early finding, few studies have investigated DORs in the PFC, and instead, most

studies have focused on the MORs, due to their more well-known neuromodulatory and behavioral effects. Future studies into the role of DORs in the PFC as well as studies examining the pharmacological effects of endorphins and enkephalins in the PFC are warranted.

MORs are expressed predominately on non-pyramidal GABAergic neurons in the frontal cortex, overlapping with enkephalin expression (Taki et al., 2000; Férézou et al., 2007). This suggests that MORs function as auto-receptors on enkephalin-expressing non-pyramidal neurons. In the PFC, activation of MORs has been shown to inhibit voltage-dependent sodium currents on non-pyramidal neurons through a PKA and PKC dependent mechanism (Witkowski and Szulczyk, 2006), an effect likely mediated by auto-receptors on non-pyramidal GABAergic enkephalin neurons. Reduced voltage-dependent sodium currents would result in decreased action potentials in GABAergic cells in the PFC, and then downstream would cause decreased inhibitory currents onto pyramidal cells. Consistent with this hypothesis, MOR agonists in other regions of the cortex have been shown to decrease GABAergic transmission and decrease inhibitory currents onto pyramidal cells (Férézou et al., 2007) and hippocampal cells (Capogna et al., 1993). These findings indicate that if this effect is consistent in the PFC, MORs may cause disinhibition of pyramidal cells whereby activation of MORs on GABAergic neurons increases pyramidal cell activity by decreasing GABAergic input onto these cells.

In addition to local disinhibition of pyramidal cells, MORs in the PFC regulates excitatory input from other regions projecting to the PFC, such as presynaptic suppression of glutamate release from the thalamus (Marek and Aghajanian, 1998; Marek et al., 2001). Importantly, MORs and DORs have been shown to synergistically interact to enhance dopamine D1 receptor-induced stimulation of adenylyl cyclase activity (Olianas et al., 2012). MORs and DORs in conjunction with their endogenous ligands are positioned to precisely coordinate neural activity both within the PFC and from other brain regions. As a result, dysregulation of this system can contribute to loss of inhibitory control over executive function and behavior as is observed in multiple psychiatric disorders (Baldo, 2016).

Stress and Anxiety (Pre-clinical Evidence)

Preclinical animal models suggest that stress exposure is linked to reduced expression of enkephalin in the PFC. Male Wistar Han rats that underwent peripubertal stress conditions (exposure to fox odor and elevated platform across post-natal days 28–42) exhibited downregulated enkephalin mRNA in the PFC (Li et al., 2018). Ninety minutes of cold and immobilizing stress in male Wistar rats resulted in significantly decreased enkephalin immunoreactivity, however, no change was observed after 30 or 180 min (Kurumaji et al., 1987). The decreased enkephalin observed only at the 90-min time point may indicate that enkephalin plays a role in adaptation to stress.

Stress has also been shown to increase MORs and decrease DORs in the PFC. Adult Swiss mice subjected to social defeat stress exhibited increased MOR protein and decreased DOR protein in the PFC in susceptible mice (Rosa et al., 2018b). Similarly, swiss mice subjected to repeated forced swim stress exhibited increased MOR expression but reduced DOR in the PFC (Rosa et al., 2018a). Neonatal handling, which is known to increase the ability to cope with stress and to decrease anxiety-like behavior is associated with increased levels of MORs in the PFC (Kiosterakis et al., 2009). This indicates that stress may lead to higher expression of MORs and decreased expression of DORs in the PFC. The increased MOR expression observed in the PFC in response to stress may be a neuroadaptation to decreased MOR ligands such as decreased enkephalin. Future work is needed to uncover the relationship between enkephalin and MORs/DORs as well as their relationship with stress.

Substance Use Disorder (Pre-clinical and Clinical Evidence)

The rewarding effects of drugs and the development of drug-seeking behavior involve changes in opioid peptides (Koob and Volkow, 2016). The current review will not cover the breadth of focus and attention on the modern opioid epidemic, covered in-depth elsewhere (Shipton et al., 2018; Skolnick, 2018; Marshall et al., 2019). Endorphin and enkephalin in the PFC contribute to inhibitory control over appetitive behaviors, and loss of control over these behaviors is known to occur in multiple psychiatric disorders such as substance use disorders (Baldo, 2016). Infusions of MOR agonists into the ventromedial PFC of male Sprague–Dawley rats resulted in increased appetitive motivation (Selleck et al., 2015, 2018). Moreover, male Wistar rats that binge ate a highly palatable diet exhibited increased preopiomelanocortin (endorphin precursor) in the PFC (Blasio et al., 2014). These findings point towards a role for endorphin and MORs in the regulation of consummatory behavior, and thus this system may contribute to excessive drug use and behaviors observed in substance use disorders.

Alcohol and nicotine have thus far not been found to affect endorphin or enkephalin in the PFC. Post mortem brains from human alcoholics show no changes in the levels of proenkephalin, MORs, or DORs in the PFC (Bazov et al., 2013). Nicotine treatment leads to changes in endorphin and its precursor (proopiomelanocortin) in limbic regions in mice; however, only moderate decreases in proopiomelanocortin have been observed in the PFC following chronic nicotine treatment (Gudehithlu et al., 2012). Other drugs, however, such as psychostimulants, have been shown to modulate enkephalin. Male Sprague–Dawley rats administered with amphetamine and then subsequently administered with the same dose 7 days later exhibited increased proenkephalin mRNA (Morales-Mulia et al., 2007). These findings indicate that endorphin and enkephalin are likely not directly regulated by alcohol or nicotine but may be regulated by other drugs such as amphetamines.

Also, substance use has been shown to regulate MOR functioning and this may contribute to behaviors involved with substance use disorders. In humans, long-term opiate and mixed opiate/cocaine abusers exhibit decreased midazoline receptor antisera-selected (IRAS)/nischarin, a putative I1-imidazoline receptor which regulates MOR trafficking (Keller et al., 2017). AA rats bred selectively for high alcohol consumption have a significantly greater proenkephalin mRNA and greater density of MORs in the PFC (Marinelli et al., 2000) which may

contribute to increased ethanol consumption, although the connection is indirect. Rats self-administering a cannabinoid receptor agonist exhibited increased MOR levels in the PFC (Fattore et al., 2007). Taken together, these results suggest endorphin/enkephalin and MORs are differentially affected by drugs of abuse, and increases in this system may be involved with specific types of substance use. Pre-clinical studies using a consistent methodology to investigate the effects of multiple different drugs of abuse on the endorphin/enkephalin system would provide a foundation for understanding how the pharmacology of the substance may contribute to the pathology of substance use disorders. Further, clinical investigations into the expression of endorphin/enkephalin in patients with various forms of substance use disorders would help to determine how these systems are differentially affected by different substances.

DISCUSSION

The PFC has been shown in both the clinical and pre-clinical literature to play a vital role in many neuropsychiatric disorders. Increasing evidence has shown that GABAergic and peptidergic neurons within the PFC, responsible for the modulation of glutamatergic inputs, local circuits, and pyramidal neuron outputs, play a key modulatory role in these disorders. More work is needed to understand the role of each of these individual peptide populations. Peptide effects vary depending on the model (e.g., acute stress vs. chronic stress models, length of manipulation, animal genetic strain, and sex differences). Also, further research should address interactions between peptide populations in the PFC—both those expressed in different and in the same, GABAerigc neurons. For instance, subpopulations of neurons within the PFC express both NPY and SST, while SST and dynorphin have also been found to co-express here. This overlap is surprising given the general opposing roles of NPY and dynorphin. Also, increasing technological advancements have allowed for the greater investigation of peptide release independent of co-expressed neurotransmitters, which will allow a greater understanding of when same-neuron peptides are released (i.e., under different neuronal firing frequencies or overall length of firing). Greater assessment of the combinatorial role of these peptides will better inform disease models and treatment. Overall, the literature suggests a strong and important role of peptides in the PFC in stress and neuropsychiatric disorders.

Future work, in addition to expanding the existing depth of literature, also needs to focus on filling the gap in the literature. Specifically, much of the pre-clinical work above has been conducted in male rodents, with very little focus on females. Others have eloquently expressed elsewhere (Shansky, 2018, 2019, 2020) the importance of: (a) investigating females as a stand-alone research question, as opposed to only in contrast to males as a baseline; and (b) the unlikeliness that female rodents have more behavioral and neurobiological variability, or that this variability is due to female-dominant sex hormones. Also, standard operating procedures may help to rectify some of the discrepancies seen. For instance, the multitude of stress models, with differing lengths of stress exposure and post-stress recovery periods, likely contributes to the variable effects seen.

Moreover, while pre-clinical evidence links neuropeptide systems to behaviors relevant to a disorder, these diseases themselves are incredibly complex. For example, the DSM-5 [American Psychological Association (APA) (2013)] includes five subtypes of depressive disorders (disruptive mood dysregulation disorder, MDD, persistent depressive disorder, premenstrual dysphoric disorder, substance/medication-induced depressive disorder, depressive disorder due to another medical condition, other specified depressive disorder, and unspecified depressive disorder). Animal models of depression understandably fail to capture this complexity, and importantly, often rely only on stress (whether social or environmental stress) and/or genetic manipulations (Krishnan and Nestler, 2011). This example holds for most neuropsychiatric disorders, in that animal models representing a *single* behavioral or biological manipulation are unlikely to fully recapitulate the complexity and range of the human disorder. Therefore, it is no surprise that animal models using a signal manipulation (i.e., forced swim) do not replicate the work of these nuanced psychiatric conditions in humans. Leading researchers in the preclinical field have agreed that animal models thus far are not fulling capturing human disorders but are nevertheless a crucial component of treatment identification and testing. These researchers identified key gaps in the literature needed for the synthesis of human and animal work: "*in vivo* experiments, precision, innovation, integration and complexity, and leadership setting the tone" (Bale et al., 2019). In this article, Bale et al. make a key and important statement supporting our overall characterization of peptides in the PFC, that this preclinical work does not directly model these disorders but, "*Rather, the goal is to achieve a better understanding of an essential biological function that is key to the illness and to ensure that our level of understanding is actionable for translation.*" Therefore, the discrepancies seen in the preclinical and clinical work for some neuropeptides provide a launching point for further investigation in humans.

Overall, though some discrepancies exist in the role of specific neuropeptides in specific disorders, their importance is clear and further investigation for each of them is paramount to understanding complex disorders in humans, and for targeting both preventions and interventions. By both filling in these gaps (investigating both sexes, consistency in models, further expanding research into humans), and expanding the focus into new questions and domains overall, a greater understanding of the role of peptidergic signaling in the PFC during stress and neuropsychiatric diseases may be obtained.

AUTHOR CONTRIBUTIONS

DB and NC wrote the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

The experiments were funded by the Brain and Behavior Research Foundation (NARSAD Young

Investigator Award; NC), The National Institute on Alcohol Abuse and Alcoholism (R21AA028008; NC), and the Penn State's Social Science Research Institute (Level 2 Award; NC).

ACKNOWLEDGMENTS

We would like to thank Malini Suresh-Nair (Crowley Lab, Department of Biology) for thoughtful comments.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Behavioral Neuroscience

REVIEW
published: 29 October 2020
doi: 10.3389/fnbeh.2020.598469

The Paraventricular Nucleus of the Thalamus Is an Important Node in the Emotional Processing Network

Jessica R. Barson, Nancy R. Mack and Wen-Jun Gao*

Department of Neurobiology and Anatomy, Drexel University College of Medicine, Philadelphia, PA, United States

The paraventricular nucleus of the thalamus (PVT) has for decades been acknowledged to be an important node in the limbic system, but studies of emotional processing generally fail to incorporate it into their investigational framework. Here, we propose that the PVT should be considered as an integral part of the emotional processing network. Through its distinct subregions, cell populations, and connections with other limbic nuclei, the PVT participates in both major features of emotion: arousal and valence. The PVT, particularly the anterior PVT, can through its neuronal activity promote arousal, both as part of the sleep-wake cycle and in response to novel stimuli. It is also involved in reward, being both responsive to rewarding stimuli and itself affecting behavior reflecting reward, likely *via* specific populations of cells distributed throughout its subregions. Similarly, neuronal activity in the PVT contributes to depression-like behavior, through yet undefined subregions. The posterior PVT in particular demonstrates a role in anxiety-like behavior, generally promoting but also inhibiting this behavior. This subregion is also especially responsive to stressors, and it functions to suppress the stress response following chronic stress exposure. In addition to participating in unconditioned or primary emotional responses, the PVT also makes major contributions to conditioned emotional behavior. Neuronal activity in response to a reward-predictive cue can be detected throughout the PVT, and endogenous activity in the posterior PVT strongly predicts approach or seeking behavior. Similarly, neuronal activity during conditioned fear retrieval is detected in the posterior PVT and its activation facilitates the expression of conditioned fear. Much of this involvement of the PVT in arousal and valence has been shown to occur through the same general afferents and efferents, including connections with the hypothalamus, prelimbic and infralimbic cortices, nucleus accumbens, and amygdala, although a detailed functional map of the PVT circuits that control emotional responses remains to be delineated. Thus, while caveats exist and more work is required, the PVT, through its extensive connections with other prominent nuclei in the limbic system, appears to be an integral part of the emotional processing network.

Keywords: anterior, anxiety, arousal, depression, fear, posterior, reward, stress

OPEN ACCESS

Edited by:
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University of Otago, New Zealand

Reviewed by:
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****Correspondence:***
Jessica R. Barson
jrb455@drexel.edu

Specialty section:
This article was submitted to Emotion Regulation and Processing, a section of the journal Frontiers in Behavioral Neuroscience

Received: *24 August 2020*
Accepted: *25 September 2020*
Published: *29 October 2020*

Citation:
Barson JR, Mack NR and Gao W-J (2020) The Paraventricular Nucleus of the Thalamus Is an Important Node in the Emotional Processing Network. Front. Behav. Neurosci. 14:598469. doi: 10.3389/fnbeh.2020.598469

INTRODUCTION

While the paraventricular nucleus of the thalamus (PVT) has for decades been acknowledged to be an important node in the limbic system (see, for example, Jayaraman, 1985; Su and Bentivoglio, 1990; Hsu et al., 2014; Colavito et al., 2015; Kirouac, 2015), studies of emotional processing, defined here as the process by which emotions are generated in response to specific stimuli, generally fail to incorporate it into their investigational framework. Here, we propose that the PVT should be considered as an integral part of the emotional processing network. According to the Two-Dimensional Theory of Emotion (Lang, 1995), affective responses can be qualified according to their placement along two axes: (1) arousal, reflecting the intensity of the stimulus; and (2) valence, reflecting the hedonic value of the stimulus. Under this framework, the PVT can be considered to participate in both major features of emotion, arousal, and valence. Thus, it is both responsive to and also influences not just arousal but also reward, motivation, depression, anxiety, stress, and fear (see below for details), generating emotional states and translating them into behavioral responses. It is involved in both conditioned responses, which require learning, and also unconditioned or primary emotional responses. Just as arousal and valence reflect two distinct dimensions of affect, however, the participation of the PVT in these dimensions may originate from different subdivisions of the PVT. Thus, the main purpose of this review is to illustrate the multiple ways in which the PVT participates in emotional processing, and also to address, where known, which specific subregions and cell populations of the PVT contribute to each facet of this phenomenon.

We note here that while emotionally laden stimuli can generate motivated behavior that is directed toward or away from those stimuli and, as such, motivated behavior can be difficult to disentangle from affective behavior, our specific focus here is on emotional processing. A growing body of literature, however, has demonstrated that the PVT also plays an integral role in motivated behavior, particularly motivated behavior that is linked to drugs of abuse. For more information on the involvement of the PVT in motivated behavior, the reader is directed to several excellent reviews (Kirouac, 2015; Millan et al., 2017; Matzeu and Martin-Fardon, 2018; Zhou and Zhu, 2019).

ANATOMICAL CHARACTERISTICS OF THE PVT

In the rodent, the PVT, a prominent nucleus in the dorsal midline thalamus that is positioned just ventral to the dorsal third ventricle, extends through a relatively long rostrocaudal axis (more than 3.2 mm in the adult rat and 2.1 mm in the adult mouse; Paxinos and Franklin, 2004; Paxinos and Watson, 2005). It is composed of at least two discrete clusters of cells, which were first distinguished by Gurdjian in 1927 as the *nucleus paraventricularis anterior* and *nucleus paraventricularis posterior* (Gurdjian, 1927). While many laboratories continue to separate the PVT into rostral and caudal halves, atlases of the rodent brain often differentiate between anterior PVT, PVT (or middle PVT), and posterior PVT (e.g., Paxinos and Franklin, 2004; Paxinos and Watson, 2005). Thus, careful attention should be paid when generating conclusions from the literature on this brain region. In this review, we distinguish between the three subregions (anterior, middle, and posterior PVT) whenever publications have explicitly made this distinction or have provided anatomical coordinates in such a way that the subregion can be determined.

As a whole, the PVT has extensive connections with the rest of the limbic system. It receives afferent inputs from multiple brain regions that process a variety of information, including defensive, visceral, nociceptive, gustatory, circadian, and executive function (Kirouac, 2015). For example, it receives serotonin from the dorsal and median raphe nuclei (Otake et al., 1995); norepinephrine from the locus coeruleus, reticular formation, and nucleus of the solitary tract (Phillipson and Bohn, 1994; Otake et al., 1995); dopamine from the hypothalamus and periaqueductal gray (Li S. et al., 2014); corticotropin-releasing factor from the amygdala and bed nucleus of the stria terminalis (BNST; Otake and Nakamura, 1995); and orexin/hypocretin from the hypothalamus (Peyron et al., 1998). In turn, it sends glutamatergic and peptidergic efferent projections to various limbic regions (Arluison et al., 1994; Csáki et al., 2000), most densely to the nucleus accumbens (Parsons et al., 2007; Dong et al., 2017), but also the BNST (Dong et al., 2017), central nucleus of the amygdala (Li and Kirouac, 2008; Dong et al., 2017), prefrontal cortex (Huang et al., 2006), and hypothalamus (Csáki et al., 2000). Of note, a specific investigation of projections from the PVT to the nucleus accumbens, BNST, and central nucleus of the amygdala has found a moderate-to-high level of collateralization (Dong et al., 2017), suggesting potential coordination in its efferent output.

While the rodent PVT subregions share many of the same afferents and efferents with each other, there are significant and notable differences in the density of these projections (**Figure 1**). For example, compared to inputs from other cortical regions, the anterior half of the PVT receives greater inputs from the ventral hippocampal subiculum and infralimbic cortex that convey information about motivational state and arousal, respectively. In contrast, compared to inputs to the anterior PVT, the posterior half of the PVT receives greater inputs from the prelimbic, infralimbic, and anterior insular cortices, that provide information about executive function, taste, and visceral sensation (Li and Kirouac, 2012; Kirouac, 2015). Moreover, while the entire PVT receives dense projections of orexin from the hypothalamus, which coveys information about arousal and stress, the posterior PVT receives heavier orexin innervation than the anterior PVT (Kirouac et al., 2005). Conversely, the anterior PVT projects widely to limbic areas, with denser projections to the suprachiasmatic nucleus (SCN), which is associated with circadian rhythm, while the more restricted projections of the posterior PVT are heavier to areas of the extended amygdala, including the BNST and central nucleus of the amygdala, which are involved in anxiety and fear (Moga and Moore, 1997; Li and Kirouac, 2008; Vertes and Hoover, 2008; Dong et al., 2017). While most neurons across the PVT project to the nucleus accumbens and a proportion of these provide collateral innervation of the BNST

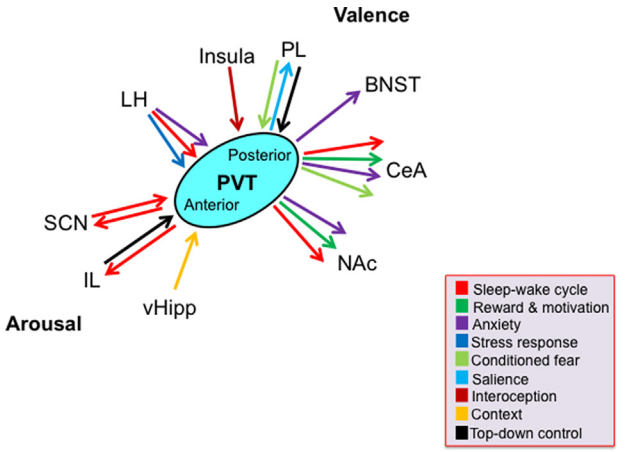

FIGURE 1 | Schematic representing key limbic brain regions and associated functions by which the paraventricular nucleus of the thalamus, across its antero-posterior axis, contributes to the two dimensions of emotion: arousal and valence. Arrows denote function(s) associated with the projections according to their color. Most, but not all, depicted functions have been directly tested, as discussed in the text. Abbreviations: BNST, bed nucleus of the stria terminalis; CeA, central nucleus of the amygdala; IL, infralimbic cortex; LH, lateral hypothalamus; NAc, nucleus accumbens; PL, prelimbic cortex; PVT, paraventricular nucleus of the thalamus; SCN, suprachiasmatic nucleus; vHipp, ventral hippocampus.

and central nucleus of the amygdala, the anterior PVT sends more projections to the dorsomedial accumbens shell, associated with appetitive behaviors, while the posterior PVT sends more projections to the ventromedial accumbens shell, associated with aversive behaviors (Dong et al., 2017). Moreover, efferent fibers have been found to travel from the anterior PVT to the posterior PVT but have not been identified in reverse, indicating that information flow may be unidirectional within the PVT (Vertes and Hoover, 2008). Together, these anatomical connections position the PVT to influence and coordinate affective behavioral responses, and they suggest that the anterior half of the PVT may have a somewhat more prominent role in arousal, while the posterior half of the PVT is more involved in valence.

Recent research has suggested that the divisions of the PVT may be more complex than previously described. Specifically, two well-defined neuronal subtypes, while largely restricted to the anterior and posterior halves of the PVT, respectively, demonstrate an antero-posterior gradient (Gao et al., 2020). Thus, while each subtype is most abundant in one half of the PVT, it is nonetheless also present in the other half, with the middle PVT subregion serving as a transition zone between the two neuronal subtypes (Gao et al., 2020). Similarly, of the PVT projections to the nucleus accumbens shell, the dorsomedial-projecting neurons show a progressive decrease from the anterior to the posterior PVT while the ventromedial-projecting neurons show the opposite gradient, decreasing from the posterior to the anterior PVT (Dong et al., 2017). These distribution gradients may explain historically conflicting findings regarding the responses and functional effects of cells throughout the PVT.

UNCONDITIONED AFFECTIVE BEHAVIOR

In tests of unconditioned affective behavior, the PVT is involved in both arousal and valence, with changes in its neuronal activity responding to and influencing indicators of arousal, reward, depression, anxiety, and stress (see below for details). While the anterior and posterior halves of the PVT both appear to be involved in these behaviors, their relative contributions to each behavior demonstrate significant variation, suggesting that there may be a gradient of cells across the antero-posterior PVT axis that participate in them.

Arousal—the PVT Regulates Arousal in a Subregion- and Cell Subpopulation-Specific Manner

The PVT shows a clear role in arousal, with slightly more evidence being generated from research investigating the anterior PVT than the posterior PVT. In work on circadian arousal, early research in rats demonstrated that levels of c-Fos (used as a marker for neuronal activity) are increased in the PVT in the dark (active) phase relative to the light phase (Peng et al., 1995), with levels peaking 4–6 h after lights-off (Mendoza et al., 2005; Ren et al., 2018). With a combination of c-Fos immunohistochemistry with retrograde tract-tracing, it has been shown that this increase occurs in both anterior PVT neurons projecting to the amygdala and posterior PVT neurons projecting to the nucleus accumbens (Peng et al., 1995). Moreover, with the preparation of slices during the dark cycle and examination using patch-clamp techniques, the anterior PVT is more likely to demonstrate spontaneous activity and increased depolarization compared to when it is prepared during the light (Kolaj et al., 2012), confirming that the anterior PVT is more active during the dark cycle. Recordings in mice have further clarified that population calcium activity in the PVT is greater during wakefulness than during sleep and that neuronal firing rate, as measured with electrophysiology, is also higher during wakefulness (Ren et al., 2018). Of note, during the transition from NREM sleep to wakefulness, terminals in the infralimbic cortex from galanin-containing PVT neurons, which in the mouse are denser in the anterior than the posterior PVT, demonstrate a decrease rather than an increase in activity, as measured by calcium transients (Gao et al., 2020). These findings suggest that cells within each PVT subregion may not uniformly demonstrate changes in activity in relation to the sleep-wake cycle; rather, specific populations of cells in the PVT are more active during behavioral arousal.

Beyond changing their activity during wakefulness compared to sleep, neurons in the PVT may themselves drive wakefulness. Investigation of population calcium activity in the mouse PVT shows that neuronal activity begins to increase before the onset of behavioral arousal (Ren et al., 2018). Further, optical stimulation of glutamatergic neurons in the PVT during NREM sleep promotes wakefulness, and this occurs *via* projections to the nucleus accumbens but not the prefrontal cortex and can be driven by orexin afferents from the lateral hypothalamus (LH; Ren et al., 2018). Conversely, chemogenetic inhibition,

or lesioning of the mouse PVT decreases wakefulness during the dark phase of the light-dark cycle, when mice are typically more active (Ren et al., 2018). On the other hand, chemogenetic activation prior to dark onset of galanin-containing PVT neurons, which in the mouse are denser in the anterior PVT, decreases wakefulness and increases NREM sleep (Gao et al., 2020). Of note, in addition to its sizeable hypothalamic orexin input (Peyron et al., 1998), which can drive behavioral arousal (Ren et al., 2018), the PVT also receives extensive afferent input from the SCN (Novak et al., 2000; Peng and Bentivoglio, 2004), which regulates the circadian clock (Luppi and Fort, 2019). Thus, neuronal activity in the PVT can promote wakefulness and the PVT itself is positioned as a major recipient of circadian and arousal signaling.

The PVT is also involved in arousal in cases that are not dependent on the light-dark cycle. Population calcium signals in the mouse middle PVT are increased by a variety of novel or unfamiliar stimuli across a range of modalities, including olfactory, visual, and auditory (Zhu et al., 2018), indicating that the PVT responds to behaviorally relevant events. In rats maintained in constant darkness, levels of c-Fos in the PVT are increased at the time of scheduled access to a palatable meal (Mendoza et al., 2005), and in neonatal rabbits, levels of c-Fos in the PVT are increased during anticipatory arousal before scheduled maternal nursing (Allingham et al., 1998). This indicates that the PVT signals not just the response to a behaviorally relevant event but also the anticipation of one. While these studies do not differentiate between anterior and posterior subregions, they indicate that the PVT overall participates not just in arousal related to the sleep-wake cycle but also in arousal related to salient stimuli and events.

Reward—the PVT Is Responsive to Reward Stimuli and Affects Reward-Related Behaviors

Research on reward suggests that the anterior portion of the PVT may be more involved in affecting reward-related behavior while the posterior PVT is more responsive to reward stimuli. An early indication for a role of the PVT in reward came in 1982, when Clavier and Gerfen demonstrated in male rats that intracranial self-stimulation could be supported by electrode placement in the middle PVT (but not the posterior PVT; Clavier and Gerfen, 1982). More recently, optogenetic self-stimulation was found to be supported when viral injections were made into the mouse PVT and fibers were placed in the nucleus accumbens (Lafferty et al., 2020), suggesting that stimulation of PVT projections to the accumbens can be rewarding. Support for a specific role of the anterior PVT in reward comes from studies in rats, which suggest that in fact inhibition of the anterior PVT promotes reward. Intracranial self-stimulation threshold, with the stimulating electrode targeted at the LH-medial forebrain bundle, is dose-dependently lowered by injection into the anterior PVT of the neuroinhibitory neuropeptide, cocaine- and amphetamine-regulated transcript peptide (CART), while injection of a CART antibody leads to the reverse effect (Choudhary et al., 2018). Similarly, time in the paired chamber in a real-time place preference paradigm is reduced by photoactivation of anterior PVT projections, both to the nucleus accumbens shell and the central nucleus of the amygdala (Do-Monte et al., 2017). It may be that animals experience reward from either inhibition of the anterior PVT (*via* its projections to the nucleus accumbens or amygdala) or stimulation of the middle PVT (also *via* projections to the nucleus accumbens). Conversely, in response to purportedly rewarding stimuli, including access to a female conspecific (for males) or thermoneutral zone, dopamine D2 receptor-expressing PVT neurons, which in the mouse are enriched in the posterior PVT, show a decrease in activity, as measured by calcium transients (Gao et al., 2020). Prior research in the mouse on single-unit excitation in the middle PVT has shown that about two-thirds of task-related neurons respond to outcomes that are both appetitive (water after water-restriction) and aversive (air puff or tail shock), while one third is specifically tuned to outcomes that are either appetitive or aversive and that these responses are proportionate with the intensity of the outcome (Zhu et al., 2018). The intermixing of these populations of cells may reflect the transition zone noted by other researchers between the anterior and posterior PVT (Dong et al., 2017; Gao et al., 2020). Altogether, these findings suggest that the PVT is both responsive to rewarding stimuli and itself can affect behavior reflecting reward and that while, to some extent, these responses may not be specific for reward or be clearly defined by PVT subregion, there may be populations of cells within the PVT that are more involved in this phenomenon.

Depression—the PVT Participates in Depression-Like Behavior

Extremely limited evidence supports a role for the PVT in depression-like behavior. Following chronic forebrain expression of a mutation of a mitochondrial DNA polymerase, which has its highest accumulation in the PVT, female but not male mice show an increased number of depressive episodes, as measured by reduced wheel running, increased levels of corticosterone, increased sleep, and greater food intake (Kasahara et al., 2016). Further, genetic inhibition of PVT synaptic output by Cre-loxP-dependent expression of tetanus toxin similarly promotes these depression-like episodes (Kasahara et al., 2016). Interestingly, seemingly opposite effects have been reported more recently by this same group. Chronic presynaptic inhibition of PVT neurons by tetanus toxin in female mice was found to reduce immobility time in a forced swim test, while long-term chemogenetic activation of the PVT increased hypoactivity as measured by reduced wheel running (Kato et al., 2019). Short-term chemogenetic modulation did not affect immobility time in a forced swim test or tail suspension test (Kato et al., 2019). Thus, PVT activity appears to participate in depression-like behavior, but the direction of these effects and the subregions and pathways through which this occurs remain to be characterized.

Anxiety—the Posterior PVT Plays a Major Role in the Regulation of Anxiety-Like Behavior

While there is discrepancy even within the same studies, the PVT also appears to participate in anxiety-like behavior,

although findings on the direction of these effects are not always in agreement. In examining the PVT overall, optogenetic stimulation of PVT projections to the central amygdala in mice is found to reduce or leave unaffected time spent in the open arms of an elevated plus-maze (Chen and Bi, 2019; Pliota et al., 2020) while inhibition of this pathway following stress somewhat increases it (Pliota et al., 2020), indicating that the PVT-to-amygdala pathway functions to promote anxiety-like behavior. Stimulation of the posterior half of the PVT generally recapitulates these findings. Microinjection into the rat posterior PVT of the neurostimulatory neuropeptide, orexin, reduces time and number of entries into the open arms of an elevated plus-maze (Li et al., 2010b; Heydendael et al., 2011) and the number of visits to the center of an open field (Li et al., 2010a); conversely, an orexin receptor antagonist reduces the latency to enter the social interaction zone in a social interaction test (Dong et al., 2015) and, following foot shock, increases the time and number of entries into the open arms of an elevated plus-maze (Li et al., 2010b). On the other hand, inhibition of rat posterior PVT neuronal activity *via* microinjection of the GABA agonists, baclofen and muscimol, similarly reduces time and entries into the open arms of an elevated plus-maze (Barson and Leibowitz, 2015). Thus, the direction of the effects of posterior PVT activity on anxiety-like behavior may depend on the specific population of cells that is affected. While support for a role of the anterior PVT in anxiety-like behavior is less robust, some studies have nevertheless demonstrated this connection. Neither photostimulation of the rat anterior PVT on its own nor its projections to the nucleus accumbens shell affects time spent in the center of an open field, but this behavior is reduced by photostimulation of the projections to the central amygdala (Do-Monte et al., 2017). Similarly, neither GABAergic inhibition of the rat anterior PVT nor photostimulation of the mouse anterior PVT-to-accumbens pathway affects time in the open arms of elevated plus maze (Barson and Leibowitz, 2015; Cheng et al., 2018). On the other hand, photostimulation of this pathway does increase time spent feeding in a novelty-suppressed feeding test and tends to increase time spent in the light chamber of a light-dark box (Cheng et al., 2018). These results suggest that, under limited circumstances, the anterior PVT may also participate in anxiety, with its projections to the amygdala promoting, and to the accumbens inhibiting, anxiety-like behavior. Overall then, the posterior PVT demonstrates a robust role in anxiety-like behavior, generally promoting but also suppressing this behavior, while the anterior PVT makes a more limited contribution, similarly promoting and suppressing this behavior *via* separate neural pathways.

Stress—the Posterior PVT Plays a Greater Role Than the Anterior PVT in the Response to Stress

A large body of evidence has connected the PVT with stress, with studies nearly uniformly demonstrating that, while the PVT responds across its antero-posterior axis to a range of purported stressors, the posterior PVT is more responsive than the anterior PVT to these stimuli. Levels of c-Fos in the whole PVT of both the rat and mouse are increased following withdrawal from alcohol (Knapp et al., 1998; Smith et al., 2019), although there is clear fluctuation in these levels throughout the withdrawal period (Smith et al., 2019). Similarly, levels of c-Fos in the mouse PVT are increased following exposure to an elevated plus-maze or foot shock (Pliota et al., 2020), and levels in the rat middle PVT are increased following a forced swim test (Zhu et al., 2011). While population calcium signaling in the rat is increased in both the anterior and posterior PVT following foot shock (Choi et al., 2019), levels of c-Fos are increased to a greater extent in the rat posterior compared to anterior PVT following noxious mechanical stimulation (Bullitt, 1990) and, in the obese Zucker rat, they can be identified at an earlier time-point following a period of food deprivation (Timofeeva and Richard, 2001). Similarly, levels of c-Fos are increased in the posterior but not anterior or middle PVT of the rat by acute restraint stress following chronic intermittent cold stress (Bhatnagar and Dallman, 1998). Moreover, calcium transients in dopamine D2 receptor-expressing PVT neurons, which are enriched in the mouse posterior PVT, are increased by aversive stimuli, including foot shock and tail suspension (Beas et al., 2018; Gao et al., 2020), and calcium events in a subset of cells in the mouse posterior PVT occur as a phasic response to footshock (Pliota et al., 2020). One exception to the greater response of the posterior PVT comes from a study that found that the anterior but not middle or posterior PVT showed elevated levels of c-Fos in mice after a novelty-suppressed feeding test, compared to mice exposed to a novel object or left naïve (Cheng et al., 2018). In light of the role of the anterior PVT in arousal, however (see "Arousal"), it may be that this test reflects differences in arousal more than it reflects stress. Overall then, the evidence as a whole supports a greater role for the posterior PVT in the response to stress.

The functional role of the posterior PVT appears to be a suppression of the stress response following chronic but not acute stressors, as demonstrated in a series of studies by Bhatnagar and colleagues. Lesioning of the rat posterior PVT blocks adaptation of the hypothalamic-pituitary-adrenal (HPA) axis (adrenocorticotropic hormone (ACTH) and corticosterone) to restraint stress, following repeated exposures to this stressor (Bhatnagar et al., 2002). Similarly, this same treatment blocks the reduction in amplitude in core body temperature rhythms after novel restraint stress in chronically cold-stressed rats but not in rats with no history of chronic stress (Bhatnagar and Dallman, 1999), and it increases the duration and height of burying an aversive stimulus in a conditioned defensive burying paradigm in chronically restraint-stressed rats but not in stress-naïve rats (Bhatnagar et al., 2003). This ability of the posterior PVT to inhibit the facilitation of the HPA axis to a novel stressor in chronically stressed rats appears to be due in part to orexin afferents from the LH. The facilitation of the HPA response to acute restraint stress is blocked by injection into the posterior PVT of an orexin receptor antagonist before chronic swim stress but not before acute restraint stress (Heydendael et al., 2011). Thus, while studies with orexin microinjections suggest that the function of the posterior PVT is to promote anxiety (see "Anxiety"), these studies suggest that it also promotes adaptation

of the HPA axis to chronic stress and dampens HPA and anxiety responses to chronic stress.

CONDITIONED AFFECTIVE BEHAVIOR

In tests of conditioned affective behavior, the PVT has been shown to involved in both appetitive and aversive behavior, with its neuronal activity changing in response to and influencing reward-seeking and fear retrieval (see below for details). While the anterior and posterior halves of the PVT both appear to be involved in these behaviors, their relative contributions to each behavior again demonstrate significant variation.

Reward and Motivation—the Anterior and Posterior PVT Differentially Regulate Reward/Motivation

Research on conditioned reward and motivation suggests that while the entire PVT is affected by and can affect the behavioral response to reward-predictive cues, activation of the anterior PVT generally inhibits motivated or seeking behavior while activation of the posterior PVT may instead promote it (but see Otis et al., 2017, 2019). Population calcium signaling in both the mouse and rat PVT is increased by stimuli that, in a Pavlovian conditioning paradigm, indicate the delivery of outcomes that are both appetitive (water after water-restriction or sucrose) and aversive (air puff, tail shock, or foot shock; Zhu et al., 2018; Choi et al., 2019). Notably, however, while a conditioned stimulus signaling the delivery of a sucrose reward stimulates activity in both the anterior and posterior PVT, activity in the anterior PVT is a weak predictor of magazine approach behavior (Choi et al., 2019). This may be because individual neurons in the anterior PVT are not uniformly activated, as it has been shown with unit-recording electrodes that firing rate can be either increased or decreased in the anterior PVT to a cue predicting sucrose availability under an operant conditioning paradigm (Do-Monte et al., 2017). In contrast, population calcium signaling in the posterior PVT serves as a strong predictor of magazine approach behavior, being increased both to a stimulus signaling the delivery of sucrose and to the consumption of that sucrose (Choi et al., 2019). Similarly, while gene expression of c-*fos* is increased in the anterior but not posterior PVT to a food-predictive cue in rats that have attributed incentive salience to the cue (Flagel et al., 2011), protein levels of c-Fos under similar conditions are elevated in posterior PVT afferents to the nucleus accumbens (Haight et al., 2017). Thus, neuronal activity in response to a reward-predictive cue can be detected throughout the PVT, but endogenous activity in the posterior PVT appears to be a more robust predictor of approach behavior.

In contrast to research on endogenous neuronal activity, research using experimenter-induced changes in neuronal activity suggests that approach behavior is affected by the anterior rather than the posterior PVT. Cue-induced sucrose magazine entries are not affected by chemogenetic inhibition of both anterior and posterior rat PVT (Choi et al., 2019) and cue-induced lever-pressing for a sucrose reward is unaffected by pharmacological inhibition of the rat posterior PVT, using microinjection of the GABA agonist, muscimol (Do-Monte et al., 2017). On the other hand, cue-induced lever-pressing for sucrose during a session where this reward is omitted (which can be conceptualized as a first extinction session) is increased by pharmacological inhibition of the rat anterior PVT and suppressed by photoactivation at cue onset (Do-Monte et al., 2017). This effect occurs *via* projections to the nucleus accumbens shell, as the inhibition of lever-pressing from PVT photoactivation is recapitulated by photoactivation of the anterior PVT-to-nucleus accumbens shell pathway and reversed by its photoinhibition (Do-Monte et al., 2017). In contrast, photoinhibition of the anterior PVT-to-central amygdala pathway, like photoactivation of the anterior PVT-to-nucleus accumbens pathway, reduces cue-induced lever-pressing for sucrose during a reward omission session (Do-Monte et al., 2017). Thus, approach or seeking behavior is affected by both the anterior and posterior PVT, but the direction of the effects of their activation may depend on the specific cell types and pathways involved.

Fear—the Posterior PVT Plays a Critical Role in Fear Retrieval

The PVT also shows a clear role in conditioned fear, particularly fear retrieval, with significant research on this topic focused on the posterior PVT. Neurons in the PVT show increased activity to a conditioned fear tone, but only starting more than 6 h following tone-shock pairings, and lasting at least 7 days. For example, in the rat PVT, more neurons show electrophysiological responses to a conditioned fear tone tested 24 h but not 2 h after conditioning, and levels of c-Fos are elevated in a fear retrieval test conducted 7 days but not 6 h following conditioning (Do-Monte et al., 2015). This same elevation in c-Fos is found when the mouse posterior PVT, rather than the whole PVT, is examined in a fear retrieval test conducted 24 h following fear conditioning (Penzo et al., 2015). It is also found 7 days following fear conditioning in rat prelimbic neurons that project to the middle-to-posterior PVT and in middle-to-posterior PVT neurons that project to the central nucleus of the amygdala (Do-Monte et al., 2015). Thus, neurons in the posterior PVT become activated during conditioned fear retrieval and this occurs *via* a prelimbic-to-PVT-to central amygdala pathway. In turn, activation of the PVT and this pathway appears to facilitate the expression of conditioned fear following this same delayed timeline. Freezing to a conditioned tone during a fear retrieval test conducted 24 h but not earlier after conditioning is diminished by pharmacological inhibition, using microinjection of muscimol into the rat dorsomedial nucleus which spreads into the PVT (Padilla-Coreano et al., 2012). Similarly, fear retrieval 1 week after conditioning is suppressed by bilateral lesions made after fear conditioning of the rat posterior PVT (Li Y. et al., 2014), and it is reduced by chemogenetic inhibition of the rat middle-to-posterior PVT when tested with an operant food reward available (Choi and McNally, 2017). It should be noted, however, that studies have not uniformly found manipulation of the PVT to affect fear recall, as freezing to a conditioned fear tone is unaffected by chemogenetic inhibition of the rat anterior

plus posterior PVT (Choi et al., 2019) and by microinjection of an orexin receptor antagonist into the rat posterior PVT (Dong et al., 2015). Despite this, there is solid evidence that activity in the prelimbic-to-PVT-to central amygdala pathway can promote the expression of conditioned fear. Fear retrieval is reduced by optogenetic silencing of prelimbic projections to the rat middle-to-posterior PVT (Do-Monte et al., 2015) and mouse PVT afferents to the central amygdala (Chen and Bi, 2019). Similarly, it is reduced by chemogenetic inhibition of mouse central amygdala-projecting PVT neurons (Penzo et al., 2015). In contrast, expression of conditioned fear is facilitated by photostimulation of mouse PVT afferents to the central amygdala (Chen and Bi, 2019). Collectively, the literature suggests that the PVT is recruited during consolidation of conditioned fear memory and, acting in response to prelimbic afferents and itself acting *via* the central amygdala, serves to promote the expression of conditioned fear.

CONCLUSIONS AND FUTURE DIRECTIONS

While much progress has been made in delineating the functional contribution of the PVT to emotional processing, some of the data as a whole remain equivocal, and, as such, many outstanding questions remain. The contribution of the specific PVT subregions does vary, based on the particular behavioral test employed and even within the same assay, suggesting that the exact role of the PVT may depend less on subregion and more on cell type. Very recent research has demonstrated the existence of antero-posterior gradients of two well-defined neuronal subtypes (Gao et al., 2020) and it is very likely that a similar pattern of distribution also exists for other neuronal subtypes within the PVT. Future studies are needed to develop efficient strategies to gain genetic access to these neuronal subtypes. The functional role of distinct PVT afferents and efferents in the regulation of specific behavioral tests of unconditioned and conditioned affective behavior are needed, and the application of genetic techniques for cell-type-specific monitoring and manipulation in the PVT will allow for rigorous testing of these questions. Of note, the discovery that many PVT projections are highly collateralized (Dong et al., 2017) has major implications for the design and interpretation of these tests. Future studies should involve well-controlled experiments for projection-specific manipulations, involving optogenetic and chemogenetic approaches, since the majority of PVT neurons project to the nucleus accumbens shell and provide collaterals to other regions, such as the BNST and central nucleus of the amygdala (Dong et al., 2017). Given the heterogeneity of PVT responses and effects on emotionally salient stimuli, future experiments should be designed to test the role of specific PVT cell types and connections in the processing of salient stimuli and subsequent behavioral output. It is especially crucial to understand how these different components of the PVT circuit (neuronal subtypes and afferent and efferent connections) act to encode distinct features of an explored environment to generate emotional fight-or-flight responses.

On the whole, however, evidence supports a strong role for the PVT in multiple aspects of emotional processing, demonstrating that it is both responsive to and itself affects both arousal and valence (**Figure 1**). The PVT, particularly the anterior PVT, can through its neuronal activity promote arousal, both as part of the sleep-wake cycle and in response to novel stimuli. The PVT is also involved in reward, being both responsive to rewarding stimuli and itself affecting behavior reflecting reward, likely *via* specific populations of cells distributed throughout its subregions. Similarly, PVT neuronal activity appears to affect depression-like behavior, through yet undefined subregions and cell populations. The posterior PVT in particular also demonstrates a role in anxiety-like behavior, generally promoting but also inhibiting this behavior. While this subregion is also especially responsive to stressors, it appears to function to suppress the stress response following chronic stress exposure. Following conditioning, the posterior PVT again plays a major role in emotional behavior. Neuronal activity in response to a reward-predictive cue can be detected throughout the PVT, but endogenous activity in the posterior PVT is a robust predictor of approach or seeking behavior. Similarly, neuronal activity during conditioned fear retrieval is detected in the posterior PVT and its activation appears to facilitate the expression of conditioned fear. Much of this involvement of the PVT in arousal and valence has been shown to occur through the same general afferents and efferents; however, a detailed functional map of the PVT circuits that control emotional responses, particularly those involving the posterior PVT, remains elusive. Afferents from the hypothalamus affect the involvement of the PVT in arousal, anxiety-like behavior, and the response to stress, and afferents from the prelimbic cortex affect its involvement in the expression of conditioned fear. In turn, through efferents to the nucleus accumbens, the PVT affects arousal, reward-related behavior, anxiety-like behavior, and motivation, and through efferents to the amygdala, it affects these same behaviors as well as the expression of conditioned fear. Finally, the PVT also affects arousal through efferents to the infralimbic cortex. It may be that some of these projections originate from the same PVT cells, but that remains to be determined. Thus, while caveats exist and more work is required to define its exact role, the PVT, through its extensive connections with other prominent nuclei in the limbic system, appears to be an integral part of the emotional processing network.

