THE ROLE OF NEUROPEPTIDES IN DRUG ADDICTION AND OTHER PSYCHIATRIC DISORDERS

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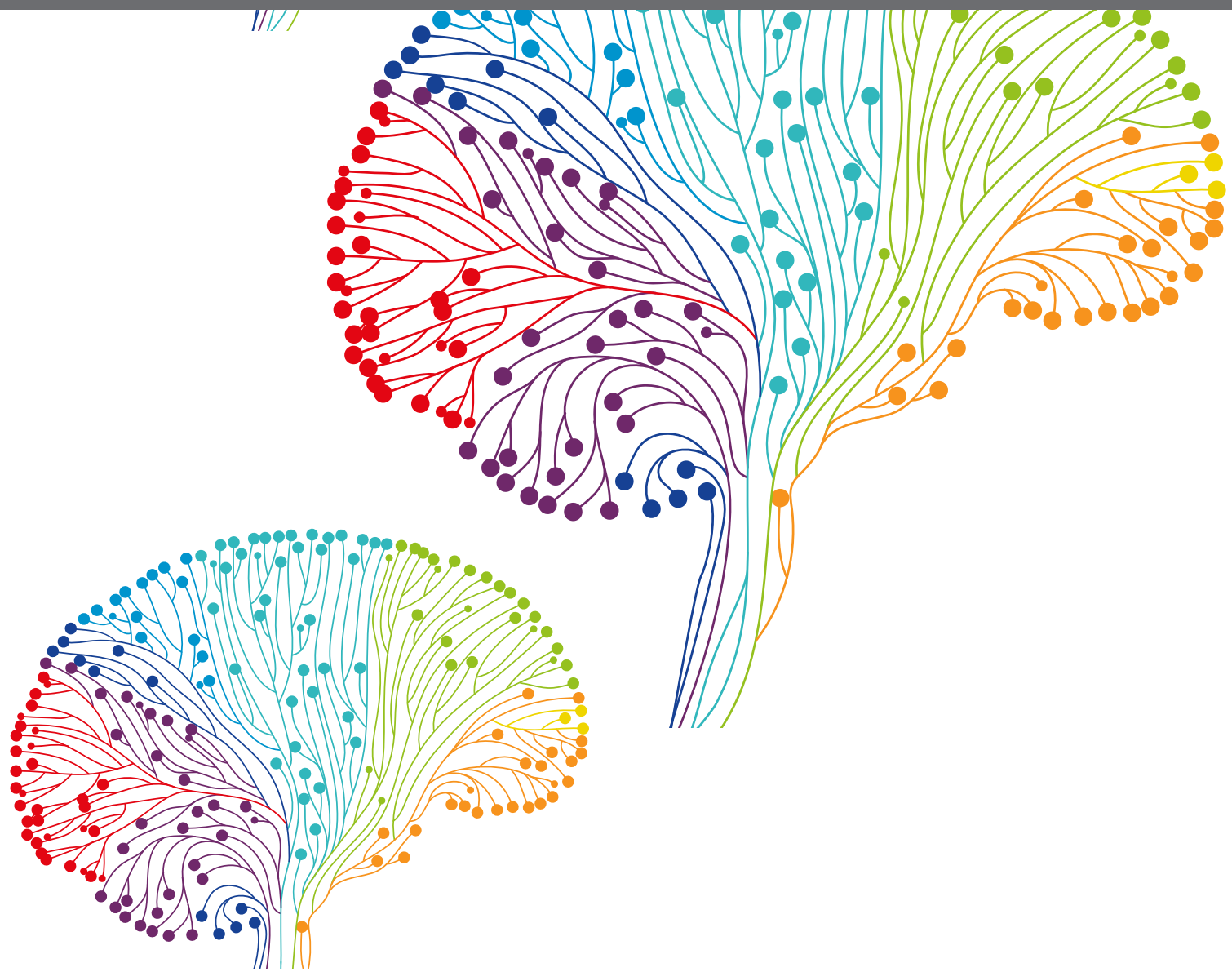

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THE ROLE OF NEUROPEPTIDES IN DRUG ADDICTION AND OTHER PSYCHIATRIC DISORDERS

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EDITORIAL
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Editorial: The Role of Neuropeptides in Drug Addiction and Other Psychiatric Disorders

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Editorial on the Research Topic

The Role of Neuropeptides in Drug Addiction and Other Psychiatric Disorders

This Research Topic was aimed to publish breakthrough findings encompassing the role of neuropeptides in addiction and other neuropsychiatric disorders. The data collected here, explain the involvement of neuropeptides in addiction-like behaviors both at the cellular and at the systemic level.

Cocaine addiction represents a major public health issue and negatively impacts society and the economy. However, there is no pharmacotherapy currently available to treat this chronic and relapsing brain disorder. Recent evidence suggests that peptides, such as glucagon-like peptide-1 (GLP-1) and leptin, which are involved in satiety and glucose and energy homeostasis, may also be functionally involved in cocaine reward and reinforcement. Zhu et al. used the place conditioning paradigm to study the effect of exendin-4, a GLP-1 agonist, on the reinstatement of cocaine-induced conditioned place preference, a model of drug reward. Reinstatement was elicited by either a priming dose of cocaine (10 mg/kg) or exposure to stress; drug and stress exposure can lead to relapse in people with cocaine use disorder. Exendin-4, given 1 h before each extinction training, attenuated reinstatement of cocaine- or stress-induced conditioned place preference. Additionally, Western Blot analysis was used to assess changes in the level of a nuclear transcription factor, NF- κB, in the nucleus accumbens in response to cocaine and stress exposure. Treatment with Exendin-4 also reduced NF-κB levels in the nucleus accumbens.

Glucagon-like peptide 1 receptors (GLP-1Rs) are highly expressed in the brain and are responsible for mediating the acute anorexigenic actions of GLP-1R agonists. In another multidisciplinary study, Zeng et al. performed a deep anatomical and neurophysiological characterization of GLP-1Rs in the central nucleus of the amygdala (CeA), where they found that GLP-1R is diffusely coexpressed in known CeA neuronal subpopulations like protein kinase, somatostatin, and tachykinin. They next mapped the anatomical positions of the GLP-1R-containing cells by using Glp1r-Cre mice and viral Cre-dependent tracing. They found that Glp1r-CeA cells are highly enriched in the medial subdivision of the CeA. By electrophysiological whole-cell recordings, the authors found that Glp1r-CeA neurons are characterized by the presence of hyperpolarized activated inward (Ih)-like currents, with a sex difference in magnitude and membrane capacitance. Future studies leveraging these data will be important to understanding the impact of GLP-1R agonists on reward and motivation, and may identify potential targets for developing pharmacotherapy to treat disorders associated with cocaine and food intake.

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In this Research Topic, a review article by Keller et al. analyzed the involvement of GLP-1 in the reward system as one of the neuropeptides that contributes to the homeostatic control system, which is often disrupted by alcohol consumption and withdrawal.

In another study, Carrette et al. examined the potential relationship between leptin and cocaine self-administration in genetically heterogeneous rats. The authors processed 120 blood samples from 60 rats. They did not find a relationship between the body weight of animals and cocaine self-administration during withdrawal and abstinence. However, baseline blood leptin levels could predict addiction-like behaviors. They observed that the higher the basal leptin levels, the lower the cocaine self-administration after cocaine withdrawal and abstinence. The authors further confirmed this finding in a separate cohort of rats, showing that leptin administration reduced cocaine seeking during cocaine protracted abstinence. Together, these results suggest that peripheral peptides involved in glucose and energy homeostasis may have a protective effect against cocaine seeking and craving.

Another neuropeptide, pituitary adenylate cyclase activating polypeptide (PACAP), implicated in homeostatic systems within the body, has been recently found to be involved in substance use disorder, specifically in alcohol use disorder. Minnig et al. investigated the potential contribution of the PAC1 receptor in the nucleus accumbens shell in alcohol drinking in rats using a viral vector-mediated knockdown approach. By using operant models of oral self-administration in rats, the authors found that the loss of function of PAC1 receptor in the nucleus accumbens shell leads to increased alcohol drinking and increased motivation for alcohol, suggesting that the PACAP/PAC1 receptor system in this brain area may act as a "brake" on excessive alcohol drinking.

Among other neuropeptides that play a functional role in motivated behaviors, β-endorphins have been implicated in cocaine reward. In this work, Singh and Lutfy investigated the role of β-endorphins in cocaine-induced conditioned place preference, extinction, and reinstatement in male and female mice lacking β-endorphin and their wild-type controls. The authors found that β-endorphin is involved in the rewarding actions of cocaine in both male and female mice. However, female mice lacking β-endorphin exhibited place preference following repeated conditioning with cocaine, whereas male mice did not. Nonetheless, re-exposure to cocaine failed to reinstate the place preference response in mice lacking β-endorphin regardless of sex. Future studies will be important to further investigate the involvement of β-endorphins in the escalation and relapse of psychostimulants in rodents.

The entactogen psychostimulant drugs, 3,4-methylenedioxym ethcathinone (methylone), 3,4-methylenedioxypentedrone (pentylone), and 3,4-methylenedioxy methamphetamine (MDMA) are commonly used substances. In this Research Topic, Khom et al. investigated whether female rats escalate self-administration of methylone, pentylone, and MDMA; they then studied the consequences of MDMA and pentylone self-administration on $GABA_A$ receptor and κ-opioid receptor signaling in the CeA. Within the class of entactogen stimulants, the propensity to have a stable self-administration pattern is variable. Importantly, to date, there are limited data available that elucidate the use liability of entactogen stimulants in female subjects, and that may be related to the limited exposure to the drug. Thus, the authors initially determined whether long access to intravenous self-administration of three entactogens leads to escalation of drug intake in female rats, as it does in males. The authors used the acquisition of self-administration under long-access conditions and post-acquisition dose substitutions under a progressive ratio schedule of reinforcement to assess potential differences in behavioral patterns in entactogen self-administration. Indeed, female rats readily acquired methylone, pentylone, and MDMA self-administration under long-access daily conditions. Lastly, they investigated the neuroadaptations in synaptic transmission in the CeA by performing whole-cell patch-clamp electrophysiology to assess changes in CeA GABA transmission and its regulation by the dynorphin/κ-opioid receptor system in female MDMA- and pentylone-exposed rats. They demonstrated a significant dysregulation of CeA neuronal activity in response to self-administration of entactogens in female rats, reinforcing the importance of performing behavioral and cellular studies in both sexes.

In a review published in this Research Topic, Cassello et al. summarized information regarding neuropeptide signaling in the prefrontal cortex (PFC) and its relevance to neuropsychiatric disorders. They reviewed data on dynorphin, enkephalin, corticotropin releasing factor (CRF), and some others. This area is an exciting emerging field of study with significant potential for clinical translation. The authors did an excellent job balancing the biological and clinical aspects of each neuropeptide, establishing knowns and unknowns, discussing the potential role of neuropeptides in neuropsychiatric disorders and animal models, and providing insights for future research.