AUTHOR CONTRIBUTIONS

JB, NM, and W-JG wrote and edited the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This research was supported by the National Institute on Alcohol Abuse and Alcoholism under Award Number R01AA028218 (JB) and by the National Institute of Mental Health under Award Number R21MH121836 (W-JG and NM). The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Behavioral Neuroscience

ORIGINAL RESEARCH
published: 12 November 2020
doi: 10.3389/fnbeh.2020.581296

A Probiotic Mixture Induces Anxiolytic- and Antidepressive-Like Effects in Fischer and Maternally Deprived Long Evans Rats

Valérie Daugé[1], Catherine Philippe[2], Mahendra Mariadassou[5], Olivier Rué[5], Jean-Charles Martin[4], Marie-Noelle Rossignol[3], Nathalie Dourmap[6], Ljubica Svilar[7], Franck Tourniaire[7], Magali Monnoye[2], Deborah Jardet[3], Marie Bangratz[8], Sophie Holowacz[8], Sylvie Rabot[2] and Laurent Naudon[1]*

[1] Université Paris-Saclay, INRAE, AgroParisTech, CNRS, Micalis Institute, Jouy-en-Josas, France, [2] Université Paris-Saclay, INRAE, AgroParisTech, Micalis Institute, Jouy-en-Josas, France, [3] Université Paris-Saclay, INRAE, MaIAGE, Jouy-en-Josas, France, [4] Université Aix Marseille, C2VN, CRIBIOM, Marseille, France, [5] Université Paris-Saclay, INRAE, BioinfOmics, MIGALE Bioinformatics Facility, Jouy-en-Josas, France, [6] UNIROUEN, UFR Médecine-Pharmacie, Inserm U 1245 Team 4, Rouen, France, [7] Université Aix Marseille, C2VN, Marseille, France, [8] PiLeJe Laboratoire, Paris, France

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Edited by:
Jessica R. Barson,
Drexel University, United States

Reviewed by:
Robert Parrish Waters,
University of Mary Washington, United States
Yuval Silberman,
Pennsylvania State University, United States

***Correspondence:**
Valérie Daugé
valerie.dauge@inrae.fr

Specialty section:
This article was submitted to Emotion Regulation and Processing, a section of the journal Frontiers in Behavioral Neuroscience

Received: 08 July 2020
Accepted: 20 October 2020
Published: 12 November 2020

Citation:
Daugé V, Philippe C, Mariadassou M, Rué O, Martin J-C, Rossignol M-N, Dourmap N, Svilar L, Tourniaire F, Monnoye M, Jardet D, Bangratz M, Holowacz S, Rabot S and Naudon L (2020) A Probiotic Mixture Induces Anxiolytic- and Antidepressive-Like Effects in Fischer and Maternally Deprived Long Evans Rats. Front. Behav. Neurosci. 14:581296. doi: 10.3389/fnbeh.2020.581296

A role of the gut microbiota in psychiatric disorders is supported by a growing body of literature. The effects of a probiotic mixture of four bacterial strains were studied in two models of anxiety and depression, naturally stress-sensitive Fischer rats and Long Evans rats subjected to maternal deprivation. Rats chronically received either the probiotic mixture (1.10^9 CFU/day) or the vehicle. Anxiety- and depressive-like behaviors were evaluated in several tests. Brain monoamine levels and gut RNA expression of tight junction proteins (Tjp) and inflammatory markers were quantified. The gut microbiota was analyzed in feces by 16S rRNA gene sequencing. Untargeted metabolite analysis reflecting primary metabolism was performed in the cecal content and in serum. Fischer rats treated with the probiotic mixture manifested a decrease in anxiety-like behaviors, in the immobility time in the forced swimming test, as well as in levels of dopamine and its major metabolites, and those of serotonin metabolites in the hippocampus and striatum. In maternally deprived Long Evans rats treated with the probiotic mixture, the number of entries into the central area in the open-field test was increased, reflecting an anxiolytic effect. The probiotic mixture increased Tjp1 and decreased Ifnγ mRNA levels in the ileum of maternally deprived rats. In both models, probiotic supplementation changed the proportions of several Operational Taxonomic Units (OTU) in the gut microbiota, and the levels of certain cecal and serum metabolites were correlated with behavioral changes. Chronic administration of the tested probiotic mixture can therefore beneficially affect anxiety- and depressive-like behaviors in rats, possibly owing to changes in the levels of certain metabolites, such as 21-deoxycortisol, and changes in brain monoamines.

Keywords: probiotic, maternal deprivation, anxiety-like behavior, depressive-like behavior, monoamines, brain, metabolites, gut junction protein RNAs

INTRODUCTION

In addition to its well-known role in the development and maintenance of digestive, metabolic, and immune functions of the host, the gut microbiota is involved in the development and functioning of the central nervous system. Bidirectional communication between the gut microbiota and the central nervous system influences the development of the hypothalamic-pituitary-adrenal axis and stress reactivity. This has been well documented by studies in germ-free animals involving antibiotic or probiotic administration (Sudo et al., 2004; Crumeyrolle-Arias et al., 2014; see review in Luczynski et al., 2016). The gut microbiota also influences anxiety-and depression-like behaviors in rodents with differing effects according to their genetic constitution and their sensitivity to acute stress. When placed in a stressful environment, germ-free Swiss, NMRI and Kunming mice, which are moderately sensitive to stress, decreased anxiety-like behavior whereas germ-free Fischer 344 rats and BALB/C mice, which are more sensitive to stress, manifest an increase in anxiety-like behavior compared to their conventional counterparts (Diaz-Heijtz et al., 2011; Neufeld et al., 2011; Nishino et al., 2013; Crumeyrolle-Arias et al., 2014). Emotional responses following antibiotic-induced disturbances in the gut microbiota depend on the type of antibiotic. A mixture of antibiotics induced anxiolytic-like effects in conventional BALB/C mice subjected to light-dark box and the step-down tests (see review in Luczynski et al., 2016). Ampicillin increased anxiety-like behavior in the elevated plus maze and helplessness behavior in the forced swimming test in conventional BALB/C mice, whereas cefoperazone, belonging to a different antibiotic family, had no effect (Ceylani et al., 2018). Furthermore, modulatory effects of probiotics have been reported in several studies. Anxiolytic-like effects in BALB/C mice subjected to various behavioral tests were observed following treatment with several *Lactobacillus* and *Bifidobacterium* probiotic strains (Bercik et al., 2011; Bravo et al., 2011; Savignac et al., 2014). Similar findings were reported in the case of Wistar rats with regard to the conditioned defensive burying test (Messaoudi et al., 2011). In addition, *Lactobacillus rhamnosus* and *Lactobacillus helveticus* strains reversed the effects of maternal separation on neural circuits underpinning fear expression and extinction in infant Sprague Dawley rats (Cowan et al., 2019). The purpose of our study was to analyze the behavioral and biochemical effects of chronic oral administration of a probiotic mixture (M) of *Lactobacillus helveticus* LA 102, *Bifidobacterium longum* LA 101, *Lactococcus lactis* LA 103, and *Streptococcus thermophilus* LA 104 (Lactibiane Référence®, PiLeJe Laboratoire, France) in two experimental models of anxiety and depression. Since gut microbiota seems to modulate emotional responses depending on how animals react to stress, we studied the effects of M in experimental models comprising rats known to be hypersensitive to stress. The first experimental model was the Fischer rat, as this rat strain shows higher stress reactivity and depressive- and anxiety-like behaviors compared to other rat strains such as Sprague-Dawley or Wistar rats (Sudakov et al., 2001; Wu and Wang, 2010). The second experimental model, concerning Long Evans rats, was a model of maternal deprivation (pups separated from their mother and their littermates). In this strain, early environmental stress, such as maternal deprivation, leads to an increase in stress reactivity and anxiety-like behavior, as shown in the open-field test, as well as to opiate dependence in adulthood (Vazquez et al., 2005a,b). In Sprague-Dawley and Wistar rats, maternal deprivation separation in place of deprivation (pups being separated from their mother but not from their littermates) also led to dysbiosis, an increase in gut permeability, colon inflammation and induced visceral hyperalgesia (Barreau et al., 2004; O'Mahony et al., 2011; Pusceddu et al., 2015; Moya-Perez et al., 2017; Murakami et al., 2017).

Despite the abundance of data related to the ability of the gut microbiota to communicate with the central nervous system, and the effect of probiotics, the pathways and brain structures involved remain poorly understood. For this reason, in the present study, in addition to analyze emotional behavior in two experimental models after chronic treatment with M, we also quantified monoamine levels in the prefrontal cortex, striatum and hippocampus to assess possible correlations between behavioral responses and monoamine levels in these brain structures specifically involved in emotional behaviors. Furthermore, the impact of M on the levels of tight junction protein and inflammatory marker mRNAs was measured in the colon and ileum as an index of gut permeability. Finally, the composition of the microbiota in the feces, and the presence of certain metabolites in the cecal content and serum, were analyzed. The results of this study showed that chronic oral administration of M reduced anxiety-like and depressive-like behaviors, and modified gut microbiota and metabolites, in both Fischer rats and maternally-deprived Long Evans rats.

MATERIALS AND METHODS

Animals

Fischer 344 male rats ($n = 23$), aged 4 weeks, were purchased from Charles River (Saint-Germain-Nuelles, France). Eleven Long Evans female rats on day 14 of gestation were obtained from Janvier (Le Genest-Saint-Isle, France) and male F1 Long Evans male rats were retained for the study ($n = 48$). Rats were housed by pairs, except for pregnant rats, which were housed individually, in a room maintained at 20–24°C with a 12-h light/dark cycle. All rats received food (RO3; Scientific Animal Food and Engineering, Augy, France) and water *ad libitum*.

Experimental procedures were carried out in accordance with EU Directive 2010/63/EU for animal experiments and approved by the Ethics Committee of the INRAE Research Center at Jouy-en-Josas and by the French Research Ministry (Approval no. 1239).

Probiotic Mixture

M is composed of *Lactobacillus helveticus* LA 102, *Bifidobacterium longum* LA 101, *Lactococcus lactis* LA 103, and *Streptococcus thermophilus* LA 104 (Lactibiane Référence®, PiLeJe Laboratoire, Paris, France). It was dissolved extemporaneously in maltodextrin solution prior

to administration. Both M and the excipient were provided by Genibio (Lorp-Sentaraille, France).

Study Design

From 6 weeks of age onwards, rats received 0.5 mL of the excipient (control rats) or M (1.10^9 CFU) by gavage with probes (Phymep, France) 5 days a week for 5 weeks for Fischer rats and 9 weeks for Long Evans rats (until euthanasia). Behavioral tests were conducted 15 days after the first gavage. They lasted 15 days for Fischer rats and 38 days for Long Evans rats. Fischer and Long Evans rats were tested during their light phase and first subjected to the novel object test, then to the black and white box, elevated plus maze, open-field and forced swimming tests (progressing from a less to more stressful environment). One or two behavioral tests were performed per week between 9:00 a.m. and 4:00 p.m. The tests and the gavage took twice as long for Long Evans rats since they were twice as many (48 rats). One week after the last test, rats were sacrificed and blood, brain, feces, cecal content and intestinal tissue were collected.

Maternal Deprivation in Long Evans Rats

Maternal deprivation was performed as described by Vazquez et al. (2005b). The day of birth was designated as day 0. On postnatal day 1, litters were cross-fostered and culled to six to seven male pups. The pups were randomly attributed to foster dams to redistribute possible effects of genetic and prenatal factors and to obtain similar litter sizes. The litters were each assigned to an experimental group. From day 1, mothers were removed from their home cage and put in a new cage for 3 h/day, the same procedure being applied at each deprivation. Neonates belonging to the maternal deprivation group (D; $n = 24$) were individually placed in temperature- (30–34°C) and humidity-controlled cages divided into compartments. Pups' cages contained 2 cm of fresh shavings covered with absorbent paper. Pups were isolated daily from day 1 to 14 from 1:00 to 4:00 p.m. They then return to their respective home cage and their mother was placed back in the cage. Pups not subjected to maternal deprivation (ND; $n = 24$) remained with their mother during this period and were handled just to change the bedding in their cages once a week. On day 22, all pups were weaned from their mothers and housed in pairs from the same litter.

Behavioral Assessments

All behavioral tests were videotaped, and the data were analyzed by two experimenters blind to treatment.

Novel Object Test

This test was performed in a dimly lit dark gray open field (90 × 70 × 60 cm) containing an object (15 cm height) fixed (Patafix®) on the floor of the box. The latency time to the first visit to the object, the time spent close to the object and the number of visits to the object were recorded for 10 min (Ennaceur et al., 2005).

Light-Dark Box Test

The plastic test box (80 × 55 × 32 cm) was divided into two compartments of the same size, one black and topped with a black cover, the other white and illuminated (130 lux). The compartments were connected by a 10 × 10 cm opening located in the center of the partition. The rat was placed in the white area. The time spent in the white area, the number of transitions and the number of attempts to exit the dark compartment (head extended out of the dark side) were recorded for 10 min (Andrade and Graeff, 2001).

Elevated Plus Maze Test

The elevated plus maze was a dark gray apparatus comprising two open arms (50 × 10 cm), and two closed arms (50 × 10 × 50 cm). The maze was elevated to a height of 70 cm. The rat was placed in the center of the maze facing an open arm (Daugé et al., 1989). The number of visits into the open and closed arms and the time spent in these, the number of visits to the end of open arms and the number of head dippings and stretchings were recorded during 5 min.

Open Field Test

The same open field used in the novel object test was strongly illuminated (500 lux). Black lines on the floor delineated 10 ×10 cm squares (Daugé et al., 1988). The rats were placed in the same corner and videotracked for 6 min. The latency time to move from the initial corner was measured, as well as the number of rearings, squares crossed, defecations, groomings and entries into the central part of the field, considered as the most aversive section of the apparatus.

Forced Swimming Test

Rats were placed in a cylinder (diameter: 20 cm, height: 46 cm) filled with water (24 ± 1°C) up to 30 cm from the bottom (Naudon and Jay, 2005). The procedure consisted of a 15 min pre-test session followed by a 5 min test phase 24 h later, during which the immobility time was measured.

Euthanasia, Fluid, and Tissue Collection

Rats were euthanized by decapitation. Brains were frozen in isopentane at −30°C. The blood was centrifuged (2,500 g, 20 min, 4°C), and the serum was frozen at −80°C. Ileal mucosa scrapings and colonic sections were collected in RNA later. The cecal content was weighed and the feces collected. All samples were stored at −80°C.

Monoamines

Serial 150 μm thick coronal sections of the prefrontal cortex (PC), striatum and hippocampus were cut at −20°C using a cryostat microtome (Leica 3050S, Leica Microsystems, Germany). Sections were punched from the PC (anteroposterior, 4.6–2.6 mm from the bregma; lateral, 0–1 mm from bregma; dorsoventral, 3–6 mm from the skull), striatum (2.2–0.6 mm from the bregma; lateral, 1–4 mm from bregma; dorsoventral, 4–8 mm from the skull) and hippocampus (−2.5–4.6 mm from the bregma; lateral, 0–4 mm from bregma; dorsoventral, 2–4 mm from the skull) and the samples were frozen at −80°C (Paxinos and Watson, 1998).

The samples of the PC, striatum and hippocampus were homogenized by ultrasonication in 0.25 ml (PC, hippocampus) or 0.3 ml (striatum) of ice-cold 0.1 N perchloric acid containing

0.1% cysteine, using a Vibra Cell Sonicator (Sonics and Materials, Newtown, CT, United States), then centrifuged at 4°C for 10 min (18,000 g). The supernatants were filtered under pressure through 0.45 μm filters (Millipore, Ireland) and then kept frozen at −80°C until analysis. The pellets were resuspended in NaOH (0.1 M) and used for protein measurement (using the Bradford method).

The levels of dopamine (DA) and its metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), 3-methoxytyramine (3-MT), norepinephrine (NE), and serotonin (5-HT) and its metabolite 5-hydroxyindoleacetic acid (5HIAA) were determined in supernatants using a reversed-phase ion pair HPLC system with electrochemical detection (Leroux et al., 2014). The HPLC system consisted of a pump (Spectrasystem P1000 XR, Thermo Fisher Scientific, France) connected to a C18 reversed phase column Supelcosil (3.0 × 150 mm, 3 μm, Sigma-Aldrich, Bellefonte, PA, United States) coupled to an electrochemical detector (Decade II, Antec, Leyden, Netherlands) with a glassy carbon electrode set at 0.75 V vs. an Ag/AgCl reference electrode. The mobile phase consisted of 50 mM KH_2PO_4, 125 mL/L methanol, 0.5 mM octan-1-sulfonic acid sodium salt and 0.15 mM Na_2EDTA at pH 3.9. The mobile phase was filtered through 0.45 μm cellulose acetate filters (Millipore) and delivered at a flow rate of 0.5 mL/min. Samples (20 μL) were injected into the HPLC system by means of an automatic device (AS3000, Thermo Electron Corporation, San Jose, CA, United States). Chromatograms were recorded and integrated by PC integration Azur software (Datalys, Le Touvet, France).

Tight Junction Protein and Inflammatory Marker RNAs

RNAs of colonic and ileal mucosa were extracted using a Quiagen kit (Qiagen, France) according to the manufacturer's instructions. RNAs were quantified and assessed for quality using an Agilent TM Bioanalyzer (Agilent, France). Reverse transcription was accomplished using an Applied Biosystems High Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific, France). Pre-amplification was performed using an Applied Biosystems TaqMan PreAmp Master Mix kit (Thermo Fisher Scientific, France) and predesigned TaqMan primers (Applied Biosystems, France) (**Supplementary Table S1**). The pre-amplified targeted genes were quantified using Q-PCR based on TaqMan gene expression assays with the predesigned TaqMan primers.

Q-PCR was performed on a Q-PCR machine StepOne Plus Applied Biosystems (Thermo Fisher Scientific, France). β-Actin was the housekeeping gene. Gene expression values were calculated using the comparative threshold cycle (Ct) method to generate ΔCt values. The relative abundance of each RNA was normalized according to the equation: Relative Quantity $RQ = 2^{-\Delta\Delta Ct}$.

Feces Microbiota

A modified version of the Godon et al. protocol (Godon et al., 1997) was used for feces DNA extraction. For each animal, 200 mg of the frozen fecal sample were resuspended in a mixture of 250 μL of guanidine thiocyanate buffer (4 M guanidine thiocyanate–0.1 M Tris (pH 7.5), 40 μL of 10% *N*-lauroyl sarcosine–0.1 M phosphate buffer (pH 8.0) and 500 μL of 5% *N*-lauroyl sarcosine; the tubes were then incubated at 70°C for 1 h. After addition of one volume (750 μL) of 0.1 mm diameter silica beads (Sigma), the tubes were shaken for 10 min at maximum speed on a Vibrobroyeur MM200 (Retsch, Germany). The tubes were then vortexed and centrifuged at 18,000 *g* for 5 min at 4°C. After recovery of the supernatant, 30 μL of Proteinase K (Chemagic STAR DNA BTS kit, Perkin Elmer, United States) were added and the samples were incubated for 10 min at 70°C at 250 rpm in a Multi-Therm (Benchmark Scientific, United States), then for 5 min at 95°C for enzyme inactivation. The tubes were then centrifuged at 18,000 *g* for 5 min at 4°C and the supernatants were transferred into the wells of a Deepwell plate. The plate was then placed on a Chemagic STAR nucleic acid workstation (Hamilton, Perkin Elmer, United States) and the DNA was extracted from the samples using a Chemagic STAR DNA BTS kit (Perkin Elmer, United States) according to the manufacturer's instructions.

The V3-V4 regions of the 16S rDNA gene were amplified from the DNA extracts during the first PCR step using the fusion primers Vaiomer 1F and Vaiomer 1R (Nadkarni et al., 2002; **Supplementary Table S2**). The PCR (Lluch et al., 2015) was performed using 2 U of a DNA-free Taq DNA Polymerase and 1xTaq DNA polymerase buffer (MTP Taq DNA Polymerase, Sigma-Aldrich, United States). The buffer was completed with 10 nmol of dNTP mixture (Sigma-Aldrich, United States), 15 nmol of each primer (Eurofins) and Nuclease-free water (Qiagen, Germany) in a final volume of 50 μL.

The PCR reaction was carried out in a T100 Thermal cycler (Biorad, United States) as follows: an initial denaturation step (94°C for 10 min) was followed by 35 cycles of amplification (94°C for 1 min, 68°C for 1 min and 72°C for 1 min) and a final elongation step at 72°C for 10 min. Amplicons were then purified using a magnetic bead CleanPCR kit (Clean NA, GC biotech B.V., Netherlands) in a 96-well format. The concentration of the purified amplicons was checked using a Nanodrop spectrophotometer (Thermo Scientific, United States) and a subset of amplicon sizes was analyzed on a Fragment Analyzer (AATI, United States) with the reagent kit ADNdb 910 (35–1,500 bp). Sample multiplexing was performed by adding tailor-made 6 bp unique indexes during the second PCR step at the same time as the second part of the P5/P7 adapters to obtain the primer Vaiomer 2F and the reverse primer Vaiomer 2R (**Supplementary Table S2**). The second PCR (Lluch et al., 2015) step was performed on 50–200 ng of purified amplicons from the first PCR using 2.5 U of a DNA-free Taq DNA polymerase and 1xTaq DNA polymerase buffer. The buffer was completed with 10 nmol of dNTP mixture (Sigma-Aldrich, United States), 25 nmol of each primer (Eurofins, Luxembourg) and Nuclease-free water (Qiagen, Germany) up to a final volume of 50 μL. The PCR reaction was carried out on a T100 Thermal cycler with an initial denaturation step (94°C for 10 min), 12 cycles of amplification (94°C for 1 min, 65°C for 1 min and 72°C for 1 min) and a final elongation step at 72°C for 10 min. Amplicons were purified as described for the first

PCR reaction. The concentration of the purified amplicons was measured using NanoDrop spectrophotometer (Thermo Fisher Scientific, United States) and the quality of a subset of amplicons (12 samples per sequencing run) was assessed on a Fragment Analyzer (AATI, United States) with the reagent kit ADNdb 910 (35–1,500 bp).

Checks were carried out to ensure that the large number of PCR cycles (35 cycles for PCR1 + 12 cycles for PCR2) had not generated significant amounts of PCR chimera or other artifacts. The region of the 16S rDNA gene to be sequenced had a length of 467 bp for a total amplicon length of 522 bp after PCR1 and of 588 bp after PCR2 (using the 16S rDNA gene of *E. coli* as a reference).

Negative controls to assess the technical background were included using Nuclease-free water (Qiagen, Germany) in place of the extracted DNA during library preparation.

All libraries were pooled to equal amounts in order to generate an equivalent number of raw reads for each library. The DNA concentration of the pool (no dilution, diluted 10x and 25x in EB + Tween 0.5% buffer) was quantified on a Qubit Fluorometer (Thermo Fisher Scientific, United States). The pool, at a final concentration between 5 and 20 nM, was used for sequencing.

The pool was denatured (NaOH 0.1N) and diluted to 7 pM. The PhiX Control v3 (Illumina, United States) was added to the pool at 15% of the final concentration as described in the Illumina procedure. Aliquots (600 μL) of this pool and the PhiX mixture were loaded onto the Illumina MiSeq cartridge according to the manufacturer's instructions, using a MiSeq Reagent Kit v3 (2 × 300 bp Paired-End Reads, 15 Gb output). FastQ files were generated at the end of the run (MiSeq Reporter software, Illumina, United States) for quality control. The quality of the run was checked internally using PhiX Control and then each paired-end sequence was assigned to its sample using the multiplexing index.

Metabarcoding of the V3-V4 region of the 16S was performed using the Illumina MiSeq sequencing technology with the universal primers F343 (CTTTCCCTACACGACGCTCT TCCGATCTACGGRAGGCAGCAG) and R784 (GGAGTTCA GACGTGTGCTCTTCCGATCTTACCAGGGTATCTAATCCT). A total of 7,074,093 pairs from 118 samples (21,065–194,968 reads per sample) were provided by the sequencing platform. Quality controls of raw data were performed using FastQC v0.11.3 (Andrews, 2010) and no problems were detected. The pairs were then merged using Flash v1.2.11 (Magoc and Salzberg, 2011), the adapters were removed using cutadapt v1.12 (Martin, 2011) and the resulting sequences were cleaned using sickle v1.33 with the following filters (length > 20, no Ns, trimming of bases with quality lower than 20). In total, 84% of the pairs passed the initial quality control filters. The remaining sequences were then dereplicated and processed using the FROGS pipeline (Escudié et al., 2017) with default parameters. OTUs were clustered using Swarm v2.1.12 (Mahé et al., 2015) with parameter d = 3 and chimeras were filtered using vsearch v1.4 (Rognes et al., 2016) in *de-novo* mode. Finally, OTUs with low abundance (<50) and/or prevalence (found in less than eight samples) were filtered out, resulting in 2,576 OTUs (corresponding to 3,310,024 sequences). OTUs were affiliated by blasting cluster seed sequences against the Silva database v128 (Quast et al., 2013), 100% of the OTUs being affiliated, with at least 80% of identity and 80% of coverage between query and target.

Short-Chain Fatty Acids (SCFA)

Cecal content samples were extracted in water and proteins were precipitated by adding phosphotungstic acid. The supernatant fraction (0.1 μL) was analyzed on a gas–liquid chromatograph (Autosystem XL; Perkin Elmer, Saint-Quentin-en-Yvelines, France) using 2-ethylbutyrate as the internal standard. Data were collected and peaks integrated using Turbochrom v. 6 software (Perkin Elmer, Courtaboeuf, France) (Lan et al., 2007).

Liquid Chromatography Mass Spectrometry (LCMS) Metabolomics

Cecal Content Samples

Samples of cecal content (25 mg) were homogenized in 150 mL cooled methanol (at −20°C) for 1 min using a ball mill (Retsh®, France) at a frequency of 50 Hz. The homogenized samples were vortexed, incubated at −20°C for 30 min, and then centrifuged for 15 min (11,000 *g*, 4°C). The supernatant was filtered through 10 kDa filter tubes by centrifuging for 45 min (11,000 g, 4°C). The extracts obtained were dried in a stream of nitrogen and frozen at −80°C. All the dried polar extracts were reconstituted with 150 μL acetonitrile/water (50:50 v:v) and analyzed using a UPLC ultimate 3000 system (Thermo Scientific), coupled to a high-resolution Q-Exactive Plus mass spectrometer (Martin et al., 2015). Metabolites were identified by reference to an in-house database, including details of more than 800 metabolites with their chromatographic retention time.

Serum Samples

Serum samples were processed essentially as described by Rosique and colleagues (Rosique et al., 2019). LCMS analysis was performed as described above.

Statistical Analyses

The data obtained in all experiments (except those concerning the microbiome and metabolomics) were analyzed using ANOVA and Student's *t*-test for pairwise comparisons in the case of a normal distribution, and the Kruskal-Wallis and Mann and Whitney tests for pairwise comparisons in the case of a non-normal distribution (GraphPad Prism software, version 5.04, La Jolla, CA, United States). An emotionality *Z*-score corresponding to behavioral modifications (head dipping and exit attempts for Fischer rats, visits to the central area of the open field and rearings for Long Evans rats) was calculated (Guilloux et al., 2011).

Data concerning the feces microbiota were analyzed using R software (R Core Team, 2017), specifically Rstudio and the following R packages: ggplot2 v1.0 (Wickham, 2016) and phyloseq (McMurdie and Holmes, 2013). The raw (OTU) table comprised a total of 2,576 OTUs from 118 samples (data not shown). Before computing alpha and beta-diversity indices, samples were rarefied to the same read depth of 11,098, corresponding to the sample with the fewest reads. Local alpha-diversity was estimated through the richness and Inverse-Simpson indices. The effect of rat strain, M supplementation,

and maternal deprivation on α-diversity was then tested by ANOVA. Beta-diversity was estimated by the Bray-Curtis index and Permanova (using the adonis function from the VEGAN package) (Dixon, 2003) was used to test the effect of rat strain, M supplementation, and maternal deprivation on beta-diversities. To limit the confounding effect of rat strains, and at the cost of statistical power, we split the samples by rat strain before performing the differential abundance analysis. We also filtered OTU based on prevalence (>50% in one combination of maternal deprivation x probiotic administration) and relative abundance (>0.1% in at least one sample) resulting in 407–437 OTUs per rat strain. Differential abundance tests were performed using DESeq2 (Love et al., 2014) and OTUs with adjusted $p < 0.1$ were declared differentially abundant. For Long Evans rats, we used DESeq2 to test (i) the effect of maternal deprivation using only control samples (i.e., from rats not supplemented with M), (ii) the effect of M and M x maternal deprivation interaction using all samples. For Fischer rats, as there was no maternal deprivation, we estimated only the effect of M supplementation.

Metabolomic data were analyzed using PLS regression methods and univariate statistics using SIMCAP12 (Sartorius, Aubagne, France) and metaboanalyst (Xia et al., 2009).

The level of significance was set at $p < 0.05$.

RESULTS

Fischer Rats

Behavioral Assessments

Novel Object Test

There were no significant differences between M-supplemented and control rats in latency to the first visit to the novel object ($U = 59.5$, $p = 0.7$), number of visits ($U = 58.0$, $p = 0.6$) or time spent visiting the object ($U = 66.0$, $p = 0.9$).

Light-Dark Box Test

There was an increase in the number of exit attempts from the black compartment to the white one made by M-supplemented rats compared to control rats ($U = 25.5$, $p = 0.04$) (**Figure 1B**). No significant effect was observed with regard to time spent in the white compartment ($U = 45.5$, $p = 0.3$) or number of transitions ($U = 42.5$, $p = 0.5$).

Elevated Plus Maze Test

Compared to control rats, M-supplemented rats showed significantly more head dippings in the open arms ($t = 2.37$, $p = 0.02$; **Figure 1A**), whereas no significant effect was observed in the percentage of visits ($t = 0.03$, $p = 0.9$), the time spent in the open arms ($t = 0.51$, $p = 0.9$), the number of stretchings ($t = 0.61$, $p = 0.5$), or the number of visits to the end of the open arms ($t = 0.29$, $p = 0.7$).

Open Field Test

There were no significant differences between M-supplemented and control rats in the number of squares crossed ($U = 57.5$, $p = 0.8$), entries into the central area ($U = 59.5$, $p = 0.9$), rearings ($U = 56.5$, $p = 0.8$), groomings ($U = 52.5$, $p = 0.6$), or defecations ($U = 58.0$, $p = 0.9$), or in the latency time ($U = 53.0$, $p = 0.6$).

Forced Swimming Test

M-supplemented rats showed a significant decrease in immobility time compared to control rats ($t = 3.0$, $p = 0.005$) (**Figure 1C**).

Brain Monoamines

There were no significant differences between M-supplemented and control rats as regards monoamine levels in the PC (not shown). M supplementation significantly decreased DA ($U = 24.0$, $p = 0.05$), DOPAC ($U = 14.0$, $p = 0.002$) and HVA levels ($U = 7.0$, $p = 0.0005$) in the hippocampus as well as DA ($U = 27.0$, $p = 0.03$) and DOPAC ($U = 11.5$, $p = 0.001$) levels and ratio 5HIAA/5HT ($U = 20$, $p = 0.009$) in the striatum (**Table 1A**). In addition, there was a negative correlation between the increase in the number of head dippings in the elevated plus maze and DA (Spearman $r = -0.81$, $p = 0.003$) and DOPAC (Spearman $r = -0.76$, $p = 0.008$) levels in the hippocampus (not shown).

Tight Junction Protein and Inflammatory Marker mRNAs in the Gut Mucosa

RNAs of tight junction protein 1 (Tjp1), occludin 1 (OCEL1) and claudin 2 (Cldn2) as well as those of the inflammatory markers interleukin 10 (Il-10) and interferon γ (Ifnγ) were detected in the ileum and colon. M supplementation induced a significant decrease in Tjp1 ($U = 15$, $p = 0.001$) and Cldn2 ($U = 15$, $p = 0.001$) mRNAs in the colon compared to the levels of these proteins in the colon of non-supplemented control rats, but no difference was seen with regard to the ileum (**Table 2A**).

Fecal Microbiota

There was no significant variation in the α- and β-diversities between the two groups of rats [ANOVA of observed richness, $F_{(1,\ 22)} = 3.68$, $p = 0.06$; Permanova, $F_{(1,\ 22)} = 1.14$, $p = 0.2$, respectively]. Nevertheless, differential abundance analysis revealed five OTUs belonging to the *Bacteroidales*, *Lachnospiraceae,* and *Ruminococcaceae* that differed between the two groups: two OTUs from the genus *Lachnospiraceae* NK4A136 and one from an unknown genus were decreased in M-supplemented rats compared to the control group whereas one OTU from the genus *Lachnospiraceae* NK4A136 and one OTU of an unknown genus were increased in the M-supplemented group compared to the control group (**Figure 2A**).

Cecal Short-Chain Fatty Acids

M-supplementation induced a significant decrease in acetate as a percentage of total SCFA levels in the cecal content ($U = 32$, $p = 0.03$). Other SCFA and total SCFA levels did not differ between the groups (**Supplementary Table S3A**).

Metabolomics

A partial Least-square discriminant analysis (PLS-DA) was performed on the 121 annotated cecal metabolites to determine the effect of M. Statistical validations are provided in **Supplementary Material**. Applying a variable selection with a variable importance in projection (VIP) factor cut-off of 1.12, a robust discriminant model was calculated with 39

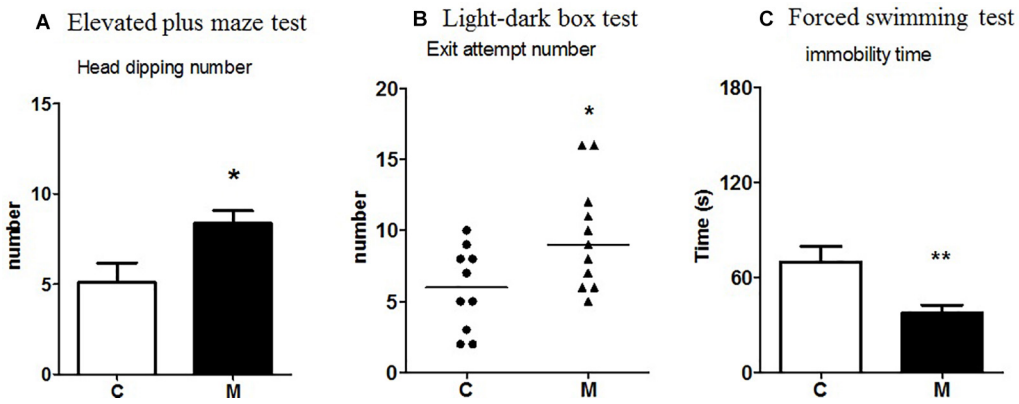

FIGURE 1 | Effect of the probiotic mixture in Fischer rats on **(A)** number of head dippings in the elevated plus maze test; **(B)** number of exit attempts in the light-dark box test; **(C)** immobility time (s) in the forced swimming test. C, control group ($n = 10$–12); M, group receiving the probiotic mixture ($n = 11$–12). The results are expressed as the mean ± SEM for the number of head dippings and immobility time and as the median and interquartile range for the number of exit attempts. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. C.

cecal metabolites ($p = 0.00056$ after cross-validation ANOVA) (**Supplementary Figure S1A**). At the individual scale, no metabolite appeared to be modified by M supplementation. Of the 39 metabolites identified, a combination of 25 was found to be associated with the *Z*-score in a PLS regression ($p = 0.01$) (**Supplementary Figure S2A**). Among these, only three were found to be associated with the *Z*-score at the individual level (**Supplementary Table S4A**), methylthioadenosine, thymidine and 2-acetamido-2-deoxy-beta-D-glucosylamine (**Supplementary Figure S2B**). No combination appeared to be related to the forced swimming behavior.

Long Evans Rats

Behavioral Assessments

Novel object test

ND and D rats did not differ significantly with regard to latency to the first visit to the novel object ($H = 4.34$, $p = 0.22$) or the number of visits ($H = 8.7$, $p = 0.03$; pairwise comparisons were not significant). In contrast, the time spent visiting the object was significantly decreased in D compared to ND rats ($U = 17.5$, $p = 0.01$). M supplementation did not change behavioral parameters.

Light-Dark Box Test

The time spent in the white compartment and the numbers of transitions and exit attempts did not differ significantly between the four groups (time spent: $H = 3.62$, $p = 0.3$; transitions: $H = 3.80$, $p = 0.2$; exit attempts: $H = 1.9$, $p = 0.3$).

Elevated Plus Maze Test

Maternal deprivation and M supplementation did not significantly affect the percentage of visits to the open arms or the percentage of time spent in these open arms [% of visits: deprivation factor: $F_{(1,\ 44} = 0$, treatment factor: $F_{(1,\ 44)} = 0.19$, $p = 0.6$, interaction: $F_{(1,\ 44)} = 0.01$, $p = 0.9$]; % of time spent: deprivation factor: $F_{(1,\ 44)} = 0.01$, $p = 0.9$, treatment factor: $F_{(1,\ 44)} = 0.13$, $p = 0.7$, interaction: $F_{(1,\ 44)} = 0.14$, $p = 0.7$), or the numbers of visits to the end of the open arms [deprivation factor: $F_{(1,\ 44)} = 0.5$, $p = 0.4$, treatment factor: $F_{(1,\ 44)} = 0.02$, $p = 0.88$, interaction: $F_{(1,\ 44)} = 1.08$, $p = 0.3$], head dippings [deprivation factor: $F_{(1,\ 44)} = 0.18$, $p = 0.6$, treatment factor: $F_{(1,\ 44)} = 0.83$, $p = 0.3$, interaction: $F_{(1,\ 44)} = 0.3$, $p = 0.5$] and stretchings [deprivation factor: $F_{(1,\ 44)} = 0.16$, $p = 0.6$, treatment factor: $F_{(1,\ 44)} = 0.32$, $p = 0.5$, interaction: $F_{(1,\ 44)} = 0.3$, $p = 0.5$].

Open field test

The number of rearings and visits to the central area were significantly different between the four groups (rearings: $H = 10.2$, $p = 0.01$; central area: $H = 13.7$, $p = 0.003$). Maternal deprivation decreased the number of rearings ($U = 19$, $p = 0.01$) and visits to the central area ($U = 22.5$; $p = 0.01$). In D rats, M increased the numbers of rearings ($U = 31.0$, $p = 0.03$) and visits to the central area ($U = 15.0$, $p < 0.001$; **Figure 3**). The latency time ($H = 3.14$, $p = 0.37$) and the numbers of squares crossed ($H = 6.48$, $p = 0.09$), groomings ($H = 4.58$, $p = 0.20$) and defecations ($H = 1.42$, $p = 0.70$) did not differ between the four groups (ND control, NDM, D control, DM).

Forced Swimming Test

There were no statistically significant differences between the four groups with respect to immobility time [deprivation: $F_{(1,\ 44)} = 0.95$, $p = 0.3$, treatment: $F_{(1,\ 44)} = 0.13$, $p = 0.7$, interaction: $F_{(1,\ 44)} = 0.04$, $p = 0.8$].

Brain Monoamines

Monoamine levels in the PC did not differ significantly between the four groups (not shown). In the hippocampus, there were significant differences between the four groups in the levels of DA ($H = 18.5$, $p = 0.0004$), DOPAC ($H = 9.3$, $p = 0.02$), HVA ($H = 13.9$, $p = 0.003$), 5HT ($H = 29.5$, $p = 0.0001$) and NE ($H = 15.3$, $p = 0.001$) and in the 5HIAA/5HT ratio ($H = 15.3$, $p = 0.001$). Maternal deprivation decreased the contents of DA ($U = 12$, $p = 0.001$), HVA ($U = 20$, $p = 0.01$), 5HT ($U = 28$, $p = 0.01$), and NE ($U = 16$, $p = 0.001$) and

TABLE 1 | Effects of the probiotic mixture on monoamine, metabolite (ng/mg proteins) and turnover (%) levels in the hippocampus and striatum of **(A)** Fischer, **(B)** maternally deprived Long Evans rats.

(A)

Hippocampus	Control	Probiotic mixture
Dopamine	2.7 (3.2)	0.83 (1.4)*
DOPAC	2.3 (2.5)	0.75 (0.8)**
HVA	6.2 (3.9)	3.3 (0.8)***
DOPAC/DA	98.7 (61.7)	83.7 (64.3)
HVA/DA	241.2 (151.8)	318.9 (199.6)
5HT	2.1 (1.1)	1.8 (0.7)
5HIAA	6.5 (3.4)	5.4 (2.3)
5HIAA/5HT	300.5 (108.1)	302.4 (57.9)
NE	3.1 (2.1)	3.0 (1.8)
Striatum		
Dopamine	145.6 (59.5)	107.2 (51.7)*
3-MT	2.8 (1.1)	2.6 (2.1)
DOPAC	13.4 (4.8)	8.9 (3.1)***
HVA	19.9 (5.4)	15.9 (9.6)
DOPAC/DA	9.3 (2.9)	8.5 (2.7)
HVA/DA	13.1 (4.4)	14.4 (5.9)
3-MT/DA	1.7 (0.4)	2.1 (1.1)
5HT	0.9 (1.6)	0.8 (1.1)
5HIAA	0.9 (1.1)	0.3 (0.7)
5HIAA/5HT	71.5 (46.5)	39.3 (28.5)**
NE	8.1 (6.5)	9.0 (6.7)

(B)

	NDC	NDM	DC	DM
Hippocampus				
DA	4.4 (6.6)	4.5 (4.6)	1.0 (0.8)***	2.4 (2.9)**$^{++}$
DOPAC	1.7 (3.0)	2.1 (3.7)	0.5 (0.6)	0.8 (1.0)$^{++}$
HVA	6.8 (5.1)	6.2 (8.4)	2.3 (2.3)**	4.0 (4.4)$^{+}$
DOPAC/DA	40.0 (24.7)	40.8 (29.9)	56.3 (49.1)	63.9 (55.9)
HVA/DA	134.4 (53.8)	150.4 (86.7)	233.5 (344.9)	211.0 (192.5)
5HT	2.6 (3.2)	2.7 (2.4)	1.2 (1.5)**	1.0 (1.0)**$^{++}$
5HIAA	7.2 (7.1)	6.4 (5.4)	4.9 (4.1)*	4.0 (3.9)**$^{+}$
5HIAA/5HT	277.0 (155.5)	275.4 (180.5)	403.2 (161.6)**	474.9 (227.3)**$^{+++}$
NE	7.4 (6.3)	7.0 (8.1)	3.4 (2.6)***	4.0 (2.1)**$^{++}$
Striatum				
DA	128.7 (55.1)	161.3 (72.8)	166.6 (72.5)*	194.1(59.2)***$^{+}$
3-MT	0.9 (0.6)	1.4 (0.8)	1.7 (0.7)***	1.7 (0.4)***$^{++}$
DOPAC	13.5 (8.9)	15.3 (10.6)	19.0 (11.0)**	28.7 (9.8)**$^{++\#}$
HVA	6.2 (4.1)	6.8 (4.3)	12.7 (8.6)**	10.9 (6.2)**$^{++}$
DOPAC/DA	11.4 (4.2)	11.7 (3.1)	11.7 (1.9)	14.7 (5.0)
HVA/DA	4.8 (2.3)	4.9 (1.6)	6.4 (3.9)	5.9 (3.9)
3-MT/DA	0.8 (0.4)	0.9 (0.3)	1.0 (0.2)	0.9 (0.2)
5HT	0.3 (0.3)	0.4 (0.4)	0.4 (0.4)	0.4 (0.3)
5HIAA	0.5 (0.3)	0.5 (0.3)	0.5 (0.4)	0.7 (0.4)
5HIAA/5HT	112.1 (117.0)	115.2 (62.8)	120.6 (70.8)	139.9 (64.9)
NE	7.0 (6.3)	4.9 (6.0)	3.6 (4.8)	3.4 (4.2)

*Mann and Whitney test, n = 11–12, *p < 0.05, **p < 0.01, ***p < 0.001 vs. C. DA, dopamine; 3-MT, 3-methoxytyramine; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid; 5HT, serotonine; 5HIAA, 5-hydroxyindolacetic acid; NE, norepinephrine. The results are expressed as median and interquartile range. ND, non deprived; D, deprived; C, control; M, probiotic mixture. Mann and Whitney test, n = 12, *p<0.05, **p < 0.01, ***p < 0.001 vs. NDC, $^{+}$p < 0.05, $^{++}$p < 0.01 vs. NDM, $^{\#}$p < 0.05 vs. DC.*

increased the 5HIAA/5HT ratio ($U = 22$, $p = 0.01$). Long Evans rats receiving M did not show significant differences compared to their control NDC and DC counterparts. In the striatum, the levels of 5HT and NE and monoamine turnover were not significantly modified but there were significant differences between the four groups in the levels of DA ($H = 12.6$, $p = 0.005$), 3-MT ($H = 17.3$, $p = 0.0006$), DOPAC ($H = 19.1$, $p = 0.0003$), and HVA ($H = 16.5$, $p = 0.0009$). Maternal deprivation induced an increase in DA ($U = 35$, $p = 0.03$), 3-MT ($U = 13.5$, $p = 0.0008$), DOPAC ($U = 31$, $p = 0.01$), and HVA ($U = 20$, $p = 0.006$) levels. M supplementation did not significantly modify the levels measured in the NDC and DC groups (**Table 1B**).

Tight Junction Protein and Inflammatory Marker RNAs

The mRNAs of Tjp1, OCEL1, Cldn2, Il-10, and Ifnγ were detected in the ileum and colon. In the colon, OCEL1 mRNAs and Tjp1 mRNAs levels were significantly modified between the four groups (OCLE1: $H = 7.88$, $p = 0.04$; Tjp1: $H = 8.59$, $p = 0.03$). In the colon, maternal deprivation induced a significant increase in OCEL1 mRNAs ($U = 31$, $p = 0.01$), M inducing a significant increase in Tjp1 ($U = 37$, $p = 0.04$) in this group. M also induced a significant increase in Tjp1 ($U = 34$, $p = 0.03$) and OCEL1 RNA levels ($U = 30$, $p = 0.01$) in the colon of ND rats. In the ileum, Tjp1 and Ifnγ RNA levels were significantly different between the four groups (Tjp1: $H = 14.5$, $p = 0.002$; Ifnγ: $H = 9.1$, $p = 0.02$). In the ileum, maternal deprivation significantly decreased Tjp1 expression levels ($U = 13$, $p = 0.0007$) and increased Ifnγ RNA levels ($U = 12$, $p = 0.009$), whereas M induced the opposite effects (Tjp1:$U = 30$, $p = 0.01$). M had no effects on RNA levels in the ileum of ND rats (**Table 2B**). In addition, there was a positive correlation between the time spent visiting a novel object and Tjp1 expression in the ileum of DM rats (Spearman r = 0.51, $p = 0.04$).

Fecal Microbiota

Analysis of β-diversity revealed significant differences between Fischer and Long Evans rats with respect to the overall composition of the fecal microbiota [Permanova, species: $F_{(1,\ 68)} = 42{,}42$, $p = 0.0001$; **Supplementary Figure S3A**].

In Long Evans rats alone, there was no significant variation in α-diversity between the four groups [ANOVA of observed richness, D: $F_{(1,\ 44)} = 3.94$, $p = 0.053$, M: $F_{(1,\ 44)} = 0.05$, $p = 0.81$, interaction: $F_{(1,\ 44)} = 0.29$, $p = 0.58$]. β-diversity analysis demonstrated that maternal deprivation had a significant impact on the overall composition of the fecal microbiota [Permanova, D: $F_{(1,\ 44)} = 2.974$, $p = 0.0006$, M: $F_{(1,\ 44)} = 1.37$, $p = 0.11$, interaction: $F_{(1,\ 44)} = 0.84$, $p = 0.64$, **Supplementary Figure S3B**].