In another review article, Matzeu and Martin-Fardon summarized the literature on the role of orexin/hypocretin neuropeptides on alcohol, opioid, nicotine, and cocaine addiction. Orexin receptor antagonists have been recognized among other potential medication targets for addiction treatment. However, it is unclear whether these compounds are equally effective across different drugs of addiction or in polysubstance use, and whether they are efficacious in specific stages of the addiction cycle. Also unknown is whether the efficacy of the orexin receptor antagonists could be sex-specific. Investigating the role of these compounds on additional models of substance use disorder and in clinical research will help elucidate how the orexin system contributes to neuropsychiatric disorders, and find novel treatments for these disorders.

Finally, Curley et al. provide an overview of recent studies regarding the molecular mechanisms related to the role of corticotropin releasing factor binding protein (CRFBP) in the progression of addiction and other psychiatric disorders, biological aging, and age-related neurodegenerative disease. Dysregulation of the CRF stress system is associated with psychiatric and neurodegenerative disorders, and CRFBP has been indicated as a novel target to mediate the contribution of stress-related dysfunction to these conditions. However, future studies on CRFBP should examine sex as a biological

variable, as well as consider the relevance of ethnic background and genetic polymorphisms that may alter susceptibility to stress-related disorders.

AUTHOR CONTRIBUTIONS

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

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REVIEW
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Corticotropin Releasing Factor Binding Protein as a Novel Target to Restore Brain Homeostasis: Lessons Learned From Alcohol Use Disorder Research

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Stress is well-known to contribute to the development of many psychiatric illnesses including alcohol and substance use disorder (AUD and SUD). The deleterious effects of stress have also been implicated in the acceleration of biological age, and age-related neurodegenerative disease. The physio-pathology of stress is regulated by the corticotropin-releasing factor (CRF) system, the upstream component of the hypothalamic-pituitary-adrenal (HPA) axis. Extensive literature has shown that dysregulation of the CRF neuroendocrine system contributes to escalation of alcohol consumption and, similarly, chronic alcohol consumption contributes to disruption of the stress system. The CRF system also represents the central switchboard for regulating homeostasis, and more recent studies have found that stress and aberrations in the CRF pathway are implicated in accelerated aging and age-related neurodegenerative disease. Corticotropin releasing factor binding protein (CRFBP) is a secreted glycoprotein distributed in peripheral tissues and in specific brain regions. It neutralizes the effects of CRF by sequestering free CRF, but may also possess excitatory function by interacting with CRF receptors. CRFBP's dual role in influencing CRF bioavailability and CRF receptor signaling has been shown to have a major part in the HPA axis response. Therefore, CRFBP may represent a valuable target to treat stress-related illness, including: development of novel medications to treat AUD and restore homeostasis in the aging brain. This narrative review focuses on molecular mechanisms related to the role of CRFBP in the progression of addictive and psychiatric disorders, biological aging, and age-related neurodegenerative disease. We provide an overview of recent studies investigating modulation of this pathway as a potential therapeutic target for AUD and age-related neurodegenerative disease.

Keywords: CRFBP, AUD, stress, neurodegeneration, HPA axis, aging

INTRODUCTION

The deleterious effect of stress, both as an acute insult or chronic exposure, has been linked to many pathophysiological changes in the human body. The brain is the central hub for the stress response, which controls both physiological and behavioral mechanisms that regulate health outcomes. Therefore, stress is considered a major contributor to the initiation and development of a variety of psychiatric disorders, including alcohol and substance use (AUD and SUD) (Koob, 2008; Sinha, 2008), anxiety (Pêgo et al., 2010; Nolte et al., 2011), and depression (Hammen, 2005). Further, stress has been implicated in the acceleration of biological aging (Epel et al., 2004; Wolf and Morrison, 2017; Yegorov et al., 2020) and in the progression of neurodegenerative diseases (Hemmerle et al., 2012; Djamshidian and Lees, 2014). Chronic psychological stress has been found to mechanistically contribute to acceleration of aging through cellular senescence (Yegorov et al., 2020) and stimulation of the pro-inflammatory response (Rea et al., 2018; Yegorov et al., 2020), which in turn may result in massive death of neurons (Bachis et al., 2008; de Pablos et al., 2014). Therefore, stress-induced acceleration of brain aging can initiate pathological processes that can lead to early onset and progression of dementia (Peavy et al., 2012), Parkinson's (Hemmerle et al., 2012), and Alzheimer's (Swaab et al., 2002; Swerdlow, 2011; Mosher and Wyss-Coray, 2014) diseases. Despite the clear evidence of the role of stress on the development of many brain diseases and in accelerating the process of biological aging, currently there are no medications that target the stress system. Furthermore, there are no therapeutic interventions designed to re-establish the homeostatic imbalance produced by acute or chronic stress insults.

In order to adapt to stress, the hypothalamic-pituitary-adrenal (HPA) axis initiates a cascade of neuroendocrine processes characterized by release of corticotropin releasing factor (CRF) from the paraventricular nucleus of the hypothalamus (PVN), which binds to two G protein-coupled receptors (GPCRs) CRF_1 and CRF_2 (Bale and Vale, 2004). After almost two decades of intense research in developing pharmacophores that target the stress system at the central nervous system (CNS) level, clinical studies utilizing CRF_1 antagonists failed to translate to the clinical setting, and lacked efficacy in trials for individuals with AUD (Binneman et al., 2008), major depression and anxiety disorders (Spierling and Zorrilla, 2017).

Determining the biochemical perturbations that disturb brain homeostasis at the cellular, tissue and organ level is critical to develop therapies for disorders that are mediated by the stress response. Recently, translational efforts in the AUD field have contributed to evaluating the role of CRF binding protein (CRFBP) as a potential target to modulate the stress system (Haass-Koffler, 2018; **Figure 1**). In preclinical settings, research has utilized *CRHBP* deficient (–/–) mice to examine phenotypic consequences of stress and the impact on stress-related behaviors (Ketchesin et al., 2017). In humans, genetic variations in CRFBP have been shown to predict antidepressant outcomes in randomized controlled trials (RCTs) (O'Connell et al., 2018). Interestingly, sequestration of CRF by CRFBP has been proposed as a target for Alzheimer's disease, due to its potential to restore normal stress functioning and improve symptoms of brain aging (Vandael and Gounko, 2019).

In this review, we will examine the role of CRFBP as a target to restore imbalances in brain homeostasis with the intent to treat brain illnesses. We will first review the role of CRFBP in the HPA axis stress response, briefly summarizing literature characterizing its actions within the central nervous system (CNS). Then, we will examine the research efforts in the AUD field, as a potential target for inhibiting stress-induced alcohol consumption. Finally, we will review the role of CRFBP as a potential target for treating other SUD, anxiety, depression, and some neurodegenerative disorders.

MECHANISTIC ROLE OF CORTICOTROPIN RELEASING FACTOR BINDING PROTEIN IN THE STRESS RESPONSE

After a stress stimulus, the initial secretion of CRF from the PVN stimulates the anterior pituitary gland to release adrenocorticotropic hormone (ACTH) in the systemic circulation (Raff, 1993). The release of ACTH then triggers the adrenal cortex to release glucocorticoid (GC) hormones, which exert inhibitory feedback on the HPA axis to regulate its function (Keller-Wood and Dallman, 1984; Tasker and Herman, 2011). Thus, the HPA axis, from central factor activation, to mobilization of hormones into the peripheral pathways, provides multiple targets that have been the focus of many research studies over the last couple of decades (Pomara et al., 2003; Du and Pang, 2015; DeMorrow, 2018; Canet et al., 2019). Preclinical studies have strongly supported CRF as a key mediator of neuroendocrine and behavioral responses to stress (Gray, 1993; Heinrichs et al., 1995; Bale and Vale, 2004), particularly in AUD and SUD (Koob and Kreek, 2007; Koob and Le Moal, 2008; Mukhara et al., 2018). While research has primarily focused on the actions of CRF and GCs, CRFBP has been suggested as a promising novel target for the stress component of AUD and stress-related illnesses. Since its discovery (Linton et al., 1988) and isolation from the human plasma (Behan et al., 1989), CRFBP has been proposed as valuable stress scavenger due to its ability to prevent the excessive stress response through limiting and regulating CRF bioavailability in the HPA axis (Potter et al., 1991, 1992; Sutton et al., 1995).

Corticotropin releasing factor binding protein is a 37 kDA soluble, secreted glycoprotein that is highly conserved from invertebrates to humans (Haass-Koffler and Bartlett, 2012; Ketchesin et al., 2017). In humans and non-human primates, CRFBP is dispersed in discrete brain regions as well as peripherally in the liver and placenta (Potter et al., 1991, 1992; Petraglia et al., 1993; Kemp et al., 1998; Slater et al., 2016) where it regulates the function and bioavailability of CRF. In other mammals including rodents, CRFBP is not expressed in peripheral tissue, which has limited research on CRFBP's full mechanistic role in the stress system in preclinical studies

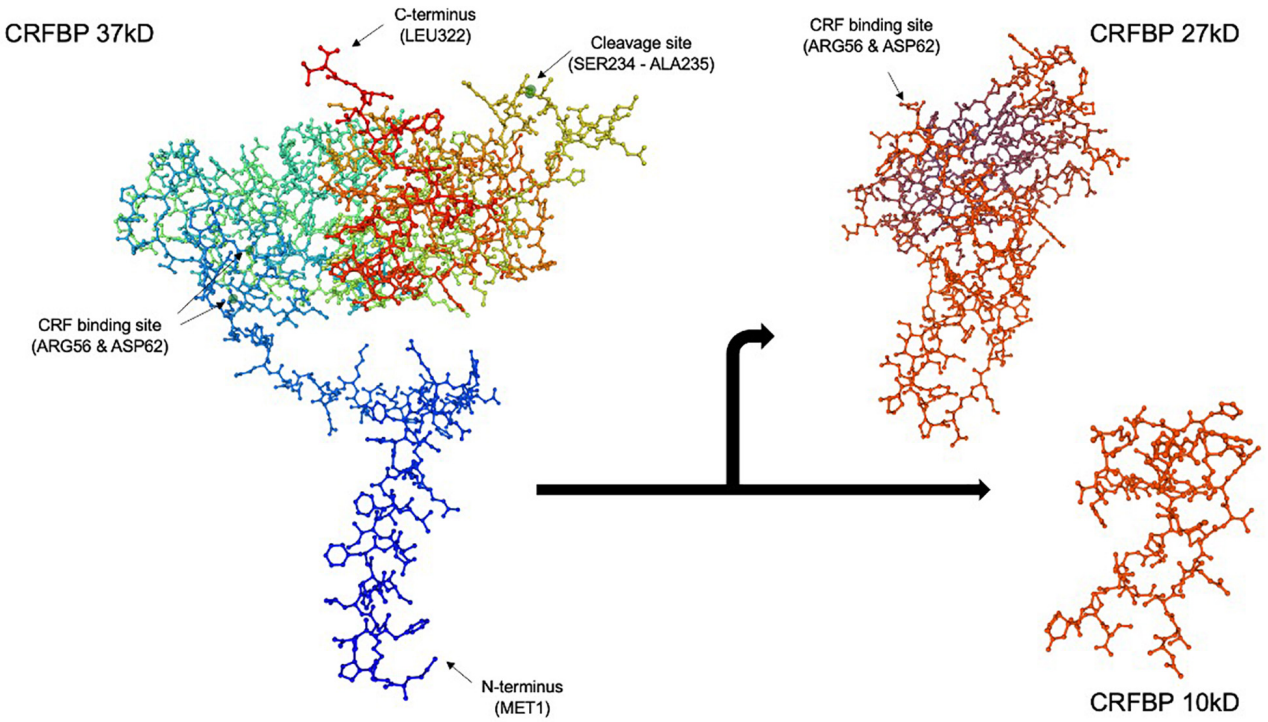

FIGURE 1 | Corticotropin releasing factor binding protein (CRFBP) 37 kD model by SWISS-MODEL protein structure homology. CRFBP is composed of 322 amino acid residues, spanning from the N-terminus at methionine 1 (MET1) to the C-terminus at leucine 322 (LEU322). Spontaneous, proteolytic cleavage occurs between amino acid residues serine 234 (SER234) and alanine 235 (ALA235), resulting in formation of the 27 kD N-terminal and 10 kD C-terminal fragments. The high affinity, CRF selective binding sites located at arginine 56 (ARG56) and aspartic acid 62 (ASP62) are retained by the 27 kD fragment. The 27 kD fragment, which acts as a scavenger of excess CRF, is believed to be responsible for the inhibitory role of CRFBP. Conversely, studies indicate the 10 kD fragment may possess excitatory functions through its selective interaction with $CRFR_2$. The full 322 amino acid CRFBP (37 kD), N-terminal fragment (27 kD) (reduced amino acid sequence 62-212), and C-terminal fragment (10 kD) (reduced amino acid sequence 45–85) figures were computed using SWISS-MODEL (Guex et al., 2009; Bienert et al., 2017; Waterhouse et al., 2018).