Differential abundance analysis indicated that maternal deprivation modified the abundance of 47 genera belonging to the Bacteroidetes, Firmicutes and Proteobacteria phyla compared to the ND control group. The D control group showed a decrease in *Lachnospiraceae* NK4A136 (1 OTU), as well as in *Oscillibacter* (1 OTU), *Parabacteroides* (1 OTU), *Roseburia*

TABLE 2 | Effects of the probiotic mixture on junction protein (Tjp1, OCEL1, Cldn2) and inflammatory marker (TNF-α, Ifnγ, Il-10) RNAs in the ileum and colon of **(A)** Fischer and **(B)** maternally deprived Long Evans rats.

(A) Fischer rats						
Ileum						
	Tjp1	**OCEL1**	**Cldn2**	**TNF-α**	**Ifnγ**	**Il-10**
C	1.0 (0.5)	0.96 (0.3)	1.0 (0.3)	1.1 (0.9)	1.1 (0.6)	0.58 (1.2)
M	0.99 (0.4)	0.97 (0.4)	1.1 (0.7)	1.1 (0.7)	1.3 (1.2)	1.3 (0.9)
Colon						
	Tjp1	**OCEL1**	**Cldn2**	**TNF-α**	**Il-10**	
C	1.0 (0.3)	0.87 (0.6)	1.0 (0.5)	1.0 (0.7)	1.1 (0.9)	
M	0.67 (0.3)***	0.81 (0.4)	0.47 (0.3)***	0.77 (0.6)	0.97 (0.7)	
(B) Long Evans rats						
Ileum						
	Tjp1	**OCEL1**	**Cldn2**	**TNF-α**	**Ifnγ**	**Il-10**
NDC	0.99 (0.2)	0.93 (0.4)	0.99 (0.5)	1.02 (0.5)	0.86 (0.8)	1.06 (0.6)
NDM	0.85 (1.3)	1.14 (0.5)	1.19 (0.4)	0.86 (0.4)	1.59 (1.6)	0.79 (0.5)
DC	0.59 (0.2)***	1.11 (0.5)	1.03 (0.3)	1.12 (0.8)	2.90 (4.9)**	0.57 (0.7)
DM	0.88 (0.4)++	1.36 (0.5)	1.18 (0.5)	1.13 (0.8)	1.69 (4.8)	0.56 (0.7)
Colon						
	Tjp1	**OCEL1**	**Cldn2**	**TNF-α**	**Il-10**	
NDC	0.88 (0.4)	0.97 (0.3)	1.01 (0.6)	0.98 (1.1)	0.78 (0.7)	
NDM	1.18 (0.7)*	1.42 (0.7)**	1.06 (1.1)	0.64 (0.8)	0.92 (1.0)	
DC	0.94 (0.3)	1.33 (0.6)**	1.15 (0.7)	0.48 (0.3)	0.59 (0.4)	
DM	1.15 (0.7)+	1.17 (1.2)	1.33 (0.6)	1.25 (1.1)	1.14 (1.2)	

*Results are expressed as $2^{-\Delta\Delta Ct}$. Mann and Whitney test. n = 11–12. ***p = 0.001 vs. C.*
*Mann and Whitney test n = 11–12, *p < 0.05, **p< 0.01, **p < 0.01 vs. NDC, +p < 0.05, ++p < 0.01 vs. DC. ND, non deprived rats; D, deprived rats; C, control; M, probiotic mixture; Tjp1, tight junction protein 1; OCEL1, occludin 1; Cldn2, claudin 2; TNF-α, tumor necrosis factor; Ifnγ, interferon γ; Il-10, interleukine 10. The results are expressed as median and interquartile of $2^{-\Delta\Delta Ct}$ divided by the median of control group.*

(2 OTUs), *Ruminiclostridium* (1 OTU), *Ruminiclostridium* 5 (1 OTU), *Ruminiclostridium* 9 (1 OTU), *Ruminococcaceae* UGC-014 (1 OTU) and unknown genera (5 OTUs). The D control group showed an increase in *Anaerotruncus* (1 OTU), *Bacteroides* (3 OTUs), *Coprococcus* 1 (1 OTU), *Desulfovibrio* (1 OTU), *Lachnoclostridium* (2 OTUs), *Lachnospiraceae* NK4A136 (11 OTUs), multi-affiliation (2 OTUs), *Oscillibacter* (1 OTU), *Prevotellaceae* UCG-001 (2 OTUs), *Ruminococcaceae* UGC-014 (2 OTUs) and unknown genera (7 OTUs) (**Supplementary Figure S4**). M supplementation did not significantly modify the abundance of any bacteria in the ND group but did so for 3 OTUs from the *Lachnospiraceae and Ruminococcaceae* family in the D group, inducing an increase in *Butyricicoccus* (1 OTU) and *Oscillibacter* (1 OTU) and a decrease in unknown genera (2 OTUs) (**Figure 2B**).

Cecal Short-Chain Fatty Acids

Maternal Deprivation Had No Significant Effect on SCFA Levels

The percentage of caproate was significantly different between the four groups ($H = 13.6$, $p = 0.003$). M induced a significant decrease in the level of caproate in the cecal content as a percentage of total SCFA level compared to the DC group ($U = 16$, $p = 0.002$). Levels of other SCFA and total SCFA levels did not differ between the four groups (**Supplementary Table S3B**).

Metabolomics

A PLS-DA was performed on 121 and 131 annotated cecal and serum metabolites to detect any effect of M. Statistical validations are provided in **Supplementary Material**. A similar analysis was performed to select metabolites associated with maternal deprivation (**Supplementary Figures S1B,C**). A total of 16 metabolites in the cecal content and serum that were associated with both maternal deprivation and sensitivity to M were retained and analyzed for correlation with the Z-score (**Supplementary Table S4B**). A combination of 6 out of the 16 metabolites was found to be significantly associated with the Z-score in a PLS regression analysis. Serum 21-deoxycortisol and pyruvic acid were found to be both collectively and individually related to both maternal deprivation and M (**Figure 4**).

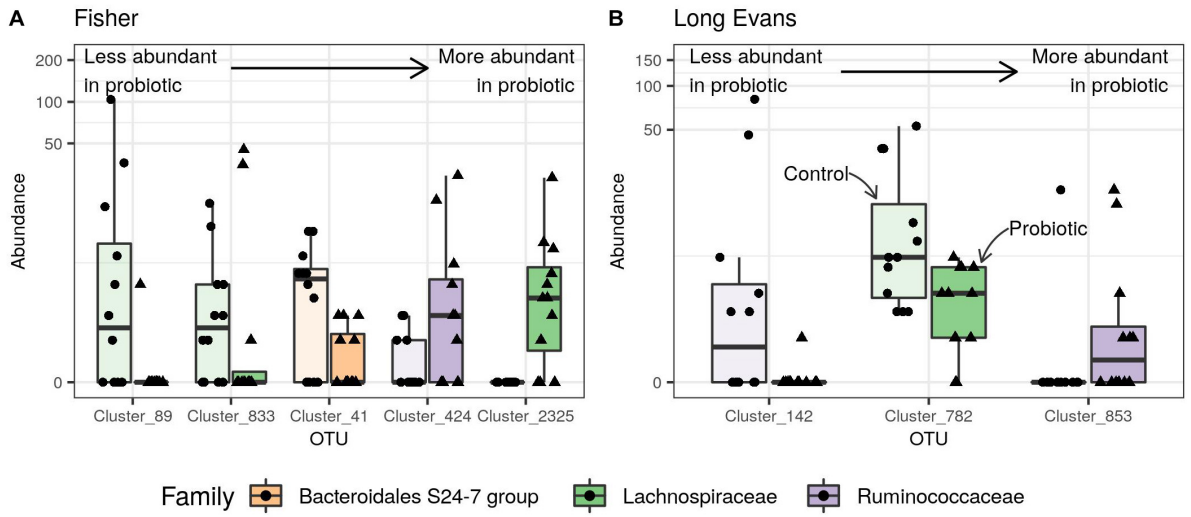

FIGURE 2 | Effect of the probiotic mixture on the normalized abundance of Differentially Abundant (DA) fecal microbial OTUs in **(A)** Fischer rats, **(B)** maternally deprived Long Evans rats. Points represent abundances in individual samples and boxplots indicate the median value and the interquartile range. Transparent boxplots correspond to control samples and opaque boxplots to samples from rats supplemented with the probiotic mixture. DA-OTUs are colored according to their rank and sorted by size effect: from less to more abundant in samples from probiotic-supplemented rats. Clusters 89, 833, and 2,325 were defined as *Lachnospiraceae* NK4A136 genus **(A)** and cluster 853 as *Butyricoccus* genus **(B)**.

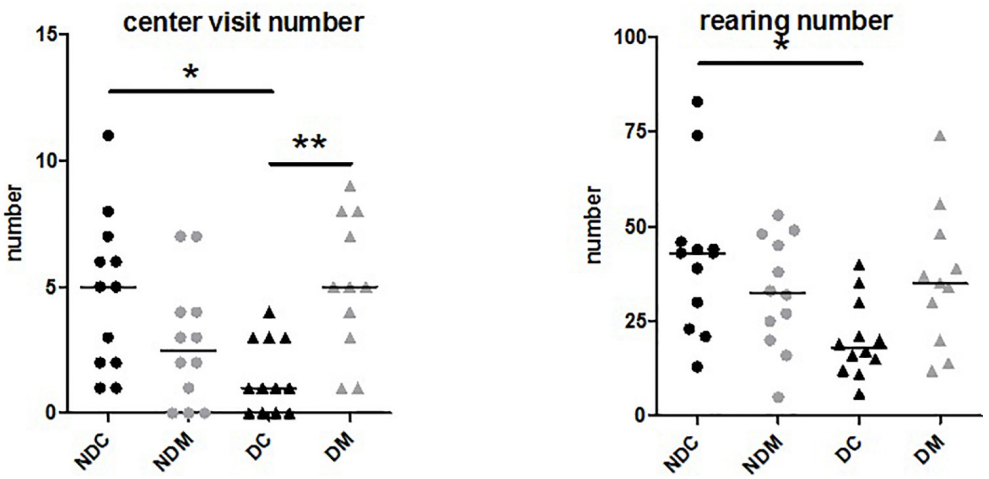

FIGURE 3 | Effect of the probiotic mixture on the number of visits to the central area and the number of rearings in the open field test in maternally deprived Long Evans rats. NDC, not maternally deprived control group ($n = 12$); NDM, not maternally deprived group receiving the probiotic mixture ($n = 12$); DC, maternally deprived control group ($n = 12$); DM, maternally deprived group receiving the probiotic mixture ($n = 12$). The results are expressed as the median and interquartile range. Number of visits to the central area ($H = 13.7$, $p = 0.003$), number of rearings ($H = 10.2$, $p = 0.01$) $^{*}p < 0.05$ vs. NDC, $^{**}p < 0.01$ vs. DC.

DISCUSSION

This study showed that chronic oral administration of M induced behavioral effects and modified gut microbiota and metabolites both in naturally stress-sensitive Fischer rats and in maternally-deprived Long Evans rats.

In Fischer rats, M did not modify the global motor activity in the elevated plus maze and in open-field test but increased the number of exit attempts in the light-dark box and head dippings in the open arms of the elevated plus maze. These findings could mean that M induced a slight decrease in anxiety-like behavior, since the classical parameters (time spent in the light compartment of the light-dark box and percentage of the number of visits and of time spent in the open arms of the elevated plus maze) were unchanged. The increase of head dippings may also suggest risk-taking behavior. An association

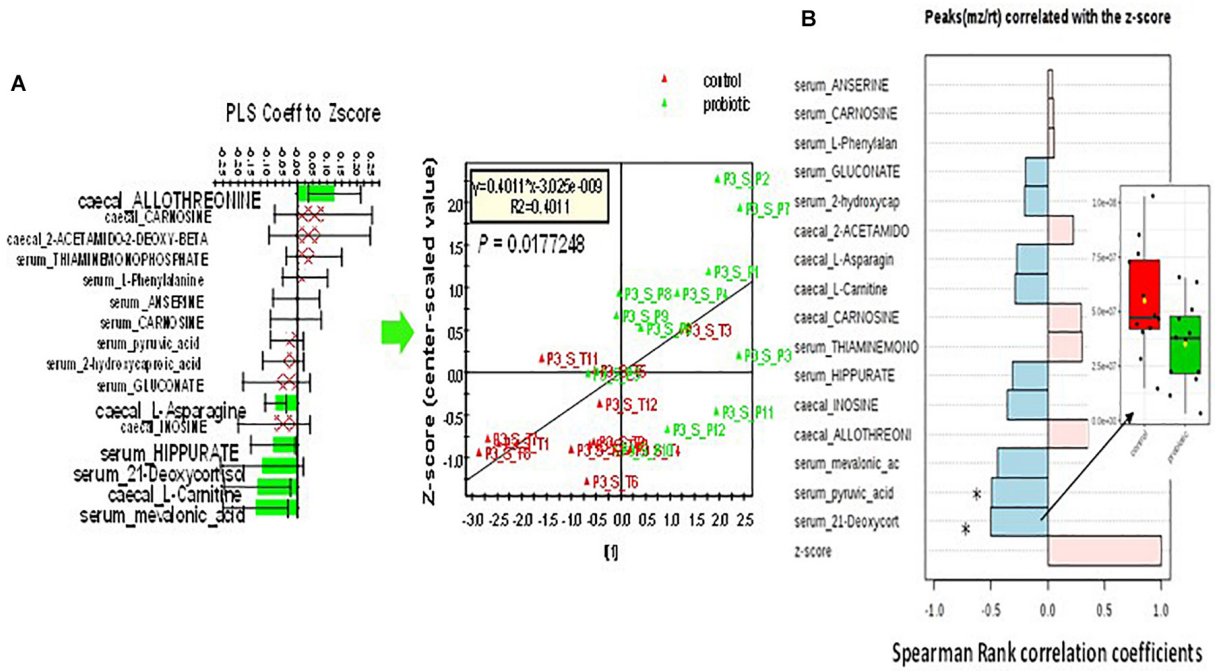

FIGURE 4 | Correlation of a selection of 16 cecal and serum metabolites in Long Evans DM rats with *Z*-score. **(A)** PLS regression of a combination of 6 out of the 16 selected metabolites to *Z*-score ($p = 0.01$ after cross-validation using ANOVA). **(B)** Individual Pearson correlation with *Z*-score. Serum 21-deoxycortisone and pyruvic acid both displayed a significant individual relationship to behavior and sensitivity to M (all adjusted values of $q < 0.1$). Insert: relative quantity of serum 21-deoxycortisol in control (red box) and treated (green box) rats.

of *Lactobacillus helveticus* (R0052) and *Bifidobacterium longum* (R0175) was also reported to decrease anxiety-like behavior in Wistar rats in the conditioned defensive burying test (Messaoudi et al., 2011). In contrast to the subtle effects of M on anxiety-like behavior, M decreased immobility time in the forced swimming test, indicating potential antidepressant-like properties.

M decreased dopaminergic transmission in the hippocampus and both dopaminergic and serotoninergic transmission in the striatum. Fischer rats are known to be hyper-reactive to stress and to present hyperactivity of the dopaminergic systems (Sziraki et al., 2001; Wu and Wang, 2010). A decrease in DA synthesis and/or the number of DA neurons induced by M could indicate a decrease in anxiety-like behavior for Fischer rats. In addition, there was a negative correlation between the increase in the number of head dippings in the elevated plus maze and DA and DOPAC levels in the hippocampus. This is in agreement with the role of the hippocampus in modulating fear and anxiety-like behaviors. This brain structure is connected to the septum, locus coeruleus, raphe nuclei, hypothalamus, amygdala and medial frontal cortex, all these cerebral regions being involved in anxiety. Furthermore, the hippocampus also receives dopaminergic afferences from the ventral tegmental area and may be substantia nigra, and both dorsal and ventral parts of the hippocampus are implicated in anxiety-like behavior through the activation of D1 and/or D2 receptors (Bast and Feldon, 2003; Nasehi et al., 2011; review in Zarrindast and Khakpai, 2015; Feng et al., 2018). Conversely, a lesion of the hippocampus or local administration of DA antagonists in the hippocampus induced a decrease in anxiety-like behavior (Zarrindast and Khakpai, 2015).

M also decreased Tjp1 and Cldn2 RNA levels in the colon. Usually, the greater the amount of tight junction proteins present, the more intestinal permeability is reduced. This seems to be the case for Tjp1 (Van Itallie and Anderson, 2014), but the opposite was found for Cldn2 (Zeissig et al., 2007). It is therefore difficult to draw any conclusion regarding this relationship without measuring both Tjp1 and Cldn2 protein levels and membrane permeability. None of the bacterial species constituting M was found in the rat feces but M treatment modified the abundance of five OTUs belonging to the Bacteroidetes and Firmicutes phyla. An increase or a decrease was observed in *Lachnospiraceae* NK4A136 abundance, as well as in that of unknown genera. The bacterial SCFA acetate level was decreased in the cecal content of M rats without any correlation with behaviors. On the other hand, the metabolites methylthioadenosine, thymidine and 2-acetamido-2-deoxy-beta-D-glucosylamine appeared to be individually correlated with the Z-score. The first two metabolites can be synthesized by *Lachnospiraceae* but as yet, no data are available concerning their behavioral effects.

Finally, M supplementation of Fischer rats induced anxiolytic- and antidepressant-like effects, which could be due in part to a decrease in dopaminergic transmission in the hippocampus and to the presence of certain host and bacterial metabolites in the intestinal content. M also acted on intestinal physiology and modified the abundance of certain bacterial strains in the gut microbiota.

In Long Evans rats, maternal deprivation, as expected, induced a decrease in the number of entries into the central part of the open-field (Vazquez et al., 2005a), and also in rearing behavior. In the novel object test, the time spent visiting the object was also reduced. We could not exclude that this could be due to a reduced movement in the arena. However, we did not observe changes in the number of squares crossed and rearing in the open-field test. The behavioral changes observed in the open-field test were completely suppressed by M, indicating anxiolytic-like properties. Although some data from the literature reported an anxiogenic-like effect in the elevated plus maze test, after maternal separation (Wang et al., 2020), we have never found such effect in our experimental conditions (deprivation model, 3h of deprivation from day 1 to 14). Lacidofil® and *Bifidobacterium pseudocatenulatum* CECT 7765 administered during the maternal separation period were reported to suppress, respectively, fear and anxiety-like behaviors in infant rodents (Cowan et al., 2016; Moya-Perez et al., 2017). Taken together, these results show that some probiotics, including M have preventive properties with regard to anxiety-like behavior but also may act on constitutive anxiety-like behavior.

As expected, maternal deprivation did not modify monoamine levels in the PC (Lejeune et al., 2013). However, it led to a decrease in catecholaminergic transmission and/or neurons and increased serotonergic neuronal activity in the hippocampus, as indicated with the increased 5HT turnover, whose ratio 5HIAA/5HT is an index. The elevated 5HT turnover could be an adaptation to compensate the decrease in other monoaminergic transmissions and/or neuronal loss. Dopaminergic transmission was also increased in the striatum. Although few data have been published regarding monoamine levels in the brain of maternally separated/deprived Long Evans rats, Matthews et al. (2001) found higher DA levels in the striatum and lower 5HT levels in the hippocampus of Lister-Hooded rats maternally separated for 6 h/day.

However, no correlation was found between behavioral changes and monoamine levels in either brain structure. M did not modify levels of monoamines and their metabolites in any of the three structures in either ND or D rats.

The higher OCEL1 mRNA levels in the colon of D rats could indicate an unexpected decrease in gut permeability but its role at tight junctions is still controversial. Notably, OCEL knockout mice have normal gut permeability and apparently normal tight junction. This protein might play an indirect role in permeability regulation (Van Itallie and Anderson, 2014). Maternal deprivation seemed to induce, as expected, a deterioration of the intestinal barrier with a decrease in Tjp1 RNA levels and an increase in expression of the pro-inflammatory marker Ifnγ in the ileum. Previous data concerning Sprague-Dawley rats showed that maternal separation increases the permeability of the intestinal barrier and also induces low-grade inflammation and greater microbial translocation, leading to functional deterioration of the intestine (Gareau et al., 2008; O'Mahony et al., 2011; Ganguly and Brenhouse, 2015). In the present study, chronic administration of M suppressed the abnormalities observed in the colon and ileum of D rats, indicating a potential protective effect on the intestinal barrier. In addition, there was a positive correlation between the time spent visiting a novel object and Tjp1 expression in the ileum of DM rats.

Maternal deprivation modified the abundance of 47 genera belonging to the Bacteroidetes, Firmicutes and Proteobacteria phyla. The maternal separation model has also been used as a model of irritable bowel syndrome, but the results obtained regarding gut microbiota composition are conflicting (Lejeune et al., 2013; Pusceddu et al., 2015; Murakami et al., 2017; Fukui et al., 2018). It is therefore conceivable that it is not the bacterial species that are important but rather their metabolic activities. In our study, none of the bacteria constituting M was found in the feces of rats but in D rats, M modified the abundance of certain bacterial species with an increase in members of the *Butyricicoccus* and *Oscillibacter* genera, and a decrease in bacteria belonging to unknown genera. *Butyricicoccus pullicaecorum* has been shown to be a potential probiotic in patients with inflammatory bowel disease and to attenuate colitis in rats (Eeckhaut et al., 2013; Steppe et al., 2014). *Oscillibacter* is known to produce anti-inflammatory metabolites (Lino et al., 2007; Man et al., 2011; Arpaia et al., 2013). There was also a decrease in caproate level in the cecal content of DM rats without any correlation with behaviors. Levels of certain metabolites in the cecal content and serum of maternally deprived rats differed according to whether the rats had been supplemented with M and these differences were correlated with behavior. Among them serum 21-deoxycortisol, a barely described metabolite in rat was reported to occur through adrenal mitochondrial CYP21A1 from 17α-hydroxyprogesterone (Maschler and Horn, 1976). 21-deoxycortisol has been shown to behave as a glucocorticoid receptor agonist (Pijnenburg-Kleizen et al., 2015) and for the first time we have shown its increase after maternal deprivation. Interestingly, its decrease in rats supplemented with M was associated with a decrease in anxiety-like behavior in D rats although cortisol was not detected and serum corticosterone levels remained unchanged (data not shown).

Finally, in Long Evans rats, M induced anxiolytic-like effects that were correlated with changes in certain host and bacterial metabolites, seemed to enhance the intestinal barrier, and modified the profile of the gut microbiota.

It is interesting to note that although Fischer and maternally deprived Long Evans rats showed distinct anxiety-like behaviors (fear of emptiness and light in Fischer rats and fear of large spaces and/or light in Long Evans rats), M was effective in reducing these behaviors in all cases.

CONCLUSION

In conclusion, the probiotic mixture tested (M) can beneficially affect anxiety- and depressive-like behaviors in both naturally stress-sensitive Fischer rats and maternally deprived Long Evans rats. M modified the composition of the gut microbiota, intestinal physiology in Fischer and Long Evans rats and brain monoamines in Fischer rats. intestinal tight junction protein expression and some cecal and serum levels of certain metabolites in Fischer and Long Evans rats were observed, reinforcing our knowledge of the

links between the gut microbiota and the brain. The next step will be to investigate the behavioral effects of methylthioadenosine, thymidine, 2-acetamido-2-deoxy-beta-D-glucosylamine and 21-deoxycortisol in our experimental models and to determine their mechanisms of action.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: https://www.ncbi.nlm.nih.gov/sra/PRJNA668659 and https://www.ebi.ac.uk/metabolights/, ID: MTBLS2126.

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethics Committee of the INRAE Research Center at Jouy-en-Josas and by the French Research Ministry (Approval No. 1239).

AUTHOR CONTRIBUTIONS

CP, MMa, OR, M-NR, LS, FT, MMo, and DJ designed the work that led to the submission, acquired data, drafted the manuscript, and approved the final version. J-CM and ND designed the work, acquired data, played a role in interpreting the results, drafted the manuscript, and approved the final version. MB and SH played an important role in interpreting the results, revised the manuscript, and approved the final version. SR and LN conceived and designed the work, played an important role in interpreting the results, revised the manuscript, and approved the final version. VD conceived and designed the work, acquired data, played an important role in interpreting the results, drafted and revised the manuscript, and approved the final version. All authors contributed to the article and approved the submitted version.

FUNDING

The reported study was supported by the French National Institute for Agronomic Research (INRA), the French National Centre for Scientific Research (CNRS), and a contract with PiLeJe.

ACKNOWLEDGMENTS

We wish to thank Emilie Caillère for rat care and gavage and for her help with the behavioral experiments. We are also grateful to Véronique Douard and Claude Blondeau for their thoughtful comments on the manuscript and to Vivien Paula Harry (freelance medical writer) for editorial assistance. We are grateful to the INRAE MIGALE bioinformatics facility (MIGALE, INRAE, 2020. Migale Bioinformatics Facility, doi: 10.15454/1.5572390655343293E12) for providing help and/or computing and/or storage resources.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2020.581296/full#supplementary-material

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Conflict of Interest: SH and MB were employees of PiLeJe Laboratoire.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Behavioral Neuroscience

ORIGINAL RESEARCH
published: 12 January 2021
doi: 10.3389/fnbeh.2020.576247

The Neurocircuitry Underlying Additive Effects of Safety Instruction on Extinction Learning

Arash Javanbakht[1], Lana Ruvolo Grasser[1], Shantanu Madaboosi[1], Asadur Chowdury[1], Israel Liberzon[2] and Vaibhav A. Diwadkar[1]*

[1] Department of Psychiatry and Behavioral Neurosciences, Wayne State University School of Medicine, Detroit, MI, United States, [2] Department of Psychiatry, Texas A&M University Central Texas, Killeen, TX, United States

Extinction learning is the dominant laboratory model for exposure therapy, a treatment involving both experience of safety near the feared object, and safety instructions relayed by a therapist. While the experiential aspect of extinction learning is well researched, less is known about instructed extinction learning and its neurocircuitry. Here, in 14 healthy participants we examined the neural correlates of, and the network interactions evoked by instructed extinction learning. Following fear conditioning to two CS+ stimuli, participants were instructed about the absence of the aversive unconditioned stimulus (US) for one of the CS+s (instructed CS; CS+I) but not the second CS+ (uninstructed CS+; CS+U). Early during extinction learning, greater activation was observed for the CS+I > CS+U contrast in regions including the vmPFC, dmPFC, vlPFC, and right parahippocampus. Subsequently, psychophysiological interaction (PPI) was applied to investigate functional connectivity of a seed in the vmPFC. This analyses revealed significant modulation of the dmPFC, parahippocampus, amygdala, and insula. Our findings suggest that the addition of cognitive instruction yields greater activation of emotion regulation and reappraisal networks during extinction learning. This work is a step in advancing laboratory paradigms that more accurately model exposure therapy and identifies regions which may be potential targets for neuromodulation to enhance psychotherapy effects.

Keywords: instructed extinction, extinction learning, fear conditioning, fear extinction, informed extinction, fMRI, PPI

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Edited by:
Yuval Silberman, Pennsylvania State University, United States

Reviewed by:
Heidi Catherine Meyer, Weill Cornell Medicine Psychiatry, United States
Mark D. Meadowcroft, Pennsylvania State University (PSU), United States

****Correspondence:***
Arash Javanbakht
ajavanba@med.wayne.edu

Specialty section:
This article was submitted to Emotion Regulation and Processing, a section of the journal Frontiers in Behavioral Neuroscience

Received: *25 June 2020*
Accepted: *14 December 2020*
Published: *12 January 2021*

Citation:
Javanbakht A, Grasser LR, Madaboosi S, Chowdury A, Liberzon I and Diwadkar VA (2021) The Neurocircuitry Underlying Additive Effects of Safety Instruction on Extinction Learning. Front. Behav. Neurosci. 14:576247. doi: 10.3389/fnbeh.2020.576247

INTRODUCTION

Fear conditioning is the established laboratory model for emotional learning (Armony et al., 1997; Craske et al., 2014). During classical fear conditioning, a neutral cue (conditioned stimulus, CS+) is repeatedly paired with an aversive stimulus (unconditioned stimulus, US), leading to the development of a fear response to the CS+ (even in the absence of the US) termed the conditioned response (CR). The CR may be defined as increased startle response, elevated electrodermal activity, and/or increased self-reported fear/anxiety/expectation of the CS-US pairing (Grasser and Jovanovic, 2020). This self-reported knowledge that an individual may have regarding the CS predicting the US is called contingency awareness (Craske et al., 2008), and contingency awareness in turn is a strong correlate of the conditioned response (Purkis and Lipp, 2007). Extinction learning is a complement to fear conditioning in which a new competing memory that is formed indicates

that the CS+ is no longer predictive of the US. This is achieved via repeated presentation of the CS+ without the US, leading to a decay in the CR (Milad and Quirk, 2012). Extinction learning is a form of safety learning (Grasser and Jovanovic, 2020) and a key mechanism (and thus a dominant laboratory model) for exposure therapy (Craske et al., 2008). Notably, abnormalities in extinction learning have been linked to fear related disorders such as phobias and post-traumatic stress disorder (Norrholm and Jovanovic, 2018).

Fear and safety learning in humans occur through a combination of direct experience (Pavlovian conditioning), observation (of others being exposed to adverse and safe events), and instruction (Olsson and Phelps, 2004). Instruction-mediated conditioning and learning is highly compelling from the perspective of top-down cortical mechanisms and relevant from the perspective of exposure therapy (Javanbakht et al., 2017). Behavioral and psychophysiology studies have demonstrated that in addition to fear conditioning solely through direct experience (as would most likely happen naturalistically), explicitly instructing participants to expect the US to follow the CS+ also leads to the development of a fear response to the CS+ (Olsson and Phelps, 2004; Raes et al., 2014; Cameron et al., 2016). The aforementioned studies have found that Pavlovian, instructed, and observational methods of fear conditioning result in similar levels of learning. In a similar vein, participants can be instructed about the *absence* of previously established contingency relationships between the CS+ and the US, thereby motivating extinction learning solely through instruction. For example, when instructed that they will no longer receive an aversive US after a previously conditioned CS+, participants show either immediate extinction (Mower, 1938; Rowles et al., 2012; Sevenster et al., 2012), or faster decay of the fear response (Koenig and Henriksen, 2005). These studies have provided valuable information regarding different learning mechanisms through which fear can be extinguished, but they have been limited by (a) between-subjects designs and (b) lack of neuroimaging data.

Complementary "bottom-up" and "top-down" mechanisms mediate brain network interactions underpinning higher-level processes such as self-referential processing (Frewen et al., 2020). "Bottom-up" mechanisms may primarily engage the salience network [insula, anterior cingulate cortex (ACC), and amygdala—see below] to alert individuals to threatening and rewarding stimuli in the environment without conscious knowledge/action. "Top-down" mechanisms may primarily engage the prefrontal cortex to consciously select and attend to stimuli in the environment based on conscious will/effort, and to regulate reactive "bottom-up" responses. Both are presumed to in part underpin psychotherapeutic efficacy (Malejko et al., 2017). Thus, studying their combined effects can inform neuroscientific theories about fear and safety, and enhance the clinical relevance of laboratory models (Javanbakht et al., 2017). In this work, we experimentally manipulated experience and instruction-based extinction learning, using an event-related fMRI design with the aim of identifying the evoked brain network profiles.

A comprehensive overview of the known neurocircuitry of fear learning is beyond our scope (Maren, 2001). Nevertheless, we succinctly sample from a wealth of neuroimaging data to provide a brief summary of key regions herein. The regions in this circuit include the dorsal anterior insula, dorsal ACC, and the amygdala (Adolphs, 2013; Yin et al., 2018), all of which comprise the salience network. The amygdala also playing a significant role in the development of extinction learning, disseminating signals across cortex, including throughout prefrontal regions, to orient and alert the brain to salient stimuli in the environment. Moreover, the structure activates both brainstem and hypothalamus to mount behavioral responses via the sympathetic adrenal medullary axis and the hypothalamic pituitary adrenal axis (Ross et al., 2017). The hippocampus and prefrontal cortex are involved in the formation and recall of extinction memories via context processing, consolidation, and retrieval (Greco and Liberzon, 2016), and the ventromedial prefrontal cortex (vmPFC) has a specific regulatory function regarding the inhibition of the amygdala and the fear response during extinction learning (Kim et al., 2003; Urry et al., 2006; Milad et al., 2007b; Ganella et al., 2017). Both the hippocampus and vmPFC are anatomically connected to the amygdala (Corches et al., 2019). Other areas involved in extinction learning include the anterior cingulate and dorsolateral prefrontal cortex (Greco and Liberzon, 2016). Preclinical studies (Fendt, 1998, 2000; Meyer et al., 2019) corroborate the findings from *in vivo* neuroimaging work in humans.

Functional imaging studies of *instructed* extinction learning are absent, though some studies have attempted to understand the neuronal bases of observational learning. For example, the ventromedial prefrontal cortex (vmPFC) is involved in vicarious learning of safety via observing others (Golkar et al., 2016) and, along with the hippocampus, context processing during extinction learning (Ahs et al., 2015; Hermann et al., 2017). *Context* plays a key role in extinction learning (Bouton, 2002; Bouton et al., 2006). Most studies focus primarily on *physical* context, but in fact context covers a broader spectrum including internal, temporal, and social/cognitive components (Bouton, 2002; Bouton et al., 2006; Liberzon and Sripada, 2008; Javanbakht, 2018). In this spectrum, *cognitive* context (operationalized as explicit instruction) may recruit the hippocampus and prefrontal cortex to judge the relevance of stimuli to memories and strategic goals (Liberzon and Sripada, 2008). Interestingly, instructed reappraisal, another method of emotion regulation, also activates the vmPFC as well as the dmPFC, the dlPFC, and the ACC (Pico-Perez et al., 2019).

In a previous behavioral study (Javanbakht et al., 2017) we examined the additive effects of safety instruction and experience on extinction learning. In the conditioning phase, participants were conditioned to two CS+ stimuli. Then, prior to extinction, they were instructed that the contingency pairing would be removed from one CS+, US contingency. The study revealed that instruction elicited a smaller fear response (measured by skin conductance) compared to the uninstructed CS+ during the *early* phase of extinction learning. These findings were the first to demonstrate the salient effects of instruction in facilitating extinction learning using a within-subject design comparing instruction + experience, vs. experience alone, but it

did not investigate the induced functional interactions within the neurocircuitry of fear.

In the present investigation and analyses, we explore how the combined effects of safety instruction and experiential learning modulated the (a) activation of brain regions and (b) network profiles in the brain's fear circuit. Notably, we hypothesized that the additive effect of instruction with experiential extinction learning would recruit the vmPFC and hippocampus, brain areas involved in emotion regulation and context processing. Psychophysiological interaction (PPI) was used as a simple framework for estimating directional (i.e., from seed-to-target) functional connectivity between a seed region (which in our case was the vmPFC) and its potential functional targets (O'Reilly et al., 2012; Silverstein et al., 2016).

MATERIALS AND METHODS

Participants

Thirty-seven (37) healthy male ($n = 17$) and female ($n = 20$) participants between the ages of 18 and 45 ($\overline{X}_{age} = 26.18$, $SD = 4.61$) were recruited for this study using approved flyers and university forum posts. Oral consent and initial eligibility screening were completed via phone interview. Six participants were excluded due to excess motion (> 4 mm) during MR imaging, leaving 31 (male = 16, female = 15, $\overline{X}_{age} = 26.18$, $SD = 4.78$) participants with usable data to include for analysis. The Institutional Review Board at WSU approved the protocol and all procedures herein. All participants gave informed consent to participate in the study and were able to tolerate small, enclosed spaces associated with fMRI data acquisition without anxiety. Exclusion criteria were: (1) lifetime psychiatric diagnoses (with the exception of history of substance related disorders more than 1 year prior), (2) serious medical or neurological illness that could compromise brain function, (3) history of significant closed head injury, (4) metal, implants, or metallic substances in the body, and/or (5) pregnant or trying to become pregnant. The Mini-International Neuropsychiatric Interview (MINI) was used to rule out psychiatric diagnoses. Consent and administration of the MINI were concurrent, on a separate screening day from the neuroimaging scans (and up to 2 weeks in advance of MRI acquisition). At the time of the screening, participants were exposed to a single trial of the US, a white noise burst described below, to ensure that they could tolerate the sound.

fMRI

Multiband gradient echo EPI fMRI was conducted on a 3T Siemens Verio system using a 32-channel volume head coil (310 vol, TR = 2 s, TE = 29 ms, multiband factor = 3, FOV = 256 × 256 × 144 mm^3, acquisition matrix = 128 × 128, 72 axial slices, pixel resolution = 2 × 2 × 2 mm^3, 10:48 mts). A high-resolution (1 mm^3) structural T_1-weighted MRI image was also collected. A scout image in each plane was acquired followed by a 3D T_1-weighted anatomical MRI image [3D Magnetization Prepared Rapid Gradient Echo (MPRAGE) sequence, TR = 2,150 ms, TE = 3.53 ms, TI-1,100 ms, flip-angle = 8°, FOV = 256 × 256 × 160 mm^3, 160 axial slices of thickness = 1 mm, pixel resolution = 1 × 1 × 1 mm^3, 4:59 mts].

Fear Conditioning and Extinction Learning Paradigm

The employed paradigm was identical to one used previously in a study of instructed fear and extinction learning (Javanbakht et al., 2017) and has been generally applied to study fear and extinction learning (Milad et al., 2005, 2007a,b, 2008, 2009; Milad and Quirk, 2012; Marin et al., 2017; Raij et al., 2018). During the task, stimuli were presented via an MR compatible projector with the paradigm controlled using ePrime 2.0 (Schneider et al., 2002). CSs included pictures of a lamp with three different colors (red, green, and yellow) placed either in an office or in a conference room (stimuli presented in **Figure 1**). The different rooms provided context such that one room was presented consistently during conditioning and the other room during extinction (Milad et al., 2005; Marin et al., 2017; Raij et al., 2018). The assignment of lamp colors and context images to participants was randomized and counterbalanced (randomly differed across participants). The US was a 95 db white noise burst (presented for 500 ms) (Sperl et al., 2016) conveyed through MRI compatible noise canceling headphones. The headphones were worn during conditioning and extinction, signaling the possibility of the US being administered throughout all phases.

The protocol consisted of habituation, conditioning, and extinction learning (protocol schematic in **Figure 1**; Milad et al., 2005, 2007a,b, 2008, 2009; Milad and Quirk, 2012; Javanbakht et al., 2017; Marin et al., 2017; Raij et al., 2018). For habituation, participants were informed that they would see a series of images, none of which would be followed by a loud noise. The purpose of the habituation phase is to remove the effects of novelty of the images that will be presented during the task. Participants saw each of the three CSs, once in the fear conditioning context and once in the extinction context, over four trials, and never heard the loud noise. Fear conditioning immediately followed. Participants were told that they would see the previous images, some of which would be followed by a loud noise. Each image was presented for 4 s with jittered inter-trial intervals between 6 and 12 s. Each CS was presented 15 times; the two CS+ stimuli were paired with the US for 10 trials (i.e., 66% reinforcement). The US was presented 3.5 s after CS+ onset. The third CS (CS-) was never paired with the US.

The session concluded with extinction learning, 10 min after fear conditioning. Prior to extinction learning, all participants were presented with instructions stating that they would *not* hear the loud noise when they saw one of the two CS+s (Instructed CS, CS+I, "You will not hear the loud noise with the red light"). The other CS+ was defined as the uninstructed CS+, CS+U. For extinction learning, each CS was presented 12 times for 4 s with jittered inter-trial intervals between 6 and 12 s, without the US.

At the end of the conditioning phase and again at the end of the extinction phase, participants were asked to verbally rate how much they expected to hear the loud noise when

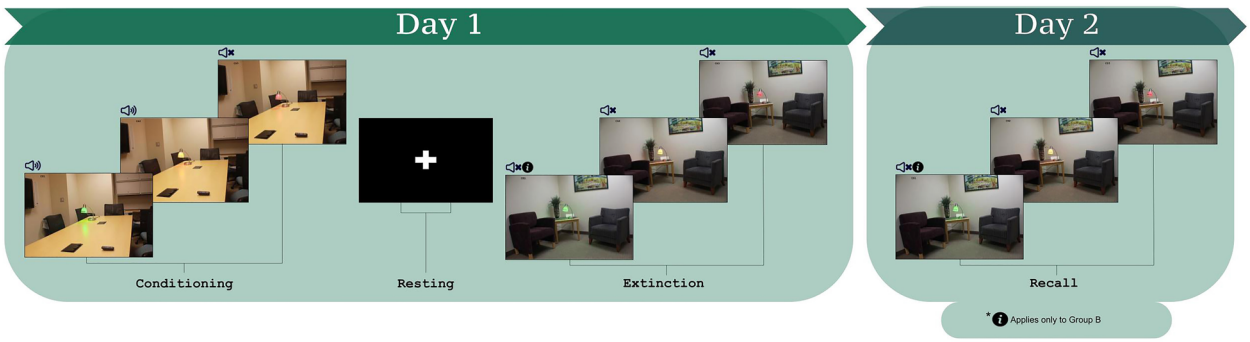

FIGURE 1 | The fear conditioning and extinction learning paradigm (Milad et al., 2005, 2007a,b, 2008, 2009; Milad and Quirk, 2012; Marin et al., 2017; Raij et al., 2018). Participants learned the CS-US contingencies during conditioning. During extinction learning, participants were instructed that for one of the CS+s, they would not hear the loud noise (CS+I). For the other CS+, no information was provided (CS+U). Three lamp colors comprise the three CSs, which were counterbalanced within and across participants as CS+I, CS+U, and CS–.

presented with each CS on a scale of 1–5, with 1 being "Not at All" and 5 being "Very Much So" (5-point likert scale). This verbally reported expectancy data was acquired for both CS+s and the CS−. Contingency awareness was defined based on expectancy data from immediately following the end of conditioning as the average of the two ratings for CS+I and CS+U being greater than that of the rating for the CS− (Tabbert et al., 2006; Javanbakht et al., 2017). Average rating for the CS+s equal to or less than the rating for the CS− was the threshold for exclusion/classification of being "unaware." Those who were not classified as "aware" of the CS-US contingency ("unaware" participants) were subsequently removed from the present analysis (Tabbert et al., 2006; Javanbakht et al., 2017). Participants for which there was no evidence of learning the CS-US contingency ("unaware") merited exclusion, given that conditioned fear must be established in order for extinction learning to be possible, and the goal of this study was to examine extinction learning.

Data Analysis

Self-Reported Expectancy

As described above, participants were asked to verbally rate how much they expected to hear the loud noise when presented with each CS on a 5-point likert scale at the conclusion of each phase—conditioning and extinction. After conditioning phase, this self-report measure of contingency/expectancy awareness was used to determine whether participants learned the CS-US contingency. We also obtained psychophysiological recordings (skin conductance response) from participants while in the scanner, but were unable to collect a sufficient amount of viable data. Measuring skin conductance in a 3T static magnetic field is a challenge both due to interference with the MRI RF. The lack of viable skin conductance data is a common problem and inherent limitation, as such data are susceptible to motion and high levels of noise (Bjorkstrand, 1990). Self-report contingency awareness data allowed us to deem participants to be "aware" of the CS-US contingency (see above).

All data were checked for out-of-range values, normality, linearity, homoscedasticity, and outliers. To compare self-reported expectancy of CS' by phase (conditioning and extinction) and CS type (CS+I, CS+U, and CS−), a 2 × 3 within-subjects repeated measures ANOVA was performed in SPSS. Mauchly's test indicated the assumption of sphericity had been met. Main effects of phase, CS type, and phase × CS type were all compared using Bonferroni corrected pairwise comparisons with 95% confidence intervals.

fMRI Preprocessing and Statistical Modeling

fMRI data were preprocessed and analyzed using standard methods in MATLAB R2013b with the Statistical Parametric Mapping toolbox (SPM12). For spatial pre-processing, the structural images were manually oriented to the AC-PC line with the reorientation vector applied across EPI image sets. Structural images were then realigned to a reference image to correct for head movement and subsequently co-registered to the structural image. The high resolution T_1 image was segmented and normalized to the MNI template, with the resultant deformations applied to the EPI image set. Low frequency components (scanner drift, physiological noises, etc.) were removed using a high-pass filter (128 s), and the EPI images were spatially smoothed using a Gaussian filter (8 mm full-width half maximum). Because of the previously observed difference between response to CS+I and CS+U during early extinction, and that at the end of the extinction CRs to both CSs fully extinguished (Javanbakht et al., 2017), we separated early extinction (first six trials of each CS+) from late extinction, as at the beginning of the extinction phase, individuals are learning that the CS+ is no longer associated with the US and forming a new, competing safety memory. At the end of the extinction phase, the assumption is (or at least the goal is to ensure) that a competing safety memory has been formed and the CR is extinguished. In first level analyses, events were modeled as regressors (duration 4 s box cars convolved with a canonical HRF) representing the CS+ (Early and Late), CS− (Early and Late)

and inter-trial intervals (ITI). Per convention, an autoregressive AR(1) model was used to account for serial correlation, and the six motion parameters (three for translation and three for rotation) were included as effects of no interest. To examine the additive effects of instruction on activation profiles of extinction learning, regressors representing early phases of CS+I > CS+U were forwarded to a second level random effects model.

A region of interest (ROI) approach was employed, restricting analyses to regions involved in extinction learning and emotion processing (Greco and Liberzon, 2016) (see section "Introduction"). The ROI approach was implemented using deterministic masks in stereotactic space (Maldjian et al., 2003). The *a priori* set of regions included the bilateral ACC and ventral PFC, bilateral amygdala, bilateral dorsal PFC, bilateral hippocampus, bilateral insula, and parahippocampal gyrus. This hypothesis driven *a priori* ROI-based approach was motivated by prior knowledge of fear circuitry (see section "Introduction").

Across all analyses, significant clusters were identified estimating the minimum cluster extent for activated voxels to be rejected as false positive (noise-only) clusters (Woodcock et al., 2016; Friedman et al., 2017). This approach performs a Monte Carlo alpha probability simulation, computing the probability of a random field of noise (after accounting for the spatial correlations of voxels based on the image smoothness within each region of interest estimated directly from the data set) to produce a cluster of a given size, after the noise is thresholded at a given level. Thus, instead of using the individual voxel probability (height) threshold alone in achieving the desired overall significance level, the method uses a combination of both probability and minimum cluster size thresholding.

RESULTS

Fifteen participants did not show evidence of the CS-US contingency awareness after conditioning (i.e., those who equally self-reported expected the US when presented with the CS+s and CS−, and those who indicated greater self-reported expectancy of the US when presented with the CS- than when presented with the CS+s). Because these participants were deemed to have failed the objective behavioral criteria for the experiment, they were excluded from the neuroimaging analyses. An additional six participants had to excluded on account of excess motion during the acquisition. Thus the final analyses included only those participants who did develop contingency awareness after conditioning *and* with viable neuroimaging data ($n = 14$).

Our subsequently presented results are organized as follows: (1) First, we present self-reported contingency awareness data for 14 participants, that reflect participant learning and task effects; (2) Next, we describe activation-based fMRI findings for each of the task conditions and comparisons (see section "Materials and Methods"); (3) These activation-based results motivated further analyses related to network effects, conducted using basic models of directional functional connectivity based on PPI (Friston et al., 1997; O'Reilly et al., 2012; Silverstein et al., 2016).