(Potter et al., 1991, 1992). CRFBP is also an extremely versatile protein that may interact, not only through sequestration of excess CRF, but by interacting with the CRF receptors in an allosteric manner. Interestingly, a potential approach to treat stress-related disorders would be by utilizing CNS-penetrant pharmacophores that may modulate the affinity of CRF for the CRF receptors, rather than blocking neural connectivities by binding to orthosteric sites (Haass-Koffler, 2018). The approach of disrupting a three-way interaction between ligand, receptor, and macromolecule, further supports the involvement of additional peptides that may influence the pathophysiology of the CRF system (Spierling and Zorrilla, 2017).

Corticotropin releasing factor binding protein is composed of 322 amino acid residues and approximately 70% undergoes proteolytic cleavage in the brain (Behan et al., 1995a; **Figure 1**). Spontaneous cleavage between amino acid residues serine 234 and alanine 235 yields an N-terminal fragment CRFBP (27 kD) and a C-terminal fragment CRFBP (10 kD), through a mechanism that has yet to be elucidated (Woods et al., 1999). The 27 kD fragment retains the CRF selective and high affinity binding site at arginine 56 and aspartic acid 62 (IC_{50} = 0.5 nM) (Woods et al., 1999). The biological role of the 10 kD fragment remains unknown.

In vitro studies indicate that CRFBP (27 kD) is responsible for the inactivation of "free" CRF, while CRFBP (10 kD) may have an excitatory role interacting selectively with $CRFR_2$ (Haass-Koffler et al., 2016). Interestingly, during stress induction, it has been hypothesized that CRFBP (27 kD) is unable to sequester the free CRF, and the CRF in excess with CRFBP (10 kD) allosterically potentiates $CRFR_2$ signaling in a three-way interaction (Haass-Koffler et al., 2016). To support this hypothesis, examination of the interaction between CRFBP and CRF_2 in cultured mesencephalic neurons demonstrated that CRFBP interacts with intracellular CRF_2 to increase presence of the receptor on the cell surface (Haass-Koffler et al., 2012), acting as a GPCR escort protein (Slater et al., 2016).

Electrophysiological studies showed that CRF potentiation of NMDAR (*N*-methyl-D-aspartate receptor)-mediated synaptic transmission in dopamine neurons of the ventral tegmental area (VTA) involves CRF_2 and requires CRFBP for potentiation (Ungless et al., 2003). In the VTA, NMDAR activity is believed to mediate stress-induced activation of the dopamine system (Overton and Clark, 1997), which is associated with alterations in behaviors underlying SUDs (Shaham et al., 2000). As such, this provides a plausible mechanism by which modulation of CRFBP can mediate stress-induced changes contributing

to SUDs. Further, double *in situ* hybridization of CRFBP in the VTA showed that CRFBP mRNA is expressed within a subset of dopaminergic neurons, but not in the neighboring substantia nigra pars compacta (SNC) (Wang and Morales, 2008). In summary, findings of *in vitro* studies indicate that complex mechanistic interactions between CRFBP, CRF, and CRF receptors play an important role in CRFBP's ability to regulate neurotransmission. The *ex vivo* studies showed that this effect may be relevant in brain regions such the VTA that have been implicated with AUD and SUD, and possibly also in neurodegenerative disorders (Ungless et al., 2003; Haass-Koffler, 2018). Of note, in mouse models of AD, degeneration of VTA dopaminergic neurons at pre-plaque stages contributes to memory deficits and dysfunction of reward processing (Nobili et al., 2017).

The development of the *CRHBP* deficient (–/–) mice allowed researchers to evaluate the impact of CRFBP on different bio-behavioral effects (Karolyi et al., 1999; Ketchesin et al., 2017). Early studies showed that *CRHBP* deficiency (–/–) in mice contributed to increased stress and anxiety-like behaviors compared to wildtype littermates (Karolyi et al., 1999). Interestingly, the same study found that *CRHBP* deficient (–/–) mice did not alter HPA axis functioning, as there were no significant differences in basal levels of corticosterone or ACTH compared to wildtype littermates (Karolyi et al., 1999). An additional transgenic mouse model which overexpressed CRFBP in the anterior pituitary gland, showed an increased motor activity, and a trend in decreased anxiety compared to wildtype littermates (Burrows et al., 1998). Similar to *CRHBP* deficient (–/–) mice, transgenic mice overexpressing pituitary CRFBP did not show any differences in basal levels of corticosterone or ACTH compared to wildtype littermates (Burrows et al., 1998; Lovejoy et al., 1998). However, significant elevation of both CRF and vasopressin in the PVN of the transgenic CRFBP overexpressing mice compared to littermates indicates that compensatory mechanisms occur in response to CRFBP overexpression in order to maintain homeostasis (Burrows et al., 1998). Further, when the HPA axis of transgenic mice overexpressing CRFBP was stimulated through injections of lipopolysaccharide, ACTH significantly decreased, indicating that higher levels of circulating CRFBP are needed to alter HPA axis function (Lovejoy et al., 1998).

Recently, it was reported sex-dependent mechanisms underlying the expression of CRFBP in rodents. A recent study in C57BL/6J mice found that hypothalamic CRFBP was upregulated in 12-month-old male mice compared to females, and conversely downregulated in female mice at 18 months (Locci et al., 2021). Similar sex-dependent changes were observed in CRFBP expression in the amygdala at different time points, in addition to sex-specific differences in acute restraint stress-induced alterations in CRFBP expression (Locci et al., 2021). This finding aligns with previous studies, which found that female C57BL/6J mice had greater CRFBP expression in the pituitary compared to male mice (Speert et al., 2002; Stinnett et al., 2015). Similarly, female adult Long Evans rats exhibit greater CRFBP expression in the medial septum compared to male rats, a brain region important for cognitive functioning (Wiersielis et al., 2019).

CORTICOTROPIN RELEASING FACTOR BINDING PROTEIN AND ALCOHOL USE DISORDER

Alcohol use disorder is characterized by habitual, excessive consumption of alcohol, leading to negative physical, emotional, and social consequences. Alcohol is unique from other drugs as it binds many different molecular targets and alters various neurotransmitter systems in the body, including endogenous opioids, γ-Aminobutyric acid (GABA), serotonin, glutamate, and dopamine (Lovinger, 1997; Trantham-Davidson and Chandler, 2015; Abrahao et al., 2017). The alcohol research field has increased its focus on the stress system, which has been identified as a major contributor to the development, progression, and reinforcement of behaviors underlying AUD (Brady and Sonne, 1999; Sarnyai et al., 2001). Importantly, researchers have found that both stress and alcohol activate the mesolimbic reward pathway, and regulate neurotransmitter activity in limbic brain regions, further supporting interest in mechanistic targets within the stress system (Haass-Koffler and Bartlett, 2012; Mukhara et al., 2018). Early studies focused on directly targeting CRF to mediate stress-induced alcohol-related behaviors (Rodriguez and Coveñas, 2017; Schreiber and Gilpin, 2018). However, more recently, researchers have examined the regulatory role of CRFBP in the CRF stress pathway as an indirect target to alter CRF levels and HPA axis activity (Haass-Koffler et al., 2016; Haass-Koffler, 2018). This interest has been further supported by findings of CRFBP expression in discrete subpopulations of dopaminergic and GABAergic neurons in the VTA (Wang and Morales, 2008), a brain pathway which is known to be important in regulating reward, reinforcement, and cognition in AUD (You et al., 2018).

Intra-VTA microinjections of the CRFBP antagonist, CRF_{6-33}, in C57BL/6J mice dose-dependently reduced ethanol consumption, suggesting that CRFBP antagonism in the VTA may contribute to increased alcohol consumption (Albrechet-Souza et al., 2015). Those data corroborate the previous electrophysiology studies that tested CRFBP's role in the VTA (Ungless et al., 2003), demonstrating that CRF requires CRFBP to potentiate NMDA receptors *via* CRF_2 (Ungless et al., 2003). A recent study that evaluated the colocalization of CRFBP and CRF2R in cultured rat mesencephalic neurons supports this early data and demonstrated that CRFBP facilitates the presence of CRF_2 on the cell surface (Slater et al., 2016).

The central nucleus of the amygdala (CeA) is an important limbic brain region relevant to AUD, as it has been identified as a hub for negative emotional circuitry and alcohol reinstatement (Roberto et al., 2012; Simms et al., 2012). However, studies investigating the role of CRFBP in the CeA have cast inconsistent results. Early findings in rats examining expression of CRFBP mRNA expression found that CRFBP is localized in key limbic regions, including the CeA (Potter et al., 1992). Pharmacological approaches, using microinjections of the CRFBP antagonist, CRF_{6-33}, into the CeA of C57BL/6J mice were found to

have no effect on ethanol intake (Albrechet-Souza et al., 2015). Downregulating CRFBP in the CeA using lentivirus vectors and sequences encoding small hairpin RNAs (shRNAs) targeting the *CRHBP* gene, decreased ethanol consumption in rats (Haass-Koffler et al., 2016). The downregulation of *CRHBP* in the CeA, however, was unable to block the subsequent challenge by yohimbine-induced ethanol self-administration (Haass-Koffler et al., 2016). This effect was reflected in a functional MRI experiment which showed that in the CeA there was a lower hemodynamic activation in *CRHBP* shRNA rats at baseline compared to controls (*Scr* shRNA) and after yohimbine administration there was an increase in hemodynamic activation in the *Scr* shRNA rats only. Those results were also corroborated by the fact that in other brain regions known to be activated after yohimbine administration (e.g., caudate putamen), there was hemodynamic activation in both *CRHBP* and *Scr* shRNA rats (Haass-Koffler et al., 2016). Altogether, these findings suggest that downregulation of CRFBP in the CeA alone may not be sufficient to mediate stress-induced alcohol consumption, particularly during noradrenergic activation (Haass-Koffler et al., 2016, 2018).