Self-Reported Contingency Awareness

As noted, self-reported expectancy information was used as a measure of contingency awareness and successful conditioning (given the absence of physiological data). Psychophysiological measures (e.g., skin conductance) are limited by large inter-individual variability and the reality that some participants do not show measurable levels of SCR during the task (Rabinak et al., 2017). Self-reported expectancy ratings of CS-US contingencies have sufficient face, diagnostic, predictive, and constructive validity, warranting them apt to assess contingency awareness (Boddez et al., 2013; Rabinak et al., 2017).

Test statistics for the repeated measure ANOVA are reported in **Table 1**. The within subjects effects of phase, CS type, and phase × CS type were all significant (ps < 0.001). Bonferroni-corrected pairwise comparisons indicated that expectancies across CS type were significantly greater following conditioning compared to extinction ($p < 0.001$). Bonferroni-corrected pairwise comparisons also indicated that expectancies across phase were significantly greater for CS+I compared to CS− and CS+U compared to CS- (p's $= 0.001$ and 0.003), but not CS+I compared to CS+U, $p = 1.00$. Therefore, the expectancy data indicated that participants had a greater expectancy of the CS-US contingency in after conditioning compared to after extinction learning and for both CS+I and CS+U compared to CS−. Participants' self-reported expectancy data indicates that they conditioned to the CS+s equally but not the CS-during conditioning, and extinguished conditioned fear to both CS+s by the end of the extinction phase (see **Figure 2**).

Regional Activation Differences

Markov chain Monte Carlo (MCMC) minimum cluster thresholds can be found in **Table 2** for all bilateral masks,

TABLE 1 | Test statistics for repeated measures ANOVA of self-reported expectancy of CS-US contingencies after conditioning and after extinction.

Main effects	*F*	*df*	Bonferroni corrected 95% CIs (LLCI, ULCI)	*P*-values
Phase	58.284	1,13	1.161, 2.077	<0.001*
CS type	14.980	2,26		<0.001*
CS+I v. CS+U			−0.567, 0.424	1.00
CS+I v. CS−			0.398, 1.530	0.001*
CS+U v. CS−			0.369, 1.703	0.003*
Phase × CS Type	29.922	2, 26		<0.001
Conditioning, CS+I			3.751, 4.677	*
Conditioning, CS+U			3.270, 4.301	*
Conditioning, CS−			0.903, 2.240	*
Extinction, CS+I			0.933, 1.353	*
Extinction, CS+U			1.140, 2.288	*
Extinction, CS−			1.146, 2.568	*

*For significance interpretation of Phase × CS Type interaction effects, the test of a significant interaction effect was significant, p < 0.001, and Bonferroni corrected 95% confidence intervals (CIs) are reported for specific effects where CIs that do not cross 0 are considered to represent significant effects, which are marked with an *.*

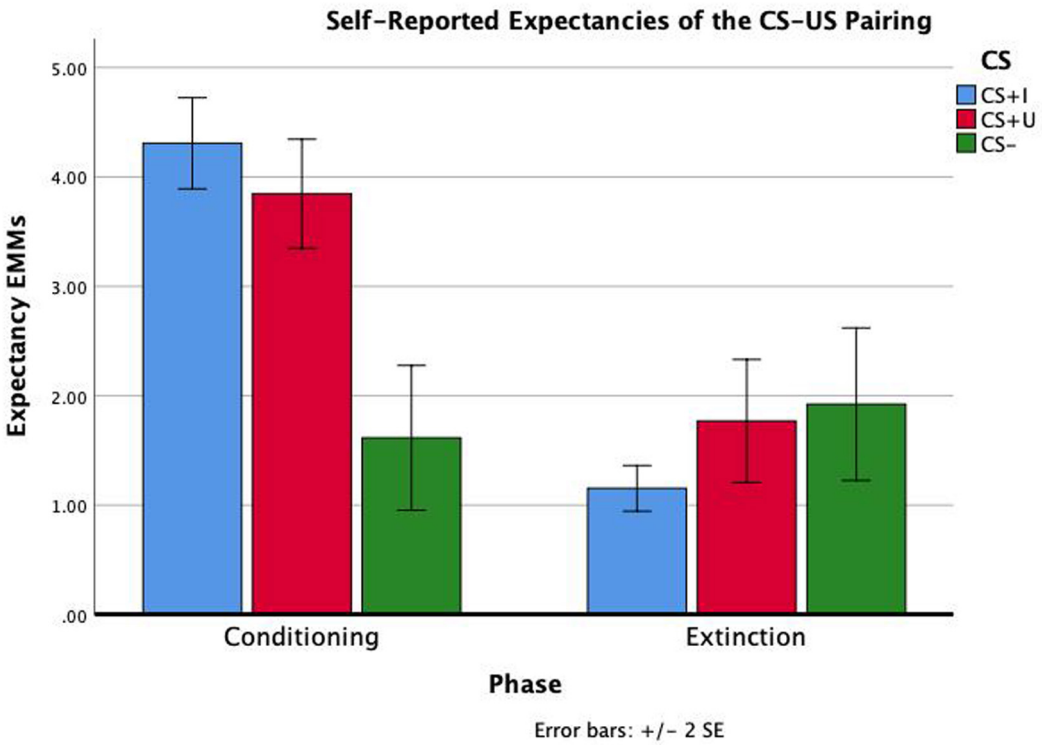

FIGURE 2 | Repeated measure ANOVA of phase × CS type on self-reported expectancies of the CS-US contingency. Self-reported contingency information was obtained at the end of conditioning and the end of extinction for each CS. There were significant effects of phase, CS type, and phase × CS type (all *p*'s < 0.01). Estimated marginal means (EMMs) reflect the mean response for each factor, adjusted for the other variables in the model. Error bars ± 2*SE* of the mean. Test results, including *post hoc* comparisons, are all reported in **Table 1**.

including those for which non-significant findings were not identified. During early *extinction* (first six trials), significant activation (CS+I > CS+U) was induced in the left vmPFC, left dmPFC, bilateral vlPFC, and right parahippocampus (**Figure 3**; test statistics available in **Table 3**), though no significant differences were observed in the converse contrast of CS+U > CS+I. Based on previous studies, our hypothesis, and our finding of involvement of vmPFC, this node was employed as an *a priori* seed in subsequent exploratory PPI analyses.

TABLE 2 | Minimum cluster thresholds by mask for Monte Carlo corrections for multiple comparisons (MCMC) simulations.

Early extinction, CS+I > CS+U	**MCMC minimum cluster threshold (regional activation; $p \leq 0.005$)**	**MCMC minimum cluster threshold (vmPFC interaction; $p \leq 0.05$)**
Anterior cingulate cortex and ventral prefrontal cortex	55.6	20.5
Amgydala	15.2	34.9
Dorsal prefrontal cortex	173	87.3
Hippocampus	33.4	42.5
Insula	38.7	44.2
Parahippocampus	28.9	35.4

Minimum cluster thresholds represent the minimum required number of voxels showing significant activation greater than that due to noise at the a priori established p-value of 0.005 for the ROI-based analysis and 0.05 for PPI with the left vmPFC as the seed. All masks, including those for which there were no significant findings, are presented. Masks are bilateral; contrast is CS+I > CS+U for the early phase (first six trials) of extinction.

Exploratory Psycho-Physiological Interactions (PPI)

Psychophysiological interaction (PPI) (Friston et al., 1997; Friedman et al., 2017) a basic model of functional connectivity was used to explore network profiles of the functionally defined vmPFC seed during extinction learning. PPI models the response of target brain regions in terms of an interaction between a linear convolution of the physiological response of the *a priori* determined seed region and the psychological contrast of interest (e.g., CS+I > CS+U).

MCMC minimum cluster thresholds can be found in **Table 2** for all bilateral masks, including those for which non-significant findings were not identified. The left vmPFC cluster derived from the activation results (CS+I > CS+U) and centered at the significance peak (coordinates: $x = -10$, $y = 50$, $z = -1$) was defined as the seed region For each participant, time series were extracted using the first eigenvariate of the weighted means of the modeled effects within a sphere (radius = 4 mm, F contrast, effects of interest, $p < 0.99$). This time series was convolved with the contrast of interest (CS+I > CS+U). The

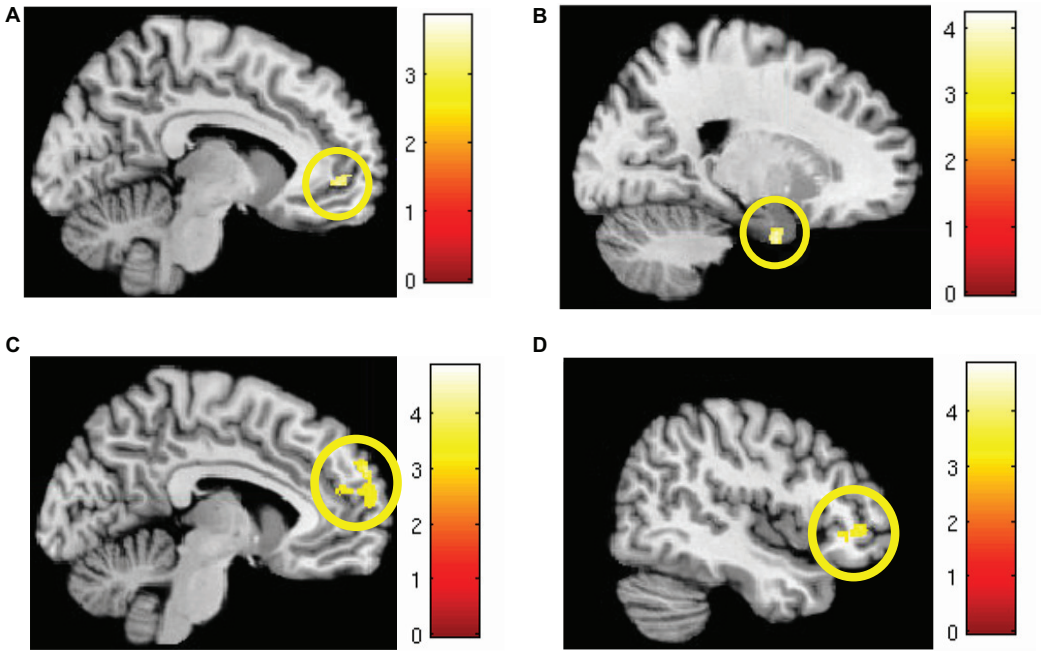

FIGURE 3 | Areas of significant regional activation during the first 6 trials of the extinction learning phase, CS+I > CS+U. **(A)** Left vmPFC; **(B)** right parahippocampus; **(C)** left dmPFC; and **(D)** bilateral vlPFC. MC corrected $p \leq 0.001$. Heat maps for each region represent *Z*-scores, which are reported alongside *p*-values in **Table 3**.

resultant PPI interaction terms models the effects of the vmPFC on any potential targets in the context of this psychological context (i.e., the contrast). Thus, each participant, contributed one first-level PPI map to a second level random effects analyses, to identify the modulatory effects of the vmPFC seed on the *a priori* network of regions (amygdala, insula, dACC, hippocampus, dmPFC, dlPFC, vlPFC, and vmPFC). Significant clusters (identified using a one-sample *t*-test) were observed in the dmPFC, parahippocampus, amygdala, and insula (**Figure 4**, statistical information in **Table 4**).

DISCUSSION

We open the Discussion by reiterating that this is the first study of the neurocircuitry evoked by the combined effects of instruction and experience on extinction learning. We reprise our results here. In assessing activation profiles for concurrently acquired fMRI data, we found increased activation in the vmPFC during the early phase of extinction learning when comparing the CS+I with CS+U, as well as increased dmPFC, vlPFC, and parahippocampal activity. PPI analysis motivated by our hypothesis and the activation results showed that the vmPFC seed significantly modulated the dmPFC, amygdala, insula, and parahippocampus during extinction learning. In the remainder of the paper, we discuss the importance of the results from the perspective of how extinction learning is represented in brain regions and networks, and conclude with implications of these results for clinical intervention.

TABLE 3 | Significant regions of interest activated during extinction learning.

Early extinction, CS+I > CS+U	**Cluster size corrected in voxels**	**Coordinates**	***P*-values (peak)**	***Z*-scores**
Left vmPFC	81	−10, 50, −1	0.001	3.09
Right parahippocampus	54	20, −1, −30	0.001	3.29
Left dmPFC	432	−9, 60, 29	<0.001	3.61
Bilateral vlPFC	184	−38, 58, −6	0.001	3.19
	190	50, 40, −1	0.001	3.07

p-values threshold: $p < 0.005$.

fMRI Effects: Activation and Network Profiles

The notably observed activation of the vmPFC is consistent with its purported role during extinction learning, social learning of safety, and cognitive reappraisal related emotion regulation. Previous studies have frequently reported vmPFC activation during extinction learning and its recall, and in general in response to safety signals (Milad et al., 2007b; Fullana et al., 2018). The vmPFC is also implicated in fear reversal studies when a CS previously associated with threat, is now safe. vmPFC is also suggested to have an inhibitory role on conditioned fear response during early extinction (for a review see Greco and Liberzon, 2016). Other works have shown involvement of the vmPFC in vicarious safety learning via social observation (Golkar et al., 2016) and reappraisal-related emotion regulation (Pico-Perez et al., 2019). A meta-analysis of a diverse group of emotion regulation tasks found vmPFC activation to be

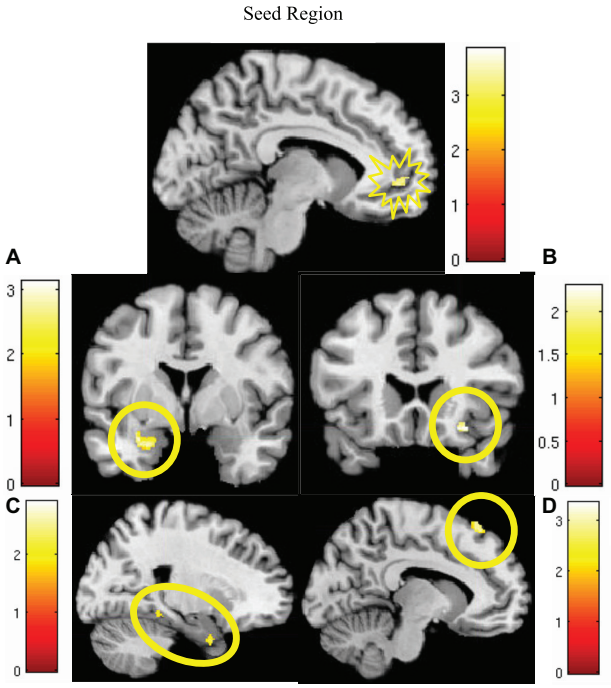

FIGURE 4 | Significantly greater activity in the left vmPFC (top; red) for the instructed CS+ (CS+I) during extinction learning, and regions of significant co-activation. Regions of significant co-activation were identified using PPI, with the vmPFC as the seed (indicated within the starburst icon; top center). During extinction learning, significant co-activation of the **(A)** amygdala (middle left), **(B)** insula (middle right), **(C)** parahippocampus (bottom left), and **(D)** dmPFC (bottom right) was observed. Contrast: CS+I > CS+U for the early phase (first six trials) of extinction learning, MC corrected $p < 0.05$. Heat maps for each region represent Z-scores, which are reported alongside *p*-values in **Table 4**.

the most consistent element of negative emotion regulation whether via extinction learning, cognitive emotion regulation (reappraisal studies), or placebo effect (Diekhof et al., 2011). Previous work has shown that instruction about the absence of CS-US contingency leads to either immediate extinction (Mower, 1938; Rowles et al., 2012; Sevenster et al., 2012), or faster decay of the fear response (Koenig and Henriksen, 2005). Thus, our findings suggest that this facilitation may happen through increased activation in the vmPFC in conjunction with other areas involved in emotion regulation (dmPFC, parahippocampus, insula, and amygdala). Moreover, the PPI analyses suggest that the vmPFC exerts network modulation that reflects the network-based signatures of instruction. In this way, instruction-mediated emotion regulation may be the meta-process that underpins extinction learning. The application of PPI allows for our resultant findings to support this theory, given that PPI implies directionality.

Other related mPFC regions, vlPFC and dmPFC showed activation during instructed extinction learning. The dmPFC also showed significant coactivation with the vmPFC. Both the vlPFC and dmPFC are involved in emotional regulation, subsequent behavioral responding, and are also activated during threat appraisal (Milad et al., 2007a) and reappraisal (Buhle et al., 2014; Helion et al., 2019), which is a relevant mechanism involved here as a function of the instruction.

The parahippocampal cortex is both anatomically and functionally connected with the medial prefrontal cortex (Baldassano et al., 2013), and in addition to the hippocampus and vmPFC, is a key brain region involved in processing contextual associations (Aminoff et al., 2013). As previously noted, context plays an important role in signaling safety of the previously conditioned cues in the environment linked with extinction learning (Maren and Quirk, 2004). Activation of the parahippocampus, and its coactivation with the vmPFC, suggests a function of relaying safety instructions as a component of the cognitive context to indicate absence of the CS-US contingency (Javanbakht et al., 2017). Previous work has suggested that similar to the physical component of the context, instruction serves as a "cognitive context" that guides reactions when the conditions of the instruction are available (e.g., red light indicating absence of the loud noise) (Maren et al., 2013). Hippocampus and prefrontal cortex that are involved in context processing, are suggested to have a role in processing cognitive information during extinction learning (Garfinkel et al., 2014). In other words, cognitive context may be manipulated by the presentation of social cues or verbal instruction (Phelps et al., 2001). By changing the expectation, this cognitive manipulation can affect fear responses during extinction learning (Hugdahl and Ohman, 1977; Olsson and Phelps, 2004). This differs from cognitive "reappraisal," as the participant is not instructed to *change/reappraise* salience of a cue, but rather is *informed* about the salience.

Clinical Implications

These findings, albeit in healthy participants who were tested in an experimental lab setting, have implications for clinical practice. The finding of increased vmPFC activity during instructed extinction learning suggests that instruction may have an additive benefit in engaging this crucial structure associated with emotion and emotion regulation. Thus, therapeutic methods that utilize instruction may enhance efficacy in patients with anxiety disorder and PTSD by modulating the activity and network profiles of the vmPFC. For instance, evidence supports efficacy of both exposure therapy (based on experiential

TABLE 4 | Significant regions of co-activation with left vmPFC seed during PPI analysis, derived from ROI-based analyses.

Early extinction, CS+I > CS+U	Cluster size corrected in voxels	Coordinates	*p*-values (peak)	*Z*-scores
Bilateral parahippocampus	93	−10, −4, −19	0.006	2.51
	64	20, −38, −13	0.009	2.73
	36	21, 4, −31	0.012	2.57
Right insula	46	30, 22, −14	0.029	2.07
Left dmPFC	155	−12, 30, 59	0.003	2.79
Left amygdala	165	−22, −1, −24	0.004	2.65

p-values threshold: $p < 0.05$.

extinction learning), and cognitive processing therapy (focused on cognitive manipulation of patient's perspective on the trauma leading to reduced fear) in treatment of PTSD (Cusack et al., 2016). However, there is a difference between laboratory models of fear conditioning and extinction learning and real clinical practice: Here fear learning happens in the laboratory and participants may give more credence to instruction provided by the experimenter, while in clinical practice fear learning has happened prior to engaging the therapist. While most laboratory models of exposure therapy are based on experiential extinction learning, such therapy involves a combination of experience and instruction. In the clinic a therapist is always signaling safety of the feared object via instruction. This is less applicable during generalization of extinction learning with self-practice outside of the clinic where the therapist is not available, although the memory of instruction is often still present. To realistically model the neurobiology of psychotherapy, we need to understand the combined role of the human social safety cue along with the experiential learning of safety. In this sense, our paradigm adds to the current laboratory models, to be more representative of the complexities of clinical work. Finally, understanding how instruction enhances extinction learning can help in individualized treatment and identifying those who may benefit from potential utilization of neuromodulation methods targeting these deficits in vmPFC to enhance response to therapy. Neuromodulation may be helpful as adjunct to therapy of conditions which have repeatedly shown deficits in vmPFC function, and which treatment involves cognitive behavioral therapy.

We also note some study limitations. The sample size for the fMRI analyses was relatively small (for reasons detailed in the section "Materials and Methods"), and the lack of viable psychophysiological data which is a general challenge (Bjorkstrand, 1990) also affected us. We were compelled to rely on self-reported expectancy as a measure of contingency awareness and successful conditioning, while noting that these measures have been shown to be associated with psychophysiological data (Indovina et al., 2011; Pohlack et al., 2012a,b). The "awareness" of fear is a clinically relevant patient experience, suggesting that contingency awareness may be an experimental surrogate of a clinically relevant "phenotype." Additionally, multiple studies note that conditioned psychophysiological responses can be acquired even in the absence of cognitive awareness of contingency awareness (Ohman and Soares, 1994; Schultz and Helmstetter, 2010; Raio et al., 2012). Therefore, while our lack of viable psychophysiology data is certainly a limitation of this, our self-report expectancy and neuroimaging data still provide a valuable and meaningful contribution to the literature. As contingency awareness data was obtained at the end of the extinction learning phase, and not after the first six trials—early phase of extinction—we lack behavioral evidence of differences between CS+I and CS+U. Future studies should place an emphasis on gathering quality psychophysiological recordings and probe contingency awareness immediately after the early phase of extinction learning (first six trials), as well as at the end. Our neuroimaging data indicate greater recruitment of prefrontal and hippocampal areas when presented with CS+I compared to CS+U in the early phase of extinction, and these regions are typically associated with inhibition of conditioned fear responses. Therefore, we would expect to see differences in self-reported expectancy data after the first six trials, such that participants would report less expectancy of the US to follow the CS+I compared to the CS+U. However, this remains to be tested. Thus these data do not capture potential differences between CS+I and CS+U, suggesting that future studies should emphasize collection of physiological recordings *and* probe contingency awareness immediately after the early phase of extinction learning, and at the conclusion. Finally, while the formal bases of PPI permits the inference of directional (and putatively asymmetric) interactions between seeds and targets (Stephan, 2004), recovering the true bases of directionality in the brain is a fundamentally challenging question (Friston et al., 2012). The challenge is (a) empirical, given that the fMRI signal is limited by the hemodynamic filter that is stages removed from neuronal processes and (b) philosophical, given that discovering directionality is in part based on discovering "causality" (Mannino and Bressler, 2015). We submit that our claims of directionality refer to previous understanding of the involved networks, and in the narrow sense to the nature of inference based on PPI, and that we cannot make strong claims about general directionality in the brain.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available although we may share the data on a case by case basis. Requests to access the datasets should be directed to AJ, ajavanba@med.wayne.edu.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Wayne State University IRB. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AJ has contributed to the design of the study, data analysis and interpretation, and writing the results. LG, SM, and AC contributed to the data collection, analysis, and interpretation, and wrote the results. IL contributed to the design of the study, data analysis, and interpretation, and wrote the results. VD contributed to the data analysis and interpretation, and wrote the results. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the State of Michigan (Lycaki/Young Funds) in collaboration with Detroit Wayne Mental Health Authority and Behavioral Health Professionals, Inc. AJ's effort was supported by R01HD099178. LRG's effort was supported by F31MH120927.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Behavioral Neuroscience

REVIEW
published: 26 January 2021
doi: 10.3389/fnbeh.2020.601111

Impact of High Fat Diet and Ethanol Consumption on Neurocircuitry Regulating Emotional Processing and Metabolic Function

*Caitlin R. Coker[1], Bailey N. Keller[2], Amy C. Arnold[2] and Yuval Silberman[2]**

[1] Biochemistry and Molecular & Cellular Biology, Georgetown University School of Medicine, Washington, DC, United States, [2] Neural and Behavioral Sciences, Penn State College of Medicine, Hershey, PA, United States

The prevalence of psychiatry disorders such as anxiety and depression has steadily increased in recent years in the United States. This increased risk for anxiety and depression is associated with excess weight gain, which is often due to over-consumption of western diets that are typically high in fat, as well as with binge eating disorders, which often overlap with overweight and obesity outcomes. This finding suggests that diet, particularly diets high in fat, may have important consequences on the neurocircuitry regulating emotional processing as well as metabolic functions. Depression and anxiety disorders are also often comorbid with alcohol and substance use disorders. It is well-characterized that many of the neurocircuits that become dysregulated by overconsumption of high fat foods are also involved in drug and alcohol use disorders, suggesting overlapping central dysfunction may be involved. Emerging preclinical data suggest that high fat diets may be an important contributor to increased susceptibility of binge drug and ethanol intake in animal models, suggesting diet could be an important aspect in the etiology of substance use disorders. Neuroinflammation in pivotal brain regions modulating metabolic function, food intake, and binge-like behaviors, such as the hypothalamus, mesolimbic dopamine circuits, and amygdala, may be a critical link between diet, ethanol, metabolic dysfunction, and neuropsychiatric conditions. This brief review will provide an overview of behavioral and physiological changes elicited by both diets high in fat and ethanol consumption, as well as some of their potential effects on neurocircuitry regulating emotional processing and metabolic function.

Keywords: obesity, binge, alcohol use disorder (AUD), mental health, neuroinflammation

OPEN ACCESS

Edited by:
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Reviewed by:
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Maria Beatrice Passani, University of Florence, Italy

***Correspondence:**
Yuval Silberman
Yus72@psu.edu

Specialty section:
This article was submitted to Emotion Regulation and Processing, a section of the journal Frontiers in Behavioral Neuroscience

Received: *31 August 2020*
Accepted: *28 December 2020*
Published: *26 January 2021*

Citation:
Coker CR, Keller BN, Arnold AC and Silberman Y (2021) Impact of High Fat Diet and Ethanol Consumption on Neurocircuitry Regulating Emotional Processing and Metabolic Function. Front. Behav. Neurosci. 14:601111. doi: 10.3389/fnbeh.2020.601111

INTRODUCTION

The prevalence of anxiety and depression has steadily increased in recent years in the United States, with over 18% of the population having anxiety (Kessler et al., 2005) and ∼6.6% having depression each year (Kessler et al., 2003). Clinical studies show a correlation between poor diet and these conditions (Bonnet et al., 2005). Rates of overweight and obesity, often due to over-consumption of high fat diets (HFD) in western culture, are also rising in the United States and are associated with an increased risk for developing psychiatric conditions such as anxiety (Petry et al., 2008; Gariepy et al., 2010) and depression (Petry et al., 2008; Preiss et al., 2013). Binge eating disorders,

which can often overlap with overweight and obesity outcomes, also show comorbidity with depression and anxiety disorders (Citrome, 2019). Thus, diet can have a strong impact on mental health. Depression and anxiety disorders are also often comorbid with alcohol (EtOH) and substance use disorders, and these interactions have been well-studied in both clinical (Kushner et al., 2000; Kingston et al., 2017) and preclinical (Pandey et al., 2005; Crews et al., 2016) research. Studies of the commonalities between binge eating disorders, overweight and obesity, and alcohol use disorders (AUD) suggest many overlapping neurological mechanisms may be involved (Rapaka et al., 2008). This brief review will discuss some of the predominant behavioral and physiological changes caused by HFD, EtOH, and the combination of the two as well as potential central mechanisms that may contribute to disruptions in emotional regulation and metabolic function. As these are very complex fields, we sought to highlight portions of the literature that may be most relevant to combined HFD and EtOH use and provided citations for further in-depth analysis when possible for topics outside the main scope of this review.

OVERLAP OF CLINICAL OUTCOMES BETWEEN OBESITY AND ALCOHOLISM

Overweight and obesity are measured by a person's body mass index (BMI), which is calculated using weight and height. Although not a measure of overall health status, in general a BMI of 25–29.9 kg/m^2 is considered overweight, while a BMI of $\geq$30 kg/m^2 is defined as obese[1]. Overweight- and obesity-related conditions are the second leading cause of preventable death in the United States, attributing to 300,000 deaths yearly (Allison et al., 1999) and having an estimated $147 billion in medical costs annually[2]. The National Health and Nutrition Examination Survey reported the prevalence of obesity among adults in the United States was 42.4% in 2017–2018, an 11.9% increase from 1999 to 2000 (Hales et al., 2017). Importantly, obesity is a primary risk factor for an array of chronic diseases including cardiovascular disease, type II diabetes, hypertension, and certain cancers (Must and McKeown, 2000).

The National Institute on Alcohol Abuse and Alcoholism (NIAAA) defines AUD as "a chronic relapsing brain disease characterized by an impaired ability to stop or control EtOH use despite adverse social, occupational, or health consequences" (National Institute on Alcohol Abuse Alcoholism, 2020). The National Survey on Drug Use and Health (NSDUH) reported over 14 million adults had AUD in 2018[3]. This is a global health issue as The Global Status Report on Alcohol and Health reported an estimated 3 million EtOH-related deaths in 2016, which made up over 5% of all deaths worldwide. Of this, over 2.3 million deaths were among men (World Health Organization, 2018). The higher level of EtOH-related deaths in men may be due to the differing prevalence of AUD between men (237 million) and women (46 million), with the highest prevalence in the European region and Americas (World Health Organization, 2018) according to the World Health Organization. Overall, EtOH misuse is the third leading cause of preventable death in the United States and attributes to over 88 thousand deaths each year (Centers for Disease Control Prevention, 2020). In 2010, excessive EtOH drinking cost the United States $249 billion, with nearly 77% attributed to binge drinking (Sacks et al., 2015). Further, chronic EtOH misuse contributes to an estimated 200 diseases and injury-related conditions. According to the World Health Organization, of the 3 million EtOH-attributable deaths in 2016, 21.3% were due to digestive diseases, 19% due to cardiovascular diseases and diabetes, 12.9% due to infectious diseases, and 12.6% due to cancers (World Health Organization, 2018). In the United States, nearly 48% of all liver cirrhosis deaths were EtOH related in 2013 (Yoon and Chen, 2016).

Not only is the prevalence of obesity and AUD on the rise separately, there is also an emerging link between developing both obesity and AUD in the United States (Petry et al., 2008; Grucza et al., 2010). Clinical data indicate chronic and excessive EtOH drinking results in an increased risk for developing metabolic dysfunction (Fan et al., 2006) and type II diabetes (Kao et al., 2001; Carlsson et al., 2003), outcomes similar to that seen with overconsumption of HFD. The shared clinical consequences of both HFD and EtOH overconsumption suggest an overlap in the mechanisms by which these insults modulate insulin action and glucose homeostasis. Furthermore, the common mental health conditions separately associated with chronic HFD and EtOH overconsumption, i.e., depression and anxiety-related disorders, suggests overlap of central mechanisms of emotional regulation that may become dysregulated by HFD and chronic EtOH use.

MODELS OF HIGH FAT DIET AND ALCOHOL INTAKE

High Fat Diet Exposure in Rodents as a Model of Obesity

Obesity in the clinical population can be attributed to both genetic and environmental factors, which can include a sedentary lifestyle and consumption of diets rich in carbohydrates and saturated fats. Rodent models involving diet manipulation can recapitulate the pathophysiology associated with diet-induced disease development in the absence or presence of predetermined genetic alterations to assess metabolic outcomes in whole-animal systems. These diet-induced obesity (DIO) models are commonly used in the preclinical setting to study the whole-body insults that occur during the progression of obesity. Rodent models of DIO have been used since the 1940s when researchers administered a highly palatable liquid diet *ad libitum* to stimulate weight gain in rats to the point of obesity (Ingle, 1949). Diets high in fat, sugar, and other nutrients have all been used in rodent models of DIO, with researchers incorporating ingredients such as Crisco

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[3]Section 5 PE Tables – Results from the 2018 National Survey on Drug Use and Health: Detailed Tables, Sections 1–3, SAMHSA, CBHSQ. Available online at: https://www.samhsa.gov/data/sites/default/files/cbhsq-reports/NSDUHDetailedTabs2018R2/NSDUHDetTabsSect5pe2018.htm#tab5-4a (accessed March 30, 2020).

(Mickelsen et al., 1955; Sclafani and Springer, 1976), chocolate (Sclafani and Springer, 1976; Burokas et al., 2018), and sucrose (Levin and Dunn-Meynell, 2002; Harzallah et al., 2016; Collins et al., 2018). This allows for comparison of the various diets found in the human population (e.g., western diets, cafeteria diets). Overall, the physiological mechanisms behind DIO models include hyperphagia of calorically dense diets, increased efficacy of dietary fat being stored in the body, a pre-diabetic phenotype (e.g., mild-modest hyperglycemia, hyperinsulinemia, insulin resistance, glucose intolerance) and alterations in the hormones involved in energy balance (Hariri and Thibault, 2010). This review will primarily focus on DIO produced by HFD feeding, as this is one of the most widely studied rodent models of obesity, particularly in terms of interactions with alcohol as well as dietary effects on depression- and anxiety-like behaviors. It is recognized, however, that some studies utilize other dietary models (e.g., high fat plus high sugar or high fructose/sucrose) as well as genetically- and pharmacologically-induced models of obesity to examine metabolic outcomes as well as measures of homeostatic and hedonic feeding behaviors as previously reviewed (Surwit et al., 1988; Pandit et al., 2012; Rosini et al., 2012; Stice et al., 2013; Hughey et al., 2014; Slomp et al., 2019). Since depression- and anxiety-like behaviors have been well-characterized as a risk factor for increased EtOH misuse, brain regions regulating these behaviors are an intriguing area of research on potential mechanisms of behavioral overlap of overconsumption of HFD and EtOH.

Animal Models of Alcohol Intake

Various EtOH exposure methods have been studied in animal models for decades. In the 1960s, researchers sought to develop a clinically relevant animal model for EtOH consumption. Dr. Charles Lieber and Dr. Leonore DeCarli created a unique model by administering EtOH as part of a nutritionally complete liquid diet (Lieber and DeCarli, 1982). This consumption model has been the forerunner in the EtOH research field as a useful tool to study pathological disorders associated with AUD, such as alcoholic liver disease (Dolganiuc et al., 2009; Bhopale et al., 2017; Guo et al., 2018). Another commonly used exposure method is EtOH vapor inhalation which is useful to maintain constant, clinically relevant blood EtOH concentrations and to induce EtOH dependence (Healey et al., 2008; Snyder et al., 2019). In addition to these passive or forced consumption models, many rodent strains will self-administer to pharmacologically relevant blood EtOH concentrations. These self-administration models often utilize operant conditioning or two-bottle choice methods. The two-bottle choice model is a voluntary consumption model providing the animal a choice between a bottle containing an EtOH solution (typically in water, but other vehicles may be used) and a second bottle containing water (or vehicle). This model allows for behavioral analysis of intake including assessment of EtOH preference and consumption, typically in a home cage setting (Coker et al., 2020). Access schedules to the EtOH solution can be readily altered to induce binge-like intake (Melendez, 2011; Thiele et al., 2014) and allows for discrete time periods for access to EtOH and HFD. The C57BL/6 strain of mice is typically used for these experiments due to their consistently high levels of EtOH consumption (Dole and Gentry, 1984; Rhodes et al., 2005; Yoneyama et al., 2008).

BEHAVIORAL AND PHYSIOLOGICAL OUTCOMES BY DIET, ALCOHOL, AND THE COMBINATION

Changes by Diet

Numerous behavioral and physiological changes have been positively linked to obesity in both clinical and preclinical studies. In clinical studies (see Robinson et al., 2020 for recent review), there is highly suggestive evidence that heavier body weight is associated with impaired executive functioning, increased impulsivity, and impaired reward-related decision making, as well as suggestive evidence of impaired self-esteem and body image concerns. The most prevalent clinical data show that heavier body weight increases the likelihood of experiencing anxiety and depression (Bonnet et al., 2005; Petry et al., 2008; Gariepy et al., 2010; Preiss et al., 2013; Robinson et al., 2020). Preclinical studies investigating anxiety- and depressive-like behaviors also show an increase in these measures in HFD-fed rodents (Sivanathan et al., 2015; Zemdegs et al., 2016; Gelineau et al., 2017). The well-known physiological consequences of obesity in the clinical population, such as insulin resistance, glucose intolerance, and increased risk for developing type II diabetes (Haslam and James, 2005), are recapitulated in rodent models. Animal studies show that both short-term and chronic HFD consumption results in glucose intolerance and insulin resistance as well as increased fasting glucose, insulin, free fatty acid, triglyceride, and leptin levels compared to regular chow diet (Hariri and Thibault, 2010; Paulson et al., 2010; Dutheil et al., 2016; Coker et al., 2020). Female mice are often shown as being more resistant to these HFD-induced physiological changes, potentially due to metabolically protective effects of estrogen (Gelineau et al., 2017). Consistent with this, a study done in female rats showed that metabolic disturbances induced by loss of estrogen due to ovariectomy (i.e., increase in weight gain, hyperleptinemia, glucose intolerance) were exacerbated by HFD. Induction of depressive-like behaviors accompanied metabolic deterioration in these rats indicating that HFD may increase vulnerability to development of depression in the absence of estrogen (Boldarine et al., 2019). It is important to recognize, however, that not all studies show that females are resistant to metabolic disturbances (White et al., 2019) and that sex as a biological variable in HFD-induced changes in metabolic function and depression or anxiety-like behavior remains to be fully elucidated. It is also important to note that diet-induced behavioral alterations, assessed with forced swim test and elevated plus maze test, can be due to many factors, including length of diet exposure. For instance, HFD in rats negatively impacted brain homeostasis and inflammation by disrupting intracellular cascades involved in synaptic plasticity, insulin signaling, and glucose homeostasis and by increasing corticosterone levels and inflammatory cytokines and was related

to anxiety- and anhedonia-like behaviors (Dutheil et al., 2016). In this same study, it was demonstrated that the consequences of HFD might be contingent on duration of exposure. While no effect was seen after 8 weeks of exposure, HFD given for 16 weeks in rats resulted in significant differences in behavioral measures of anxiety (assessed using novelty suppressed feeding test, open field test, elevated plus maze test, and novel object recognition test) and anhedonia (determined using sucrose preference test and female urine sniffing test) (Dutheil et al., 2016). Overall, the impact of HFD and other obesogenic diets on anxiety, depression and other behaviors remains in important area of research.

Changes by Alcohol

The comorbidity between EtOH consumption and anxiety has been well-studied in both clinical (Kushner et al., 2000) and preclinical (Pandey et al., 2005; Crews et al., 2016) populations across the entire lifespan. Depressive disorders are also commonly comorbid psychological conditions in people with AUD (Grant et al., 2004), with their co-occurrence leading to increased severity of symptoms (Hasin et al., 2002). Epidemiologic data on the physiological consequences of EtOH intake differ depending on intake patterns. Moderate EtOH consumption appears protective against insulin resistance in both clinical (Davies et al., 2002; Koppes et al., 2005; Yokoyama, 2011; Bonnet et al., 2012; Traversy and Chaput, 2015) and preclinical (Paulson et al., 2010) studies, while acute and chronic/binge consumption is associated with insulin resistance in non-obese humans (Fan et al., 2006; Papachristou et al., 2006; Ting and Lautt, 2006) and rodents (Dhillon et al., 1996; Lindtner et al., 2013). As a result, the relationship between EtOH consumption and insulin sensitivity is often described as an inverted U-shape (Villegas et al., 2004; Koppes et al., 2005; Ting and Lautt, 2006).

The effects of EtOH on glucose tolerance and leptin levels in healthy humans and rodents are also inconsistent. Standard oral glucose tolerance tests in the clinical population have shown no change (Singh et al., 1988; Beulens et al., 2008) or improvement (McMonagle and Felig, 1975) in glucose tolerance following moderate EtOH consumption, and impaired glucose tolerance in subjects who have chronically consumed EtOH (Andersen et al., 1983). Animal studies show moderate EtOH consumption can lead to no change (Hong et al., 2009; Paulson et al., 2010; Gelineau et al., 2017) or improvement (Hong et al., 2010) in glucose tolerance, while chronic consumption promotes glucose intolerance (Feng et al., 2010) in mice and rats. Additionally, chronic EtOH consumption has been shown to elevate (Nicolas et al., 2001; Obradovic and Meadows, 2002) or decrease (Hiney et al., 1999) leptin levels in rodent models, while moderate consumption produced no change in mice and humans (Beulens et al., 2008; Gelineau et al., 2017). Overall, the discrepancies on the impact of EtOH consumption on metabolic parameters appear to be due to differences in patterns of EtOH intake or percentage of EtOH in solution used. This can make interpretation of results across studies difficult and suggests standardized models of EtOH effects on metabolic and other physiological functions may be important in future studies.

Changes by Combination of Diet and Alcohol

Chronic consumption of both HFD and EtOH likely has combinatorial effects on overall mental and physiological health. For instance, patients diagnosed with anxiety or depression are more likely to be obese and to binge drink (Strine et al., 2008). Preclinical studies show that concurrent EtOH and HFD consumption increases anxiety measures in the light/dark box in predominantly in female mice (Gelineau et al., 2017). The epidemiologic evidence described in the previous sections for potential beneficial physiological effects of moderate EtOH consumption on insulin sensitivity may only occur in non-obese patients (Yokoyama, 2011). Most preclinical studies, however, show that moderate EtOH consumption mitigates HFD-induced metabolic dysfunction (Hong et al., 2009; Paulson et al., 2010; Gelineau et al., 2017). These studies, specifically, show that moderate EtOH consumption improves insulin sensitivity and glucose tolerance in mice on HFD, without affecting HFD induced changes in body mass or circulating insulin and leptin levels. As discussed above, many of the discrepancies in the HFD and EtOH co-consumption literature may be due to differences in methodology of consumption between studies. To address this possibility, studies by our lab (Coker et al., 2020) show that differences in the scheduling of EtOH and HFD access mediate the amount of EtOH consumed and the resultant impact on insulin and glucose function. This study showed that moderate and binge EtOH consumption does not improve insulin sensitivity or glucose tolerance in HFD-fed mice. Additionally, binge consumption of both HFD and EtOH promoted insulin resistance and glucose intolerance in the absence of an overweight phenotype. Clinically, such metabolic dysregulation in lean individuals is linked to increased risk for more severe type II diabetes as well as increased total and cardiovascular-related mortality compared with overweight diabetic individuals (George et al., 2015; Olaogun et al., 2020). Thus, there may be an unrecognized clinical population that may be seemingly healthy due to overall lower BMI but have underlying metabolic dysfunction that may not be fully considered. Overall, these findings suggest there are various factors that may affect how EtOH and HFD interact to influence metabolic disturbances, such as frequency and duration of access. Standardization of HFD and EtOH co-consumption models may improve upon discrepancies in the field.

POTENTIAL CENTRAL MECHANISMS OF HIGH FAT DIET AND ALCOHOL BEHAVIORAL INTERACTIONS

Clinical and Preclinical Studies of Mechanisms of High Fat Diet Modulation of Central Neurocircuits

Two systems interact in the regulation of feeding behavior – homeostatic systems and brain reward systems (Kenny, 2011a). The homeostatic system involves metabolic mechanisms including hormonal regulators from the periphery (e.g., ghrelin, insulin, leptin) that control hunger, satiety, and adiposity. Energy

balance is maintained at homeostatic levels by these hormones acting on various hypothalamic (paraventricular nucleus, PVN; arcuate nucleus, ARC; lateral nucleus; dorsomedial nucleus) and hindbrain (nucleus of the tractus solitarius; raphe nucleus) feeding circuits. Research on the integrative signaling of homeostatic and hedonic feeding in the central nervous system (CNS) suggests that endocrine signals from the periphery, such as hormonal regulators of appetite, can act on other CNS regions outside of the traditionally studied hypothalamic and brainstem regions, such as dopaminergic (ventral tegmental area, VTA) and limbic (amygdala, hippocampus, cortex) regions (Stice et al., 2013). While these two systems interact to influence food intake, it has been shown that the hedonic properties of food can override the homeostatic system to stimulate feeding behavior resulting in hyperphagia even after energy requirements have been met. Consequently, stimulation of the reward systems to promote overconsumption of palatable food can lead to overweight and obesity (Kenny, 2011a,b). The HFD-induced sensitization of these pathways does not appear to normalize following return to standard diet in rodent models (Mazzone et al., 2020), suggesting long term consequences to consummatory pathways is not easily resolved and may trigger compulsive eating behaviors (Moore et al., 2017a,b). For more detailed information on these well-defined neurocircuits, we direct readers to the following reviews that outline how appetite and energy homeostasis are regulated by various hypothalamic circuits (Cassidy and Tong, 2017; Sternson and Eiselt, 2017; Timper and Brüning, 2017; Chowen et al., 2019) and reviews that focus on the different subdivisions of the amygdala and reward (Baxter and Murray, 2002; Gilpin et al., 2015; Janak and Tye, 2015; Wassum and Izquierdo, 2015; Daviu et al., 2019).

Clinical and Preclinical Studies of Mechanisms of Alcohol Use Disorders Across the Lifespan

In addition to highly prevalent AUD risk in adult populations, EtOH is the most commonly used substance of abuse among adolescents and is a major public health concern in the United States[4]. Underage drinking makes up 11% of all EtOH consumed nationwide and over 90% of this is in the form of binge drinking[5]. The adolescent brain is particularly vulnerable to many effects of EtOH (Guerri et al., 2009) and there are countless long-lasting negative consequences associated with underage binge drinking, including an increased risk for developing alcohol/substance use disorders (Grant and Dawson, 1997; DeWit et al., 2000; Guerri and Pascual, 2016) and other mental health issues later in life (Spear, 2016). Animal models recapitulate many of the findings of the clinical literature, suggesting long lasting perturbations in neurobiological functions following adolescent EtOH exposure in rodents (Hiller-Sturmhöfel and Spear, 2018; Spear, 2018; Crews et al., 2019) that lead to increased susceptibility to EtOH effects in adulthood. For instance, it has been shown that intermittent EtOH exposure in adolescent rats, as a model for binge drinking behaviors, produces anxiety-like behaviors and is associated with increased EtOH consumption in adulthood (Pandey et al., 2015). Preclinical studies exploring activity of the mesocorticolimbic dopamine reward system in response to adolescent EtOH consumption report an increase in dopamine neurotransmission compared to EtOH-naïve rats (Sahr et al., 2004), as well as an increase in extracellular dopamine levels in the nucleus accumbens (NAc) of EtOH-treated adolescent rats compared to EtOH-treated adult rats (Pascual et al., 2009) which can continue into adulthood (Badanich et al., 2007). Remodeling changes in the neural circuits that make up the mesocorticolimbic pathway and alterations in dopamine function in these circuits occur throughout adolescence (Spear, 2000). Thus, it is plausible that sensitization of the mesocorticolimbic dopaminergic pathway following adolescent EtOH exposure mediates the long-term susceptibility of developing AUD later in life, although many other pathways have been suggested to be involved in this phenomenon (Crews et al., 2019).