In preclinical alcohol behavioral studies, the impact of binge drinking on CRFBP expression across relevant brain regions was examined in C57BL/6J mice using repeated drinking-in-the-dark (DID) cycles. *In situ* hybridization findings indicated that repetitive binge drinking significantly decreases CRFBP mRNA expression in the prelimbic (PL) and infralimbic medial prefrontal cortex (mPFC) after three cycles of DID, and in the PL mPFC after six cycles (Ketchesin et al., 2016). Notably, there were no observed changes in CRF or CRF_1 mRNA in the mPFC, VTA, bed nucleus of the stria terminalis, or amygdala (Ketchesin et al., 2016). The DID paradigm was also paired to the CRFBP transgenic mouse model, showing that alcohol consumption was significantly higher in *CRHBP* deficient (–/–) mice compared to their wildtype littermates. This effect was statistically significant after repeated DID sessions with a strong distinction after the sixth DID exposure (Haass-Koffler et al., 2016). Interestingly, studies examining acute restraint stress observed increased CRFBP mRNA expression in the rodent pituitary (McClennen et al., 1998; Stinnett et al., 2015) and amygdalar regions (Lombardo et al., 2001; Herringa et al., 2004; Roseboom et al., 2007); however, chronic restraint was associated with decreases in amygdalar CRFBP mRNA expression (Pisarska et al., 2000). These findings highlight different mechanisms through which acute and chronic stress regulate central expression of CRFBP.

Overall, these findings represent promising preliminary evidence to support further preclinical investigation of CRFBP as a target for alcohol-related behaviors. Specifically, future studies should focus on examining robust models of behavioral and pharmacologically-induced stress, to further elucidate the mechanistic role of CRFBP in AUD, and its ability to mediate dysregulation of the stress response within preclinical models of AUD.

As CRFBP is still a novel therapeutic target undergoing investigation, clinically relevant research examining CRFBP in AUD remains limited to human genetic studies. Translation of *in vitro* findings to human genetics research has led to preliminary evaluation of several single nucleotide polymorphisms (SNPs) of the *CRHBP* gene. Studies that evaluated SNPs that reside in the *CRHBP* (10 kD) gene have shown to be associated with stress-induced craving in non-treatment-seeking individuals who drink heavily (Ray, 2011; **Table 1**). A study examining individuals with AUD found associations between three SNPs (*rs10062367, rs7718461,* and *rs10055255)* and alcohol and anxiety-related phenotypes (Haass-Koffler et al., 2016; **Table 1**). One of the SNPs (*rs10062367)* was associated with increased risk of these phenotypes in African and European individuals, while the other two SNPs (*rs7718461* and *rs10055255)* were found to be linked to decreased anxiety-related phenotypes in Europeans (Haass-Koffler et al., 2016; **Table 1**).

Furthermore, polymorphism in the CRFBP10 kD has been observed in individuals with diagnosis of AUD (Enoch et al., 2008) and suicide risk (Roy et al., 2012). In individuals with AUD, two SNPs (*rs10474485* and *rs1715747*) in the *CRHBP* gene were significantly associated with increased depressive symptoms scores (Kertes et al., 2011; **Table 1**). A subsequent study found that *CRHBP* genotype (*rs10055255*) moderated the relationship between stress-induced negative affect, and the negative consequences of drinking in young adults who drink heavily (Tartter and Ray, 2012; **Table 1**). These studies further support the role of CRFBP not only in AUD, but other mental health outcomes.

Although findings linking genetic polymorphisms to stress and AUD are promising for the development of diagnostic markers, prognostic tools and therapeutic development, additional translational research is needed to identify clinically translatable approaches for targeting CRFBP in humans.

POTENTIAL ROLE OF CORTICOTROPIN RELEASING FACTOR BINDING PROTEIN IN OTHER STRESS-RELATED PSYCHIATRIC DISORDERS

As stress has been well established to play a role in the pathogenesis of various neuropsychiatric disorders, many studies over the years have focused on targeting the CRF system to mediate dysregulation of the HPA axis (Binder and Nemeroff, 2010; Haass-Koffler and Bartlett, 2012). However, despite promising preclinical findings, clinical trials targeting CRF and CRF_1 have shown limited or negligent success in treating a range of psychiatric conditions, including SUD (Lijffijt et al., 2014), anxiety and depressive, and neurodegenerative disorders (Sanders and Nemeroff, 2016; Spierling and Zorrilla, 2017). Thus, targeting of CRFBP may similarly hold promise for the treatment of the stress-related components of these disorders.

The role of CRFBP in seeking of other drugs has also undergone limited investigation within rodent models. For example, the role of VTA CRFBP in stress-induced reinstatement of cocaine seeking was examined in Long Evans rats (Wang et al., 2007). The study found that perfusing CRF-like agonists (CRF, UCN I, and mouse UCN II) which avidly bind CRFBP, increased glutamate and dopamine levels within the VTA, and

TABLE 1 | Association between single nucleotide polymorphisms (SNPs) of the *CRHBP* gene, psychiatric and neurodegenerative disorders.

CRHBP SNP	Allele	*N*	Ancestry	Diagnosis	Outcome	Risk	References
rs10062367	A	476	Full	AUD	Alcohol consumption	↑	Haass-Koffler et al., 2016
		206	African			↑	
		86	European		Anxiety symptoms	↑	
rs7718461	A	220	European		Anxiety symptoms	↓	
rs1053989	C	223			Neuroticism	↓	
		221			Anxiety symptoms	↓	
rs10474485	–	554	European	AUD	Depressive symptoms	↑	Kertes et al., 2011
rs1715747	G				Depressive symptoms	↑	
rs10055255	T	41	Full	AUD	Stress-induced negative affect and negative consequences of drinking	↑	Tartter and Ray, 2012
rs1500	C	351	NS*	OUD and CUD	Cocaine and benzodiazepine use	↑	Peles et al., 2019
rs3792738	A	336	Asian	HUD	Stress symptoms	↓	Su et al., 2018
rs1875999	T	177	European	MDD and matched controls	MDD symptoms	↑	Claes et al., 2003
rs7728378							
rs28365143	G	636	Full	MDD	Depression symptoms	↓	O'Connell et al., 2018
rs10473984	T	1953	Full	MDD with anxious depressive symptoms	Treatment response	↓	Binder et al., 2010
		399	African and Hispanic				

CUD, cocaine use disorder; HUD, heroin use disorder; MDD, major depressive disorder; NS, not specified; OUD, opioid use disorder.*

also reinstated cocaine seeking (Wang et al., 2007). However, intra-VTA infusions of agents which do not bind CRFBP (stressin I, UCN III, and ovine CRF) did not increase the neurotransmitter levels, nor did they reinstate cocaine seeking (Wang et al., 2007). The finding that CRFBP agonism in the VTA increases drug seeking compliments research in the AUD field, which found that antagonism of CRFBP in the VTA reduces alcohol consumption (Albrechet-Souza et al., 2015).

In individuals with concurrent opioid and cocaine use disorder who underwent methadone maintenance therapy for at least a year, the *CRHBP* SNP (*rs1500* C allele) was linked with cocaine and benzodiazepine consumption (Peles et al., 2019). The *rs1500* C allele was a predictor of cocaine and benzodiazepine 1 year into treatment (Peles et al., 2019; **Table 1**).

Similarly, associations between the stress pathway and heroin use have been identified through analysis of polymorphisms in *CRHBP* (Su et al., 2018). A study examining nine SNPs in stress-related genes in abstinent individuals with heroin use disorder and healthy controls, found no genotypic differences between these groups, however, multivariate regression analysis identified one *CRHBP* variant (*rs3792738)*, as a predictor risk of heroin relapse in patients with chronic stress (Su et al., 2018; **Table 1**).

Identification of variations in the *CRHBP* gene and stress pathway have been associated with development of several SUDs and relapse susceptibility (Levran et al., 2014, 2018), which holds promise for the development of personalized therapeutic treatments. However, those results are limited to human genetic study targeting individual SNPs. Extensive genome-wide association studies (GWAS) approaches within different SUDs are necessary to further elucidate the mechanistic role of CRFBP in this population.

The link between the CRF system and anxiety and depressive disorders has been well supported in preclinical studies (Arborelius et al., 1999). As CRFBP is found in all CNS CRF-related pathways and the pituitary, research investigating the role of CRFBP specifically in anxiety disorders to support development of pharmacotherapies is of great interest. Early studies in patients with major depressive disorder (MDD) identified several SNPs associated with vulnerability for major depression (Claes et al., 2003; Van Den Eede et al., 2007). In an isolated population of Swedish individuals with recurrent MDD and matched controls, two SNPs (*rs1875999* and *rs7728378*) in the *CRHBP* gene were found to be significantly associated with occurrence of MDD in the patients with the variant compared to control individuals (Claes et al., 2003; **Table 1**). In a follow up study, an extended sample of Swedish patients and an independent sample of Belgian patients with MDD (and their respective matched controls) were examined to replicate and confirm the original findings (Van Den Eede et al., 2007). Unfortunately, the findings could not be replicated in the independent Belgian population, and the results of the extended Swedish population differed from the original study in that only an overall trend was observed. However, analysis of sex differences identified significant associations of three *CRHBP* SNPs (*rs7728378*, *rs1875999,* and *rs1052967*) with occurrence of MDD in Swedish males (Van Den Eede et al., 2007; **Table 1**). These observed sex differences in humans are in line with preclinical findings, which indicated sexual dimorphisms exist in the expression of *CRHBP* in the pituitary of mice (Speert et al., 2002).

More recently, researchers have expanded investigation of the *CRHBP* gene to assess the role that genetic variants play in response to antidepressant medications. In individuals with non-psychotic MDD, without comorbid substance or psychiatric conditions, 16 candidate HPA axis SNPs were investigated as predictors of treatment outcomes for three

antidepressants (escitalopram, sertraline and venlafaxine-XR) (O'Connell et al., 2018). Only one of the 16 SNPs, (*CRHBP rs28365143*) was found to significantly predict reduction in Hamilton Depression Rating Scale scores (O'Connell et al., 2018; **Table 1**). Individuals with the *rs28365143* homozygous G allele, reported significantly better outcomes than A allele carriers, notably only for the escitalopram and sertraline antidepressants (both selective serotonin reuptake inhibitor, SSRIs), while no association was found with venlafaxine-XR treatment (serotonin-norepinephrine reuptake inhibitor, SNRIs) (O'Connell et al., 2018; **Table 1**). There has long been controversy within the field as to whether SSRIs or SNRIs are more effective in treating depressive disorders, as findings have been highly variable (Thase, 2008). Genetic variations in the *CRHBP* gene could provide additional insight into these differential responses, supporting the development of personalized medicine approaches for pharmacological treatments.

This approach can be further informed through investigation of *CRHBP* genetic variations among diverse populations. For example, a study of European American, African American, and Hispanic patients with non-psychotic MDD examined associations of genetic variants in 10 genes that regulate the CRF and arginine vasopressin systems with antidepressant treatment response (citalopram, SSRI) (Binder et al., 2010). Of these 10 genes, only one SNP *rs10473984* within the *CRHBP* locus was significantly associated with treatment outcome (remission and reduction in depressive symptoms) in response to citalopram (Binder et al., 2010). In African Americans and Hispanics, *rs10473984* T allele was associated with worse treatment outcomes compared to European Americans (although population stratification and admixture among ethnic groups present possible confounding variables) (Binder et al., 2010; **Table 1**). This observation may position ethnic background as an important factor to be considered in CRFBP's role in antidepressant treatment response.

Another relevant consideration is the high occurrence of comorbid depressive disorder and SUD (Currie et al., 2005; Brière et al., 2014). Investigation of *CRHBP* genetic polymorphisms can provide additional insight on the role that *CRHBP* variants play in genetic risk for comorbid depression and other psychiatric conditions. For example, a study examining the history of depressive symptoms in individuals with AUD analyzed markers in 120 candidate genes relevant to SUD's and anxiety or depression (Kertes et al., 2011). Of the 1,350 SNPs assessed, only three met or exceeded the significance threshold for association with depressive symptom scores (*CRHBP rs1715747, GABRB1 rs4315750, and OPRM1 rs650245*), although interestingly none of the analyzed markers were associated with AUD symptom scores (Kertes et al., 2011; **Table 1**).

Overall, these findings support a link between CRFBP and anxiety and depressive disorders. They also highlight the importance of investigating additional population variables, such as ethnic background and sex to inform the development of personalized medicine. Future studies should seek to further utilize preclinical models to examine the mechanistic contributions of CRFBP to the development of anxiety and depressive illnesses.

POTENTIAL ROLE OF CORTICOTROPIN RELEASING FACTOR BINDING PROTEIN IN OTHER STRESS-RELATED NEURODEGENERATIVE DISORDERS

Chronic stress exposure is believed to accelerate biological aging through mechanistic changes leading to cellular senescence (Yegorov et al., 2020). A review of preclinical and clinical studies suggests that psychological stress increases neuroinflammation, enhancing pro-inflammatory cytokine signaling (Salim, 2016). Increased pro-inflammatory cytokine levels may contribute to oxidative stress and cellular damage through promoting production of reactive oxygen species (ROS) (Salim, 2016), which has been linked to accelerated aging (El Assar et al., 2017). Further, shortening of telomeres, which is considered a hallmark of aging (Jiang et al., 2007), is associated with oxidative stress (Epel et al., 2004). Thus, similar to the proposed role of CRFBP in mediating stress-induced alcohol behaviors, CRFBP may also hold potential for mediating stress-induced acceleration of aging and age-related disorders.

Corticotropin releasing factor binding protein has been suggested as a molecular mechanism linking stress and age-related neurodegenerative disorders, particularly Alzheimer's disease (AD) (Vandael and Gounko, 2019). Early *in vitro* studies showed that astrocytes express and regulate the release of CRFBP (Behan et al., 1995c; Maciejewski et al., 1996; McClennen and Seasholtz, 1999). Importantly, by contiguously connecting the CNS and regulating neuronal metabolism and maintaining brain homeostasis, astrocytes also have a well-established role in the pathogenesis of neurodegenerative disorders (Li et al., 2011; Ben Haim et al., 2015; Phatnani and Maniatis, 2015). Thus, targeting of astrocytic CRFBP provides a plausible mechanism to mediate the contribution of astrocytes to neurodegenerative diseases (Vandael and Gounko, 2019).

An early study in the *postmortem* brain tissue of individuals with AD and healthy controls found that there was no difference in CRFBP levels measured between groups, except for a significant decrease in CRFBP observed in Brodmann area (BA) 39 (Behan et al., 1997). They did, however, find that both complexed CRF (BA 8, BA 9, BA 22, BA 39, nucleus basalis, and globus pallidus) and free CRF (A 4, BA 39, and caudate) levels were significantly decreased in AD patients compared to controls (Behan et al., 1997). This finding holds importance for targeting of CRFBP to treat AD, as modulating CRFBP could mediate the progression of AD pathology through increasing available CRF. This hypothesis was explored in another study, which again examined *postmortem* tissue of individuals with AD and healthy controls, and further utilized a CRFBP antagonist (CRF_{6-33}) to dissociate CRF from CRFBP in a rodent model (Behan et al., 1995b). Immunoassay analysis of standardized brain regions from *postmortem* human tissue similarly showed no difference in CRFBP expression between AD and control brains, however, CRF was significantly decreased in the frontal, parietal, and temporal brain regions of AD patients compared to control (Behan et al., 1995b). In rats, administration of the CRFBP antagonist (CRF_{6-33}) was found to significantly improve

learning and memory (Morris water maze), without significantly altering stress (elevated plus maze) (Behan et al., 1995b). Another study using transgenic mice, overexpressing CRFBP in the pituitary, observed an increase in CRF and increased motor activity, which could provide a method to mediate HPA axis dysfunction associated with AD (Burrows et al., 1998).

Together, these findings further implicate modulation of CRFBP as a possible target to diminish age-related dysfunction in the brain, accelerated by chronic stress. However, research within preclinical rodent models of AD is necessary to better represent the impact of CRFBP on the AD pathology. Additionally, targeting CRFBP to alter neurotransmission in the dopaminergic neurons of the VTA may hold promise for mediating AD associated alterations in these neurons, which contribute to memory and reward dysfunction (Nobili et al., 2017).

Similarly, the implication of stress in the development of Parkinson's disease (PD) supports potential targeting of CRFBP to mediate the development and progression of PD (Miller and O'Callaghan, 2008). PD is a progressive neurodegenerative disease characterized by degeneration of dopaminergic neurons in the striatum, leading to motor dysfunction (DeMaagd and Philip, 2015). Targeting CRFBP may reduce progression of PD and symptoms, through altering dopaminergic signaling and function in key brain regions. Particularly relevant are preliminary findings of CRFBP overexpression in the pituitary of mice, which resulted in increased motor activity (Burrows et al., 1998). Future research should examine potential mechanisms by which modulation of CRFBP may alter neuronal survival in neurodegenerative conditions, as well as the ability of CRFBP to mediate the consequences of chronic stress which contribute to age-related disorders.

Studies examining age- and disease-related neurodegeneration are particularly important to AUD research as well, as there is high comorbidity between AUD and dementia, yet the elderly remain largely overlooked within AUD research (Caputo et al., 2012).

CONCLUSION

Dysregulation of the CRF stress system is highly associated with psychiatric and neurodegenerative disorders. Over the years, various peptides within the CRF system have been investigated as potential targets for treatment, however, have shown limited efficacy in clinical translation. CRFBP is a novel target to mediate the contribution of stress related dysfunction to these conditions. Although a majority of the research on CRFBP within disorders has been focused on AUD, preliminary findings are also relevant to other stress-related psychiatric conditions and age-related neurodegenerative diseases. Further, there is a common occurrence of comorbidity between stress-related disorders and psychiatric conditions, which implicates CRFBP as a therapeutic target to treat the common mechanisms underlying many illnesses (Brady et al., 2000; McCauley et al., 2012; Flory and Yehuda, 2015). Given the promising preliminary findings, further preclinical and clinical research elucidating the role of CRFBP in stress- and alcohol-related behaviors underlying AUD are necessary to provide support for CRFBP as a pharmacological target to treat AUD. This review provides an overview of CRFBP in other stress-related psychiatric disorders, including SUDs, anxiety and depressive conditions, and age-related neurodegenerative disorders. However, the reviewed literature highlights several knowledge gaps pertaining to our understanding of CRFBP. First, findings in discrete brain regions indicate that CRFBP's actions within the CNS may be both inhibitory and excitatory, requiring further examination to elucidate brain region-specific mechanisms which alter functioning. Additionally, in-depth investigation of CRFBP within preclinical models of SUDs and stress-related psychiatric and neurodegenerative disorders is required to better understand the role of CRFBP in mediating cognitive and memory deficits. Furthermore, there is a dearth of literature critically examining the sex-dependent mechanisms of CRFBP. In order to better inform personalized medicine approaches, future studies on CRFBP should include examination of sex as a biological variable, as well as consider the relevance of ethnic background and genetic polymorphisms as factors that may alter susceptibility to stress-related disorders. Advancements within the AUD research field relevant to this neuropeptide have the potential to inform further understanding and treatment for many conditions that are contributed to by chronic stress pathology.

AUTHOR CONTRIBUTIONS

DC and CH-K wrote the manuscript. CH-K supervised the project. AW and DS contributed to writing the manuscript. All authors approved the final version of the manuscript and agreed to be accountable for the content of the work.

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Viral-Mediated Knockdown of Nucleus Accumbens Shell PAC1 Receptor Promotes Excessive Alcohol Drinking in Alcohol-Preferring Rats

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Alcohol use disorder (AUD) is a chronic, relapsing disorder whose genetic and environmental susceptibility components are not fully understood. Neuropeptidergic signaling has been repeatedly implicated in modulating excessive alcohol drinking, especially within sub-regions of the striatum. Here, we investigated the potential involvement of the selective receptor for pituitary adenylate cyclase-activating polypeptide (PACAP), PAC1R, in the nucleus accumbens shell (NAcc Shell) in excessive alcohol drinking in alcohol-preferring rats, an established animal model of the genetic propensity for alcoholism. Scr:sP alcohol-preferring rats were trained to operantly self-administer alcohol and then either an AAV virus short-hairpin RNA (shRNA) targeted to knockdown PAC1R, or an AAV control virus were microinfused into the NAcc Shell. NAcc Shell PAC1R shRNA knockdown virus was confirmed to significantly decrease PAC1R levels in the NAcc Shell. The effects of NAcc Shell PAC1R shRNA knockdown on ethanol self-administration were investigated using a Fixed Ratio (FR) 1 and a Progressive Ratio (PR) schedule of reinforcement. The effect of PAC1R knockdown on self-administration of an alternative reinforcer, saccharin, was also assessed. The results showed that the reduction in PAC1R in the NAcc Shell led to excessive ethanol drinking, increased preference for ethanol, and higher motivation to drink. NAcc Shell PAC1R shRNA knockdown did not comparably increase saccharin self-administration, suggesting selectivity of action. These data suggest that NAcc Shell PAC1R may serves as a "brake" on alcohol drinking, and thereby the loss of function of PAC1R leads to excessive alcohol consumption. Therefore, the PACAP/PAC1R system may represent a novel target for the treatment of AUD.

Keywords: ethanol, self-administration, alcohol use disorder (AUD), PAC1R, pituitary adenylate cyclase activating polypeptide (PACAP), compulsive, addiction, neuropeptide

INTRODUCTION

Alcohol use disorder (AUD) is a chronic, relapsing disorder that affects over 14 million adults in the US (Substance Abuse and Mental Health Services Administration (SAMHSA), 2019), and is estimated to be responsible for 5.3% of global deaths (Shield et al., 2020). Both environmental and genetic factors contribute to AUD susceptibility, with the hereditability estimated at 50–60% of the total phenotypic variability (Reilly et al., 2017). Animal models of hereditary preference for alcohol are an invaluable tool for investigations into the genetic component of AUD (Ciccocioppo and Hyytia, 2006; Crabbe, 2014; Gessa, 2016). One such model, a line of alcohol-preferring rats genetically selected for heavy alcohol consumption, the Sardinian alcohol-preferring rats, was utilized in this study (Colombo et al., 2006; Sabino et al., 2007, 2011, 2013a,b; Blasio et al., 2015).