The likelihood of developing AUD is not limited to early onset of EtOH consumption. Chronic and binge-like EtOH exposures can increase subsequent EtOH intake in adult clinical research and in preclinical models, suggesting a gradual transition from social drinking to excessive EtOH consumption and diagnosis of AUD can be modeled preclinically. This progressive transition has successfully been simulated in rodent models of intermittent access to EtOH in a two-bottle choice paradigm (Carnicella et al., 2014). For example, intermittent access to EtOH in rats has been shown to escalate subsequent self-administration of EtOH (those in a post-dependent state) compared to those given continuous access to EtOH or with no history of EtOH (Kimbrough et al., 2017). Additionally, the increase in self-administration of EtOH observed in rats with a history of EtOH dependence induction persists even after weeks of withdrawal (Roberts et al., 2000). This model of EtOH dependence may be due to allostatic changes in reward function due to modifications in neurotransmitter systems known to be involved in regulating the neurobiology of the positive reinforcing effects of EtOH (Koob, 2003).

The compulsive-like drug seeking effect associated with AUD is thought to involve, among other mechanisms, corticotropin-releasing factor (CRF) signaling in the extended amygdala (i.e., central amygdala, CeA and bed nucleus of the stria terminalis, BNST), which mediates the negative affective states typical of EtOH withdrawal and post-abstinence craving behaviors (Koob and Volkow, 2016; Zorrilla and Koob, 2019). CRF release increases within the CeA and BNST of EtOH dependent rats during withdrawal (Olive et al., 2002; Funk et al., 2006), which is thought to drive the increase in subsequent self-administration of EtOH. Studies indicating the CRF system is critical for the increase in self-administration in the post-dependent state show that treatment with CRF receptor antagonists reduces subsequent EtOH self-administration in these animals, specifically when injected in the CeA (Funk et al., 2006), with no effect in non-dependent animals (Funk et al., 2007).

[4] *Underage Drinking | National Institute on Alcohol Abuse and Alcoholism (NIAAA)*. Available online at: https://www.niaaa.nih.gov/publications/brochures-and-fact-sheets/underage-drinking (accessed June 16, 2020)

[5] Underage Drinking | CDC. Available online at: https://www.cdc.gov/alcohol/fact-sheets/underage-drinking.htm (accessed June 16, 2020)

Clinical and Preclinical Studies of High Fat Diet-Induced Alcohol Intake

Numerous clinical studies show increased desire, cravings, and intake of high fat foods during and after EtOH drinking episodes (Caton et al., 2004; Breslow et al., 2013; Piazza-Gardner and Barry, 2014). Epidemiologic data report 433 kcal excess consumption in men on drinking days vs. non-drinking days but only 61% of the excess calories is made up by the EtOH itself (Breslow et al., 2013), suggesting EtOH intake can drive increased food intake. Findings also suggest the effect of EtOH on food intake is modulated by increasing appetite and delaying the sensation of satiety (Caton et al., 2004). Less is known clinically, however, about the potential for HFD consumption to increase EtOH intake. Cross-sectional studies report that binge eating behaviors, which can be associated with increased propensity for obesity, are not only associated with increased intake of food higher in energy density, but also increased consumption of EtOH (Muñoz-Pareja et al., 2013; Bogusz et al., 2021). There are many shared mechanisms between binge eating disorders and drug addiction, including alterations in reward signaling and emotional processing (Carlier et al., 2015; Schulte et al., 2016; Moore et al., 2017a,b), which suggests that binging may be part of an overall compulsive behavioral phenotype that can straddle reward modalities. These findings may also suggest, therefore, that binge HFD intake may be a risk factor for escalation of EtOH intake. In the preclinical setting, a similar positive relationship for EtOH-induced increases in HFD intake has been shown in some animal models (Barson et al., 2009). While the inverse relationship of acute HFD exposure stimulating EtOH intake has been suggested in some animal models (Carrillo et al., 2004), the majority of findings indicate that HFD access decreases EtOH consumption in rodent models (Feng et al., 2012; Gelineau et al., 2017; Sirohi et al., 2017a,b). These discrepancies may be due, at least in part, to differences in HFD access periods.

Our recent work indicates that an intermittent HFD model using repeated acute HFD access periods once per week and intermittent, limited access to EtOH on days HFD is not given significantly increases EtOH intake in mice compared to both continuous control and HFD diet access (Coker et al., 2020). This is in contrast to other studies that show intermittent high fat/high sucrose diets given twice a week or more reduce EtOH intake in rats (Sirohi et al., 2017b). Additional data from our lab suggest that multiple HFD access days per week reduces EtOH intake and preference in mice at lower concentrations (10%), but this reduced intake and preference is not observed at higher EtOH concentrations (20%) compared to mice given HFD access only one time per week. These findings suggest that HFD may alter the overall reward value of EtOH, which is dependent on how many HFD access periods are available. Overall, it appears that access schedules mediate the interaction between binge HFD and binge EtOH intake in preclinical models. These differences also appear to have clinical relevance that may distinguish the ability of diets to induce binge eating disorders, substance use disorders, and their combination. These data, along with the findings discussed above showing palatable diets may decrease EtOH intake under certain conditions, suggest that dietary modifications during treatment for alcohol or drug use disorders may be an important avenue for future research to improve both behavioral and metabolic clinical outcomes.

Central Mechanisms of High Fat Diet and Alcohol Overlap

As described in previous sections, emerging evidence suggests similarities in the regulation of the overconsumption of palatable diets and drugs of abuse. For example, a review by Barson et al. (2011) focused on the similarities in hypothalamic and mesocorticolimbic circuits which act to regulate the intake of both food and EtOH. Interestingly, feeding research has historically focused on the hypothalamus, as its various nuclei are known to modulate food intake. The focus of feeding research has now expanded to include mesocorticolimbic regions that also regulate hedonic food intake. Specifically, studies show an increase in dopamine within the NAc while feeding or in the presence of food, an effect that lasted after consumption ceased. This increase was observed in mice made underweight but fed prior to the study as well as in mice mildly food deprived and trained to bar press for food (Hernandez and Hoebel, 1988; Smith and Schneider, 1988). Although it has long been recognized that EtOH can impact hypothalamic circuits regulating feeding behaviors (Amit et al., 1975), EtOH research has historically examined mesolimbic dopaminergic and other emotional regulatory circuits such as the extended amygdala. There is renewed interest in recent years, however, regarding EtOH effects on neuropeptide systems typically examined in food intake pathways (Leibowitz, 2007; Olney et al., 2014; Carlier et al., 2015; Alhadeff et al., 2019). Additionally, there are neuronal networks that extend between hypothalamic and mesocorticolimbic regions that may regulate the consumption of palatable food and drugs of abuse. Hypothalamic neurons can project to other hypothalamic subregions as well as to extra-hypothalamic regions, such as from the PVN to the VTA (Rodaros et al., 2007). Projections from the lateral hypothalamus extend to amygdala, NAc, VTA, and prefrontal cortex, and these regions also project back to the hypothalamus (Beckstead et al., 1979; Kita and Oomura, 1981; Fadel and Deutch, 2002; Kampe et al., 2009). Interestingly, aside from overlapping neurocircuit and neurochemical systems, endocrine signals from the periphery involved in the homeostatic control of feeding (e.g., leptin, ghrelin, insulin) also have direct effects on dopaminergic function and the reward value of food (Stice et al., 2013). Studies show satiety hormones, such as leptin and insulin, decrease food reward, dopamine release, and dopamine neuronal excitability (Figlewicz et al., 2004, 2006; Figlewicz and Benoit, 2009; Mebel et al., 2012), whereas the hunger hormone ghrelin increases food reward and dopaminergic function (Overduin et al., 2012; PerellóPerelló and Zigman, 2012). Overall, these findings suggest multiple overlapping connections between feeding and reward circuitry.

The overlap in hedonic intake mechanisms between both palatable food and drugs of abuse suggests overconsumption of these two reinforcers may share common mechanisms

contributing to central dysfunction. The mesolimbic dopamine reward system, which projects from the VTA to the NAc and other limbic regions, is a key focus when studying the overlapping neurobiology of substance misuse and palatable diet overconsumption (Stice et al., 2013; Volkow et al., 2017). Studies show that consumption of both palatable food and EtOH stimulate dopaminergic neuron firing in the VTA and dopamine release in the NAc and prefrontal cortex (Yoshida et al., 1992; Wilson et al., 1995; Martel and Fantino, 1996; Volkow et al., 2002; Gambarana et al., 2003; Yan et al., 2005; Liang et al., 2006; Morzorati et al., 2010; Ding et al., 2011). The role of insulin on dopaminergic neurons within various mesolimbic brain regions is noteworthy. In the VTA, insulin has been shown to decrease dopaminergic neuron firing (Labouèbe et al., 2013) and is thought to aid in the termination of food intake (along with homeostatic signaling in the hypothalamus), whereas in the NAc insulin potentiates release and reuptake at dopamine terminals and is thought to have influence on regulation of the rewarding characteristics of food intake (Stouffer et al., 2015). Studies show that HFD-induced insulin resistance alters dopamine terminal function in the NAc, which is reversed by restoring insulin signaling (Fordahl and Jones, 2016), suggesting central insulin resistance after HFD exposure may be an important factor in hedonic food intake and impaired satiety.

The amygdala, a limbic region that acts as an integrative center for emotions, memory, and motivation, also regulates food intake and reward. Clinical studies using functional magnetic resonance imaging (fMRI) show an increase in amygdala activity in overweight and obese adults (Ho et al., 2012) and children (Boutelle et al., 2015) in response to visual food cues and appetitive taste even in the postprandial state, indicating impaired satiety. Early preclinical studies investigating the role of the amygdala on feeding behavior indicate hyperphagia, increased weight gain, and hyperinsulinemia following bilateral lesions of the amygdala (King et al., 1994, 1996). Furthermore, studies show an increase in dopamine turnover in the amygdala following food intake in lean rats, which in turn is involved in reducing food intake (Anderberg et al., 2014). The anorexigenic hormone, glucagon-like peptide 1 (GLP1), was also shown to increase dopamine transmission in these animals and consequently reduce food intake (Anderberg et al., 2014). Thus, dopamine signaling is thought to be an important mechanism behind the regulation of food intake in the amygdala. Interestingly, GLP1 receptor agonists have recently been utilized in preclinical models to reduce reinstatement of drug seeking behaviors (Douton et al., 2020), suggesting drug targets that can improve metabolic outcomes in obesity (Iepsen et al., 2020) may be useful in the treatment of substance use disorders. Additionally, insulin receptor signaling in the amygdala is similar to that of the hypothalamus, which results in reduced food intake. This signaling is disrupted in rodents on an obesogenic diet resulting in insulin resistance in the amygdala (Areias and Prada, 2015). Such HFD-induced disruptions to amygdala signaling may enhance anxiety- and depressive-like behaviors in rodent models and potentially in the clinical population. The aforementioned findings suggest that insulin and other anorexigenic hormones may engage multiple mechanisms to promote satiety and influence reward salience, specifically by acting on various brain regions including the mesolimbic dopamine reward system, amygdala, and hypothalamus.

Recent evidence has identified additional overlapping brain regions, such as the nucleus of the tractus solitaries (NTS), that are involved in reward processing of palatable food and drugs of abuse (Kenny, 2011b). The NTS is well-known for receiving afferent information from the gastrointestinal tract triggered by gastric distension following ingestion of food (Garcia-Diaz et al., 1988), then projects to the hypothalamus sending satiety signals which collectively makes up the homeostatic regulation of feeding behavior. The NTS also receives afferent information from chemosensory neurons in the oral cavity that detect the palatability of food. All incoming information is then processed by various neuronal populations in the NTS, such as those expressing tyrosine hydroxylase (TH), pro-opiomelanocortin (POMC), and GLP1 (Sumal et al., 1983; Appleyard et al., 2005; Hayes et al., 2009). These neurons in turn project to various brain regions involved in detecting the hedonic properties of food and drugs of abuse, including limbic regions involved in reward processing (i.e., NAc, CeA, BNST), hypothalamic and thalamic regions, and prefrontal cortex. These numerous central and peripheral systems interact to control food and drug intake and may act as locations for sensitization of rewarding properties of HFD and EtOH or other drugs.

While this review focuses on the shared central mechanisms between binge eating disorders and drug addiction, suggesting the possibility binge eating disorders might represent an addiction-like behavioral state toward food, it is important to discuss criticism the theory of food addiction has received. Binge eating has been conceptualized as a form of addictive behavior due to overlapping properties shared with other accepted addictive behaviors (i.e., AUD), such as the loss of control over consumption and repeated engagement in binge behaviors despite negative consequences (Ferriter and Ray, 2011), as well as overlapping symptoms and genetic factors (Davis, 2013, 2017; Munn-Chernoff and Baker, 2016). A review by Rogers (2017) meticulously outlines the similarities and differences in appetites for foods and drugs. While there are clearly many similarities, one important difference between food and drugs of abuse is that food is necessary for survival while drugs of abuse are not. Furthermore, drugs of abuse are more potent reward signals than food in general, which likely leads to stronger levels of plasticity in brain circuitry controlling intake. Rogers concludes that while binge eating disorders may fulfill some key criteria of addictive behavior, broadening the definition of addiction to include food intake in general is likely detrimental to the impact of current research to both binge eating disorders and substance use disorders. Therefore, while there are many similarities between binge eating and binge drug and alcohol intake, including overlap of neurocircuitry and the potential for cross-sensitization of binge behaviors across modalities described above, "food addiction" as a generalized term is inaccurate and does not fully explain the continued high prevalence of overweight and obesity (Rogers, 2017).

NEUROIMMUNE FUNCTION

Overall, the shared neurobiology of addiction and obesity may be attributed to common brain regions and neurotransmitter systems involved in the regulation of consumption of palatable food and drugs of abuse, suggesting overlapping central dysfunction may be involved. The effects of palatable diet and drugs of abuse on reward circuits, such as the mesolimbic dopamine reward system, may be a key focus of this overlap. The influence of peripheral hormones on these reward systems are also noteworthy. As discussed in this section, one potential mechanism for the increase of disease susceptibility (i.e., diet-induced obesity and alcoholism) involving these brain regions may be neuroimmune function.

High Fat Diet Effects on Hypothalamic Neuroinflammation/Microglia

Microglia are the resident immune cells of the brain and play a key role in HFD-induced neuroinflammation. "Resting" or "ramified" microglia mainly exist under non-pathological conditions with their highly branched processes surveying the CNS. Upon stimulation, including detection of neuronal damage or systemic inflammation, microglia undergo morphological changes and rapidly convert from the resting state to the more amoeboid, non-ramified "active" state allowing for phagocytosis of pathogens or cellular debris (Kreutzberg, 1996; Ransohoff and Cardona, 2010; Cerbai et al., 2012; Salter and Beggs, 2014). Preclinical studies show hypothalamic inflammation following HFD is marked by a rapid increase in microglia activity which is thought to be a critical regulator of susceptibility to DIO (Valdearcos et al., 2017).

It is well-characterized that HFD exposure is closely associated with enhanced peripheral and central inflammation and this is a common feature of obesity, insulin resistance, and type II diabetes. However, it is now been shown that neuroinflammation due to HFD consumption can precede the onset of body mass increases or peripheral inflammation in animal models. Hypothalamic insulin resistance appears to be an antecedent to peripheral insulin resistance, as the development of inflammatory responses and insulin resistance to HFD occurs much more rapidly in this brain region compared to peripheral tissues (Prada et al., 2005; Valdearcos et al., 2015). Rodent studies show that even a single day of HFD exposure induces neuroinflammation in the hypothalamus (Waise et al., 2015). This process occurs before an increase in adiposity or peripheral inflammation, suggesting increased body mass is not required for development of insulin resistance in the hypothalamus (Rorato et al., 2017). Importantly, long-lasting changes in hypothalamic neuroinflammation occur in rodent models of developmental HFD exposure, leading to persistent changes in peripheral metabolic function independent of body mass (Cai and Liu, 2011). Together, these findings indicate that neuroinflammation in the hypothalamus may be a key driver to both central and peripheral alterations in insulin receptor function and metabolic outcomes following HFD intake.

High Fat Diet Effects on Extra-Hypothalamic Neuroinflammation

HFD-induced neuroinflammation has typically been studied in the hypothalamus, due to its distinct neuronal populations that are specific to regulating food intake and energy expenditure. Neuroinflammation brought on by various obesogenic diets has also been observed in multiple brain regions such as the amygdala, hippocampus, and cerebellum (Guillemot-Legris and Muccioli, 2017). Studies show that HFD-induced obesity increases expression of the pro-inflammatory cytokine interleukin-1 beta (IL-1β) (Almeida-Suhett et al., 2017) and induces insulin resistance in the amygdala through increased activation of the pro-inflammatory transcription factor nuclear factor-kappa B (NF-κB) (Castro et al., 2013). Animal studies indicate enhanced NF-κB inflammatory signaling (Lu et al., 2011; Kang et al., 2016), increased inflammatory markers (i.e., IL-1β, IL-6, and TNFα) (Lu et al., 2011; Miao et al., 2013), and heightened glial activation (i.e., astrogliosis and microgliosis) (Lu et al., 2011; Rivera et al., 2013; Kang et al., 2016) in the hippocampus following HFD exposure. In the mesolimbic reward pathway of non-human primates, emerging evidence indicates an obesogenic diet alters dopamine signaling and functional connectivity between the NAc and prefrontal cortex related to an increase in inflammatory markers (Godfrey et al., 2020). Thus, diet-induced inflammatory signaling may alter dopaminergic reward systems, increasing HFD intake, and potentially modulate intake of other rewards, such as drugs of abuse. Increased adiposity in DIO models may also lead to sensitization of stress mediated pro-inflammatory cytokine release from adipose cells (Qing et al., 2020), potentially leading to a feed-forward mechanism for enhanced central dysfunction in reward, stress, and metabolic regulatory neurocircuitry.

Alcohol Effects on Neuroinflammation/Microglia

EtOH consumption leads to a host of complications involving the CNS including inflammation and impairment of neuroimmune responses. A single dose of EtOH (5 g/kg intraperitoneal) has been shown to increase pro-inflammatory mRNA expression in mouse brains (Qin et al., 2008). Microglia are becoming recognized as critical modulators of EtOH-induced neurotoxicity (Henriques et al., 2018; Melbourne et al., 2019) since there is an increase in their activation markers in post-mortem brains of human alcoholics (He and Crews, 2008). Research shows there are alterations in CNS innate immune signaling upon exposure to EtOH, which increases activation of microglia and in turn exacerbates the neurotoxicity of EtOH. As a result, this EtOH-induced microglia pro-inflammatory activation leads to increased production of inflammatory cytokines, such as IL-1β, IL-6, and TNFα through the activation of the pro-inflammatory transcription factor NF-κB (Zou and Crews, 2010, 2012; Zhao et al., 2013; Hernandez et al., 2016). Excessive production of these cytokines and other pro-inflammatory factors, such as reactive oxygen species (ROS), by activated microglia contribute to EtOH-related pathologies (Alfonso-Loeches and Guerri, 2011).

The extent of the aforementioned effects of EtOH on neuroimmune responses and neurotoxicity by microglia activity differ depending on length of EtOH exposure, age of exposure, and brain region examined (Chastain and Sarkar, 2014; Perkins et al., 2019). Studies report microglia are partially activated following binge EtOH exposure which may cause increased production of anti-inflammatory factors (Marshall et al., 2013), whereas chronic intermittent EtOH exposure fully activates microglia which increases production of pro-inflammatory cytokines and ROS thereby resulting in neurotoxicity (Qin and Crews, 2012a,b; Zhao et al., 2013). These data align with the concept of microglial activation being a graded response resulting in various active microglial phenotypes (Melbourne et al., 2019).

EtOH-induced microglia activation is also considered in the development of AUD through microglia priming. It is hypothesized that adult chronic EtOH exposure can sensitize the immune system, specifically microglia, long-term thus increasing the risk for developing EtOH-related disorders (Chastain and Sarkar, 2014). Similar long-term immune sensitization is seen following prenatal EtOH exposure. This hypothesis is supported by findings of a greater increase in microglia activation in response to a systemic immune challenge in mice with a history of chronic EtOH (Qin and Crews, 2012a) and a study that reported prenatal EtOH exposure had a long-term effect on immune function in rats signified by increased severity of inflammation in a rheumatoid arthritis animal model (Zhang et al., 2011). Additionally, studies show that EtOH-induced neuroinflammation (measured by microglia activation) is not reversed by long-term withdrawal (Cruz et al., 2017), indicating long-term microglia sensitization.

Interestingly, EtOH administration in binge-like patterns induces whole-body insulin resistance and disruptions in glucose homeostasis by impairing hypothalamic insulin receptor function (Lindtner et al., 2013). These findings suggest that effects of EtOH on hypothalamic neuroimmune function are also critical for modulation of insulin and glucose homeostasis, similar to effects of HFD on hypothalamic neuroimmune function. Overall, these studies indicate that neuroimmune signaling and function may be a key point of cross-sensitization between HFD and EtOH to regulate aspects of peripheral and central metabolic function as well as emotional processing behaviors. Future studies will be needed to directly examine this possibility.

SUMMARY

The increased prevalence of obesity, AUD, and psychiatric conditions such as anxiety and depression in the United States has become a major public health concern. The shared clinical consequences of chronic and binge HFD intake and chronic EtOH misuse suggest an overlap in the mechanisms by which these factors modulate whole body physiology and central pathologies related to emotional regulation pathways indicated in psychiatric conditions. While we present evidence that HFD and EtOH intake may cross-sensitize binge behaviors and metabolic consequences via overlapping central mechanisms, a better mechanistic understanding of these processes may allow alteration of diets to be of benefit during AUD treatment, as has been suggested in other studies (Brutman et al., 2020; Shah et al., 2020). An important topic not discussed in this review is the effect of HFD and EtOH on microbiome gut-brain interactions. Since research is just beginning to understand the important roles of the gut microbiome on HFD-induced metabolic dysregulation and in EtOH-related behaviors individually, it is too early to properly assess how the gut microbiome might be impacted by a combination of HFD and EtOH intake. We refer readers to these other reviews that have discussed aspects of the gut microbiome on regulation of metabolic function, neurocircuits, and behaviors (e.g., Schellekens et al., 2012; Torres-Fuentes et al., 2017; Jerlhag, 2019; Cryan et al., 2020). In summary, we highlight numerous potential mechanisms behind cross-sensitization of HFD and EtOH effects in both behaviors and metabolic function, including alterations in neurotransmitter systems and neuroimmune function in shared neurocircuitry regulating emotional processing and peripheral signaling that will be important for future investigations.

AUTHOR CONTRIBUTIONS

All authors wrote and edited the manuscript.

FUNDING

This work was supported by NIH grants: AA026865, AA027697, TR002016, and TR002014.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Behavioral Neuroscience

REVIEW
published: 09 February 2021
doi: 10.3389/fnbeh.2021.613025

Extended Amygdala Neuropeptide Circuitry of Emotional Arousal: Waking Up on the Wrong Side of the Bed Nuclei of Stria Terminalis

William J. Giardino[*†] *and Matthew B. Pomrenze*[*†]

Department of Psychiatry and Behavioral Sciences, Stanford University School of Medicine, Stanford, CA, United States

OPEN ACCESS

Edited by:
Yuval Silberman, Pennsylvania State University, United States

Reviewed by:
Dennis Sparta, University of Maryland, Baltimore, United States
Antonio Figueiredo, University of Maryland, Baltimore, United States in collaboration with reviewer DS
Yanhua Huang, University of Pittsburgh, United States

***Correspondence:**
William J. Giardino
willgiar@stanford.edu
Matthew B. Pomrenze
pomrenze@stanford.edu

[†]*These authors have contributed equally to this work*

Specialty section:
This article was submitted to Emotion Regulation and Processing, a section of the journal Frontiers in Behavioral Neuroscience

Received: *01 October 2020*
Accepted: *15 January 2021*
Published: *09 February 2021*

Citation:
Giardino WJ and Pomrenze MB (2021) Extended Amygdala Neuropeptide Circuitry of Emotional Arousal: Waking Up on the Wrong Side of the Bed Nuclei of Stria Terminalis. Front. Behav. Neurosci. 15:613025. doi: 10.3389/fnbeh.2021.613025

Sleep is fundamental to life, and poor sleep quality is linked to the suboptimal function of the neural circuits that process and respond to emotional stimuli. Wakefulness ("arousal") is chiefly regulated by circadian and homeostatic forces, but affective mood states also strongly impact the balance between sleep and wake. Considering the bidirectional relationships between sleep/wake changes and emotional dynamics, we use the term "emotional arousal" as a representative characteristic of the profound overlap between brain pathways that: (1) modulate wakefulness; (2) interpret emotional information; and (3) calibrate motivated behaviors. Interestingly, many emotional arousal circuits communicate using specialized signaling molecules called *neuropeptides* to broadly modify neural network activities. One major neuropeptide-enriched brain region that is critical for emotional processing and has been recently implicated in sleep regulation is the bed nuclei of stria terminalis (BNST), a core component of the *extended amygdala* (an anatomical term that also includes the central and medial amygdalae, nucleus accumbens shell, and transition zones betwixt). The BNST encompasses an astonishing diversity of cell types that differ across many features including spatial organization, molecular signature, biological sex and hormonal milieu, synaptic input, axonal output, neurophysiological communication mode, and functional role. Given this tremendous complexity, comprehensive elucidation of the BNST neuropeptide circuit mechanisms underlying emotional arousal presents an ambitious set of challenges. In this review, we describe how rigorous investigation of these unresolved questions may reveal key insights to enhancing psychiatric treatments and global psychological wellbeing.

Keywords: bed nuclei of the stria terminalis, extended amygdala, neuropeptide, arousal, circuit, sleep, wakefulness, bed nucleus of stria terminalis (BNST)

INTRODUCTION

Precise control of wakefulness ("arousal") is essential for generating the adaptive forms of reward-seeking and stress resilience that encourage healthy survival (Tsujino and Sakurai, 2009; Eban-Rothschild et al., 2017). Thus, neuronal wakefulness systems were shaped by evolution to confer high sensitivity for detecting, interpreting, and acting upon emotional stimuli.

Healthy sleep/wake cycles are essential for optimal cognition and emotion, and poor sleep quality can lead to deleterious changes in the physiological function of brain circuits that gate behavioral responses to emotional stimuli (Koob and Colrain, 2020). In mental health conditions of addiction, anxiety, and depression, maladaptive responses to hedonically-valenced stimuli are linked to distinct activity patterns in brain arousal pathways. Indeed, stress is a major factor driving *insomnia* (inability to sleep), and various sleep-related disturbances are common among individuals enduring stress-related psychiatric conditions. On the other hand, experiencing pleasure and anticipating future reward can also extend wakefulness and prevent healthy sleep, highlighting the ability of both positive and negative hedonically-valenced stimuli to shift the thresholds of arousal (Eban-Rothschild et al., 2018). A further fascinating example of the link between emotion and arousal is the sleep disorder *narcolepsy with cataplexy*, in which powerful feelings of euphoria or aversion can interrupt wakefulness by triggering rapid intrusion of a sleep-like state (Adamantidis et al., 2020). These profound neuroscientific mysteries hint at the commonalities among (and/or interactions between) brain pathways that calibrate wakefulness, process emotional information, and generate motivated behaviors.

Intriguingly, many emotional arousal circuits use specialized modulatory signaling molecules called *neuropeptides* to fine-tune the coordination of broad neural network activity (Ryabinin et al., 2012; Schank et al., 2012; Giardino and de Lecea, 2014; Kash et al., 2015; Li et al., 2017). One major neuropeptide-enriched emotional processing network is the *extended amygdala* (an anatomical term referring to neurons spanning the bed nuclei of stria terminalis (BNST), central and medial amygdalae (CeA, MeA), nucleus accumbens shell (NAcSh), and the transition zones betwixt; Alheid, 2003). While communication *via* neuropeptide signaling likely allows the BNST to perform sophisticated control of emotional arousal circuitry, the primary mechanisms underlying changes in synthesis, storage, and release of peptide neuromodulators from BNST neurons remain largely undescribed. This is due in part to the complex patterns of more than 10 discrete neuropeptides that are distributed in varying combinations of multi-neuropeptide co-expression amongst up to forty unique cellular subpopulations (Moffitt et al., 2018; Welch et al., 2019; Rodriguez-Romaguera et al., 2020). The BNST encompasses a particularly astonishing diversity of cell types that differ along spectrums of several features, including spatial organization, molecular signature, biological sex and hormonal milieu, synaptic input, axonal output, neurophysiological messaging, and functional role (Kash et al., 2015; Lebow and Chen, 2016; Vranjkovic et al., 2017; Ch'ng et al., 2018; Beyeler and Dabrowska, 2020).

Historically, an all-encompassing framework for the BNST cell groups and connections driving emotional behaviors was limited by existing pharmacological and neurochemical approaches. Recent advances in genetic, optical, and computational tools for mapping, manipulating, and monitoring brain activity have revolutionized functional annotation of behavioral neurocircuits (Saunders et al., 2015; Nectow and Nestler, 2020; Xia and Kheirbek, 2020). Nevertheless, comprehensive elucidation of the BNST neuropeptide mechanisms underlying emotional arousal presents an ambitious set of challenges. In this review, we describe how rigorous investigation of these unresolved questions may reveal key insights to enhancing psychiatric treatments and global psychological wellbeing.

SPATIALLY-DEFINED BNST CELL TYPES

The BNST is a ventromedial forebrain complex surrounded on all sides by the hypothalamus, thalamus, striatum, septum, and lateral ventricles. Given the wide-ranging descriptions of "BNST", we primarily discuss the multiple distinct neuronal populations corresponding to those encompassed within adult mouse (*Mus musculus*) brain stereotaxic coordinates approximately +0.45 to −0.35 mm anterior/posterior (A/P), 0.40 to 1.20 mm medial/lateral (M/L) bilaterally off the midline, and −4.0 to −5.0 mm dorsal/ventral (D/V). Various systems of nomenclature have been proposed for labeling unique BNST subcompartments, but the classification of BNST cellular populations based solely on spatial location remains unstandardized and highly subjective (Bota et al., 2012; Lebow and Chen, 2016; Barbier et al., 2021). This persisting lack of consensus for definitive BNST spatial subdivisions reflects the challenges faced by early anatomists, who first divided BNST on the M/L axis, only to be challenged by developmental biologists who inferred a predominantly A/P axis, followed by synaptic physiologists, neurochemists, and others who emphasized a D/V axis (corresponding to divergent patterns of monoaminergic innervation, for example; De Olmos and Ingram, 1972; Krettek and Price, 1978; Weller and Smith, 1982; Bayer and Altman, 1987; Dong et al., 2000; Egli and Winder, 2003; Bota et al., 2012; McElligott et al., 2013; Radley and Johnson, 2018). Although functional associations with BNST divisions across each of the anatomical axes have sparked valuable hypotheses, variability in the degree to which unique BNST features differ across distinct spatial dimensions limits the holistic impact of relying solely on such descriptors to functionally parcellate the BNST.

For example, the term "ventral BNST" commonly refers to neurons located directly ventral to (beneath) the anterior commissure (a prominent white matter tract that forms a wide horizontal band when viewed in the coronal plane). However, pioneering neuroanatomists acknowledged more than 30 years ago that, while the commissure may be a useful landmark for dividing general areas of the BNST, "it does not necessarily always define strict cytoarchitectonic boundaries, since a component of the dorsal area may well be separated and come to lie in the ventral area" (Ju and Swanson, 1989). In other words, the commissure forms a wide horizontal shape only at certain points along the rodent BNST A/P axis, and the commissure's departure from view in the caudal BNST reveals contiguous cellular populations that may have been "divided" on a D/V axis in rostral sections purely incidentally. Indeed, Ju and Swanson (1989) noted that "Immunohistochemical studies with antisera to several peptides also indicate very similar staining patterns within these (D/V) regions. It seems clear to us, therefore,

that the anterior commissure *simply passes through* the BNST" (Ju and Swanson, 1989).

Upon eschewing the commissure as a monolithic landmark, Swanson and colleagues identified at least five different BNST cellular populations residing ventrally to the commissure (Ju and Swanson, 1989; Ju et al., 1989; Dong et al., 2001a,b; Dong and Swanson, 2003, 2004a,b, 2006a,b,c). Although they originally used the abbreviation "vBNST" to refer only to a particular ventralmost subnucleus within the ventral BNST complex (Ju and Swanson, 1989; Ju et al., 1989; Dong et al., 2000, 2001a,b; Dong and Swanson, 2003, 2004a,b, 2006a,b,c), modern widespread usage of the term "vBNST" generally translates to "ventral BNST writ large", and the commissure remains a major dividing line for ascribing any readily identifiable characteristics that may be distinguished along the D/V BNST axis. **Supplementary Table 1** displays the incongruity of stereotaxic coordinates used to target the mouse "ventral BNST" in recent behavioral neuroscience publications, reflecting the limitations of relying on ambiguous spatial descriptors (Jennings et al., 2013b; Dedic et al., 2018; Kim et al., 2018; Hardaway et al., 2019; Chen et al., 2020; Girven et al., 2020). Especially given the renewed interest in adjacent bordering structures (i.e., ventral pallidum, substantia innominata, preoptic area; McHenry et al., 2017; Gordon-Fennell et al., 2019; Ottenheimer et al., 2019; Stephenson-Jones et al., 2020), investigators may decide to refine their definitions when examining ventrally-located BNST neuronal populations. Of course, "ventral BNST" is simply one of many instances of imperfect BNST anatomical nomenclature. Numerous additional examples of incongruous systems for spatially labeling neuronal subtypes serve only to further strengthen the rationale for adopting a BNST framework that heavily incorporates non-spatial defining features (Ju and Swanson, 1989; Jennings et al., 2013a,b; Kim et al., 2013; Giardino et al., 2018; Barbier et al., 2021).

Although beyond the scope of this review, potential differences in the spatial organization of BNST cell types between various rodent and primate species also require serious consideration. In addition to the anatomical literature cited above, we refer the reader to foundational work from Bales, Blackford, Fox, Fudge, Luyten, Shackman, Trainor, Zahm, and others (Zahm, 1998; Fudge and Haber, 2001; Zahm et al., 2003; Hostetler et al., 2011; Avery et al., 2014, 2016; Fox et al., 2015; Luyten et al., 2016; Shackman and Fox, 2016; Fudge et al., 2017; Oler et al., 2017; Raymaekers et al., 2017; Reichard et al., 2017; Theiss et al., 2017; Duque-Wilckens et al., 2018; Fox and Shackman, 2019; Luyck et al., 2019, 2020; Flook et al., 2020; Luyten, 2020). Indeed, most *in vivo* data generated from monkey and human BNST thus far has been collected using methods with a low spatial resolution like functional magnetic resonance imaging and deep brain stimulation. Within these mental health contexts, reliable *in vivo* parcellation of human BNST subcompartments remains a lofty goal. Keeping this in mind, we encourage others to acknowledge the possibility that acquiring enhanced spatial resolution of the BNST in human patients may turn out to be largely inconsequential for improving overall psychiatric and neurological outcomes. In doing so, we posit that emphasis on *non-spatial* aspects of BNST neurons (such as molecular markers, physiological features, long-range projection targets, and sources of upstream neural inputs) may hold the key for accelerating discovery on functional contributions of BNST circuitry to behavior and sleep/wake arousal states.

MOLECULARLY-DEFINED BNST CELL TYPES

Similar to the rest of the extended amygdala, the BNST contains many cell types marked by a myriad of neurotransmitters, neuropeptides, receptors, enzymes, and regulatory proteins (Bota et al., 2012). The BNST primarily consists of subpopulations of inhibitory neurons marked by the GABA transporter *Vgat*, as well as excitatory populations marked by the glutamate transporter *Vglut2*, and a mixed excitatory/inhibitory population marked by co-expression of *Vgat* and *Vglut3* (Kudo et al., 2012; Jennings et al., 2013b). Although these various GABAergic and glutamatergic populations are widely dispersed, mapping molecularly-defined cell types to different spatial areas of the BNST may help clarify how different subregions regulate emotional arousal behaviors (**Figure 1**).

To survey BNST neurons defined by molecular markers other than GABA/glutamate transporters, multiple studies first used Cre driver rodent lines with Ai9/Ai14 tdTomato fluorescent reporter mice or viral labeling techniques and combined anatomical analyses with immunohistochemical staining (Chen et al., 2015; Pomrenze et al., 2015; Nguyen et al., 2016; Giardino et al., 2018; Walker et al., 2019). The global population of *Vgat*-BNST neurons was found to encompass several molecularly-defined subgroups, including neurons expressing genetic and protein markers for the neuropeptides corticotropin-releasing factor (*Crf*; Dabrowska et al., 2013, 2016) and cholecystokinin (*Cck*; Giardino et al., 2018). *Crf* and *Cck* are non-overlapping GABAergic subpopulations that occupy separate lateral vs. medial subdivisions, co-residing in adjacent compartments throughout the middle of the BNST A/P axis, approximately +0.2 to −0.2 mm from Bregma in the mouse. In addition to *Crf* and *Cck*, the neuropeptides dynorphin (*Pdyn*), enkephalin (*Penk*), neurotensin (*Nts*), neuropeptide Y (*Npy*), nociceptin (*Pnoc*), somatostatin (*Sst*), substance P (*Tac1*), neurokinin B (*Tac2*), and vasopressin (*Avp*) are also found in neurons throughout the BNST (Malsbury and McKay, 1987; Walter et al., 1991; Poulin et al., 2009; Kudo et al., 2014; Crowley et al., 2016; Ahrens et al., 2018; Giardino et al., 2018; Zelikowsky et al., 2018; Kovner et al., 2019; Rigney et al., 2019; Rodriguez-Romaguera et al., 2020; Smith et al., 2020; Whylings et al., 2020; Xiao et al., 2020; **Figure 1A**). Labeling for the calcium-binding protein calretinin appears selective for the dorsolateral (dl)BNST, whereas dopamine receptor type-1 (*Drd1*) and protein kinase C delta (*Pkcd*) neurons cluster more specifically within the oval nucleus (ovBNST, a discrete subnucleus within the larger dlBNST subregion; Kim et al., 2013; Nguyen et al., 2016; Pomrenze et al., 2019a; Wang et al., 2019).

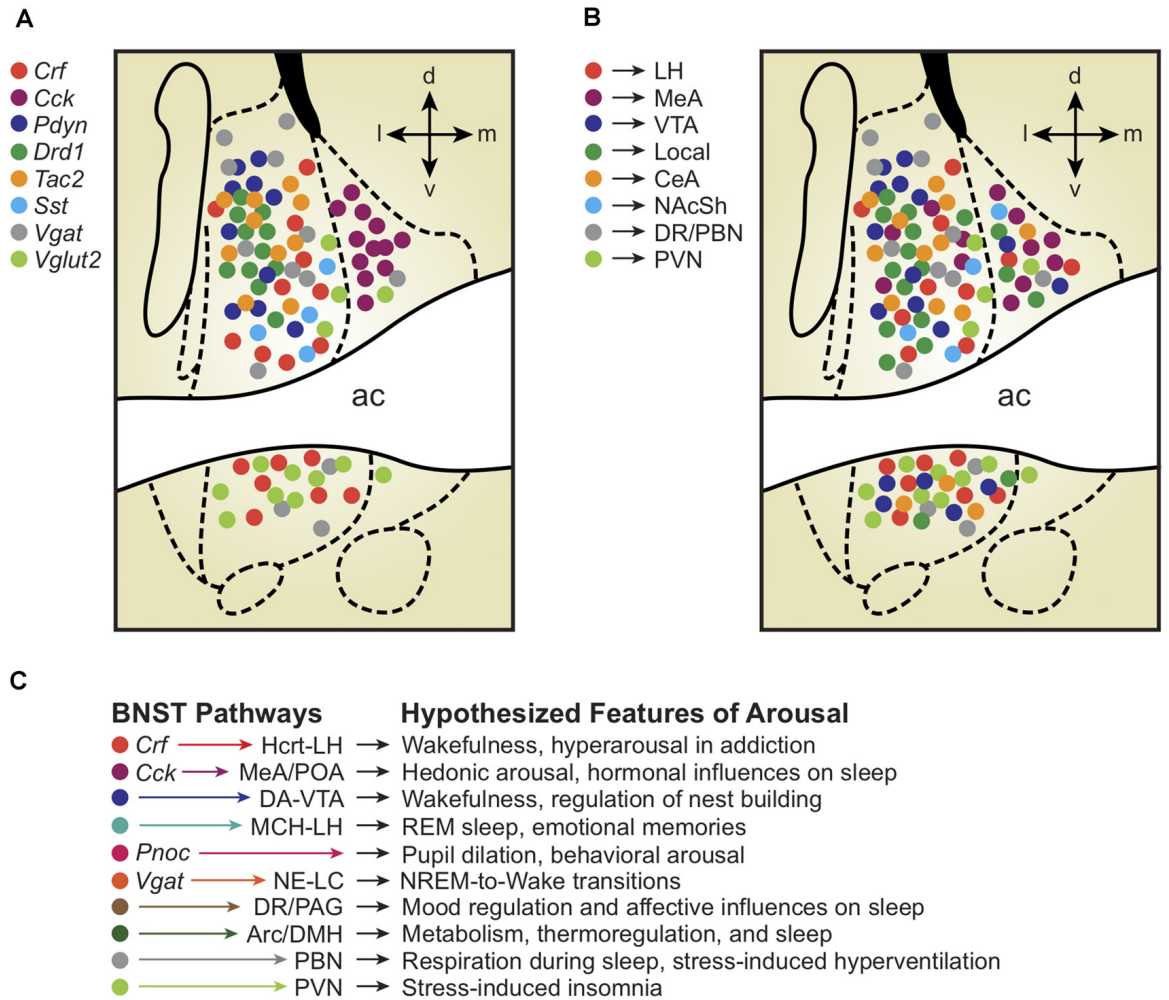

FIGURE 1 | (A) Depiction of molecularly-defined bed nuclei of stria terminalis (BNST) cell types and their distribution across BNST subregions. Note the remarkable compartmentalization of some cell-types compared with others (*Cck* vs. *Crf* vs. *Vglut2*). **(B)** Depiction of projection-defined BNST cell types and their approximate distributions across BNST subregions. Interestingly, some (but not all) projection-defined cell types roughly map onto corresponding molecularly-defined subpopulations. **(C)** Depiction of BNST cell types, pathways, and their hypothesized relationships with distinct features of arousal and sleep/wake regulation. Colors of each hypothesized pathway reflect the molecularly- and projection-defined BNST cell types when consistent with panels **(A)** and **(B)**. Distinct colors represent hypothesized BNST pathways with unknown molecular traits and spatial distributions across the BNST.

Breakthrough efforts to characterize the entire genetic diversity of the BNST and surrounding regions at the single-cell level provided evidence for up to 37 distinct neuronal subtypes, although an exhaustive discussion of this data is beyond the scope of our review (Moffitt et al., 2018; Welch et al., 2019). A more recent single-cell RNA sequencing study targeted specifically in the dorsal (d)BNST identified several neuronal clusters, including those marked by expected genes (e.g., *Pkcd*, *Sst*, *Npy*), but also some surprising markers (e.g., *Lmo4*; Rodriguez-Romaguera et al., 2020).

Numerous BNST neurons (particularly in the posteromedial [pm]BNST) express markers for actions of gonadal steroid hormones, including the androgen receptor (AR), progesterone receptor (PR), estrogen receptors, and aromatase (Aro), the enzyme that converts androgens to estrogens (Bayless and Shah, 2016). Aro-BNST neurons have been well-studied in contexts of sexually dimorphic behaviors (Bayless et al., 2019), and pmBNST neurons expressing AR and PR are more numerous in males vs. females (Juntti et al., 2010; Yang et al., 2013), highlighting the importance of sex differences and hormonal interactions when studying BNST contributions to emotional arousal (Bangasser and Shors, 2008; Bangasser et al., 2019).

Concerning the potential wake-modulating effects of molecularly-defined BNST subpopulations, the reported pupil dilatory effects of *Pnoc*-BNST neuron stimulation (Rodriguez-Romaguera et al., 2020) suggest that such rapid arousal responses may also regulate sleep/wake state transitions, but this has not been explicitly tested. Furthermore, while genetic markers in the BNST have thus far been *primarily used to simply define the type of cell* rather than determine the role of the corresponding

protein product, future progress will shift toward understanding the physiological actions of the molecule itself. For example, it will be essential to assess whether modern neurotechnological approaches used to *stimulate CRF-expressing BNST neurons* will be sufficient to accurately recapitulate the sleep/wake changes resulting from the *natural release of the CRF neuropeptide from BNST neurons*.

PROJECTION-DEFINED BNST CELL TYPES

A major property of the BNST is its connections with a plethora of downstream target brain regions, providing a useful framework for conceptualizing the potential contributions of BNST neurons to distinct features of sleep/wake arousal states (**Figure 1B**). For example, by examining BNST projection neurons that form synapses with cells in limbic regions regulating emotional behavior, One landmark article characterized the role of three different BNST pathways in separate features of anxiety (Kim et al., 2013). The authors showed that neurons in the anterodorsal (ad)BNST target the lateral hypothalamus (LH), ventral tegmental area (VTA), and parabrachial nucleus (PBN) to drive anxiolysis, reward, and decreased respiratory rate, respectively. Retrograde tracing determined minimal overlap between cells projecting to the three different downstream regions, collectively implying unique anxiety-relevant functions for different BNST cell types based in part on their projection targets. Given the rich literature describing roles for the LH (Li et al., 2017, 2018), VTA (Eban-Rothschild et al., 2016, 2020), and PBN (Kaur and Saper, 2019) in various aspects of sleep/wake regulation, we speculate that such BNST axonal outputs controlling separable components of emotional behavior may also modulate distinct physiological aspects of sleep/wake arousal states (**Figure 1C**).

In earlier studies of BNST neuroanatomy in rodents, VTA-projecting BNST neurons had been characterized by the Watanabe group, who identified a double-inhibitory pathway in which GABAergic BNST neurons preferentially target VTA-GABA neurons (Kudo et al., 2012). Kudo et al. (2012, 2014) also identified VTA-projecting BNST neurons marked by the glutamate transporters *Vglut2* and *Vglut3*, as well as the opioid peptide *Penk*, providing multiple diverse mechanisms for modulating the activity of GABA and dopamine (DA) neurons in the VTA. Jennings et al. (2013b) reported that *Vgat* and *Vglut2* neurons specifically in the ventral (v)BNST synapse onto GABA and DA VTA neurons, where they control distinct motivational states. Numerous other studies also focused on VTA-projecting BNST neurons, including those labeled by *Crf* and *Pdyn* (Briand et al., 2010; Silberman et al., 2013; Marcinkiewcz et al., 2016; Pina and Cunningham, 2017; Rinker et al., 2017; Companion and Thiele, 2018; Dedic et al., 2018; Fellinger et al., 2020).

Given recent evidence that VTA-DA neurons drive wakefulness and regulate nest-building (a critical sleep preparatory sequence in mice), BNST→VTA-DA circuits may therefore be a key mechanism influencing ethologically-relevant behavioral arousal (Eban-Rothschild et al., 2016, 2017; Eban-Rothschild and de Lecea, 2017). Furthermore, the activity of the VTA-GABA neuron population was found to be positively correlated with high-frequency gamma signal (30–80 Hz) during wakefulness, providing another measure of physiological arousal likely impacted by BNST→VTA projections (Eban-Rothschild et al., 2020).