The neuropeptide pituitary adenylate cyclase activating polypeptide (PACAP) belongs to the neuropeptide superfamily that includes vasoactive intestinal peptide (VIP), growth hormone-releasing hormone (GHRH), and secretin (Sherwood et al., 2000). PACAP has been implicated in a large variety of homeostatic systems within the body, including energy metabolism, food intake, body temperature, neuronal survival, reproduction (Gray et al., 2002; Inglott et al., 2011; Resch et al., 2011, 2014; Iemolo et al., 2015; Ross et al., 2018), as well as in the body's response to stress and in several neuropsychiatric disorders (Hammack et al., 2009, 2010; Ressler et al., 2011; Dore et al., 2013; Mustafa et al., 2015; Seiglie et al., 2019; Ross et al., 2020; Varodayan et al., 2020, reviewed in Hammack and May, 2015; Lutfy and Shankar, 2019).

The PACAP system has recently been implicated in substance use disorders more generally, and in AUD specifically (for review, see Gargiulo et al., 2020; Stojakovic et al., 2020). Acute ethanol leads to an increase in the mRNA levels of the PACAP-selective receptor, PAC1R, via the Receptor for Activated C Kinase 1 (RACK1) scaffolding protein in cell lines (He et al., 2002). PACAP modulates the hypothermic effects of alcohol (Szabó et al., 1998; Tanaka et al., 2004), and tolerance to acute ethanol-induced ataxia (Moore et al., 1998). Recent reports provide evidence that PACAP-27 and PACAP-38 administration modulate home cage alcohol drinking (Gargiulo et al., 2021), and that ethanol exposure increases PACAP expression in the paraventricular nucleus of the thalamus (Gupta et al., 2018) and in the bed nucleus of the stria terminalis (Ferragud et al., 2021). In human studies, a single nucleotide polymorphism of the gene coding for PACAP, ADCYAP1, was linked to higher levels of alcohol intake in a Finnish population of social drinkers (Kovanen et al., 2010), and a variant of PAC1R was found to be associated with problematic alcohol use in women (Dragan et al., 2017).

The nucleus accumbens (NAcc) Shell is a key substrate for the actions of drugs and alcohol, playing a critical role in the establishment of their acute reinforcing effects and in incentive salience (Koob and Volkow, 2010). The NAcc Shell receives input from a number of other limbic and midbrain structures, including the infralimbic (IL) subregion of the medial prefrontal cortex, a projection proposed to be part of a "stop" circuitry in the context of drug and alcohol seeking (Koob and Volkow, 2016). Interestingly, immunoreactivity for PACAP is found in the NAcc Shell, as well as expression of PAC1R (Cauvin et al., 1991; Ghatei et al., 1993; Palkovits et al., 1995; Zhang et al., 2021).

This study aimed to investigate a potential contribution of the PAC1R specifically in the NAcc Shell in alcohol drinking in rats, using a viral vector-mediated knockdown.

MATERIALS AND METHODS

Animals

Subjects in this study were male rats derived from TSRI Sardinian alcohol-preferring rats (Scr:sP, 29–30th generation[1]) which were derived through intra-line breeding at The Scripps Research Institute from sP rats (32nd generations of selective breeding, courtesy Prof. G.L. Gessa, University of Cagliari, Italy). These animals were maintained without further selective breeding at Boston University, and housed in an AAALAC-approved vivarium with a 12 h light-dark cycle. Experiments were performed during the dark cycle. Regular rodent chow and water were available *ad libitum*. All procedures were approved by Boston University Medical Campus Institutional Animal Care and Use Committee and followed National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and *Principles of Laboratory Animal Care* guidelines.

Drugs

Ethanol (10% *w/v*) and saccharin (0.02% *w/v*) solutions were prepared in tap water using 95% ethyl alcohol and saccharin sodium salt hydrate (Sigma Aldrich, St. Louis, MO), respectively. Adeno-associated viruses (AAV) containing either a custom-designed PAC1R short-hairpin RNA for PAC1R with a green fluorescent protein or only a green fluorescent protein were: AAV1-CAG-rADCYAP1R1-shRNAmir-GFP ("AAV-PAC1R KD," Vigene Biosciences, Rockville, MD) and AAV1-CAG-GFP ("AAV-CTRL," Addgene, Watertown, MA).

Intracranial Surgery

Animals were intracranially injected bilaterally with either an AAV-PAC1R KD or an AAV-CTRL, via a 2 μl, 22-gauge syringe (Hamilton, Reno, NV). Coordinates used for the NAcc Shell injections were (in mm): AP: +1.45, ML: ±2.5 (6-degree angle), DV: −7.1, and a volume of 500 nl per side was infused. For all surgeries, the incisor bar was set to −3.3 mm from the interaural line (flat skull), as per the Paxinos atlas (Paxinos and Watson, 2007).

Apparatus for Operant Oral Self-Administration

The test chambers used for operant oral self-administration (Med Associates, Inc., St. Albans, VT) were located in

[1]http://rgd.mcw.edu/rgdweb/report/strain/main.html?id=2302666

sound-attenuating, ventilated cubicles (66 × 56 × 36 cm) (Blasio et al., 2015; Ferragud et al., 2021). Syringe pumps (Med Associates, St. Albans, VT) dispensed ethanol (or saccharin) and water into two stainless steel drinking cups mounted 2 cm above the grid floor in the middle of one side panel. Two retractable levers were located 3.2 cm to either side of the drinking cups. Fluid delivery and operant responses were controlled by microcomputers with 10 ms resolution.

Fixed Ratio-1 Schedule Self-Administration Procedure

FR-1 Training: Rats (n = 8/group) were first allowed continuous (24-h/day) home cage two-bottle choice access to ethanol (10% *w/v*, prepared using ethyl-alcohol and tap water) for 1 week, and then moved to limited access (2 h/day) for 4 days. During two-bottle choice training, rats always had access to both ethanol and water. Rats then were allowed two-choice operant self-administration access to ethanol and water for 1–3 overnight sessions (16 h, with food available *ad libitum*) until they reached approximately 100 lever presses for ethanol within a session. Subsequently, animals performed daily self-administration sessions (30 min) until stable responding was reached (<20% variation across three consecutive sessions). Across all sessions, lever presses had no scheduled consequences for 2.01 s after the activation of the pumps to avoid double-lever hits (Sabino et al., 2006, 2009).

FR-1 following AAV Infusion: Following stable performing in FR-1 operant sessions as described above, rats were matched based on baseline alcohol intake and body weight into two groups. Either AAV-PAC1R or AAV-GFP virus was infused into NAcc Shell bilaterally as described above. After 3 weeks of viral incubation, a time-course commonly used in rodent brain tissue to ensure sufficient viral infection (Reimsnider et al., 2007; Aschauer et al., 2013), 30 min self-administration sessions resumed for a total of 14 post-injection sessions (2 weeks). The preference for ethanol reported from these self-administration sessions is calculated as (total infusions (ml) for ethanol/[total infusions (ml) for ethanol + total infusions (ml) for water]) × 100.

Progressive Ratio Schedule Self-Administration Procedure

Following FR-1 self-administration, rats were allowed to self-administer 10% *w/v* ethanol under a PR schedule of reinforcement, where the number of responses required to deliver an ethanol reinforcer increases with successive deliveries [progression: response ratio = $4 \times (e^{\#\text{of reinforcer} \times 0.1}) - 3.8$, rounded to the nearest integer (Sabino et al., 2011)]. The session began with the completion of the first ratio, with the latency to complete the first ratio set to a maximum of 2 h or when rats had not completed a ratio for 14 min, as previously reported (Gilpin et al., 2008; Sabino et al., 2011). Three responses were required to start the session to avoid unintentional starts (PR schedule: 3, 1, 2, 2, 3, 3, 4, 5, 6, 7, 8, 9, 11, 12, 14, 16, 18, 20, 23, etc.). The last completed ratio was defined as the breakpoint. Responses on the inactive lever were also recorded.

Fixed Ratio-1 Saccharin Self-Administration Procedure

At the end of the PR ethanol self-administration experiment, rats were allowed to perform self-administration sessions as described above, but with a choice between a saccharin solution (0.02% *w/v*) and water. This concentration was chosen because it maintains responding rates that are the same as 10% *w/v* ethanol. One rat had to be euthanized prior to saccharin intake, reducing the PAC1R KD group to n = 7.

Immunohistochemistry

One hour after the final operant session, rats were deeply anesthetized with isoflurane and perfused transcardially with cold phosphate-buffered saline (PBS), followed by ice cold 4% paraformaldehyde (PFA). The brains were dissected, stored in 4% PFA overnight, and then placed in a 30% sucrose solution for 48 h. Coronal brain sections were cut at 40 μm using a cryostat. Every 6th section through the NAcc Shell (range: +2.28 to +0.84) was collected and used for immunohistochemistry to verify protein knockdown in a semi quantitative manner (non-calibrated to known protein concentration), as done previously (Musatov et al., 2006; Scheimann et al., 2019; You et al., 2019). Following Tris buffered saline (TBS) washes, sodium citrate buffer antigen retrieval was performed at 95 °C for 10 min. Sections were then incubated in a blocking solution for 1 h (5% normal goat serum, 0.2% Triton X-100 in TBS) and then incubated in primary antibodies in blocking solution for 48 hr at 4 °C (rabbit anti-PAC1R, AVR-003, 1:250, Alomone Labs, Jerusalem, Israel; chicken anti-GFP, ab13970, 1:1,000, Abcam, Cambridge, MA; for immunizing peptide validation see Supplementary Figure 1 in Varodayan et al. (2020). After TBS washes, secondary antibody incubation (anti-rabbit AF555, A21429 1:500, Invitrogen, Carlsbad, CA; anti-chicken AF488, 103-545-155 1:500, Jackson ImmunoResearch, West Grove, PA) was performed for 2 h at room temperature. Sections were then mounted and coverslipped with DAPI Hardset mounting medium (Vector Laboratories, Burlingame, CA).

Quantification of Immunohistochemistry

Representative images of staining were taken at a 20× magnification on an Olympus BX-51 microscope. On a subset of rats (n = 6/group), unbiased stereological counts of cells expressing PAC1R, GFP, or both in the NAcc Shell were performed as previously described (Dore et al., 2013; Ferragud et al., 2021). The area was outlined using an Olympus PlanApo N x2 objective with numerical aperture 0.08, and counting was performed with an Olympus UPlanFL N x20 objective with numerical aperture 0.75. Cells were counted by an experimenter blind to experimental group using the Optical Fractionator Workflow module in Stereo Investigator software (MBF Biosciences, Williston, VT). In this workflow, the following parameters were used: the grid frame and counting frame were 250 × 250 μm, the guard zone was 2 μm and the dissector height was 20 μm. The Stereo Investigator software was used to estimate the average mounted thickness of sections, and this value was used to estimate the total volume of the counted region, as well as

the total number of PAC1R+ cells. GFP staining of viral injection site was imaged at 2× on an Olympus BX-51 microscope for viral placement verification. One animal was excluded for incorrect placement (unilateral injection). The center of injection sites is included below in **Figure 1**, all included animals had expression contained to the NAcc Shell.

Statistical Analysis

Data from FR-1 and PR experiments were analyzed using two-way split plot analyses of variance (ANOVAs), with Group as a between-subjects factor, and Session as a within-subject factor. Pairwise *post hoc* comparisons were made using the Student–Newman–Keuls tests, Student's *t*-test was used when comparing two groups. Significance was set at $p \leq 0.05$. The software/graphic packages used were Statistica 7.0, and GraphPad 9.2.

RESULTS

NAcc Shell PAC1R shRNA Knockdown Induces Excessive Drinking in Scr:sP Alcohol-Preferring Rats

Following the FR-1 ethanol training sessions, rats were split into two groups, matched for body weight and average ethanol intake over the final three sessions before surgery [baseline: AAV-CTRL group 1.1 ± 0.1 g/kg, AAV-PAC1R KD group 1.0 ± 0.1 g/kg, $t(14) = 0.07$, $p = 0.95$]. Body weight remained relatively stable across sessions, and percent bodyweight change did not differ between groups [AAV-CTRL: 7.7 ± 1.56%, AAV-PAC1 KD: 5.1 ± 1.57%, $t(14) = 0.26$, n.s.]. Scr:sP rats with virally mediated NAcc Shell PAC1R shRNA knockdown drank significantly more ethanol [**Figure 2A**, Group: $F(1, 14) = 11.82$, $p \leq 0.01$, Group*Session: $F(13, 182) = 1.03$, n.s.] and displayed a higher ethanol preference [**Figure 2E**, Group: $F(1, 14) = 12.69$, $p \leq 0.01$, Group*Session: $F(13, 182) = 0.27$, n.s.], compared to rats infused with a control virus. This higher drinking was evident when comparing the cumulative alcohol intake across all sessions [**Figure 2B**, $t(14) = 3.44$, $p \leq 0.01$], and the average preference across all sessions [**Figure 2F**, $t(14) = 3.56$, $p \leq 0.01$]. Correspondingly, the AAV-PAC1R KD rats pressed for ethanol a greater number of times per session compared to AAV-CTRL [AAV-CTRL: 37.9 ± 3.51, AAV-PAC1 KD: 56.1 ± 5.46, $t(14) = 3, 38$, $p \leq 0.01$, data not shown]. NAcc Shell AAV-PAC1R KD rats drank significantly less water than AAV-CTRL rats across sessions [**Figure 2C**, Group: $F(1, 14) = 6.25$, $p \leq 0.05$, Group*Session: $F(13, 182) = 0.48$, n.s.], and cumulatively [**Figure 2D**, $t(14) = 2.500$, $p \leq 0.05$]. This decreased water drinking did not compensate for the high levels of ethanol intake by NAcc Shell PAC1R shRNA knockdown rats in terms of total fluid intake, which was, therefore, also increased compared to controls [Group: $F(1, 14) = 9.54$, $p \leq 0.001$, Group*Session: $F(13, 182) = 0.85$, n.s., data not shown].

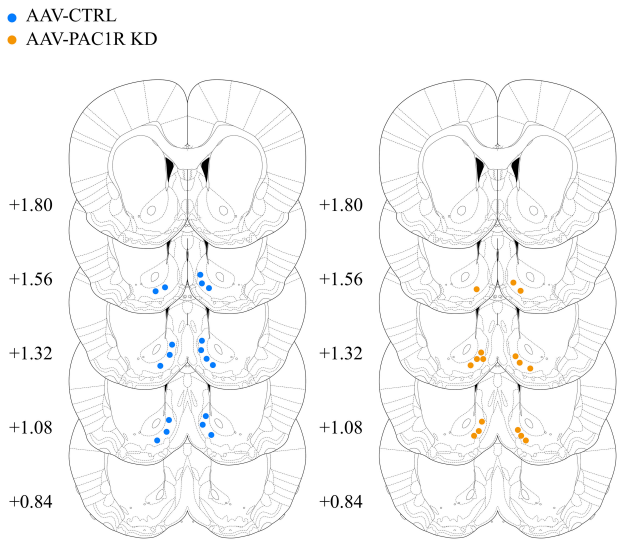

FIGURE 1 | Center of AAV injection sites in AAV-CTRL and AAV-PAC1R KD groups. All injections were restricted to the NAcc Shell.

NAcc Shell PAC1R shRNA Knockdown Increases Motivation of Scr:sP Rats to Drink Alcohol

As shown in **Figure 3**, NAcc Shell PAC1R knockdown increased the motivation to drink ethanol, as measured by breakpoint in a PR schedule of reinforcement [**Figure 3A**, Group: $F(1, 14) = 6.95$, $p \leq 0.05$, Group*Session: $F(2, 28) = 1.24$, n.s., **Figure 3B**, $t(14) = 2.36$, $p \leq 0.05$]. The total ethanol lever presses were also significantly higher in NAcc Shell PAC1R KD rats, compared to AAV-CTRL rats [**Figure 3C**: Group: $F(1, 14) = 6.15$, $p \leq 0.05$, Group*Session: $F(2, 28) = 0.85$, n.s., **Figure 3D**, $t(14) = 2.48$, $p \leq 0.05$]. The AAV-CTRL rats pressed the inactive lever more frequently than the AAV-PAC1 KD rats [**Figure 3E**, Group: $F(1, 14) = 5.36$, $p \leq 0.05$, Group*Session: $F(2, 28) = 0.70$, n.s., **Figure 3F**: $t(14) = 2.32$, $p \leq 0.05$].

NAcc Shell PAC1R shRNA Knockdown Does Not Affect Saccharin Self-Administration in Scr:sP Rats

Saccharin intake was not affected by NAcc Shell PAC1R shRNA knockdown, as shown in **Figure 4A** [Group: $F(1, 13) = 1.75$, n.s., Group*Session: $F(4, 52) = 1.83$, n.s.]. Cumulative saccharin intake across all session was also unaffected [**Figure 4B**, $t(13) = 1.32$, n.s.], as well as lever presses for saccharin [AAV-CTRL: 26.8 ± 4.30, AAV-PAC1 KD: 43.3 ± 11.00, $t(13) = 1.47$, n.s., data not shown]. Water intake during saccharin self-administration sessions also did not differ between groups [**Figure 4C**, Group: $F(1, 13) = 1.26$, n.s., Group*Session: $F(4, 52) = 0.79$, n.s., **Figure 4D**, $t(13) = 1.12$, n.s.].

Confirmation of NAcc Shell PAC1R shRNA Knockdown

As shown in **Figure 5**, NAcc Shell PAC1R knockdown was successful in reducing the number of PAC1R+ cells in the NAcc Shell [**Figure 5C**, $t(9) = 6.54$, $p \leq 0.001$]. The number of cells expressing PAC1R that were virally infected (GFP+) was

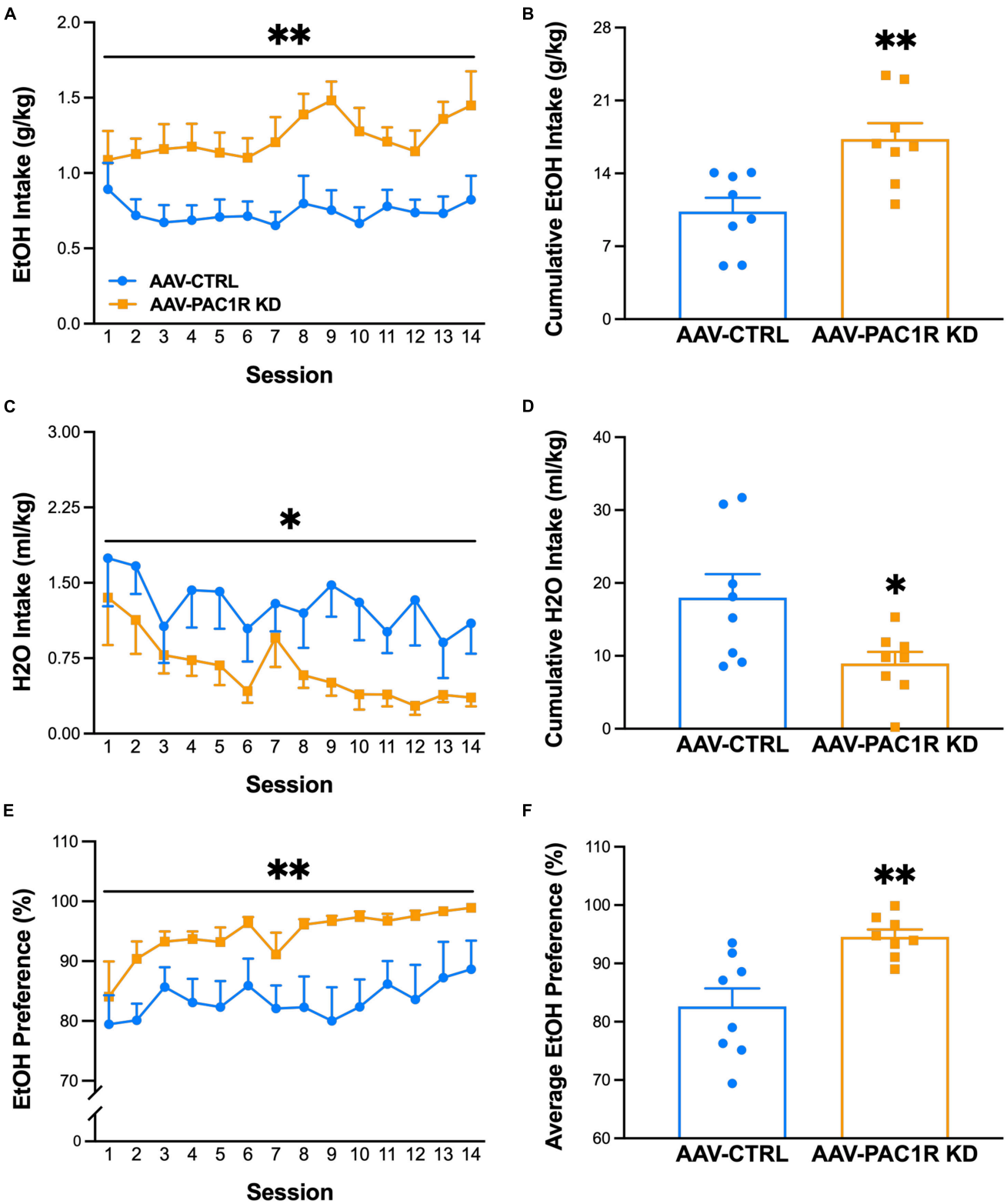

FIGURE 2 | Effect of AAV-mediated short-hairpin virus knockdown of PAC1R in the NAcc Shell in Scr:sP rats on ethanol self-administration in an FR-1 schedule of reinforcement (n = 8/group). **(A,B)** Rats with NAcc Shell PAC1R shRNA knockdown ("AAV-PAC1R KD") drank significantly more ethanol than control AAV rats ("AAV-CTRL"). **(C,D)** NAcc Shell AAV-PAC1R KD rats drank less water in later sessions. **(E,F)** NAcc Shell AAV-PAC1R KD rats also showed a higher preference for ethanol compared to AAV-CTRL rats. Data represent Mean ± SEM. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$.