Separate efforts on LH-projecting neurons identified a large *Vgat*-BNST population that preferentially targeted *Vglut2*-LH neurons downstream (Jennings et al., 2013a). Later studies determined that LH-projecting *Vgat*-BNST neurons include both *Crf* and *Cck* subpopulations that exhibit divergent preferences for downstream target cell types, with *Crf*-BNST neurons displaying a particularly high level of connectivity with LH neurons containing the arousal-promoting neuropeptide hypocretin (*Hcrt*; also known as orexin; Giardino et al., 2018). *Hcrt*-LH neurons comprise a subset of glutamatergic LH neurons, consistent with the interpretation that *Crf*-BNST→*Hcrt*-LH connections represent a subset of the larger *Vgat*-BNST→*Vglut2*-LH pathway. González et al. (2016) also investigated BNST→LH connectivity, finding that *Vgat*-BNST neurons synapse onto both Hcrt/orexin and melanin-concentrating hormone (MCH) neurons of the LH. This discovery of BNST neurons interacting with the MCH system is notable based on several studies showing that *Mch*-LH neurons promote rapid eye movement (REM) sleep (Bandaru et al., 2020).

In addition to their LH projections, *Crf* and *Cck* BNST neurons innervate (to varying degrees) the paraventricular nucleus of the hypothalamus (PVN), medial amygdala (MeA), CeA, medial preoptic area (mPOA), NAcSh, ventral premammillary nucleus (PMv), and ventrolateral periaqueductal gray (vlPAG; Dabrowska et al., 2016; Giardino et al., 2018). BNST projections to the PVN are particularly relevant given recent findings that glutamatergic neurons in this region are critical for the control of wakefulness (Liu et al., 2020). Further pursuing questions of BNST→hypothalamus connectivity, Barbier et al. (2021) traced the long-range projections of dlBNST and dorsomedial (dm)BNST neurons in exquisite detail, finding that the dlBNST projects especially to the LH and tuberomammillary nucleus (TMN; the site of wake-promoting histamine neurons; Barbier et al., 2021). In contrast to the dlBNST, the dmBNST preferentially innervates the PVN, the arcuate nucleus (Arc), and dorsomedial hypothalamus (DMH) regions that may influence sleep pressure *via* regulation of metabolic and thermoregulatory processes (Barbier et al., 2021).

In addition to targeted investigations, serendipitous retrograde labeling of upstream neurons has yielded surprising BNST outputs and circuit motifs. For example, monosynaptic rabies tracing from either "patch" or "matrix" neurons in the dorsal striatum revealed a major input from the dBNST to patch neurons that were largely absent from matrix neurons (Smith et al., 2016). These dBNST neurons form inhibitory synapses with striatal patch neurons who in turn project to DA neurons in the substantia nigra. Similarly, a GABAergic projection from *Sst*-BNST neurons was recently characterized and shown to form synapses with parvalbumin interneurons in the NAcSh (Xiao et al., 2020). Therefore, in addition to directly targeting the VTA, the BNST is capable of modulating mesolimbic DA

function through indirect mechanisms at sites of midbrain innervation in the striatum.

Monosynaptic rabies tracing also identified BNST neurons that project directly to serotonin and GABA neurons in the dorsal raphé nucleus (DR; Weissbourd et al., 2014). Interestingly, BNST→DRN neurons preferentially target GABA cells over serotonin cells. While the majority of the BNST→DR cells were GABAergic at mouse coordinates +0.14 mm A/P from Bregma, a smaller set of BNST→DR neurons were labeled by *Vglut2* at +0.02 mm A/P from Bregma. Separate rabies tracing studies also revealed BNST inputs to noradrenergic locus coeruleus neurons (Schwarz et al., 2015), DA and GABA VTA neurons (Beier et al., 2015), and CRF receptor 1 (CRFR1) PVN neurons (Jiang et al., 2018).

Beyond its rich collection of long-range projection neurons, the BNST contains several species of short-range interneurons. The Deisseroth group showed that ovBNST neurons project locally between subregions of the adBNST where they release GABA (Kim et al., 2013). In line with this, ovBNST neurons secrete dynorphin to inhibit excitatory basolateral amygdala (BLA) fibers innervating the adBNST (Crowley et al., 2016). Neurons in ovBNST also send dense axons to the vBNST (Dong et al., 2001; Wang et al., 2019). Aside from inhibitory local connections, many studies raised the possibility that neuropeptide modulators are also released locally from within the BNST. For example, NPY enhances inhibitory transmission in *Crf*-BNST neurons (Pleil et al., 2015) and although this study did not identify the source of NPY, the BNST contains several NPY-expressing neurons capable of local release. Another study from the Kash group found a complex microcircuit in the BNST comprised of *Crf* interneurons that are modulated by serotonin and regulate the activity of separate long-range BNST projection neurons (Marcinkiewcz et al., 2016). Collectively, these studies demonstrate the complexity of both long-range and local connectivity in the BNST and point to another dimension of inherent organization arising from gene expression and neurotransmitter phenotype.

PHYSIOLOGY-DEFINED BNST CELL TYPES

The BNST contains multiple neuronal cell types that have been classified according to their electrophysiological properties, most prominently in rats by Hammack and others (Hammack et al., 2007; Hazra et al., 2011; Dabrowska et al., 2013; Rodríguez-Sierra et al., 2013; Silberman et al., 2013; Nagano et al., 2015; Yamauchi et al., 2018). While originally described primarily within the ovBNST, these three types (Type I, II, and III) have also been identified in the non-oval areas of adBNST, as well as anteroventral (av)BNST. Type I neurons exhibit an I_h-like current in response to hyperpolarizing current injection and a regular firing pattern in response to depolarizing current. Type II neurons exhibit a similar I_h-like current but burst fire in response to depolarizing current. Type III neurons do not exhibit an I_h-like current but instead, show a fast rectification in response to hyperpolarizing current and exhibit a regular firing pattern when depolarized. Coupled with the diverse synaptic inputs, these physiological characteristics likely play a strong role in modulating varied forms of BNST output.

Regarding distinct output modes of neurophysiological communication, most outgoing physiological signals from the BNST have been recorded simply as GABAergic/glutamatergic inhibitory/excitatory postsynaptic currents. However, the existence of co-expressed neuropeptides in *Vgat*-BNST neurons suggests the likelihood of multiplexed modes of signaling in which single cells may influence downstream activity *via* a combination of slow-acting neuropeptide release and fast-acting classical neurotransmitter release. Indeed, a growing suite of fluorescent sensor tools for detecting receptor signaling with the cell-type resolution at rapid timescales may prove revolutionary for elucidating the sleep/wake mechanisms of neuropeptide release and actions across synaptic and extrasynaptic sites of the BNST circuitry (Gizowski et al., 2016; Patriarchi et al., 2018; Sun et al., 2018).

FUNCTIONALLY-DEFINED BNST CELL TYPES

Hedonic Valence

The BNST is known to impact both positive and negative states of reward and stress, and early studies relied primarily on electrolytic and neurochemical lesions to generate several opposing hypotheses regarding the bi-valent emotional behaviors generated by activity in the BNST (Bangasser et al., 2005; Pezuk et al., 2008; Resstel et al., 2008). Access to molecularly-defined cell types enabled by modern neurotechnology has ushered in several recent advances in understanding the sources of hedonic valence in the BNST, beginning with studies from the Stuber group showing that optical stimulation of *Vgat*-BNST and *Vglut2*-BNST neurons produced approach and avoidance behaviors, respectively (Jennings et al., 2013a,b).

Going beyond the separation of large populations of GABAergic vs. glutamatergic neurons, additional tools for recording and modulating cell-specific neural activity *in vivo* revealed that dlBNST *Crf* neurons preferentially respond to aversive stimuli and drive behavioral avoidance, whereas dmBNST *Cck* neurons are activated by rewarding stimuli and generate behavioral preference/approach (Giardino et al., 2018). Mirroring this lateral/medial functional distinction, *Drd1* neurons in the ovBNST/dlBNST and *Six3* neurons in the dmBNST also drive avoidance and approach, respectively (Giardino et al., 2018). Because activation of the global *Vgat*-BNST population promotes positive valence (Jennings et al., 2013a,b; Giardino et al., 2018), we hypothesize that *Crf* marks a specialized subgroup of lateral *Vgat*-BNST neurons with opposing functional properties from the larger set of combined lateral and medial *Vgat*-BNST cells. In other words, global *Vgat*-BNST stimulation may more closely resemble activation of medial *Vgat*-BNST neurons (like *Cck*) rather than lateral *Vgat*-BNST neurons (like *Crf*).

It should be noted that the vast majority of data on BNST circuits driving hedonic valence has been generated only

from male mice. Given that the BNST is a major site for integrating signals from centrally-circulating gonadal steroids, intense investigation of hormonal influences on the function of extended amygdala pathways may be key for understanding sex differences in the stress response and reward sensitivity. Future studies may also seek to titrate levels of stressor exposure, drug consumption, and other variables to determine whether such experiential factors can influence the hedonic valence associated with mobilization of particular BNST subpopulations.

Anxiety

Despite the distinct hedonic valences associated with stimulating *Crf* and *Cck* BNST neurons, optogenetic or chemogenetic activation of either *Crf* or *Cck* BNST neurons led to increased indices of anxiety-like behavior in multiple paradigms (Giardino et al., 2018), suggesting that standard measures of "anxiety" in rodents may reflect generalized arousal states independent of hedonic valence *per se*. Consistent with this interpretation, activation of *Vgat*-BNST neurons (Mazzone et al., 2018), *Drd1*-ovBNST neurons (Kim et al., 2013), *Pdyn*-BNST→VTA neurons (Fellinger et al., 2020), dlBNST→CeA neurons (Yamauchi et al., 2018), and *Crf*-CeA→dlBNST neurons (Pomrenze et al., 2019b) all increased anxiety-like behaviors. Yet, separate studies found that stimulation of adBNST→LH neurons (Kim et al., 2013), *Vgat*-vBNST→VTA neurons (Jennings et al., 2013b), and *Sst*-BNST→NAcSh neurons (Xiao et al., 2020) had the opposite effect of reducing anxiety, revealing some unresolved issues regarding how BNST microcircuits and long-range pathways regulate stress-related "anxiety" phenotypes. Anxiogenic circumstances like fear learning and stress-induced social deficits also strongly engage the BNST (Bjorni et al., 2020; Emmons et al., 2021), providing multiple perspectives for approaching the study of BNST in anxiety-related emotional arousal.

THE BNST IN SLEEP, WAKE, AND EMOTIONAL AROUSAL

Only a handful of studies have explicitly investigated the BNST within the context of sleep/wake arousal states and corresponding neurophysiological rhythms. Beginning in 1995, *in vivo* recordings of 63 single units in the BNST of cats revealed that 72% of neurons fired more frequently during wakefulness and rapid eye movement (REM) sleep than during "quiet sleep" (presumably non-REM/slow-wave sleep; NREM; Terreberry et al., 1995). These findings are consistent with more recent data indicating that excitatory projections from the BNST may activate REM-active neurons in the sublaterodorsal tegmental nucleus (SLD) of the brainstem (Boissard et al., 2003; Rodrigo-Angulo et al., 2008). In the BNST of rats, sleep deprivation increased cFos and CRF protein expression (Duan et al., 2005; Deurveilher et al., 2008) and M3 muscarinic acetylcholine receptor gene expression (Kushida et al., 1995), suggesting possible neuropeptidergic and cholinergic mechanisms for homeostatic sleep drive in the BNST.

In 2014, an intriguing arousal-related physiological signature was discovered in the extended amygdala by Haufler and Pare (2014), who recorded high-frequency oscillations (HFOs; 110–160 Hz local field potentials) that appeared with a significantly higher incidence in the BNST and CeA relative to surrounding areas (striatum, pallidum, septum; Haufler and Pare, 2014). HFOs in hippocampal, cortical, and subthalamic brain regions are thought to have functional consequences in memory-processing, epileptic seizures, and symptoms of Parkinson's disease, respectively. Whereas the role of HFOs in the BNST remains unclear, their ability to entrain large populations of neurons (with greater power during REM vs. non-REM sleep; Haufler and Pare, 2014) provides a fascinating example of the network-level changes in extended amygdala activity that might profoundly influence discrete states of arousal. Future endeavors will revolutionize understanding of how arousal state transitions may be aligned to physiological activity changes and/or receptor signaling events in a BNST cell type-specific manner by implementing combinatorial approaches like EEG/EMG sleep monitoring in tandem with next-generation neural recording technologies (fiber photometry, miniscope, and two-photon imaging).

To directly investigate the BNST as a potential node in the arousal circuitry, Kodani et al. (2017, 2019) utilized optogenetic methods and reported that they could generate immediate transitions from NREM sleep to wakefulness by stimulating the global GABAergic BNST population in *Gad67*-Cre mice. *Gad67*-BNST arousal was associated with activation of norepinephrine neurons in the locus coeruleus (NE-LC), an established wakefulness-promoting center. *Gad67*-BNST arousal was also associated with activation of Hcrt-LH neurons and required signaling of Hcrt receptors (Kodani et al., 2017), consistent with the reported contributions of BNST→LH circuits to emotional arousal and related behaviors (Giardino and de Lecea, 2014; González et al., 2016; Giardino et al., 2018; Barbier et al., 2021). Of course, the precise directionalities of the relationships between endogenous BNST activity, emotional behaviors, and changes in sleep and wakefulness remain mostly uncharacterized. For example, whereas states of stress might be hypothesized to drive insomnia *via* chronically elevated BNST hyperactivity, additional experiments are required to determine whether excess BNST activity is truly a contributing factor or rather an indirect consequence of stress-induced arousal.

In addition to providing *outputs* to wake-promoting downstream targets in the LH, VTA, and PVN, the BNST also receives *inputs* from arousal-promoting neurons such as calretinin cells of the paraventricular thalamus (PVT; Hua et al., 2018). Hua et al. (2018) recently found that starvation promotes a state of hyperarousal *via* activation of the PVT-calretinin circuit, which can be blocked by chemogenetic inhibition of the PVT→BNST pathway. Despite this exciting progress, a complete understanding of the arousal-promoting abilities of the global GABAergic BNST population requires additional studies. Future experiments will aim to determine whether distinct molecularly-defined or pathway-specific BNST outputs are necessary and/or

sufficient for regulating various forms of behavioral arousal and natural sleep-to-wake transitions (see hypothesized functions in **Figure 1C**).

On this note, it will be important to causally determine which BNST neurocircuits have experience-dependent effects on sleep/wake as a function of negative vs. positive hedonic valence. This can be accomplished by monitoring the activities of extended amygdala pathways and corresponding changes in arousal following emotional experiences with ethologically aversive or rewarding stimuli. For example, the discovery that reward-promoting Cck-BNST neurons are densely connected with the medial amygdala (MeA; Giardino et al., 2018) suggests that these pathways powerfully influence arousal changes linked to innately rewarding consummatory behaviors. In contrast to the Cck-BNST→MeA circuit, Crf-BNST→Hcrt-LH connections likely drive stress-induced insomnia following psychosocial challenges.

Given the well-established role of the BNST in drug-seeking behavior (Vranjkovic et al., 2017), attention should be placed on the idea that addiction-related sleep disturbances are driven by specific extended amygdala pathways. Core features of drug addiction (e.g., craving, relapse, and withdrawal) are each associated with maladaptive neuroplasticity in homeostatic circuits that regulate sleep/wake cycles and stress sensitivity, illustrating how addiction can be viewed as a condition of pathological hyperarousal (Koob, 2013; Koob and Colrain, 2020). Substance use disorders are highly co-morbid with other arousal-related psychiatric conditions (e.g., insomnia, anxiety, PTSD, panic), and the BNST may be a common substrate linking dysregulated hedonic processing to chronic sleep disruption.

Finally, the BNST may be an important and overlooked link in emotional arousal about the sleep disorder *narcolepsy with cataplexy* (in which powerful feelings of euphoria or aversion can interrupt wakefulness by triggering rapid intrusion of a sleep-like state). Intriguingly, narcolepsy is associated with selective loss of Hcrt neurons in the LH. Based on existing evidence for BNST→LH connections driving motivated behaviors, detailed studies are warranted on BNST circuit contributions to emotionally-triggered arousal destabilization. Of course, the existence of putative sleep-promoting BNST neurons remains to be seen. However, BNST connections with hypothalamic nuclei regulating the release of prolactin, oxytocin, and vasopressin suggest that experiments linking BNST activity to arousal changes following binge eating ("food coma") and sexual behavior (mating) may be successful in revealing the neurocircuitry of mysterious phenomena like post-ingestive sleep, post-copulatory sleep, and narcolepsy with cataplexy.

CONCLUSIONS

Altogether, our understanding of extended amygdala circuits regulating affective behaviors will have direct relevance to therapeutic strategies aimed at modulating motivation and sleep/wake regulation. Before the advent of tools permitting cell type-specific and pathway-specific labeling and manipulation, the inherent complexity of extended amygdala pathways hindered progress in understanding their roles in emotional arousal. As BNST circuits continue to be disentangled at the genetic, synaptic, and systems levels, new anatomical and functional frameworks are taking shape that illustrates the multifaceted nature of the BNST. Data of this kind are extremely valuable for developing new therapeutic approaches for a range of neuropsychiatric conditions, highlighting the extraordinary relevance of emotional arousal circuits to researchers and clinicians across the fields of neuroscience, genetics, psychology, and mental health interventions. We anticipate that as studies on BNST function become more precise, effective clinical treatment strategies will be successfully developed.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

This study received funding from NIH/NIAAA K99/R00 AA025677 (WG) and NIH/NIDA T32 DA035165 (MP).

ACKNOWLEDGMENTS

We thank D. W. Bayless, J. R. Knoedler, D. C. Castro, T. L. Kash, L. R. Halladay, and others for insightful discussions.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2021.613025/full#supplementary-material.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Behavioral Neuroscience

REVIEW
published: 04 March 2021
doi: 10.3389/fnbeh.2021.601275

A Role for the Amygdala in Impairments of Affective Behaviors Following Mild Traumatic Brain Injury

*Taylor A. McCorkle[1], Jessica R. Barson[1,2] and Ramesh Raghupathi[1,2]**

[1] Graduate Program in Neuroscience, Graduate School of Biomedical Sciences and Professional Studies, Drexel University College of Medicine, Philadelphia, PA, United States, [2] Department of Neurobiology and Anatomy, Drexel University College of Medicine, Philadelphia, PA, United States

Mild traumatic brain injury (TBI) results in chronic affective disorders such as depression, anxiety, and fear that persist up to years following injury and significantly impair the quality of life for patients. Although a great deal of research has contributed to defining symptoms of mild TBI, there are no adequate drug therapies for brain-injured individuals. Preclinical studies have modeled these deficits in affective behaviors post-injury to understand the underlying mechanisms with a view to developing appropriate treatment strategies. These studies have also unveiled sex differences that contribute to the varying phenotypes associated with each behavior. Although clinical and preclinical studies have viewed these behavioral deficits as separate entities with unique neurobiological mechanisms, mechanistic similarities suggest that a novel approach is needed to advance research on drug therapy. This review will discuss the circuitry involved in the expression of deficits in affective behaviors following mild TBI in humans and animals and provide evidence that the manifestation of impairment in these behaviors stems from an amygdala-dependent emotional processing deficit. It will highlight mechanistic similarities between these different types of affective behaviors that can potentially advance mild TBI drug therapy by investigating treatments for the deficits in affective behaviors as one entity, requiring the same treatment.

Keywords: mild TBI, depression, anxiety, posttraumatic stress disorder, basolateral amygdala, central amygdala, GABA, CRF

OPEN ACCESS

Edited by:
Gregg Stanwood, Florida State University, United States

Reviewed by:
Cathy W. Levenson, Florida State University, United States
Adel Helmy, University of Cambridge, United Kingdom

***Correspondence:**
Ramesh Raghupathi
rr79@drexel.edu

Specialty section:
This article was submitted to Emotion Regulation and Processing, a section of the journal Frontiers in Behavioral Neuroscience

Received: *31 August 2020*
Accepted: *29 January 2021*
Published: *04 March 2021*

Citation:
McCorkle TA, Barson JR and Raghupathi R (2021) A Role for the Amygdala in Impairments of Affective Behaviors Following Mild Traumatic Brain Injury. Front. Behav. Neurosci. 15:601275. doi: 10.3389/fnbeh.2021.601275

INTRODUCTION

Traumatic brain injury (TBI) is a serious public health concern that affects over 1.5 million people each year and results in the death and disability of thousands, with mild TBI comprising the greatest proportion of these cases (Kay et al., 1993; Coronado et al., 2011; McMahon et al., 2014). In the civilian population, mild TBI occurs largely because of playing contact sports such as American football, soccer, and ice hockey, whereas military personnel are exposed to mild blast TBI (Prins et al., 2013; Baldwin et al., 2018). Although sports-related concussions and mild

blast TBI occur through different means, they share a similar prevalence of about 15–20% in civilian and veteran populations, as well as similar behavioral deficits (McKee and Robinson, 2014; Gardner and Yaffe, 2016).

Depression, anxiety, and fear/post-traumatic stress disorder (PTSD) are affective behavioral impairments that are among the most frequently reported behavioral problems that manifest in the chronic period following mild TBI (Baldassarre et al., 2015; Ellis et al., 2015; Horn et al., 2016; Bunt et al., 2020). The severity of these deficits is known to vary by sex, in that women are more prone to exhibiting affective disorders post-injury (Broshek et al., 2005; Bunt et al., 2020). Interestingly, the heightened response women exhibit following mild TBI is not consistently seen following moderate or severe TBI (Lavoie et al., 2017). Clinical studies used qualitative methods to diagnose the emotional state of an individual in conjunction with brain imaging tasks allowing for the identification of potential brain regions involved in affective disorders (Lange et al., 2016; Chong and Schwedt, 2018; Bunt et al., 2020; Teymoori et al., 2020). In preclinical models, validated behavioral assays have become the mode of quantifying what are usually thought to be subjective, affective states (Can et al., 2011, 2012; Calhoon and Tye, 2015; Eagle et al., 2016; Liu et al., 2018). Through these assays and the accompanied use of other biological techniques, mechanistic underpinnings of each disorder have been identified, as well as sex differences in deficit expression. However, limitations in the effectiveness of current drug regimens for affective disorders suggest that deficits following mild TBI should be examined in a new light (Xiong et al., 2013). Neurobiological studies have suggested that the amygdala is a key brain region involved in emotional processing (Phelps and Ledoux, 2005; Pessoa, 2010; Kim et al., 2011; Korgaonkar et al., 2019), and its impairment is associated with the manifestation of the affective disorders seen after TBI (Han et al., 2015; Hoffman et al., 2019). Understanding the mechanistic underpinnings of dysfunction in this brain region following injury will advance the field of TBI research. This review will discuss the current methods, corresponding mechanisms, and drug therapies used to identify and treat affective behavioral deficits in human and animal models of mild TBI. Because of the high comorbidity and mechanistic similarities across depression, anxiety, and fear, we suggest that they should be considered as a collective deficit in emotional processing stemming from impairment within the amygdala.

HUMAN STUDIES

Definition and Epidemiology of Mild TBI

Mild TBI is often used interchangeably with concussion (Sussman et al., 2018) and affects approximately 15–20% of the population (McKee and Robinson, 2014; Gardner and Yaffe, 2016). Further, about 50% of people fail to report their injury suggesting that a majority of mild TBI incidents may be repetitive (Vargas et al., 2015; Pryor et al., 2016; Baldwin et al., 2018). Typically, mild TBI is defined by a Glasgow Coma Scale score between 13 and 15 along with a lack of, or very minimal, impairment in the level of consciousness. The two main types of mild TBI are contact-based such as sports-related concussions where some form of physical contact occurs, and blast-based which usually occurs as a result of exposure to an explosion (blast wave) during combat (Prins et al., 2013; Baldwin et al., 2018). Moreover, although it can happen at any age, mild TBI is particularly prevalent amongst adolescents who participate in contact sports (Baldwin et al., 2018), and young adults, who represent most enlisted military personnel (Lange et al., 2016). The risk for mild TBI is greater in boys and men due to sports including football and ice hockey and the fact that the military is a male-dominant field. However, girls are more prone to concussion when looking at gender-comparable sports (Baldwin et al., 2018).

Behavioral Consequences of Mild TBI

Immediately after a concussion, patients experience headache, dizziness, nausea, sensitivity to noise, motor impairments, and deficits in executive functioning (Szczupak et al., 2016). Typically, these somatic signs and symptoms are documented using either the Sport Concussion Assessment Tool (SCAT) or the Immediate Post-concussion Assessment of Cognitive Testing (ImPACT). These acute symptoms resolve within 7-10 days in a vast majority of patients but can exist for 90 days or more in those that are then diagnosed with post-concussion syndrome (Ryan and Warden, 2003). In addition, and to a greater extent in those that have suffered multiple concussions, brain-injured patients can develop deficits in learning and memory and affective behavior disorders such as depression, anxiety and fear which can persist for years post-injury (Manley et al., 2017). Military personnel who suffer from mild blast TBI experience similar acute symptoms as individuals suffering from sports-related concussion and develop chronic problems such as impaired cognition (Waid-Ebbs et al., 2014), depression and post-traumatic stress disorder, which largely consists of anxiety and fear-related behaviors (Shively and Perl, 2012). In the chronic post-concussion phase, both cognitive and affective behaviors are assessed using more specific and sensitive measures such as the Wechsler Intelligence Scale, the Tower of London test, the Patient Health Questionnaire (PHQ), the Generalized Anxiety Disorder-7 (GAD-7) scale and the Clinician Assessment of PTSD each with its defined threshold for determining the severity of the deficit (**Tables 1**, **2**). Although previous research has found that both sexes exhibit some degree of affective impairment, the consensus of literature states that women experience significantly exacerbated symptoms (Broshek et al., 2005; Sufrinko et al., 2017). Thus, the prevalence of depression, anxiety, and fear is increased in women compared to men (Bunt et al., 2020). Moreover, studies suggest that the severity of behavioral deficits in women depends on their menstrual phase (Bazarian et al., 2010; Stein, 2013; Wunderle et al., 2014). For example, women injured when their progesterone concentrations were high had lower health scores 1-month following injury (Wunderle et al., 2014). Another study showed that menstrual phase had no effect on concussion symptom severity (Mihalik et al., 2009); however, this study was conducted using healthy collegiate athletes. Combined, these

TABLE 1 | Clinical studies that evaluated cognition (such as executive function, memory, and concentration) and emotional (such as anxiety, depression, and PTSD) behaviors following mild TBI.

Study description	Behaviors evaluated	
	Cognitive	**Emotional**
Veterans (*N* = 10) diagnosed with repeated mild blast TBI (Waid-Ebbs et al., 2014)	Executive functions measured using Tower of London, Behavior Rating Index of Executive Function-Adult Version and Delis Kaplan Executive Function System	Depression using the Beck Depression Inventory II (> 29 = severe depression) PTSD using the PTSD Checklist-Military Version (> 50 = PTSD)
Veterans 18 years or older deployed in OIF/OEF conflicts (Baldassarre et al., 2015)	Poor concentration and difficulty making decisions using the Neurobehavioral Symptom Inventory	Depression using the Beck Depression Inventory II (score ≥ 17) Anxiety using the Beck Anxiety Inventory (score ≥ 8) PTSD using the ClinicianAdministered PTSD Scale (lenient (F1/I2), moderate (F1/I2 plus total severity ≥ 45), and stringent (F1/I2 plus total severity ≥ 65)
Veterans of OIF/OEF conflicts (*N* = 2235, Schneiderman et al., 2008)	Memory and post-concussion symptoms	PTSD using the PTSD Checklist (> 50 = PTSD)
Active-duty Marines and Navy Corpsmen (*N* = 825, Glenn et al., 2017)	DID NOT TEST	PTSD using the clinician administered PTSD Scale (one criterion A event, one cluster B symptom, two cluster C symptoms, and two cluster D symptoms) and Fear-Potentiated Startle
Former NFL players (Roberts et al., 2019)	Cognition-related QOL using QOL in Neurological Disorders: Applied Cognition	Depression and Anxiety using PHQ-4 (score ≥ 3 for each behavior)
Active semiprofessional and professional football players (Pryor et al., 2016)	DID NOT TEST	Depression using the Center for Epidemiologic Studies Depression Scale (score range from 0 = little/no depression to 60 = major depression)
NFLPA retired players section (*N* = 1617, Schwenk et al., 2007)	DID NOT TEST	Depression using PHQ-9 (0–9 = no-to-mild; 10–27 = moderate-to-severe)
Male and female patients (*N* = 491) ages 12–18 with a diagnosed SRC within 30 days of a clinic visit (Bunt et al., 2020)	Memory and concentration using the SCAT-5 scale	Depression using GAD-7 Anxiety using PHQ-9
Male and female collegiate athletes (*N* = 84) with concussion (Vargas et al., 2015)	Reading, memory and concentration using the Wechsler Test of Adult Reading and the ImPACT score	Depression using the Beck Depression Inventory-Fast Screen (score ≥ 4)
Pediatric patients (19 and younger) referred to Pan Am Concussion Program (Ellis et al., 2015)	DID NOT TEST	Depression and Anxiety using the PCSS emotional sub scores (range of 0–24)
Mild TBI patients (18 years or older, *N* = 238) from the Northern California TBI Model Systems of Care database (Lavoie et al., 2017)	DID NOT TEST	Depression using PHQ-9 (0-9 no-to-mild, 10–27 moderate-to-severe)
Mild TBI patients (18 years and older) following their first head injury ages (Rao et al., 2010)	Attention, learning, delayed recall and memory using the MMSE, National Adult Reading Test and the Hopkins Verbal Learning Test-Revised	Depression using the Structured Clinical Interview for DSM-IV Axis 1 disorders Social Functioning Examination
Patients (18 years and older) who sustained TBI at least 3 months prior (*N* = 101, Mohammad Farris Iman Leong Bin Abdullah et al., 2018)	DID NOT TEST	Depression and Anxiety using the Structured Clinical Interview for DSM-IV Axis I Disorders Research Version
Patients (16 years or older) who sustained a TBI at least 6 months prior (Teymoori et al., 2020)	DID NOT TEST	Depression and anxiety using the PHQ subsection for Anxiety and Depression (scores of 8 and 10 used as cutoff, respectively) Anxiety using the GAD-7 (score of 8 used as cutoff) PTSD using the PTSD Checklist for DSM-V (score of 33 used as cutoff)

Whereas certain studies evaluated both sets of behaviors, a few only reported the incidence of emotional behaviors. The studies described in this table did not assess structural alterations in the brains of mild TBI patients.
GAD-7, Generalized Anxiety Disorder-7; ImPACT, Immediate Post-concussion Assessment and Cognitive Testing; MMSE, MiniMental State Examination; OIF/OEF, Operation Iraqi Freedom/Operation Enduring Freedom; PCSS, Post-Concussion Symptom Scale; PHQ, Patient Health Questionnaire; PTSD, Post-Traumatic Stress Disorder.

limited and conflicting studies highlight the need for further investigation into the impact menstrual cycle has on behavioral consequences of mild TBI in women. Moreover, men also show a degree of affective impairment as well as significant cognitive deficits post-injury (Roberts et al., 2019). Since affective behavioral disorders have cognitive components to them, this can serve as a way through which men exhibit greater signs of psychological impairment.

TABLE 2 | Clinical studies that evaluated structural/functional alterations (using imaging techniques) in patients that were tested for cognitive (such as executive function, memory, and concentration) and emotional (such as anxiety, depression, and PTSD) behaviors following mild TBI.

Study description	Structural/functional changes		Behavioral changes	
	Amygdala	**Other regions**	**Cognitive**	**Emotional**
Combat veterans from Iraq or Afghanistan with mild TBI only (N = 15) or mild TBI + PTSD (*N* = 17, Shu et al., 2014)	ND	Event-related potentials in the dorsal anterior cingulate cortex	DID NOT TEST	Depression using the Beck Depression Inventory II PTSD using the clinician administered PTSD Scale (>65)
US Service Members (*N* = 153, Tate et al., 2016	Changes in shape (using MRI) of right anterior amygdala as a function of time after injury	Changes in surface areas (using MRI) of anterior medial accumbens and left anterior medial caudate Increased	Memory and decision-making using the Neurobehavioral Symptom Inventory	PTSD using the clinician administered PTSD Scale (DSM-IV Criteria)
Football players (N30) with acute concussion (Mustafi et al., 2018)	ND	Increased mean diffusivity (using DTI) in corpus callosum, superior longitudinal fasciculus and corona radiata	SCAT	Depression and Anxiety using Brief Symptom Inventory
Active and former professional rugby players with history of concussion (*N* = 24, Wojtowicz et al., 2018)	Smaller left amygdala volumes using MRI	Smaller bilateral hippocampi in the absence of differences in whole brain cortical thickness using MRI	Learning and memory using the Rey Auditory Verbal Learning Test, the Rey Complex Figure Test and the Long Delay Recall Task	Depression, Anxiety, and Stress (comprised of 7-item scales) Alcohol Use Disorders Identification Test
Patients with mild TBI (*N* = 42, Giguère et al., 2019)	ND	ND	Executive function and working memory using Repeatable Battery for the Assessment of Neuropsychological Status and the Delis-Kaplan Executive Function System ColorWord inference test and the Digit Span task	Hospital Anxiety and Depression Scale (pathological scores are 11–21)
Chronic TBI patients (*N* = 54, Han et al., 2015)	Enhanced bilateral amygdala connectivity (using MRI) in TBI patients exhibiting depression group	No change in somato-motor cortex connectivity using MRI	Memory and executive functions using Immediate and Delayed Recall Tasks, the Full Scale Intelligent Quotient-2, the Wechsler Abbreviated Scale of Intelligence, the Wechsler Test of Adult Reading, and the Delis-Kaplan Executive Function System	Depression using the Beck Depression Inventory II (score up to 13 = minimal depression and 14–63 = mild to severe) PTSD using PTSD Checklist Stressor specific for DSM-IV
Patients with closed head injury (*N* = 91, Jorge et al., 2004)	ND	Volumes for the entire brain, the orbitofrontal cortex, medial-frontal cortex and lateral prefrontal cortex determined using MRI	Learning, memory, executive functions using the MMSE, the Rey Auditory Verbal Learning Test, the Rey Complex Figure Test and the Multilingual Aphasia Examination	Depression, anxiety, aggression Two semistructured interviews (Present State Examination) along with Structured Clinical Interview for DSM-IV diagnoses Hamilton Depression Rating Scale Hamilton Anxiety Scale Overt Aggression Scale

Most studies that have evaluated structural/functional changes using advanced imaging techniques have focused on the cortex and hippocampus along with various white matter tracts. A few studies have evaluated alterations in structure and function of the amydgala.
DTI, Diffusion Tensor Imaging; MRI, Magnetic Resonance Imaging; PTSD, Post-Traumatic Stress Disorder; SCAT, Sport Concussion Assessment Tool.

A search of clinical studies in PubMed using search terms such as mild TBI and cognition or mild TBI and depression or anxiety or fear revealed almost twice as many publications focused on cognitive impairments after mild TBI. However, recent studies revealed that patients develop depression, anxiety, and fear years after injury, which greatly contributed to a decreased quality of life (Baldassarre et al., 2015; Giguère et al., 2019; **Tables 1**, **2**). Affective disorders are reported in approximately 20% of brain-injured individuals (Schwenk et al., 2007; McKee and Robinson, 2014; Scholten et al., 2016; Brassil and Salvatore, 2018), and can impact other behavioral outputs. For example, depression increases the bias to aversive memory recall (Leal et al., 2017), anxiety decreases the ability to handle stress as it directly involves the hypothalamic-pituitary-adrenal (HPA) axis (Maeng and Milad, 2015), and the expression of fear is linked to aggression (Gao et al., 2014).

Post-traumatic depression is characterized by alterations in affect, diminished interest in pleasurable activities, negative and intrusive thoughts, reductions in physical movement, decreased executive functioning, and loss of energy (Lavoie et al., 2017). Depression is evaluated based on the results from the Beck Depression Inventory II, the PHQ-9, or the Structured Clinical Interview for DSM-IV/V Axis 1 Disorders (criteria are described in **Tables 1**, **2**). Approximately 15% of individuals with mild TBI and mild blast TBI suffer from post-injury depression (Schwenk et al., 2007; McKee and Robinson, 2014). This is a gross underestimation because more than 50% of individuals with a mild TBI fail to report symptoms (Rao et al., 2010; Vargas et al., 2015; Pryor et al., 2016). About 21% of individuals with mild TBI experience some form of anxiety disorder within the first year of injury which increases to 36% after the first year due to high long-term prevalence (Scholten et al., 2016); anxiety was measured using the Beck Anxiety Inventory, the GAD-7 scale or the PHQ (criteria are described in **Tables 1**, **2**). The manifestation of fear-related behaviors ("avoiding similar situations") is a hallmark of PTSD which also includes difficulty sleeping and flashbacks (Brewin et al., 2017). In the civilian population, PTSD occurs at a prevalence rate of less than 25%, whereas over 35% of military personnel exposed to mild blast TBI exhibit symptoms of PTSD (Schneiderman et al., 2008; Shu et al., 2014; Brassil and Salvatore, 2018). Individuals exposed to mild blast TBI experience heightened stress responses and emotional memory associations due to the nature of their situation. In addition, repeated traumatic events occurring during deployment results in strong, conditioned fear learning and expression of fear which may be the basis for the high risk of PTSD in this group (Glenn et al., 2017). Tests used to detect PTSD and their criteria are described in **Tables 1** and **2**.

Depression, anxiety, and fear-related behaviors following mild TBI are comorbid conditions (**Tables 1**, **2**; Jorge et al., 2004; Ellis et al., 2015; Mohammad Farris Iman Leong Bin Abdullah et al., 2018; Teymoori et al., 2020). A study of over 100 individuals with a mild TBI used a Structured Clinical Interview for DSM-IV axis I disorders to determine that 25% showed signs of depression and 14% had anxiety, with fear-evoked PTSD representing a major contributor to this anxiety. Importantly, about half of these individuals had comorbid anxiety and depression (Mohammad Farris Iman Leong Bin Abdullah et al., 2018). In another study, the same structured interview was utilized, as well as the Hamilton Depression and Anxiety Scales to show that 33% of mild TBI patients exhibited symptoms of depression within the first year and within this group, 76% also had anxiety (Jorge et al., 2004).

Structural Changes Following Mild TBI

The primary structural pathology of mild TBI is damage to white matter tracts. Post-concussion white matter changes are largely present in the frontal, parietal, and temporal lobes of the brain (Chong and Schwedt, 2018). An advanced MRI study demonstrated significantly altered diffusion properties of white matter tracts in concussed athletes, which were correlated with acute symptoms post-injury and functional deficits. Specifically, an increase in mean diffusivity in the corpus callosum, superior longitudinal fasciculus, and the corona radiata of the concussed group compared to healthy controls has been reported (Mustafi et al., 2018). Structural impairment can persist in adolescents and individuals who suffer multiple concussions, contributing to prolonged behavioral impairment (Chong and Schwedt, 2018). Furthermore, a study investigating former high school athletes that suffered repeated concussions showed higher mean diffusivity in the anterior limb of the internal capsule over 20 years post-injury (Terry et al., 2019).

Functional MRI studies have shown changes in hippocampal connectivity following repeated collisions in collegiate football players (Slobounov et al., 2017; Wojtowicz et al., 2018). A study of professional rugby players with a history of concussion demonstrated no significant differences between cortical thickness, but smaller whole brain hippocampus and left amygdala volumes compared to healthy controls (Wojtowicz et al., 2018). Neuroimaging studies of mild blast TBI patients tend to be understudied; however, one study showed that injury to the hippocampus in veteran populations results in globally decreased gray matter volumes compared to controls (Bhattrai et al., 2019). Alterations in hippocampal connectivity relate directly to cognitive and affective behavior deficits (Belujon and Grace, 2011; Leal et al., 2017). In military personnel who had suffered at least one mild TBI, the radial distance of the amygdala was positively correlated with time since injury, meaning individuals further removed from injury showed greater amygdala thickness (Tate et al., 2016). Moreover, imaging performed on individuals with moderate TBI and depressive symptoms demonstrated that these individuals have increased amygdala connectivity relative to healthy controls, and this increased connectivity was associated with affective disorders (Han et al., 2015). The amygdala is a key hub of emotional processing (Phelps and Ledoux, 2005; Pessoa, 2010; Kim et al., 2011; Korgaonkar et al., 2019), therefore, changes in its size or connectivity can lead to affective behavior impairments seen post-injury (Han et al., 2015; Hoffman et al., 2019). Structural impairments in the hippocampus and amygdala post-injury provide avenues through which affective behavioral disorders can manifest in individuals; however, these structures are not widely investigated in neuroimaging studies following mild TBI. Sex differences in the structural impairments following mild TBI are also understudied and filling this gap in literature

would aid in understanding the relationship between behavioral outcome and structural alterations. Most studies that have evaluated chronic behavioral deficits in concussed patients typically do not provide assessment of structural or functional alterations (**Table 1**). The preponderance of studies that have included imaging correlates for behavioral changes focus on structures that may be more vulnerable to concussions such as white matter tracts and cortical areas at or near the sites of impact (**Table 2**), making it difficult to determine specific relationships between behaviors and intracerebral alterations.

ANIMAL STUDIES

Definition and Models of Mild TBI

Multiple animal models of mild TBI that effectively reflect structural changes (axonal injury and neurodegeneration) and impairments in motor, cognitive, and affective behaviors typically reported in brain-injured clinical populations, have been developed (Malkesman et al., 2013; Bodnar et al., 2019). Mild TBI can be induced through impact to the intact skull, head, or exposure to a blast wave. Impact to the intact skull by way of an extended piston tip or a weight drop that does not result in skull fracture or hematoma is defined as "mild" (Ma et al., 2019). Because both sports-related concussions and mild blast TBI are characterized by their repetitive nature, the greatest challenge in developing a clinically appropriate animal model of contact or blast TBI is selecting the best approach and variability across the animal models exists in impact site, frequency, and severity (Weber, 2007). When performing repetitive mild TBI, impact frequency must be translational, realistically modeling the frequency in the human condition. Moreover, despite the immense variability that can exist between models of mild TBI, validation must be based on the structural and behavioral changes of mild TBI seen in human populations. To reduce variability in the site and magnitude of impact, animals are typically restrained and therefore require anesthesia such as isoflurane or ketamine (Rowe et al., 2014). Mild blast TBI has been performed with (Rowe et al., 2014) or without (Uddin et al., 2019) anesthesia, using a shock tube and cranium only blast injury apparatus, respectively (Kuehn et al., 2011; Ma et al., 2019; Uddin et al., 2019). Anesthetized animals exhibit similar behavioral and structural deficits as seen in awake rodent and clinical mild TBI models; therefore, the protective effects of the anesthetics are often minimal, but must be considered when deciding on which preclinical mild TBI model to utilize (Rowe et al., 2014). Moreover, it is important to note that the use of anesthesia limits complete fidelity of animal models to reflect concussion in humans, as humans are not anesthetized during injury.

Behavioral Changes Following Mild TBI

Preclinical models of mild TBI exhibit short-and-long-term behavioral deficits post-injury. Acutely, brain-injured animals show impaired locomotion, working memory, and anxiety (Wright et al., 2017). Repetitive brain injury has been shown to lead to chronic deficits in social behavior, spatial working memory, spatial learning, and other prefrontal cortex and hippocampal-associated functions (Cheng et al., 2014; Nolan et al., 2018). The worsening or development of affective behavioral deficits is frequently reported in preclinical models of single and repetitive TBI (Teutsch et al., 2018; Popovitz et al., 2019; Beitchman et al., 2020), and mild TBI, and include spatial memory impairments, depression, anxiety, and impairments in fear-related behaviors, as seen in clinical populations (Petraglia et al., 2014; Wright et al., 2017). Moreover, animals subjected to repetitive brain injuries are at an increased risk of experiencing prolonged behavioral impairments post-injury (Petraglia et al., 2014). Affective behavioral impairments are also understudied in preclinical models of mild TBI, which often highlight the presence of these disorders without uncovering their mechanistic basis or examining potential therapies. Due to these issues, there are numerous gaps in the field of post-traumatic depression, anxiety, and fear, and these gaps will be explored in upcoming sections of the review.

Male and female brain-injured animals demonstrate impairment in both cognitive and affective behaviors, but male animals typically exhibit exacerbated cognitive impairments and female brain-injured animals show exacerbated affective disorders (Wright et al., 2017). TBI disrupts the female estrous cycle, causing imbalances in estrogen and progesterone levels and makes female animals more vulnerable to the negative effects of stressors (Wright et al., 2017; Fortress et al., 2019). Preclinical data investigating the effects of the estrous cycle on injury-induced behavioral outcomes are more extensive and conclusive than the current clinical data. Despite this knowledge on sex differences in behavioral outcomes, most preclinical models of mild TBI solely utilize male animals. However, although sex differences in structural and behavioral outcome of mild TBI are historically understudied, they have been gaining more attention in recent years.

Pathology

Structural changes observed in animals following mild TBI closely reflect those seen in humans exhibiting minimal damage, mostly present as traumatic axonal injury in white matter tracts (Mierzwa et al., 2015; Kikinis et al., 2017; Hoogenboom et al., 2019). Imaging studies demonstrate decreased mean diffusivity in the genu of the corpus callosum and increased fractional anisotropy in white matter tracts in injured animals one-week post-injury (Kikinis et al., 2017; Hoogenboom et al., 2019) as well as decreased axial diffusivity in the corpus callosum 2 weeks-post repetitive mild TBI (Wright et al., 2017). Diffusion tensor imaging showed decreased expression of myelin basic protein in the corpus callosum and high fractional anisotropy in white matter tracts immediately after injury which are normalized in the months following injury in most cases (Herrera et al., 2017). MRI in a mouse model of repetitive mild TBI demonstrated microgliosis in white matter tracts acutely post-injury (Robinson et al., 2017). Sex differences in pathology following mild TBI are largely understudied. However, one study demonstrated that following single and repetitive mild TBI, male animals showed increased astrocyte reactivity in the corpus callosum only after mild TBI, whereas female animals showed increased reactivity only following repetitive mild TBI (Wright et al., 2017). The

results of this study would suggest that potential sex differences in pathology should be further investigated.

Preclinical studies have also investigated structural deficits in regions such as the hippocampus and amygdala (**Table 3**). Single and repetitive closed head injury in young adult rats found significant changes in fractional anisotropy in the central amygdala (CeA, Kulkarni et al., 2019). These changes were seen at 7-8 weeks post-injury and were associated with impairments in affective behavior regulation. This study also demonstrated that repetitive mild TBI showed greater amygdala alterations than single injury; however, both injury models exhibited impairments compared to controls (Kulkarni et al., 2019). Moderate TBI in adult male rats resulted in dendritic hypertrophy in the basolateral (BLA) amygdala at 7- and 28-days post-injury, demonstrating the long-term structural alterations of the amygdala (Hoffman et al., 2017). Increased dendritic spine density in the amygdala, as well as increased dendritic branching in dendrites further from the soma regions have been found in a mouse model of mild blast TBI. This may be indicative of increased communication within amygdala circuitry following blast injury (Ratliff et al., 2019). Repeated mild blast TBI has shown alterations in the transcriptome within the BLA and CeA subregions which were associated with anxiety-like behaviors (Blaze et al., 2020), and single blast injury has led to a decrease in the number of BLA pyramidal neurons (Heldt et al., 2014). An impairment in inhibitory transmission in the BLA following mild TBI as indicated by a decrease in GABAergic neurons has also been reported (Almeida-Suhett et al., 2014) as well as decreased network excitability in the amygdala (Palmer et al., 2016). Repeated mild TBI was associated with decreased microglia in the hippocampus and BLA of brain-injured animals (Cheng et al., 2019) and hypoconnectivity in the hippocampus (Kulkarni et al., 2019). These studies highlight the need to further

TABLE 3 | Structural (using histology and/or imaging) and behavioral (cognition, anxiety, depression, fear) alterations in animal models of mild TBI.

Study description	Structural alterations		Behavioral alterations	
	Amygdala	**Other regions**	**Cognitive**	**Emotional**
Single or repeated mild TBI in adult male rats (Kulkarni et al., 2019)	Central amygdala altered diffusivity following single and repeated impact using diffusion weighted imaging	Changes in diffusivity in the caudate putamen, white matter, basal ganglia, brainstem, cerebellum. Hypoconnectivity in the hippocampus, midbrain dopamine system, hyperconnectivity in olfactory system	Novel Object Recognition and Barnes maze test to demonstrate deficits in working memory and spatial memory	DID NOT TEST
Midline fluid percussion brain injury in adult male rats (Hoffman et al., 2017)	Dendritic hypertrophy and activated astrocytes in the basolateral amygdala	Neurodegeneration and axonal injury in the cortex, hippocampus, thalamus and corpus callosum	DID NOT TEST	DID NOT TEST
Mild blast TBI in adult male mice (Ratliff et al., 2019)	No difference in dendritic length but increased spine density and branching at distal dendrites of amygdala neurons	ND	Deficits in spatial working memory using Novel Object Recognition Test	Anxiety-like behavior using the Staircase Test
Repeated mild blast TBI in adult male rats (Blaze et al., 2020)	Alterations in transcriptome within the BLA and CeA subregions in brain-injured rats	ND	No deficits in working memory using Novel Object Recognition	Anxiety-like and fear behaviors using the Light/Dark Box, Elevated Zero Maze, and Fear Conditioning tests
Repeated mild TBI in adult male wild-type and APP/PS1 mutant mice (Cheng et al., 2019)	Reduction in microglia within the BLA of brain-injured mutant mice	No difference in microglial reactivity between WT and mutant mice in the PFC, parietal cortex, corpus callosum, optic tract-white matter. Reduced microglial reactivity in hippocampus of mutant mice	Impaired spatial learning using the Barnes Maze	Risk-taking behaviors using the Elevated Plus Maze Impaired fear memory using the Passive Avoidance Test
Mild TBI in adolescent male rats (Almeida-Suhett et al., 2014)	Loss of GAD67-positive neurons in BLA of injured animals and impairment in inhibitory transmission in the BLA	ND	DID NOT TEST	Anxiety-like behaviors using the Open Field Test
Mild blast TBI in adult male C57BL/6 mice (Heldt et al., 2014)	No evidence of reactive glia in the BLA of brain-injured Neuronal loss in the BLA	No neuronal loss in cerebral cortex or striatum	DID NOT TEST	Anxiety using Open Field test Depressive behaviors using Tail Suspension Fear related behaviors using Acoustic Startle, Test, Prepulse Inhibition, Fear acquisition and extinction tests
Lateral fluid percussion brain injury in adult male C57BL/J6 mice (Palmer et al., 2016)	Decreased evoked EPSPs in the BLA Decreased network excitability in lateral amygdala	ND	DID NOT TEST	Impairments in fear acquisition using Cued Fear Conditioning

investigate both circuit-based dysfunction (e.g., alterations in the excitatory/inhibitory balance) and cellular alterations (neuronal damage, glial activation) within the various regions of the amygdala.

CURRENT TREATMENT STRATEGIES FOR POST-TRAUMATIC AFFECTIVE DISORDERS

Human Studies

Current treatment strategies for depression, anxiety and fear/PTSD in brain-injured patients utilize best practices that are in place for treating these disorders in patients that have not suffered a TBI (Plantier and Luauté, 2016; Gupta et al., 2019; Silverberg and Panenka, 2019). Thus, serotonin reuptake inhibitors (SSRIs) are the most utilized therapy for post-traumatic depression (Kraus et al., 2017; Silverberg and Panenka, 2019). However, a meta-analysis of clinical trials using SSRIs following concussion found that these drugs have no benefit for post-traumatic depression in some cases, but rather their effects stem from robust placebo effects (Silverberg and Panenka, 2019). Alternate therapeutic strategies include cognitive behavioral therapy (CBT) which helps modify negative thought patterns and has been used extensively in military veterans (Cooper et al., 2015; Fann et al., 2015; Ponsford et al., 2015). In addition to CBT, which is focused on information processing and memory, motivational interviewing has also been used on brain-injured military veterans suffering from PTSD and civilian populations with post-traumatic anxiety (Cooper et al., 2015). Melatonin, which can improve sleep quality following mild TBI, has been effective in attenuating anxiety in brain-injured individuals (Grima et al., 2018). Sedatives such as benzodiazepines are used to treat anxiety in individuals with mild TBI by working as positive allosteric modulators that increase the activity of the GABA-A receptor although the effects of benzodiazepines are transient and variable (Flower and Hellings, 2012; Plantier and Luauté, 2016).

Animal Studies

Therapies for affective disorders that are currently utilized on brain-injured patients have typically not been tested in clinically relevant animal models of TBI (Cryan et al., 2002). Rather, pharmacologic interventions aimed at reducing acute neurodegeneration after TBI use depression, anxiety, and fear behavior as outcome measures. For example, both hyperbaric oxygen therapy and lithium (which inhibits the activity of the apoptosis regulator glycogen synthase kinase-3) have been used in animal models of TBI to reduce depression-like symptoms (Shapira et al., 2007; Lim et al., 2017). Similarly, antagonists of either the glutamate receptor, glucocorticoid receptor, or the corticotropin releasing factor (CRF) receptor 1 ameliorated post-traumatic anxiety-like behaviors (Fox et al., 2016; Kosari-Nasab et al., 2019) or PTSD-like behaviors (Perez-Garcia et al., 2018). Together with the clinical data, the pre-clinical studies highlight the disconnect between the bench and the bedside when it comes to treating affective disorders in TBI patients.

AN INTEGRATED APPROACH TO POST-TRAUMATIC AFFECTIVE BEHAVIORS: THE ROLE OF THE AMYGDALA

The Significance of the Amygdala

Human and animal studies of mild TBI have demonstrated that affective behavioral disorders are frequent long-term consequences of injury but have insufficient treatment options. Therefore, this section will highlight the idea that deficits in affective behavior following TBI, and specifically mild TBI, may depend heavily on neuronal projections and neurochemicals originating within the amygdala (Han et al., 2015; Horn et al., 2016; Ratliff et al., 2019; Beitchman et al., 2020). The function, structure, and connectivity of the amygdala is disrupted following TBI suggesting that this region may be a source of behavioral impairment. For example, hyperactivity of the amygdala is suggested to be involved in the manifestation of affective disorders in brain-injured patients (Han et al., 2015). Clinical and preclinical neurobiological studies have supported the idea that the amygdala, a major component of the limbic system, may be a hub for regulating affective behaviors (Phelps and Ledoux, 2005; Etkin et al., 2010; Pessoa, 2010; Calhoon and Tye, 2015; Korgaonkar et al., 2019). The amygdala sends projections to multiple brain regions thereby involving it in the various circuits responsible for the expression of affective behaviors and is also home to a diverse array of neurotransmitters and neuropeptides which boosts its potential to mediate impairments in affective behaviors (Phelps and Ledoux, 2005; Etkin et al., 2010; Pessoa, 2010; Felix-Ortiz and Tye, 2014; Calhoon and Tye, 2015; McGarry and Carter, 2016; Paretkar and Dimitrov, 2018; Korgaonkar et al., 2019).

Anatomic Pathways: The Basolateral and Central Amygdala

The BLA and CeA are the two primary subregions of the amygdala widely known for their involvement in depression and fear and anxiety, respectively. Their projections to various brain regions aid in the regulation of these disorders, in that disruption of these pathways can lead to disorder expression (Kim and Jung, 2006; Calhoon and Tye, 2015; Leal et al., 2017). This section will examine how the manifestation of depression, anxiety, or fear can occur through divergent circuitry arising from the various subregions of the amygdala.

Disruptions in the BLA-derived circuitry drive the expression of both cognitive and emotional aspects of depression-like behaviors. Preclinical studies show that the BLA exhibits neuronal hypertrophy in response to TBI (Hoffman et al., 2017) and increased dendritic branching following mild blast TBI (Ratliff et al., 2019). Animal models of depression exhibit impaired processing through the expression of anhedonia, despair, and prefrontal and hippocampal-dependent cognitive

deficits (Paul et al., 2005; Der-Avakian and Markou, 2012). The BLA is also the main input nucleus receiving sensory information from the thalamus which is imbued with emotional value and associations are made between neural stimuli and outcomes of positive or negative valence (Janak and Tye, 2015). Cognitive components of depression are driven by the BLA coordinating emotional learning and memory via projections to the prefrontal cortex and the ventral hippocampus (McGarry and Carter, 2016; Leal et al., 2017). Disruptions in these pathways in humans lead to impaired emotional memory, creating a bias toward negative thoughts (Leal et al., 2017). Activation of BLA neurons following mild TBI in animals potentiates glutamatergic targets leading to excitotoxicity in other brain regions including the hippocampus (Reger et al., 2012). The BLA also projects to the nucleus accumbens shell, where it inhibits dopamine release leading to low motivational states (McGarry and Carter, 2016). A decrease in dopamine release and reuptake in the nucleus accumbens has been reported in the chronic period following TBI in animals (Chen et al., 2017) which may underlie decreased motivation that leads to a depressive phenotype. Moreover, activation of the BLA during REM sleep, which is reduced in brain-injured patients, is critical for sleep-dependent emotional processing (Mcgaugh, 2004; Mantua et al., 2017; Clark et al., 2020). When this processing is impaired, there is an increase in arousal to negative words and experiences seen in patients (Liu et al., 2012). Collectively, these data provide a basis for the BLA, via its connections to the prefrontal cortex, hippocampus, and nucleus accumbens to regulate the expression of post-traumatic depression.

The BLA also aids in recruiting CeA neurons in response to threatening cues (Calhoon and Tye, 2015). Brain imaging studies in rodents found significant alterations in the CeA following single and repetitive mild TBI which corresponded to impairments in affective behavior (Kulkarni et al., 2019). The coordinated activity of the CeA, the bed nucleus of the stria terminalis (BNST), the ventral hippocampus, and the prefrontal cortex is required for the interpretation of stimuli as threatening to produce an anxiety-like response. Threatening cues induce anxiogenic behavior because of increased activation of the CeA and the BNST, whereas hyperexcitation within the CeA promotes arousal and hypervigilance and leads to the association of non-threatening factors with potential danger (Calhoon and Tye, 2015). Information regarding potential threats flows forward from the CeA to the BNST, then to the hippocampus and prefrontal cortex, and back from the prefrontal cortex and the hippocampus to the CeA (Calhoon and Tye, 2015); dysfunction in the hippocampus and the prefrontal cortex also contributes to anxiety-like behaviors (Cominski et al., 2014). Fear and anxiety-provoking circuits share numerous similarities, and contextual fear conditioning requires strong input from the hippocampus to relate contextual cues to an aversive stimulus (Kim and Jung, 2006; Calhoon and Tye, 2015). Moreover, medial septum cholinergic inputs into the hippocampus are needed for appropriate processing of contextual cues as background information (Calhoon and Tye, 2015; Staib et al., 2018). The CeA also projects to the paraventricular nucleus of the hypothalamus (Beaulieu et al., 1986) and hyperactivity in the CeA leads to disruption of the hypothalamic-pituitary-adrenal (HPA) axis and anxiety-like behaviors and alterations in the natural fear response (Flandreau et al., 2012; Calhoon and Tye, 2015). In animals, TBI induces disruption in HPA circuitry resulting in compromised emotional regulation and, specifically, anxiety (Tapp et al., 2019). These data suggest that the CeA may be a likely nucleus mediating post-traumatic anxiety and fear behaviors.

Neurochemical Pathways: γ-Amino Butyric Acid and CRF

Whereas different subregions of the amygdala may be more responsible for the expression of one affective disorder over the other, neurochemical transmission within the amygdala may contribute to the comorbid expression of depression, anxiety, and fear-related behaviors. These neurochemicals consist of the primary excitatory neurotransmitter glutamate, the primary inhibitory neurotransmitter γ-amino butyric acid (GABA), and CRF, the peptide hormone largely known to be involved in the stress response. Alterations in the concentration or activity of these neurochemicals have been linked to the manifestation of all three affective behavioral disorders, suggesting that they serve as points of integration of mechanisms underlying the convergence of depression, anxiety and fear.

Disruptions in the concentrations of GABA and glutamate are associated with deficits in affective behaviors (Calhoon and Tye, 2015; Jie et al., 2018). Activation of GABAergic neurons within the amygdala prevents the propagation of excitatory input to downstream regions and keeps anxiety responses in check (Calhoon and Tye, 2015). Either due to reduced GABA transmission or increased activity within the BLA following mild TBI, potentiation of glutamatergic activity may be associated with fear-related behaviors (Reger et al., 2012; McGuire et al., 2018). Anxiety-like behaviors following TBI in animals has been associated with decreased GAD expression in the BLA (Popovitz et al., 2019) or decreased evoked glutamate release and slower glutamate clearance within the CeA (Beitchman et al., 2020). Depression also manifests through imbalances in glutamate and GABA concentrations (Luscher et al., 2011; Luscher and Fuchs, 2015; Duman et al., 2019). For example, a postmortem study showed decreased levels of GABA expression in the BLA of patients with major depression disorder (Douillard-Guilloux et al., 2017). Thus, overall, reductions in GABA levels and excitotoxicity of glutamate, specifically within the amygdala, are major contributors to all three affective disorders, serving as a potential point of mechanistic convergence.

The expression of CRF is increased in the amygdala following brain injury (Narla et al., 2019; Tapp et al., 2019) and blockade of CRFR1 receptor 1 within the HPA axis attenuates post-traumatic stress and anxiety-like behaviors (Kosari-Nasab et al., 2019). The activity of CRF in extrahypothalamic regions contributes to depression, anxiety, and fear-related behaviors (Binder and Nemeroff, 2009; Sanford et al., 2017; Paretkar and Dimitrov, 2018). The CeA contains CRF-positive neurons (Rodaros et al., 2007), whereas the BLA contains numerous CRF receptor-positive projection neurons (Roozendaal et al., 2002). Increased CRF in the BLA can impair memory consolidation

(Narla et al., 2019), whereas increased CRF in the nucleus accumbens leads to depressive behaviors likely by modulating extracellular acetylcholine (Chen et al., 2012); CRF receptor 1 antagonists exhibit antidepressant activity (Overstreet and Griebel, 2004). Neurons within the CeA that express CRF have been investigated for their role in perpetuating anxiety behaviors following chronic stress (Paretkar and Dimitrov, 2018; Hupalo et al., 2019) and promote fear learning by regulating acquisition recall (Sanford et al., 2017). Norepinephrine can promote GABA release from neurons thereby enhancing GABA-mediated inhibition of CRF neuronal activity (Levy and Tasker, 2012); norepinephrine is reduced following TBI suggesting that post-traumatic affective behaviors may be linked to CRF activity (McGuire et al., 2018). Together, these data suggest that hyperactivity of CRF neurons within the amygdala following injury may be a viable mechanistic underpinning of all three affective disorders.

Integration of Amygdala Structure and Function

In psychology, emotional processing describes the way in which an individual successfully responds to emotional stimuli or stressful events, and impairment in processing can lead to signs of depression, anxiety, and fear (Rachman, 1980) which have a high incidence of comorbidity in a subset of brain-injured patients (Jorge et al., 2004; Ellis et al., 2015; Mohammad Farris Iman Leong Bin Abdullah et al., 2018; Teymoori et al., 2020). The amygdala is well-known for its involvement in emotional processing based on observations of structural and functional alterations in patients that develop affective disorders such as depression and fear (Belujon and Grace, 2011; Douillard-Guilloux et al., 2017). Thus, a decrease in the number of somatostatin-labeled neurons within the BLA of the amygdala was observed in patients with major depression (Douillard-Guilloux et al., 2017). Additionally, in patients with generalized anxiety disorder, functional MRI during an emotional conflict task revealed greater activation in the amygdala (Etkin et al., 2010). Similarly, patients with PTSD exhibited an increase in amygdala activation detected using either positron emission tomography (Bremner et al., 2005) or blood oxygen level dependent functional MRI (Protopopescu et al., 2005), particularly when exposed to negative stimuli. The subregions of the amygdala, specifically the BLA and the CeA, may be the points of divergence in that impairment in each subregion is associated with a specific deficit. Thus, disruption in the BLA can result in the expression of depressive-like behaviors (Douillard-Guilloux et al., 2017), whereas impairment to the CeA can result in anxiety or fear-related behaviors (Calhoon and Tye, 2015). Because it is structurally and functionally impaired following mild TBI, the amygdala has been highlighted as critical to the development of post-traumatic deficits in affective behaviors. The true integration of these disorders lies in the connectivity between, and the neurochemical interactions within these two subregions of the amygdala. Therefore, impairment in the circuitry of BLA and CeA as well as disruptions in GABA or CRF concentrations post-injury have the potential to lead to comorbid expression of depression, anxiety, and fear-related behaviors. In these instances, the behavioral consequence exhibited would be one of a collective impairment in emotional processing.

FUTURE DIRECTIONS

Mild TBI is a public health concern that greatly impacts the lives of adolescents who suffer from sports-related concussion and young adults who experience mild blast TBI. A primary consequence of mild TBI is the development of long-term deficits in affective behaviors which have been reported in both humans and animals. It is important to note that the comorbidity of depression, anxiety, and fear-related behaviors are only observed in a subset of patients, and the appearance of these behaviors in preclinical models depends on the impact site and frequency of the repetitive injury. These behaviors have been largely understudied and there are few treatments in use currently that attenuate post-traumatic depression, anxiety, and fear/PTSD. Future clinical and preclinical studies need to examine the impairment in amygdala circuitry and neurotransmission following mild TBI. One of the major structural impairments following mild TBI is axonal injury and brain imaging must examine which projections to and from the different subregions of the amygdala are disrupted. In addition, alterations in the expression and activity of specific neurotransmitters must also be evaluated for the behavioral deficit being studied. Treatment paradigms therefore need to examine structural and functional alterations in the amygdala concomitantly with disruption in specific neurotransmitters facilitating an integrated approach, potentially ameliorating depression, anxiety, and fear-related behaviors together.

AUTHOR CONTRIBUTIONS

TM, JB, and RR wrote and edited the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported, in part, by grants from the National Institutes of Health [R01 NS110898 (RR) and AA028218 (JB)] and Commonwealth Universal Research Enhancement from the Pennsylvania Department of Health [SAP 410-007-9710 (JB, RR) and SAP 410-007-7079 (RR)].

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Behavioral Neuroscience

ORIGINAL RESEARCH
published: 26 March 2021
doi: 10.3389/fnbeh.2021.621751

The Role of Parvalbumin Interneuron GIRK Signaling in the Regulation of Affect and Cognition in Male and Female Mice

*Eden M. Anderson, Skyler Demis, Hunter D'Acquisto, Annabel Engelhardt and Matthew Hearing**

Department of Biomedical Sciences, Marquette University, Milwaukee, WI, United States

OPEN ACCESS

Edited by:
Nicholas Michael Graziane, Pennsylvania State University, United States

Reviewed by:
Fereshteh S. Nugent, Uniformed Services University, United States
Yaoying Ma, Binghamton University, United States

***Correspondence:**
Matthew Hearing
matthew.hearing@marquette.edu

Specialty section:
This article was submitted to Emotion Regulation and Processing, a section of the journal Frontiers in Behavioral Neuroscience

Received: *26 October 2020*
Accepted: *19 January 2021*
Published: *26 March 2021*

Citation:
Anderson EM, Demis S, D'Acquisto H, Engelhardt A and Hearing M (2021) The Role of Parvalbumin Interneuron GIRK Signaling in the Regulation of Affect and Cognition in Male and Female Mice. Front. Behav. Neurosci. 15:621751. doi: 10.3389/fnbeh.2021.621751

Pathological impairments in the regulation of affect (i.e., emotion) and flexible decision-making are commonly observed across numerous neuropsychiatric disorders and are thought to reflect dysfunction of cortical and subcortical circuits that arise in part from imbalances in excitation and inhibition within these structures. Disruptions in GABA transmission, in particular, that from parvalbumin-expressing interneurons (PVI), has been highlighted as a likely mechanism by which this imbalance arises, as they regulate excitation and synchronization of principle output neurons. G protein-gated inwardly rectifying potassium ion (GIRK/Kir3) channels are known to modulate excitability and output of pyramidal neurons in areas like the medial prefrontal cortex and hippocampus; however, the role GIRK plays in PVI excitability and behavior is unknown. Male and female mice lacking GIRK1 in PVI (Girk1$^{flox/flox}$:PVcre) and expressing td-tomato in PVI (Girk1$^{flox/flox}$:PV^{Cre}:PVtdtom) exhibited increased open arm time in the elevated plus-maze, while males showed an increase in immobile episodes during the forced swim test (FST). Loss of GIRK1 did not alter motivated behavior for an appetitive reward or impair overall performance in an operant-based attention set-shifting model of cognitive flexibility; however it did alter types of errors committed during the visual cue test. Unexpectedly, baseline sex differences were also identified in these tasks, with females exhibiting overall poorer performance compared to males and distinct types of errors, highlighting potential differences in task-related problem-solving. Interestingly, reductions in PVI GIRK signaling did not correspond to changes in membrane excitability but did increase action potential (AP) firing at higher current injections in PVI of males, but not females. This is the first investigation on the role that PVI GIRK-signaling has on membrane excitability, AP firing, and their role on affect and cognition together increasing the understanding of PVI cellular mechanisms and function.

Keywords: G protein-gated inwardly-rectifying K$^+$, GIRK, parvalbumin, prefrontal cortex cognition, affect

INTRODUCTION

Cognitive flexibility is the ability to adapt behavior in response to changing environmental contingencies and is a critical component of everyday life. As such, impairments in flexibility increase susceptibility to negative life events (e.g., stress), reduce emotional control, and promote the development of maladaptive behaviors that can disrupt the capacity of individuals to engage in their lives effectively (Lange et al., 2017; Waltz, 2017; Gabrys et al., 2018). Neuropsychiatric disorders such as major depressive disorder (MDD), obsessive-compulsive disorder (OCD), autism, and schizophrenia share common symptomology including cognitive inflexibility, reduced inhibitory control, and impaired working memory (Marazziti et al., 2010; Moghaddam and Javitt, 2012; Diamond, 2013; Etkin et al., 2013; Remijnse et al., 2013; Dajani and Uddin, 2015); however what contributes to these impairments is not well understood.

Optimal regulation of affect (i.e., emotion) and flexible decision-making require a balance of excitation and inhibition (E/I) in numerous cortical and subcortical circuits including the medial prefrontal cortex (mPFC; Pantazopoulos et al., 2006; Kehrer et al., 2008; Yizhar et al., 2011; Gandal et al., 2012a; Murray et al., 2015). Pathological alterations in GABA transmission, in particular, that of parvalbumin-expressing interneurons (PVI), has been highlighted as a likely mechanism by which E/I imbalances and associated symptomology arises (Cardin et al., 2009; Sohal et al., 2009; Gandal et al., 2012b; Murray et al., 2015; Wöhr et al., 2015; Kim et al., 2016a; Ferguson and Gao, 2018). Analysis of postmortem human brain tissues revealed decreased expression of parvalbumin and parvalbumin mRNA in patients affected by schizophrenia or autism (Hashimoto et al., 2003; Curley and Lewis, 2012; Lewis, 2014; Hashemi et al., 2017). Similarly, loss of parvalbumin in rodents promotes autism- (Wöhr et al., 2015) and depression-like symptoms (Fogaça and Duman, 2019), together suggesting a role for parvalbumin and PVI in related neuropsychiatric disorder symptomatology.

Within cortical regions such as the mPFC, PVIs are powerful coordinators of network activity (Markram et al., 2004; Klausberger and Somogyi, 2008; Rudy et al., 2011; Kepecs and Fishell, 2014), with fast-spiking PVIs comprising approximately 50% of cortical interneurons (Kawaguchi and Kubota, 1997). PVIs target the soma and perisomatic compartments of principle output pyramidal neurons where they regulate excitation and synchronize firing (Celio and Heizmann, 1981; Kubota and Kawaguchi, 1994; Atallah et al., 2012; Kvitsiani et al., 2013; Hu et al., 2014; Ferguson and Gao, 2018) to orchestrate cortical information flow (Sohal et al., 2009; Murray et al., 2015; Kim et al., 2016a). In the mPFC, PVIs are highly recruited by afferent excitatory signaling; however intrinsic cellular properties (e.g., membrane excitability) dictate how cells respond to this excitatory drive.

G protein-gated inwardly rectifying potassium ion (GIRK/Kir3) channels produce a slow hyperpolarizing current which modulates neuron excitability and spike firing (Hearing et al., 2013; Marron Fernandez de Velasco et al., 2015; Nimitvilai et al., 2017) acting through inhibitory G protein-coupled receptors including $GABA_BR$ (Glaaser and Slesinger, 2015; Luján and Aguado, 2015). The role of mPFC and forebrain GIRK channels on pyramidal neuron excitability and behavioral outcomes has been established (Hearing et al., 2013; Victoria et al., 2016); however, while GIRKs are known to reside in PVIs and likely contribute to $GABA_BR$-mediated signaling in the hippocampus (Booker et al., 2013), their role on mPFC PVI excitability and output is not known. Increasing evidence suggests that drugs that target G protein inhibitory signaling may serve as clinically relevant therapeutic strategies to treat both cognitive and affect-related symptoms (Mombereau et al., 2004; Slattery et al., 2005; Gandal et al., 2012a; Kumar et al., 2013; Lecca et al., 2016). Thus, identifying key modulators of this signaling and how it varies across cell-types remains an important step. Similarly, while PVIs have become a target of recent therapies, there is a need to better understand the cellular mechanisms that regulate their function before progress can be made in this regard (Hu et al., 2014). Accordingly, this study focuses on inhibitory signaling mediated by PVI GIRK channels by determining the role this signaling has on the output of PVI in the prelimbic cortex and its relevance to prefrontal cortex-dependent regulation of affect and cognitive flexibility in both male and female mice.

MATERIALS AND METHODS

Animals

Girk1$^{flox/flox}$ stock mice were generated as described (Signorini et al., 1997; Kotecki et al., 2015), and donated by Dr. Kevin Wickman (University of Minnesota). Male *Girk1*$^{flox/flox}$ mice were bred with female mice purchased from Jackson Laboratories expressing cre recombinase in parvalbumin-expressing neurons (B6.129P2-Pvalbtm$^{1(cre)Arbr}$/J; Stock No:017320) then backcrossed to create *Girk1*$^{flox/flox}$. Female *Girk1*$^{flox/flox}$ mice positive for PVcre were then bred with male mice purchased from Jackson Laboratories expressing tdtomato in parvalbumin-positive neurons (C57BL/6-Tg(Pvalb-tdtomato)15Gfng/J; Stock No: 027395) and backcrossed to generate experimental *Girk1*$^{flox/flox}$ mice hemizygous for PVcre and PVtdtomato. For all experiments, *Girk1*$^{flox/flox}$ without cre recombinase present were used as controls whereas *Girk1*$^{flox/flox}$ with cre recombinase was used as the experimental group. For behavioral experiments, a subset of control and experimental mice also expressed tdtomato in parvalbumin-positive neurons. For electrophysiology experiments, mice also expressed tdtomato in parvalbumin-positive neurons. Mice were housed in a temperature and humidity-controlled room with a 12/12 h light/dark cycle with food and water available *ad libitum* except throughout attention set-shifting and the progressive ratio test. Male and female experimental mice were PD78 $\pm$ 2 days at the start of visual cue testing. Behavioral procedures were conducted in the light phase. Experiments were approved by the Institutional Animal Care and Use Committee at Marquette University.

Behavioral Testing Timeline

Mice were handled for 3 days then tested in the elevated plus-maze (EPM), followed by attention set-shifting training and testing, progressive ratio, and forced swim test (FST; **Figure 1A**). The battery of behavioral tests was conducted in this order to reduce potential effects of stress on later behavioral tests. EPM was run first, followed by attention set-shifting and progressive ratio during which mice were food-deprived which has been used as a stressor during chronic unpredictable stress protocols and has been shown to elevate corticosterone levels in rats (for reviews see Carr, 2002; Antoniuk et al., 2019). To food-deprive, mice were also single housed which has been shown to elicit endocrine changes (for review see Mumtaz et al., 2018) and early life isolation has been shown to influence EPM behavior in rats (Wright et al., 1991). FST was assessed last because inescapable swim stress has been used as a physical and psychological stressor to elicit a coping response (for review see Molendijk and de Kloet, 2015; de Kloet and Molendijk, 2016). Despite efforts to run behavioral tests from least to most stressful, it should be noted that carryover effects from one behavioral test to the next cannot be excluded. A subset of mice were then used for slice electrophysiology; however, not all mice used for slice electrophysiology underwent behavioral assessments.

Elevated Plus Maze

Mice were tested for anxiety-like behaviors using the EPM as previously described (Anderson et al., 2019) under low light conditions (50 lux at the center of the maze). AnyMaze (Stoelting Company) tracking software was used to record and analyze behavior. Percent time in the open arms was calculated as total time in the open arms divided by total time in the maze.

Attention Set-shifting

Attention set-shifting training and tests were conducted in operant conditioning chambers (Med Associates, Inc.) and were modified and based on methods previously described (Brady and Floresco, 2015). Following EPM, mice were food-deprived to 85–90% of their free-feeding weight. For all attention set-shifting training and testing, correct responses were rewarded with the presentation of 50% liquid Ensure® diluted in tap water. During *food training*, a fixed ratio 1 schedule was used, whereby a response on either the left or the right lever resulted in the delivery of a reward. The initial food training session was 3 h in length and repeated daily until the mouse earned at least 50 rewards. The next day, mice had 30 min to receive at least 50 rewards and this training session was also repeated daily until the mice did so.

During *lever training*, retractable levers were pseudorandomly presented with no more than two consecutive extensions of each lever. Mice were required to press the lever within 10 s of lever extension to receive a reward; the absence of a lever press was deemed as an omission. Each lever training session consisted of 90 trials (45 of each the left and right lever), with each trial followed by a 20 s time out. Mice were required to reach criterion of $\leq$5 omissions total on two consecutive days to move forward. Once the lever training criterion was reached, *lever bias* was assessed during which both the right and left lever were presented and reinforced on a fixed ratio of one for a total of seven trials. If a mouse did not have $\leq$5 omissions for two consecutive days within 15 days of lever training, training was ceased and the mouse did not progress through testing ($N = 12$).

The following day, *visual cue testing* was conducted until 150 trials or 10 consecutive correct responses were reached with a minimum of at least 30 trials. If the criterion was not reached on the first day of testing, a second or third day of testing was conducted. During visual cue testing, an illuminated cue light was presented above either the left or right lever in a pseudorandomized order. A response on the lever underneath the illuminated cue light resulted in reward delivery and a 20 s time out; incorrect responses resulted in only the time out. Omissions were counted as stated above and were not counted towards a trial to criterion (i.e., neither correct nor error) when data was analyzed but counted towards the daily 150 trials. The next day, *extradimensional shift testing* was conducted, during which the reinforced lever was always the opposite lever of the previously assessed lever bias. The cue light was presented in a manner/order similar to that during the visual cue test; however, it was not associated with the correct response. Mice were run in the extradimensional shift test until 10 consecutive responses or 150 trials were conducted with a minimum of 30 trials. The day after the extradimensional shift test criterion was reached (i.e., 10 consecutive correct responses), reversal testing was conducted during which the reinforced lever was always that of the previously assessed lever bias; however, the cue light was presented identically to that used during the visual cue test. For set-shifting experiments, all tests for a mouse were excluded if >15 omissions in a given test were recorded as this may reflect mechanical issues, motoric, or motivational issues and may influence responding on subsequent tests.

For further behavioral assessment, the types of errors were analyzed based on previously published methods (Brady and Floresco, 2015). Tests were divided into blocks of 16 trials, not including trials with omitted responses. For visual cue testing, *initial errors* were errors that were made within each block until there were less than six in a single block. Once there were less than six errors in a single block, errors in all subsequent blocks were characterized as *regressive errors*. For the extradimensional set shift test, tests were divided into bins of 16 trials. Errors that were made based on the previous visual cue test rule used, such that the response was made on the lever under the illuminated cue light, were considered *perseverative* until less than six errors in a single bin were made. Errors in the next bin and subsequent bins were considered regressive errors. *Never reinforced errors* were those that were incorrect but the response was not on the lever underneath the illuminated cue light. For the reversal test, perseverative and regressive errors were assessed as described above; however, errors were considered perseverative until less than 10 errors in a bin were made. Separately, errors were also divided into errors that were made towards the cue light distractor and away from the cue light distractor. Response latencies were measured as the amount of time from the extension of the lever until a response was made.

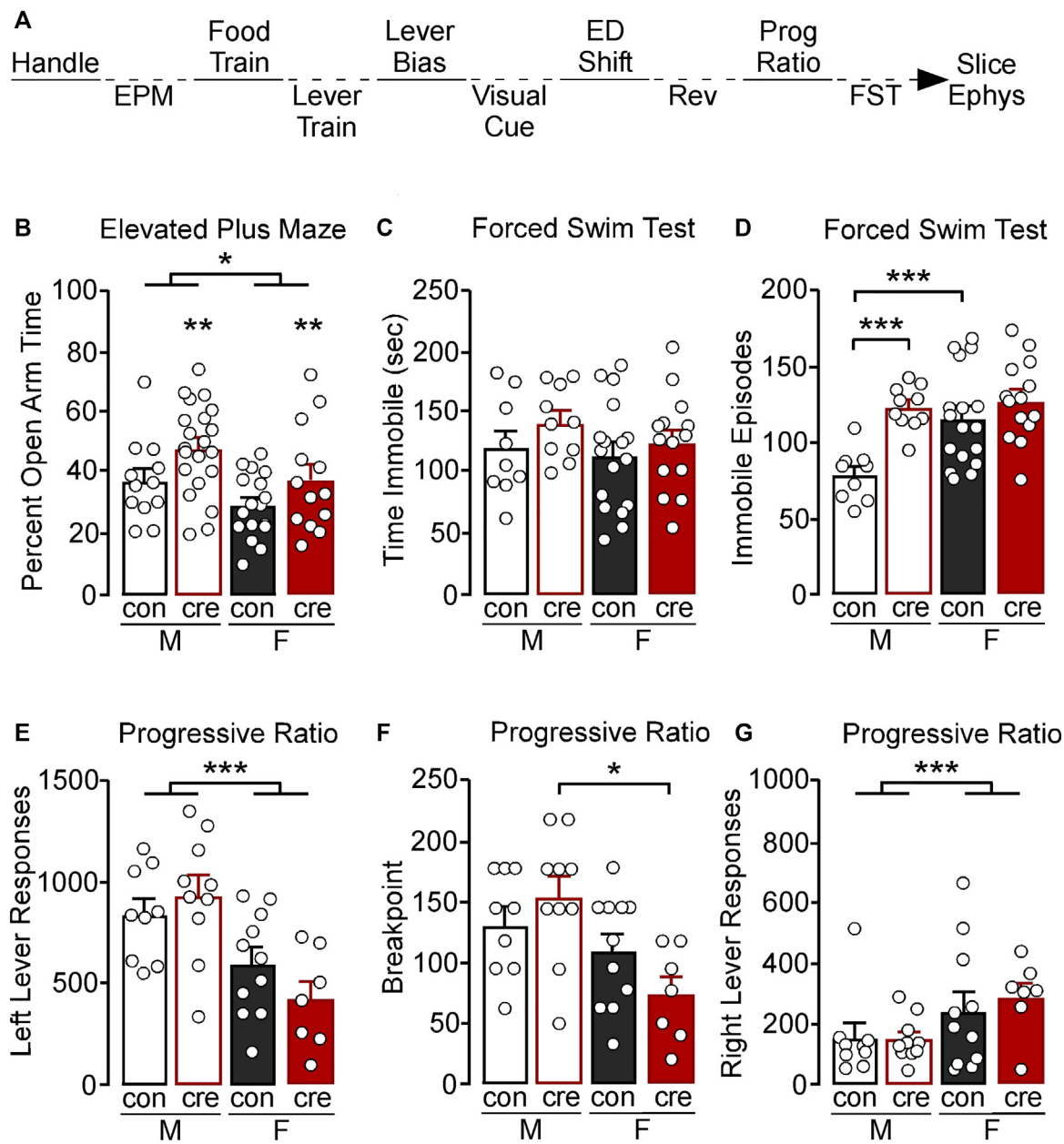

FIGURE 1 | (A) Experimental timeline. Mice were handled for at least 3 days then tested in the elevated plus-maze (EPM), food and lever trained, followed by an assessment for a lever bias. Mice were then tested in a visual cue test, an extradimensional shift (ED shift), and a reversal (REV) test, followed by an assessment of motivation in a progressive ratio (Prog Ratio) test. Mice were then tested in the forced swim test (FST). Whole-cell slice electrophysiology was conducted in a subset of mice; however not all mice that had slice electrophysiology underwent behavior. **(B)** Males spent a greater percentage of time in the open arm compared to females (*$p < 0.05$). Cre-positive mice had a significant increase in percent open arm time (**$p < 0.01$). **(C)** Male and female control and cre-positive mice had a similar amount of immobile time in the FST. **(D)** Female control mice had a greater number of immobile episodes compared to male control mice (***$p < 0.001$). Male cre-positive mice had a greater number of immobile episodes compared to control males (***$p < 0.001$). **(E)** During the progressive ratio, males had a greater number of left lever active presses compared to females (***$p < 0.001$). **(F)** Female cre-positive mice had a significantly lower breakpoint compared to male cre-positive mice (*$p < 0.05$). **(G)** Female mice had a greater number of right lever inactive presses compared to males, regardless of condition (***$p < 0.001$).

Progressive Ratio

Following attention set-shifting, mice were then tested for motivation using liquid Ensure® as the reinforcer. Responses on the left lever were reinforced whereas responses on the right lever were inactive and resulted in no consequences. Responses required to obtain each subsequent reward progressively increased. The schedule of reinforcement was $(5e^{0.2*n})$-5 (Richardson and Roberts, 1996) and testing lasted for a total of

90 min. Following progressive ratio testing, food was returned *ad libitum*.

Forced Swim Test

A transparent glass beaker 7″ in diameter was filled with 25 ± 2°C to a depth that prevented the mouse from touching the bottom. Mice were individually placed in the water and habituated for 2 min. Behavioral assessment was recorded during the subsequent 4 min during which immobile time and number of episodes were tracked. Following testing, mice were immediately dried and kept in a warm holding cage. Behaviors were recorded using a side-mounted camera and assessed using AnyMaze tracking software. Immobility sensitivity was set at 85% and a minimum of 250 ms to be counted as an immobility episode.

Slice Electrophysiology

Mice were anesthetized with isoflurane (Henry Schein), decapitated, and the brain removed and put in ice-cold 95% O_2 5% CO_2 oxygenated sucrose solution (229 mM sucrose, 1.9 mM KCl, 1.2 mM NaH_2PO_4, 33 mM $NaHCO_3$, 10 mM glucose, 0.4 mM ascorbic acid, 6 mM $MgCl_2$, and 0.5 mM $CaCl_2$). Coronal slices (300 μm) containing the mPFC were collected using a Leica VT1000S vibratome. Slices were immediately incubated at 31°C for 10 min in a solution containing 119 mM NaCl, 2.5 mM KCl, 1 mM NaH_2PO_4, 26.2mM $NaHCO_3$, 11 mM glucose, 0.4 mM ascorbic acid, 4 mM $MgCl_2$, and 1 mM $CaCl_2$. Slices were then removed, allowed to cool to room temperature, and incubated further for a minimum of 35 min.

Whole-cell recordings were performed as previously described (Hearing et al., 2013; Anderson et al., 2019). Oxygenated ACSF (125 mM NaCl, 2.5 mM KCl, 25 mM $NaHCO_3$, 10 mM glucose, 0.4 mM ascorbic acid, 1.3 mM $MgCl_2$, and 2 mM $CaCl_2$) was gravity perfused at a temperature of 29–33°C at a flow rate of ∼1.5–2.5 ml/min. Sutter Integrated Patch Amplifier (IPA) with Igor Pro (Wave Metrics, Inc.) was used for the data acquisition software. Recordings were filtered at 2 kHz and sampled at 5 kHz. Layer 5/6 PVI were identified based on the presence of td-tomato fluorescence. For all recordings, adequate whole-cell access ($Ra < 40$ MΩ) was maintained. Borosilicate glass pipettes were filled with 140 mM K-Gluconate, 5.0 mM HEPES, 1.1 mM EGTA, 2.0 mM $MgCl_2$, 2.0 mM Na_2-ATP, 0.3 mM Na-GTP, and 5.0 mM phosphocreatine (pH 7.3, 290 mOsm). For ML297 recordings, a baseline with <20% fluctuation current was obtained, followed by bath application of 10 μM ML297 (David Weaver, Vanderbilt) in 0.04% DMSO (Sigma–Aldrich). Evoked currents were reversed using bath application of 0.30 mM barium chloride (Thermo Fisher Scientific). For rheobase and action potential (AP) frequency, a 20 pA current-step injection was used (0–300 pA, 1 s current injections). Capacitance, membrane resistance, and resting membrane potential (RMP) were taken as a simultaneous value after obtaining whole-cell access. AP duration was measured as the time to reach half the amplitude for the first action potential. Afterhyperpolarization (AHP) amplitude was measured from the spike threshold equipotential point to the maximum amplitude of the first action potential hyperpolarization. Sample sizes are denoted as *n* for the number of recordings/cells and *N* for the number of mice.

Statistical Analysis

Data are presented as mean ± SEM. SigmaPlot 11.0 was used to perform statistical analyses. A 2 (male, female) × 2 (control, cre) analysis of variance was used for all comparisons except assessment of progressive ratio breakpoint which violates parametric assumptions, and therefore a Kruskal–Wallis test was used. The Student–Newman–Keuls method for multiple *post-hoc* comparisons was used when applicable. Statistical outliers (±2 SD) from behavioral tests were excluded from analyses (two data points from EPM; five total mice from all attention set-shift tests; one data point from progressive ratio; two action potentials from action potential and afterhyperpolarization analyses). For electrophysiology data, cell-based and animal-based analyses were conducted during which each recording was a single data point or recordings from each animal were averaged and counted as a single data point, respectively.

RESULTS

Effects of GIRK1 Knockout in PVI on EPM, Forced Swim Test, and Motivation

Constitutive knockout of GIRK signaling, including channels expressing the GIRK1 subunit, has been shown to alter anxiety- and depression-like behavior, learning, and memory, as well as motivation for appetitive rewards (Pravetoni and Wickman, 2008; Wydeven et al., 2014; Llamosas et al., 2015; Victoria et al., 2016). However, recent work has shown that cell-type-specific ablation of GIRK signaling has unique effects on behavior (Victoria et al., 2016), making straightforward interpretation of these phenotypes difficult. As GIRK channels are known to be present in PVI, and alteration of PV-dependent GABA neuron activity is known to regulate affect and cognitive control (Sohal et al., 2009; Rossi et al., 2012; Sparta et al., 2014; Murray et al., 2015; Kim et al., 2016b; Page et al., 2019), we aimed to determine whether GIRK1-dependent signaling in PVI is necessary for normal affect, motivation, and cognitive control.

To examine the behavioral relevance of PVI GIRK1 signaling, we assessed affect-related behavior using the EPM and FST. Individual differences in locomotor activity were controlled for by assessing the percent of time spent in the open arm divided by the total time assessed. A main effect of sex was identified, with males having increased percent time in the open arm compared to females ($F_{(1,59)} = 5.91$, $p = 0.018$). There was also a main effect of condition, with cre-positive mice having increased percent open arm time compared to control mice ($F_{(1,59)} = 7.05$, $p = 0.010$); however no sex by condition interaction was observed ($F_{(1,59)} = 0.07$, $p = 0.789$; **Figure 1B**).

During the FST, there were no differences in the total time spent immobile (sex: $F_{(1,46)} = 0.87$, $p = 0.356$; condition: $F_{(1,46)} = 1.91$, $p = 0.174$; interaction: $F_{(1,46)} = 0.20$, $p = 0.657$; **Figure 1C**). There was, however, a significant sex by condition interaction when comparing number of immobile episodes during testing ($F_{(1,46)} = 4.78$, $p = 0.034$), with male cre-positive mice having significantly more immobile episodes than control

counterparts ($p < 0.001$), whereas there were no differences between conditions in female mice ($p = 0.200$). Female controls also had increased immobile episodes compared to the male controls ($p < 0.001$), whereas male and female cre-positive mice did not differ ($p = 0.619$; **Figure 1D**).

To examine if the loss of GIRK1 in PVI impacts motivation and to control for any potential differences observed in our set-shifting experiments (see below), we assessed responding for an appetitive liquid reward (Ensure®) using a progressive ratio model. Responses on the left reinforced lever, the breaking point at which the mouse would no longer respond, and the right nonreinforced lever were recorded. Males had an overall greater number of left lever presses compared to females, regardless of condition, whereas controls and cre-positive mice had similar number of responses (sex: $F_{(1,33)} = 18.50$, $p < 0.001$; condition: $F_{(1,33)} = 0.22$, $p = 0.640$; interaction: $F_{(1,33)} = 2.43$, $p = 0.128$; **Figure 1E**). A Kruskal–Wallis nonparametric test detected a significant difference on breakpoint during progressive ratio ($H_{(3)} = 10.76$, $p = 0.013$), with breakpoints in cre-positive males greater than cre-positive female mice, and no other significant *post-hoc* comparisons (**Figure 1F**). Notably, similar to the left lever, a main effect of sex was observed for responding on the right non-reinforced lever ($F_{(1,33)} = 5.44$, $p = 0.026$). In this case, females displayed a higher number of presses; however no effect of condition or interaction was observed (condition: $F_{(1,33)} = 0.15$, $p = 0.702$; interaction: $F_{(1,33)} = 0.26$, $p = 0.614$; **Figure 1G**).

Effects of PVI GIRK1 Knockout on Cognitive Flexibility

Our recent work has shown that disruption of GIRK1 signaling in mPFC prelimbic pyramidal neurons impairs cognitive performance in an attentional set-shifting model of cognitive flexibility (Anderson et al., 2020). Given prior research highlighting a role for PVI activity in cognitive control, we next assessed whether GIRK1 signaling in PVI also influences cognitive control. Before attention set-shift testing, mice had to have two consecutive days with ≤5 omissions during lever training during which there was no effect of sex ($F_{(1,34)} = 0.94$, $p = 0.340$), condition ($F_{(1,34)} = 1.64$, $p = 0.210$), nor an interaction ($F_{(1,34)} = 1.59$, $p = 0.216$; data not shown) on the number of days to reach this criterion. Once mice passed lever training criterion and were assessed for a side bias, testing in the visual cue was conducted. There was an effect of sex on the total trials to reach criterion during the visual cue test ($F_{(1,34)} = 8.32$, $p = 0.007$) but no effect of condition ($F_{(1,34)} = 0.42$, $p = 0.522$) or a sex by condition interaction ($F_{(1,34)} = 0.41$, $p = 0.527$; **Figure 2A**). Similarly, there was an effect of sex on the total errors made to reach criterion during the visual cue test ($F_{(1,34)} = 8.32$, $p = 0.007$) but no effect of condition ($F_{(1,34)} = 0.31$, $p = 0.583$) nor a sex by condition interaction ($F_{(1,34)} = 0.41$, $p = 0.710$; **Figure 2B**).

In a more refined investigation into errors, we divided the error type based on errors made before the mice received less than six errors in one 16 trial bin (i.e., initial errors) and those after (regressive errors), the latter of which reflects an inability to maintain the rule strategy. For initial errors, there was significant sex by condition interaction ($F_{(1,34)} = 7.72$, $p = 0.009$; **Figure 2C**) with *post-hoc* comparisons indicating no significant difference between male control and cre-positive mice ($p = 0.154$), whereas female cre-positive mice had fewer initial errors compared to control mice ($p = 0.012$). Notably, control females had significantly more initial errors compared to control males ($p = 0.027$), whereas male and female cre-positive mice did not differ ($p = 0.116$). Examination of regressive errors showed no main effect of condition

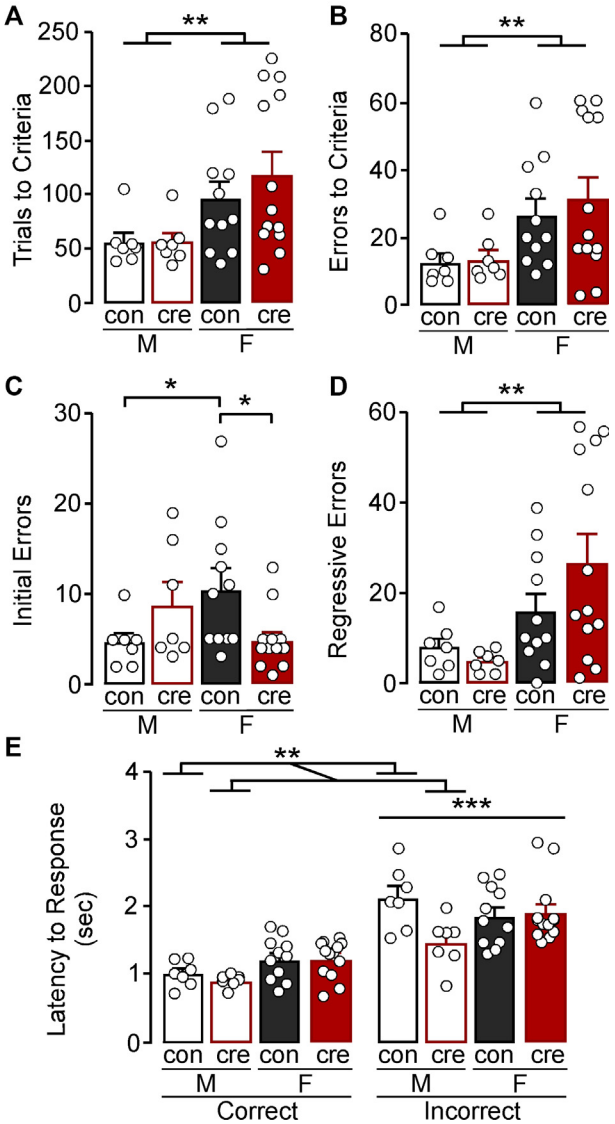

FIGURE 2 | Visual cue test. **(A)** Females took more trials to reach the criterion compared to males (**$p < 0.01$). **(B)** Females made more errors to reach criterion compared to males (**$p < 0.01$). **(C)** Female control mice made more initial errors compared to male control mice while female cre-positive made fewer errors compared to female control mice (*$p < 0.05$). **(D)** Females made more regressive errors compared to males (**$p < 0.01$). **(E)** The latency to make an incorrect response was higher than the latency to make a correct response (***$p < 0.001$). Cre-positive male mice, regardless of response type, took less time to respond compared to control mice (**$p < 0.01$).

($F_{(1,34)}$ = 0.61, p = 0.441) or a condition by sex interaction ($F_{(1,34)}$ = 1.98, p = 0.169); however females had significantly more regressive errors compared to males ($F_{(1,34)}$ = 9.09, p = 0.005; **Figure 2D**). Together, further analyses of the error type revealed that regressive errors drive the increased errors to criterion in females, while assessment of the initial errors indicate female control mice have greater difficulty at the beginning of the test compared to male controls and female cre-positive mice.

The speed of processing and general motor function of each mouse can be measured by taking the average latency to respond after the lever was presented (Brady and Floresco, 2015). A 2 (sex) × 2 (condition) × 2 (response type) ANOVA was used to compare latency to respond during the visual cue test. There was a main effect of response type with latency for mice to respond being significantly greater for incorrect compared to correct responses ($F_{(1,68)}$ = 76.88, $p < 0.001$; **Figure 2E**). There was also significant sex by condition interaction ($F_{(1,68)}$ = 6.01, p = 0.017) with *post-hoc* comparisons indicating that male cre-positive mice took less time to respond compared to male controls (p = 0.006), while females, regardless of condition, had similar response latencies (p = 0.758). There were no other significant interactions (sex by response type: $F_{(1,68)}$ = 1.06, p = 0.307; condition by response type: $F_{(1,68)}$ = 2.09, p = 0.153; sex by condition by response type: $F_{(1,68)}$ = 3.06, p = 0.085).

During the extradimensional shift test, there were no differences in trials to reach criterion comparing sex ($F_{(1,34)}$ = 0.84, p = 0.365), condition ($F_{(1,34)}$ = 0.21, p = 0.653), or sex by condition ($F_{(1,34)}$ = 0.00, p = 0.978; **Figure 3A**). There were also no differences in errors to reach criterion comparing sex ($F_{(1,34)}$ = 2.22, p = 0.145), condition ($F_{(1,34)}$ = 0.79, p = 0.381), or sex by condition ($F_{(1,34)}$ = 0.37, p = 0.551; **Figure 3B**). However, females had overall more perseverative errors than males ($F_{(1,34)}$ = 4.34, p = 0.045), while there were no differences of condition on number of perseverative errors ($F_{(1,34)}$ = 0.71, p = 0.404) or a sex by condition interaction ($F_{(1,34)}$ = 0.03, p = 0.882; **Figure 3C**). There was no main effect of sex ($F_{(1,34)}$ = 0.72, p = 0.402), main effect of condition ($F_{(1,34)}$ = 0.01, p = 0.910), nor a sex by condition interaction ($F_{(1,34)}$ = 0.01, p = 0.094) on regressive errors during the extradimensional shift test (**Figure 3D**). There were also no differences on number of errors made that had previously never been reinforced (sex: $F_{(1,34)}$ = 2.27, p = 0.141; condition: $F_{(1,34)}$ = 0.03, p = 0.857; interaction: $F_{(1,34)}$ = 1.28, p = 0.265; **Figure 3E**). There was a significant effect of sex on latency to respond, regardless of condition and response type, with females taking longer to respond compared to males ($F_{(1,68)}$ = 7.39, p = 0.008; **Figure 3F**) but no other differences (condition: $F_{(1,68)}$ = 0.21, p = 0.645; response type: $F_{(1,68)}$ = 0.08, p = 0.772; sex by condition: $F_{(1,68)}$ = 0.00, p = 0.974; sex by response type: $F_{(1,68)}$ = 0.00, p = 0.973; condition by response type: $F_{(1,68)}$ = 0.64, p = 0.427; sex by condition by response type: $F_{(1,68)}$ = 0.26, p = 0.614).

During the reversal test, females took more trials to reach criterion compared to males ($F_{(1,33)}$ = 8.03, p = 0.008), but there were no differences comparing conditions ($F_{(1,33)}$ = 1.59, p = 0.216) or a sex by condition interaction ($F_{(1,33)}$ = 0.04, p = 0.849; **Figure 4A**). Similarly, females had more errors during the reversal test compared to males ($F_{(1,33)}$ = 4.54, p = 0.041); however there was no difference comparing the conditions ($F_{(1,33)}$ = 0.24, p = 0.630) or a sex by condition interaction ($F_{(1,33)}$ = 0.07, p = 0.793; **Figure 4B**). There were no differences in perseverative errors (sex: $F_{(1,33)}$ = 0.16, p = 0.695; condition: $F_{(1,33)}$ = 0.02, p = 0.882; sex by condition: $F_{(1,33)}$ = 0.00, p = 0.969; **Figure 4C**) or regressive errors (sex: $F_{(1,33)}$ = 3.94, p = 0.056; condition: $F_{(1,33)}$ = 0.12, p = 0.727; sex by condition: $F_{(1,33)}$ = 0.14, p = 0.709; **Figure 4D**). Errors were next analyzed as being either toward or away from the cue distractor. There was a significant effect of sex on errors towards the distractor ($F_{(1,33)}$ = 5.30, p = 0.028) with females making more errors towards the distractor (**Figure 4E**), but there were no differences between the two conditions ($F_{(1,33)}$ = 1.11, p = 0.299) or a sex by condition interaction ($F_{(1,33)}$ = 0.00, p = 0.988). There were also no differences in number of errors made away from the distractor (sex: $F_{(1,33)}$ = 1.43, p = 0.241; condition: $F_{(1,33)}$ = 0.24, p = 0.629; interaction: $F_{(1,33)}$ = 0.31, p = 0.582; **Figure 4F**). Finally, there were no differences in latency to make a response during the reversal test (sex: $F_{(1,66)}$ = 2.17, p = 0.146; condition: $F_{(1,66)}$ = 1.81, p = 0.184; response type: $F_{(1,66)}$ = 0.44, p = 0.510; sex by condition: $F_{(1,66)}$ = 1.00, p = 0.322; sex by response type: $F_{(1,66)}$ = 0.45, p = 0.506; condition by response type: $F_{(1,66)}$ = 0.00, p = 0.944; sex by condition by response type: $F_{(1,66)}$ = 0.79, p = 0.377; **Figure 4G**). The extradimensional shift and reversal tests suggest that cre-positive mice have similar cognitive flexibility to control mice, and also reveal that females tend to persist on the initial visual cue test rule (i.e., increased perseverative errors during the extradimensional shift test and increased errors towards distractor during the reversal test) despite the changing contingencies.

Characterization of GIRK1 Knockout in Prelimbic Cortex PVI

Past work has identified an important role for GIRK1 signaling in hippocampal PVI (Booker et al., 2013). Given known contributions of the prelimbic region of the mPFC in regulating the aforementioned behaviors, we confirmed the presence of GIRK1 in prelimbic PVI and characterized knockout in *Girk1*$^{flox/flox}$ mice. To facilitate the electrophysiological evaluation of PVI neurons in PVCre:*Girk1*$^{flox/flox}$ mice, we crossed this line with transgenic mice expressing tdtomato under the control of the PV promoter. To determine if the knockout was reducing GIRK1 in PVI, voltage-clamped whole-cell slice recordings were performed to assess GIRK1-specific changes in somatodendritic currents using the GIRK1 selective agonist, ML297 (Wydeven et al., 2014; **Figure 5A**). Bath application of ML297 produced an outward current (I_{ML297}) that correlated with a decrease in input resistance (not shown) and was blocked by subsequent application of barium chloride (0.3 mM). Comparison across sex and condition (con vs. cre) showed that ML297-mediated currents were not different in PVI from males vs. females ($F_{(1,21)}$ = 0.26, p = 0.616) nor was there a sex by condition interaction ($F_{(1,21)}$ = 0.00, p = 0.972). PVI recorded from cre-positive mice showed a significant reduction in ML297-induced current compared to PVI from control mice ($F_{(1,21)}$ = 10.23, p = 0.004; **Figure 5B**) indicative of a

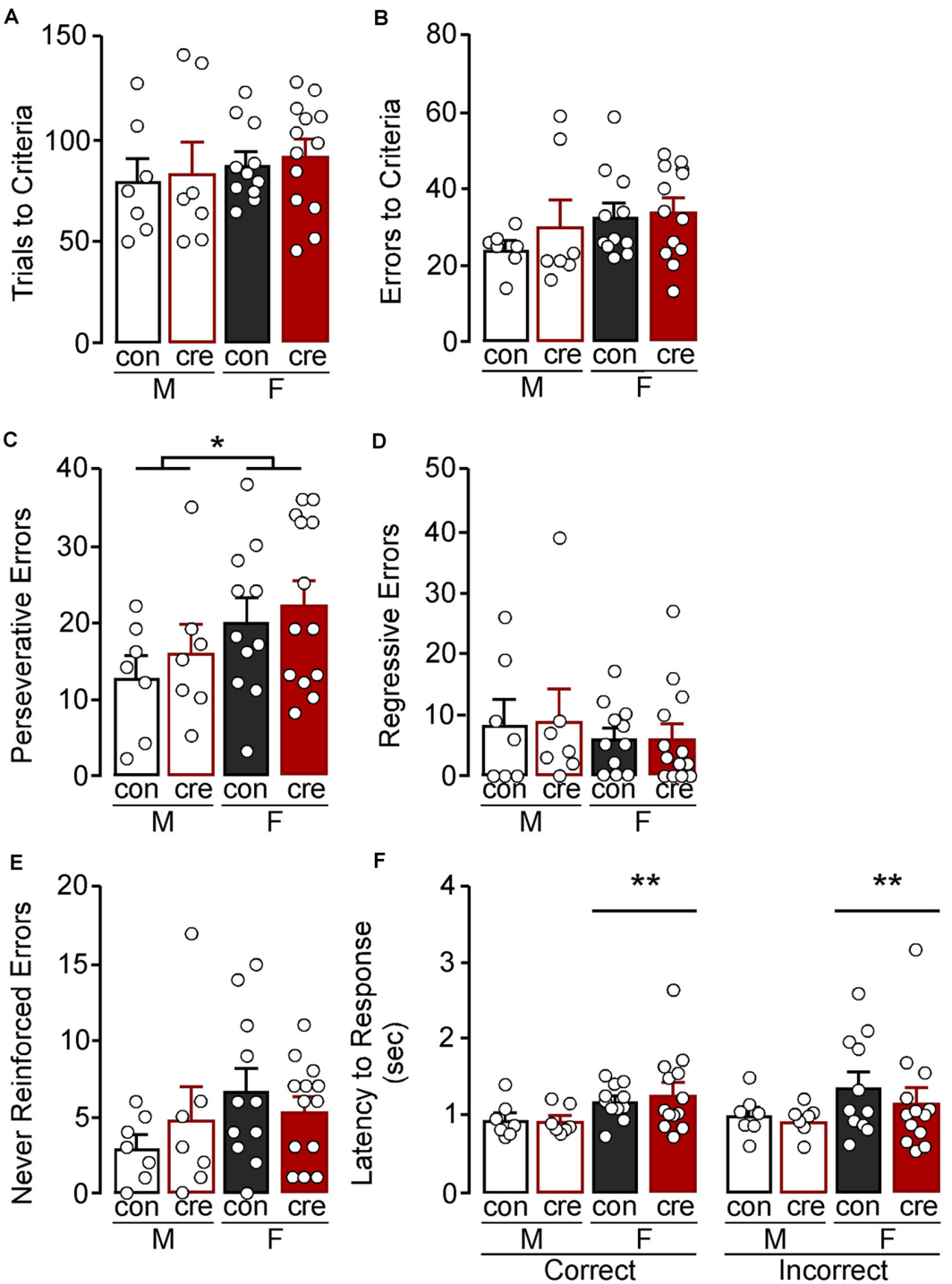

FIGURE 3 | Extradimensional shift test. **(A)** Trials criterion and **(B)** errors to criterion were similar for all groups. **(C)** Females made more perseverative errors compared to males (*$p < 0.05$) while the number of **(D)** regressive and **(E)** never reinforced errors were similar for all groups. **(F)** Females took longer to respond compared to males (**$p < 0.01$) regardless of condition and response type.

reduction in GIRK1 signaling (male control $n = 6/N = 4$; male cre $n = 8/N = 4$; female control $n = 6/N = 5$; female cre $n = 5/N = 3$). Similarly, animal-based analysis during which data points were averaged for each animal show no significant effect of sex ($F_{(1,12)} = 0.89$, $p = 0.364$) nor a sex by condition interaction ($F_{(1,12)} = 0.00$, $p = 0.951$); however

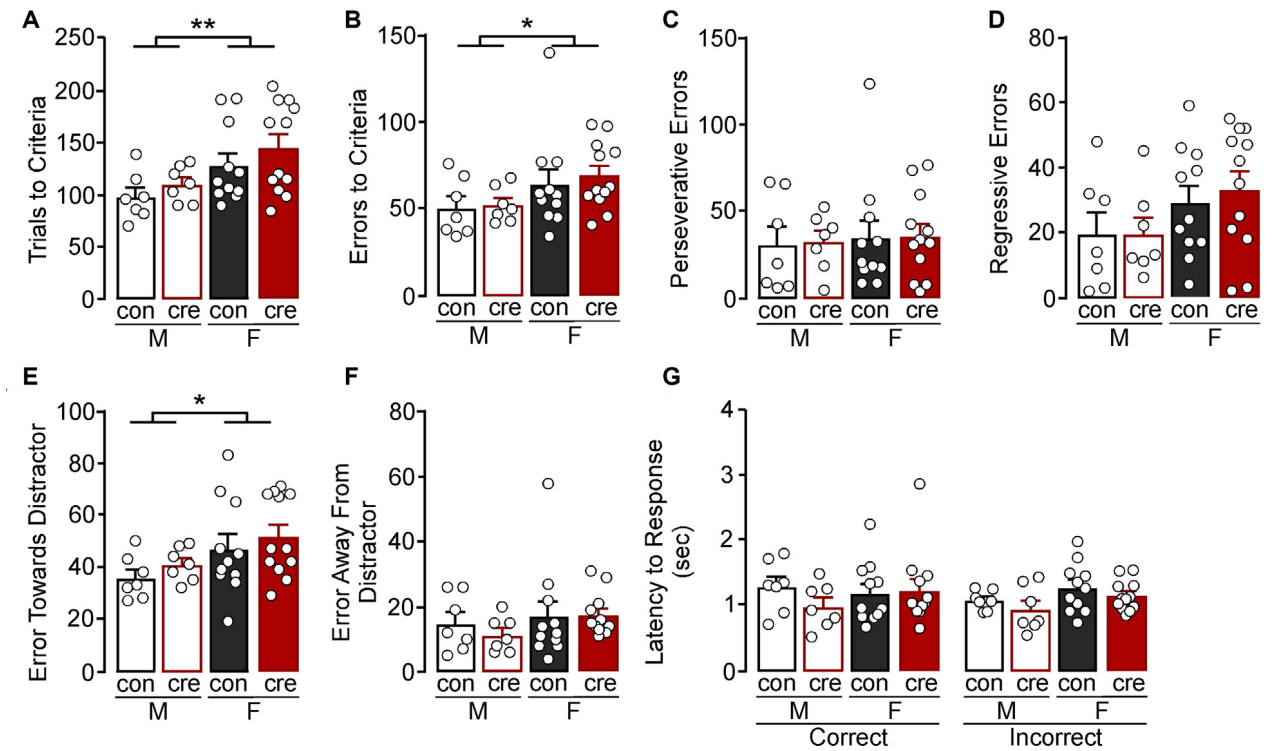

FIGURE 4 | Reversal test. **(A)** Females took more trials (**$p < 0.01$) and **(B)** errors to criterion (*$p < 0.01$) compared to males. **(C)** All groups had a similar number of perseverative and **(D)** regressive errors. **(E)** Females made more errors towards the cue distractor compared to males (*$p < 0.05$). **(F)** The number of errors away from the distractor was similar for all groups. **(G)** All groups had similar latency to respond, regardless of response type.

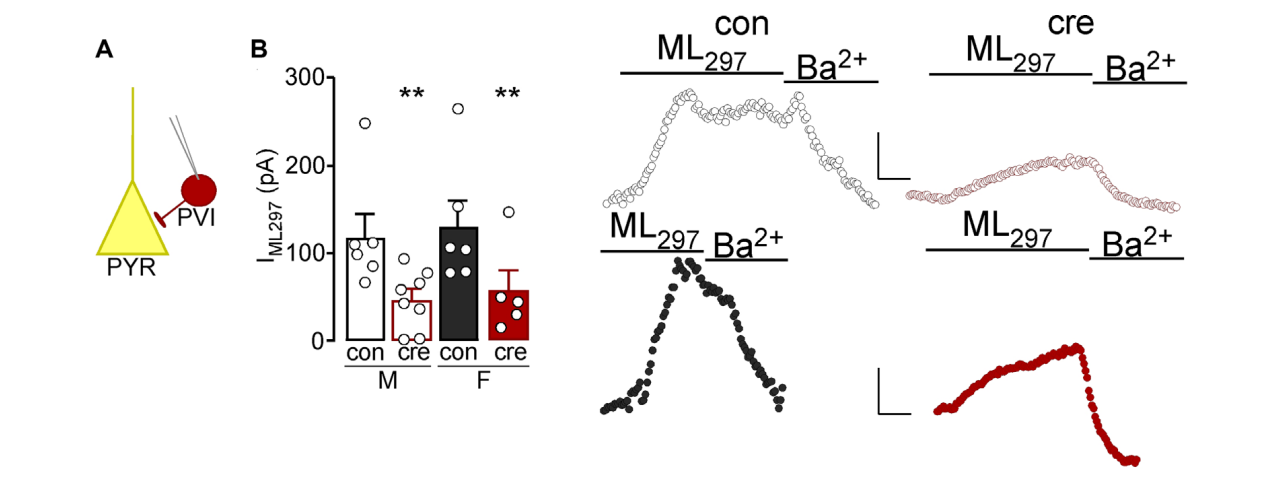

FIGURE 5 | **(A)** Whole-cell slice electrophysiology was used to record from parvalbumin interneurons (PVI) labeled with td-tomato in the prelimbic cortex. **(B)** The current induced by bath application of ML297 was lower in PVI from cre-positive mice (right representatives) compared to those from control mice (left representatives; **$p < 0.01$). Scale bar: 50 pA/180 s.

there was a main effect of condition ($F_{(1,12)} = 4.83$, $p = 0.048$). Similar to initial findings, recordings from cells in cre-positive mice had a significant reduction in ML297-induced current compared to cells from cre-negative mice indicating a reduction in GIRK1 signaling.

To determine how loss of GIRK1 signaling impacts membrane properties we next accessed capacitance, membrane resistance, RMP, threshold to fire an action potential, rheobase, amplitude and duration of initial action potential, AHP amplitude, and spike firing frequency in response to increasing

current injections. For cell-based analyses, there were no differences in capacitance (sex: $F_{(1,35)} = 0.22$, $p = 0.641$; condition: $F_{(1,35)} = 0.05$, $p = 0.826$; interaction: $F_{(1,35)} = 2.10$, $p = 0.157$; **Figure 6A**) or resistance (sex: $F_{(1,35)} = 0.43$, $p = 0.518$; condition: $F_{(1,35)} = 0.05$, $p = 0.821$; interaction: $F_{(1,35)} = 0.00$, $p = 0.980$; **Figure 6B**; male control $n = 9/N = 6$; male cre $n = 8/N = 4$; female control $n = 11/N = 5$; female cre $n = 11/N = 5$). RMP was more negative in females compared to males ($F_{(1,26)} = 5.13$, $p = 0.032$) however there was no main effect of condition ($F_{(1,26)} = 0.02$, $p = 0.898$) nor a sex by condition interaction ($F_{(1,26)} = 0.61$, $p = 0.442$; **Figure 6C**; male control $n = 9/N = 6$; male cre $n = 8/N = 4$; female control $n = 6/N = 4$; female cre $n = 7/N = 4$). The threshold to fire an action potential was also more negative in females compared to males ($F_{(1,26)} = 13.46$, $p = 0.001$) with no main effect of condition ($F_{(1,26)} = 3.86$, $p = 0.060$) nor a sex by condition interaction ($F_{(1,26)} = 0.09$, $p = 0.773$; **Figure 6D**; male control $n = 8/N = 6$; male cre $n = 8/N = 4$; female control $n = 7/N = 4$; female cre $n = 7/N = 4$). Rheobase (i.e., the amount of current injected for the cell to fire an action potential) did not differ based on sex or condition (sex: $F_{(1,27)} = 0.21$, $p = 0.651$; condition: $F_{(1,27)} = 1.02$, $p = 0.322$; interaction: $F_{(1,27)} = 0.59$, $p = 0.449$; **Figure 6E**; male control $n = 9/N = 6$; male cre $n = 8/N = 4$; female control $n = 7/N = 4$; female cre $n = 7/N = 4$). The action potential amplitude (sex: $F_{(1,26)} = 3.51$, $p = 0.072$; condition: $F_{(1,26)} = 4.16$, $p = 0.052$; interaction: $F_{(1,26)} = 0.88$, $p = 0.356$; **Figure 6F**) and duration at 50% the amplitude did not differ between groups (sex: $F_{(1,26)} = 0.07$, $p = 0.797$; condition: $F_{(1,26)} = 0.00$, $p = 0.988$; interaction: $F_{(1,26)} = 2.75$, $p = 0.109$; **Figure 6G**; male control $n = 8/N = 6$; male cre $n = 8/N = 4$; female control $n = 7/N = 4$; female cre $n = 7/N = 4$). There was a significant effect of condition on the afterhyperpolarization amplitude, with cre-positive mice having greater afterhyperpolarizations compared to control mice ($F_{(1,25)} = 7.23$, $p = 0.203$). However, there were no differences in sex ($F_{(1,25)} = 1.36$, $p = 0.255$) nor a sex by condition interaction ($F_{(1,25)} = 1.71$, $p = 0.203$; **Figure 6H**; male control $n = 8/N = 6$; male cre $n = 8/N = 4$; female control $n = 6/N = 3$; female cre $n = 7/N = 4$). Lastly, during the current-step injection in PVI from males, there was a significant current by condition interaction ($F_{(15,225)} = 2.01$, $p = 0.016$) with cre-positive cells firing significantly more action potentials at higher currents (240–300 pA) compared to controls ($p < 0.05$; **Figure 6I**). Conversely, there was no main effect of condition ($F_{(1,12)} = 0.69$, $p = 0.423$) or a condition by current interaction ($F_{(15,180)} = 0.75$, $p = 0.735$; **Figure 6J**) in female PVI (male control $n = 9/N = 6$; male cre $n = 8/N = 4$; female control $n = 7/N = 4$; female cre $n = 7/N = 4$). Together, these data indicate that loss of GIRK1 does not impact activation threshold but does increase neuronal firing of male PVI.

Animal-based statistics, during which recordings from each animal were averaged, show similar findings to the cell-based statistics. There were no differences in capacitance (sex: $F_{(1,16)} = 0.03$, $p = 0.867$; condition: $F_{(1,16)} = 0.19$, $p = 0.669$; interaction: $F_{(1,16)} = 2.72$, $p = 0.119$) or resistance (sex: $F_{(1,16)} = 0.13$, $p = 0.728$; condition: $F_{(1,16)} = 0.04$, $p = 0.836$; interaction: $F_{(1,16)} = 0.01$, $p = 0.937$). Similar to the cell-based statistics, RMP was more negative in females compared to males ($F_{(1,14)} = 4.59$, $p = 0.050$) however there was no main effect of condition ($F_{(1,14)} = 0.09$, $p = 0.775$) nor a sex by condition interaction ($F_{(1,14)} = 0.52$, $p = 0.484$). The threshold to fire an action potential was also more negative in females compared to males ($F_{(1,14)} = 13.19$, $p = 0.003$) with no main effect of condition ($F_{(1,14)} = 3.82$, $p = 0.071$) nor a sex by condition interaction ($F_{(1,14)} = 0.73$, $p = 0.406$). Rheobase did not differ based on sex or condition (sex: $F_{(1,14)} = 1.06$, $p = 0.321$; condition: $F_{(1,14)} = 1.71$, $p = 0.212$; interaction: $F_{(1,14)} = 1.06$, $p = 0.321$). Unlike cell-based statistics which showed no difference in action potential amplitude, animal-based statistics revealed a significant difference with females having significantly greater amplitude compared to males (sex: $F_{(1,14)} = 5.94$, $p = 0.029$; condition: $F_{(1,14)} = 2.79$, $p = 0.117$; interaction: $F_{(1,14)} = 1.18$, $p = 0.296$). The duration at 50% the amplitude did not differ between groups (sex: $F_{(1,14)} = 0.07$, $p = 0.794$; condition: $F_{(1,14)} = 0.15$, $p = 0.706$; interaction: $F_{(1,14)} = 2.63$, $p = 0.127$). Unlike cell-based statistics, there was no significant effect on the afterhyperpolarization amplitude (sex: $F_{(1,13)} = 1.30$, $p = 0.275$; condition: $F_{(1,13)} = 4.50$, $p = 0.054$; interaction: $F_{(1,13)} = 1.25$, $p = 0.284$). Lastly, during the current-step injection in PVI, there were no significant effects of condition or condition by current interactions in males (condition: $F_{(1,8)} = 1.80$, $p = 0.217$; interaction: $F_{(14,112)} = 1.24$, $p = 0.255$) or females (condition: $F_{(1,6)} = 1.83$, $p = 0.225$; interaction: $F_{(14,84)} = 1.39$, $p = 0.174$). Similar to cell-based statistics, these data indicate that loss of GIRK1 does not impact activation threshold however also does not alter neuronal firing in males or females, despite significantly reducing GIRK1 signaling.

DISCUSSION

The current study evaluated the impact of selectively ablating GIRK channels expressing the GIRK1 subunit in PVI on affect and cognitive flexibility. We found that loss of PVI GIRK1 signaling in males and females increased the percent time spent in the open arm during the EPM, suggestive of reduced anxiety-related behavior. Notably, effects on anxiety-like behavior in EPM or open field were not previously observed with conditional knockout of GIRK2 channels in GABA neurons using GAD-Cre transgenic mice (Victoria et al., 2016), suggesting that more selective targeting of GABA neuron subpopulations may yield discrete changes. Loss of GIRK1 selectively in males also increased immobile episodes during the FST, suggesting an increase in active coping. Although not directly comparable, these findings are in alignment with recent work showing that increased activation of mPFC PVI results in anxiety-like behaviors (Page et al., 2019). Moreover, the lack of effect on motivation during the progressive ratio is in agreement with previous research showing that optogenetic stimulation of mPFC PVI does not alter appetitive reward consumption (Sparta et al., 2014). Past *in vivo* work has shown that increased spike firing of PVI cells reduces the output of pyramidal neurons in the mPFC (Sparta et al., 2014), thus it is possible that any observed changes in behavior, albeit discrete, reflect reductions in prelimbic cortex pyramidal neuron activity.

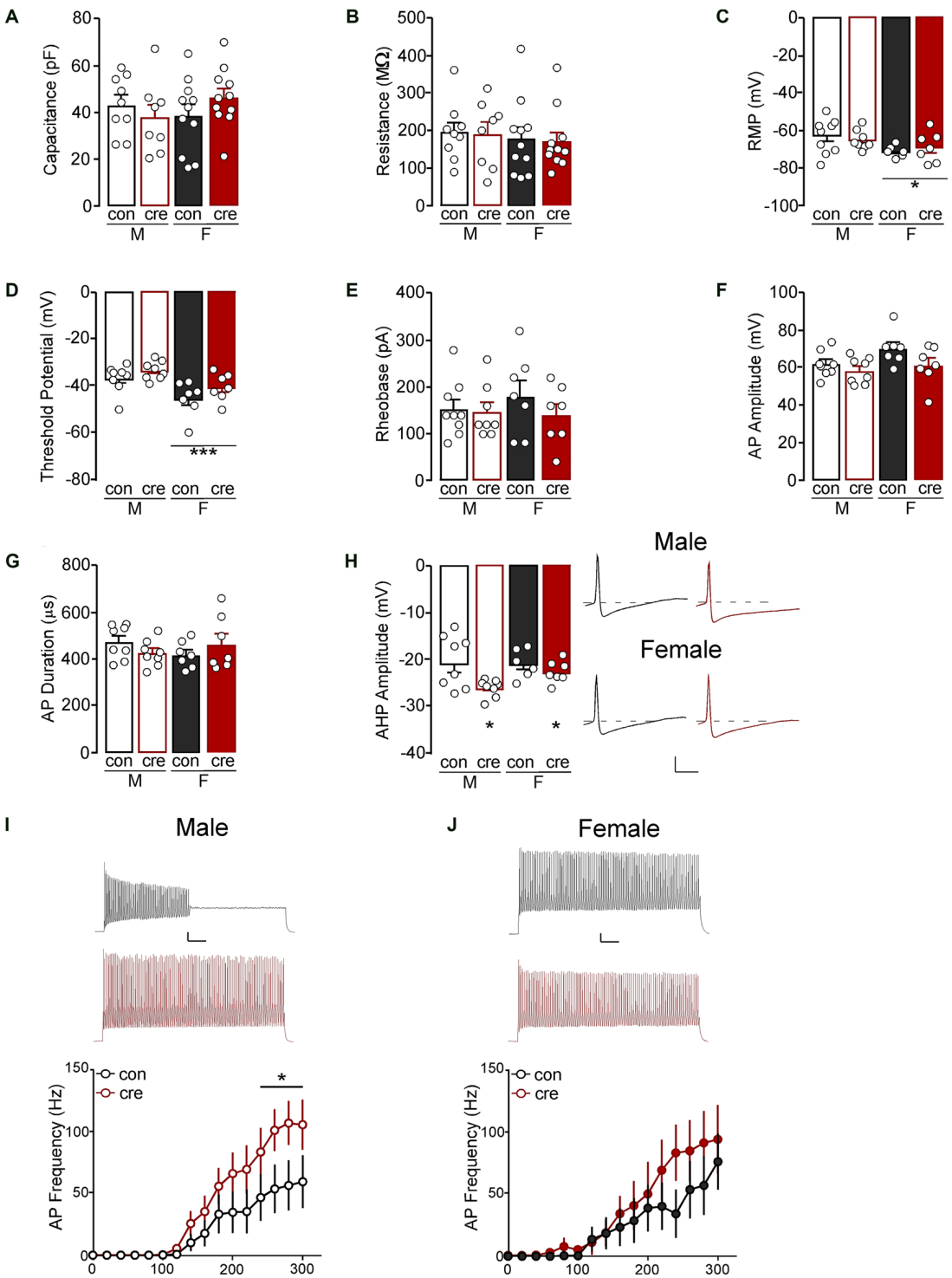

FIGURE 6 | (A) The capacitance and **(B)** resistance of PVI neurons were similar for all groups. **(C)** The resting membrane potential (RMP) of PVI from females, regardless of condition, were more negative than those from males (*$p < 0.05$). **(D)** The threshold potential before firing an action potential was also more negative in PVI from females compared to males (***$p = 0.001$). **(E)** The current required to evoke an action potential was similar for all groups. **(F)** The action potential (AP) amplitude and **(G)** duration was similar for all groups. **(H)** Afterhyperpolarization (AHP) amplitude for the first action potential from cre-positive PVI, regardless of sex, was more negative compare to PVI from cre-negative mice (*$p < 0.05$; scale: 20 mV/5 ms). **(I)** The number of action potentials fired at 240–300 pA was higher in PVI from cre-positive male mice compared to control male mice (*$p < 0.05$; scale 10 mV/100 ms, traces at 300 pA). **(J)** PVI had similar action potential frequency in cre-positive and control female mice (scale 10 mV/100 ms, traces at 300 pA).

Given the span of literature highlighting a critical role for PVI in the regulation of mPFC-dependent cortical processing (Ferguson and Gao, 2018) and previously identified roles for GIRK signaling in regulating affect and cognition (Pravetoni and Wickman, 2008; Lazary et al., 2011; Cooper et al., 2012; Yamada et al., 2012; Wydeven et al., 2014; Lecca et al., 2016; Victoria et al., 2016), we predicted that effects of GIRK1 ablation would be evident in measures of cognitive flexibility. These predictions were based on studies using approaches that reduced PVI-dependent signaling, however, the impact of increased PVI output on cortical processing and cognition is far less clear. For example, cre-inducible channelrhodopsin activation of mPFC PVI at high frequencies has been shown to promote delayed alternation impairments (Rossi et al., 2012) and accelerate the extinction of cue-reward behavior (Sparta et al., 2014). Conversely, chemogenetic and optogenetic activation of mPFC PVI does not alter performance in a novel object recognition test of working memory or fear conditioning (Yizhar et al., 2011; Page et al., 2019). In the present study, loss of PVI GIRK1 signaling did not result in behavioral deficits during an overall performance in tests of visual cue-based discriminative learning or flexibility during the extradimensional shift and reversal test—tasks which are dependent on the mPFC (Ragozzino et al., 1999; Floresco et al., 2008) and orbitofrontal cortex (Ghods-Sharifi et al., 2008), respectively. However, during the visual cue test, cre-positive males had a reduced latency to respond compared to their control counterparts, which may be indicative of the increased speed of processing. These findings align with a reduction in attentional processing following PVI silencing (Kim et al., 2016b). Although the alterations in speed of processing did not correspond to differences in the trials or errors to criterion, others have shown that PVI inactivation produces deficits in cognitive flexibility in the water maze (Murray et al., 2015). The outcomes of these studies are difficult to compare to the current study, as they determined the role of acute and intermittent increases in PVI activity, rather than chronically altering PVI activity specifically through reductions in GIRK signaling. Regardless, the present study in combination with past work suggests that while the loss of PVI signaling promotes critical deficits in cognitive control, the effects of increased PVI output are far more complex. In agreement, a recent report (Caballero et al., 2020) has suggested that there may be a threshold of PVI expression through which cortical dysfunction becomes evident. Thus, possibly the loss of GIRK signaling alone does not meet that threshold, as other inhibitory signaling may be decreased to compensate for changes in PVI excitability and output.

GIRK1 Knockout Effects on PVI Excitability

Past studies have shown that perisomatic inhibition of hippocampal PVI is likely driven by activation of GIRK channels (Booker et al., 2013) and that while GIRK1–3 subunits were present in PVI, GIRK1 was the most prominently expressed. Similarly, our past work has shown that GIRK1-expressing GIRK channels are the primary mediators of GIRK signaling in prelimbic cortex pyramidal neurons, however, to our knowledge this is the first study to assess the role of GIRK signaling in prelimbic cortex PVI. Whole-cell recordings with the selective GIRK1 agonist ML297 showed that GIRK1-containing channels are indeed present in prelimbic PVI and that GIRK1-dependent current do not differ in males or females. The presence of cre-recombinase reduced GIRK1-mediated currents by ∼60%, with residual current likely driven by vehicle (DMSO). While GIRK1 knockout produced a leftward shift in the current-spike relationship (increased firing frequency) in PVI from males (albeit only when analyzed using cell-based statistics), to a lesser non-significant extent in females, it unexpectedly did not alter rheobase.

Examination of intrinsic membrane properties using cell-based statistics showed no baseline sex differences or effects of GIRK1 knockout on capacitance, membrane resistance, action potential amplitude, or action potential duration. These data align with previous work from our lab and others showing no significant changes in these measures in prelimbic pyramidal neurons with GIRK1 knockout (Hearing et al., 2013) and a lack of GIRK contribution to the regulation of firing properties (Llamosas et al., 2017; but see Imbrosci and Mittmann, 2013). Conversely, unlike our past findings in prelimbic pyramidal neurons, PVI from cre-positive mice show increased afterhyperpolarization amplitude compared to cre-negative PVI. Further, while not impacted by GIRK1 knockout, RMP was more hyperpolarized in females, and while rheobase did not differ in males and females, more sensitive assessments showed that action potentials are initiated at more hyperpolarized potentials in females. Together, these data highlight effects of GIRK1 ablation on intrinsic physiology that may be both unique to PVI, and also sex-specific, and demonstrate previously uncharacterized sex differences in PVI excitability.

Although analysis of data through the use of animal-based statistics resulted in similar findings for capacitance, membrane resistance, RMP, the threshold to fire an action potential, and action potential duration, there were some inconsistencies. There were no differences in action potential amplitude when analyzed using cell-based statistics, however, animal-based statistics found a significant effect of sex. It should be noted that animal-based statistics assume that each recording is from a similar cell-type. Given noted subpopulations of cells within a given cell-type throughout the brain, including PVI (chandelier vs. basket), and known differences in the electrophysiological characteristics of each cell-type, taking the average of cells from each mouse may increase the risk of a Type 1 error by reducing variability in the statistical analysis. Frequently, each animal contributes one to three recordings within a dataset, therefore, the use of animal-based statistics to average recordings also significantly reduced the sample size which may result in a Type 2 error. Although cell-based statistics revealed a significantly greater afterhyperpolarization amplitude in cre-positive compared to controls, there were no differences when analyzed using animal-based statistics. Similarly, while cell-based statistics revealed greater action potential firing from cre-positive mice compared to controls in males, there were also no differences when analyzed using animal-based statistics. The lack of findings using animal-based statistics may be the result of decreased power due to reductions in sample size from averaging all recordings within each animal.

The lack of a consistent effect on traditional measures of membrane excitability (rheobase, current-spike relationships), as well as lack of a robust behavioral phenotype, may reflect a variety of factors. First, the knockout of PVI GIRK-signaling was already in effect during critical development periods which may have led to compensatory changes in non-GIRK effectors more readily modulating membrane excitability. Although a viral vector to specifically target PVI GIRK-signaling is not readily available, it would be beneficial for future research to target PVI GIRK channels specifically during adulthood to reduce the potential for compensation of other signaling during development. Further, neuronal GIRK channels can be homo- and heterotetrameric complexes formed primarily by the assembly of GIRK1, GIRK2, and GIRK3 subunits (Karschin et al., 1996; Hearing et al., 2013), it is possible that loss of GIRK1 leads to upregulation of homotetrameric GIRK2-expressing channels. However, such a phenomenon has not been observed with constitutive and conditional knockout of GIRK1 or GIRK2 in other cell-types in areas such as the hippocampus, prefrontal cortex, and ventral tegmental area (Hearing et al., 2013; Kotecki et al., 2015; Victoria et al., 2016). Third, mPFC PVIs include basket and chandelier type cells that differ in structure, physiology, and GPCR agonist response (Kawaguchi and Kubota, 1997; Booker et al., 2013). Thus, decreasing GIRK-signaling in both cell-types may have diminished any opportunity for behavioral changes that may have arisen should GIRK-signaling have been reduced in only one cell-type. Finally, GIRK channels may play a role in regulating membrane excitability in PVI, and differences in membrane properties of GIRK1 channel knockout PVI may become evident by blocking fast synaptic transmission. Similarly, although beyond the scope of these studies, PVI GIRK channels may exhibit differences in coupling efficiency or regulators of G protein signaling (Labouebe et al., 2007). Given these and several other limitations of this study, including a lack of anatomical and temporally precise knockout of GIRK1, future research investigating the role that PVI GIRK1 KO has on postsynaptic Gi-GPCR signaling as well as using more targeted manipulations (i.e., viral-mediated) is warranted.

Relatedly, the global knockout approach used also assumedly resulted in reductions in PVI GIRK1-signaling in brain regions other than the prelimbic cortex which may have resulted in changes to PVI excitability in these regions and alterations in behavior paradigms that were not assessed in the current series of experiments. Unfortunately, data on the role of PVI GIRK-signaling in other brain regions on behavior is nonexistent; however the role of PVI in regions such as the amygdala (e.g., Wolff et al., 2014; Luo et al., 2020), hippocampus (Tucker et al., 2019), and striatum (Monteiro et al., 2018) are well-studied.

Sex Differences in Cognitive Flexibility and Motivation

Although not the main focus of the study, we unexpectedly identified differences in male and female performance across the set-shifting task that were largely independent of GIRK ablation. During the initial visual cue test, females required more trials and errors to reach the criterion compared to males, regardless of genotype. In controls, the increased number of errors in females reflects an equal balance between initial and regressive errors. However, loss of PVI GIRK appears to shift this towards an increase in regressive error types and a reduction in initial errors, suggesting an impaired ability to maintain a newly learned rule.

During the extradimensional shift, while females do not require more trials to reach criterion than males, they perseverate more on the rule associated with the prior day visual cue test (they continue to follow the cue light) and take longer to respond compared to males. Females also require more trials to reach criterion in a test of reversal learning. Further investigation of the error types shows a greater number of errors on the lever underneath the cue (i.e., more errors towards the distractor) indicating that they are still perseverating on the visual cue rule, despite learning a new rule during the extradimensional shift. To our knowledge, this is the first study to investigate sex differences in an operant based attentional set-shifting model of cognitive flexibility. It should be noted that although gonadal hormones influence cognition (Luine and Frankfurt, 2020; Taxier et al., 2020), the estrous cycle does not appear to influence attention set-shifting performance (Workman et al., 2013), and these sex differences may not be replicated in C57BL/6J mice. The day following the reversal test, motivation for an Ensure reward was measured using a progressive ratio test during which only the left lever was reinforced. Although male mice had a significantly greater number of left lever active responses, females exhibited greater responses on the right inactive lever. As progressive ratio testing only occurred on a single day, the increased right lever responses in females may suggest a lack of task understanding with a shift to right lever responding at higher breakpoints. Although interesting future directions arise from these measurable sex differences in a variety of behaviors, it should be noted that the efficiency of the cre-recombinase knockdown was not measured in the current study. Recombination in the PVI:cre strain has been noted in >90% of cells (Jackson Laboratory) however there are examples of incomplete recombination (i.e., cre mosaicism) and differences in efficiency in males and females in other cre strains (Jackson Laboratory) indicating that the potential of sex differences in cre efficiency related to the current findings should not be disregarded.

Findings from the current study suggest that PVI GIRK1 signaling does not mediate membrane excitability to the extent that it does in principle pyramidal neurons and may differentially impact firing frequency in males and females. Further, while reductions in PVI GIRK1 signaling influenced anxiety-like behaviors, effects on cognitive performance were more nuanced. While outside the scope of this study, the demonstration that GIRK channels are present in prelimbic PVI requires further investigation into their function, as past work has shown that differences in subunit expression and coupling dictate responsivity to various Gi-coupled GPCR agonists, and thus may inform future drug therapies targeting GIRKs or Gi-coupled GPCRs. Further, the current findings have provided unexpected insight into how biological sex impacts cognitive processing associated with an operant-based model of cognitive flexibility which may have

important implications for treating pathological deficits in cognitive control.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Marquette University Institutional Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

EA and MH designed, discussed, planned all experiments, and wrote the manuscript. EA, AE, SD, HD'A, and MH performed experiments and analyzed data. All authors contributed to the article and approved the submitted version.

FUNDING

These studies were supported by funding from the Brain and Behavior Research Foundation (#26299), Marquette University Regular Research Grant, and the Charles E. Kubly Mental Health Research Foundation at Marquette University.

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