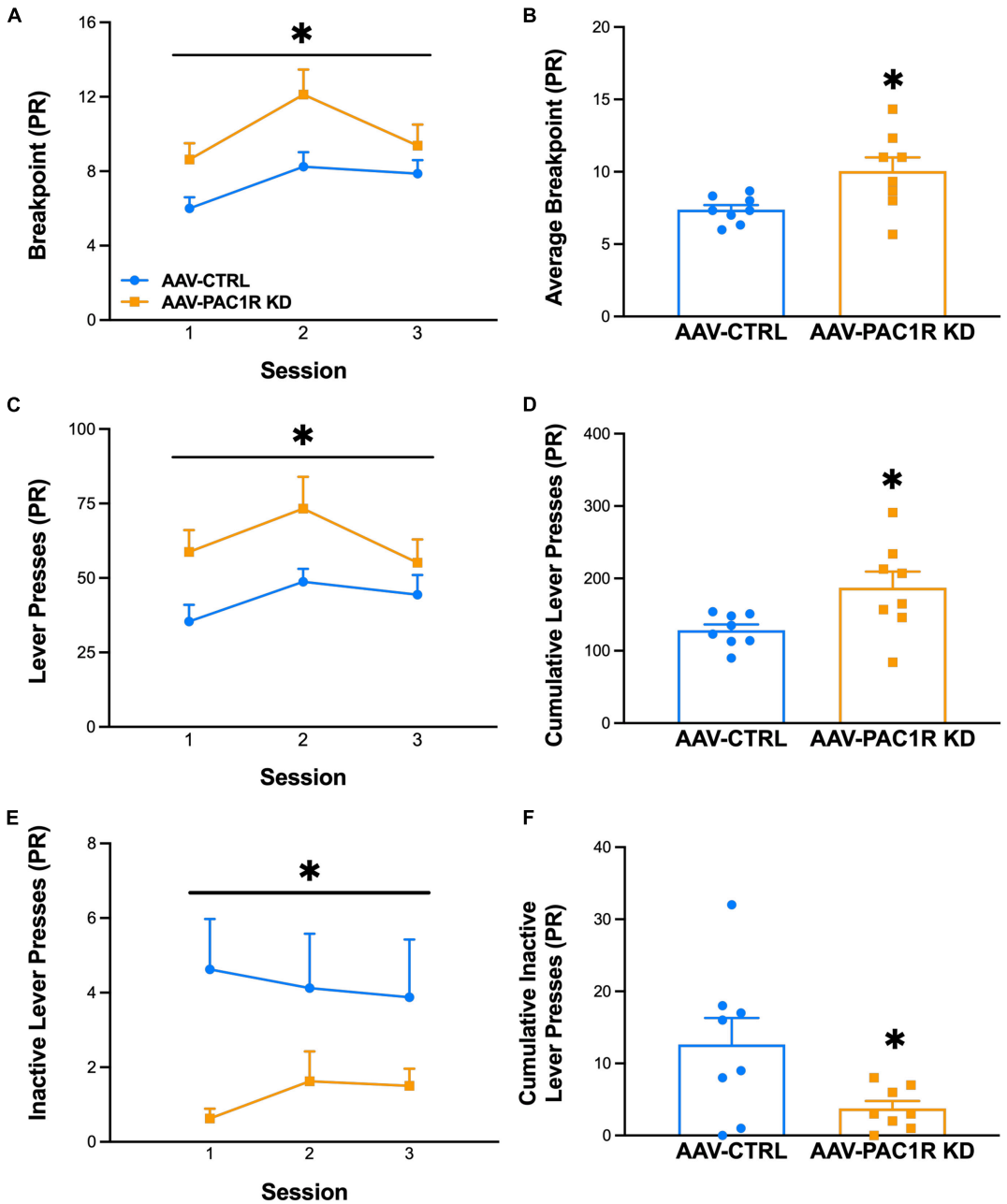

FIGURE 3 | Effect of NAcc Shell PAC1R shRNA knockdown on motivation to drink alcohol in a progressive ratio schedule of reinforcement (n = 8/group). **(A,B)** Rats with NAcc Shell PAC1R shRNA knockdown ("AAV-PAC1R KD") showed a higher breakpoint in the PR schedule, compared to control rats ("AAV-CTRL"). **(C,D)** NAcc Shell AAV-PAC1R KD rats also displayed higher lever presses for ethanol, compared to AAV-CTRL rats. **(E,F)** AAV-CTRL rats pressed the inactive lever more often than NAcc Shell AAV-PAC1R KD rats. Data represent Mean ± SEM. $^*p \leq 0.05$.

also significantly reduced in AAV-PAC1R KD rats compared to AAV-CTRL [**Figure 5D**, $t(9) = 9.00$, $p \leq 0.001$].

DISCUSSION

The main findings of this study were that NAcc Shell PAC1R knockdown via an AAV-shRNA led to: (1) higher self-administration of, and preference for, alcohol; (2) increased motivation to drink alcohol; (3) no significant change in self-administration of the alternative reinforcer saccharin; and (4) a decrease in PAC1R+ cells as well as in virally infected cells expressing PAC1R in the NAcc Shell, as compared to a control virus infusion. These findings suggest an important role for the PACAP/PAC1R system in the NAcc Shell in the control of alcohol drinking.

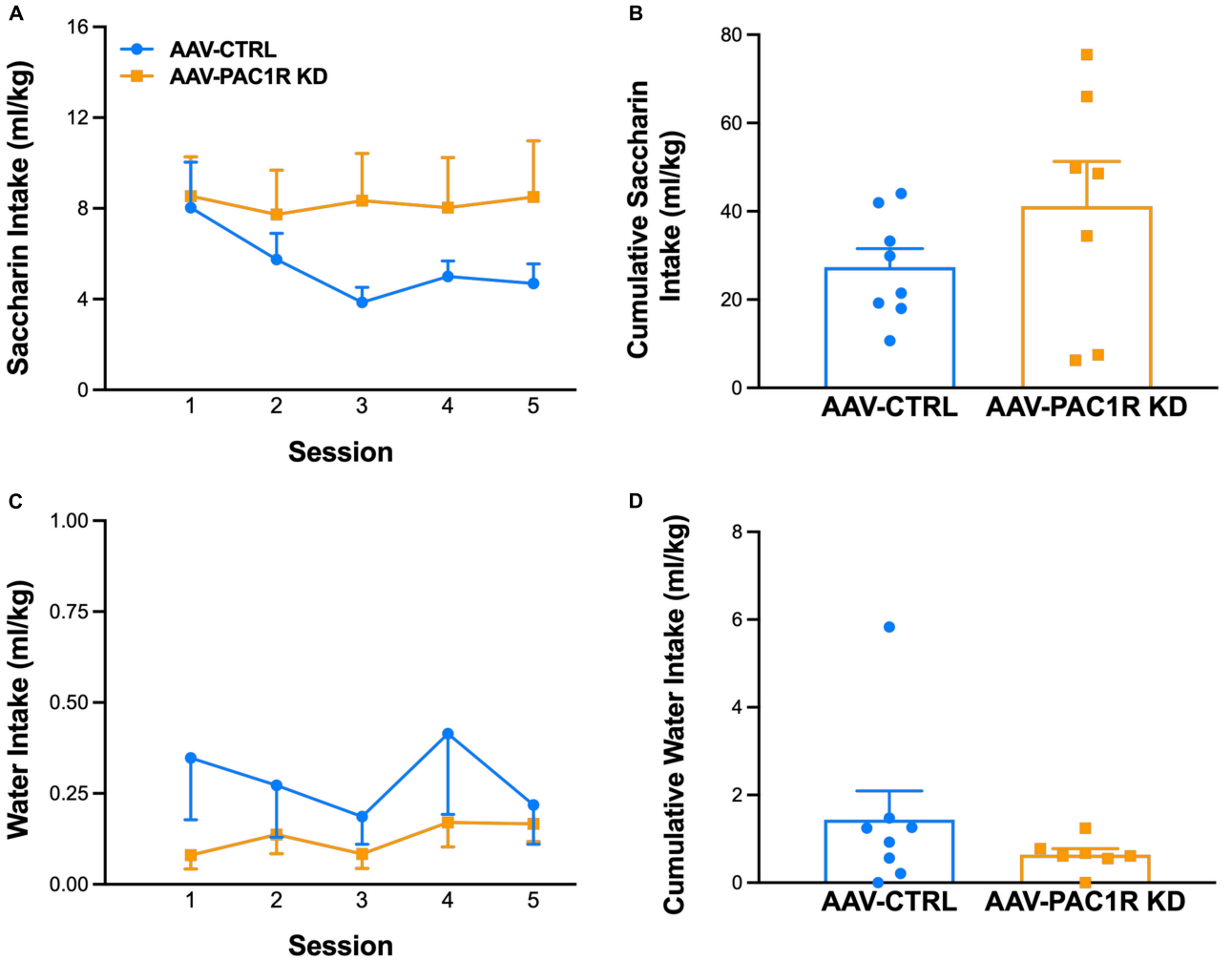

FIGURE 4 | Effect of NAcc Shell PAC1R shRNA knockdown on saccharin self-administration (FR-1 schedule) (*n* = 7–8/group). **(A,B)** Rats with NAcc Shell PAC1R shRNA knockdown ("AAV-PAC1R KD") and control rats ("AAV-CTRL") showed no significant differences in self-administration of the alternative reinforcer saccharin, or of the concurrently available water **(C,D)**. Data represent Mean ± SEM.

The Scr:sP rat line used in this study descends from the original Sardinian alcohol-preferring (sP) rat line, which was selectively bred from Wistar rats for its preference for alcohol, and it represents an established model of hereditary excessive alcohol drinking (Colombo et al., 2006; Sabino et al., 2011, 2013a,b; Blasio et al., 2015).

NAcc Shell shRNA PAC1R knockdown in Scr:sP rats led to significantly higher alcohol drinking and preference for alcohol in an FR-1 schedule of reinforcement, compared to rats with a control virus infusion. NAcc Shell PAC1R knockdown rats drank more and had a higher preference for alcohol compared to control rats beginning on the second and third daily session, respectively, and the two groups maintained this difference in ethanol intake over the course of the 14-day observation period. It should be noted that the control group started with one initial self-administration session similar to the shRNA PAC1R knockdown group following the 3-week AAV incubation period, and then stabilized at a lower level for the rest of the sessions, which is consistent with an alcohol deprivation effect, commonly seen in rats, including alcohol-preferring rats, after a period of abstinence (Heyser et al., 1997; Rodd et al., 2003; Vengeliene et al., 2003; Schroeder et al., 2005; Bell et al., 2006).

Interestingly, NAcc Shell PAC1R knockdown decreased water intake in the later sessions, which can be interpreted as a compensatory behavior for the increased ethanol intake to try and maintain the total fluid intake. The interpretation that water reduction was not a specific effect is supported by the data obtained in the saccharin experiment, where water intake was unaffected by the NAcc Shell PAC1R knockdown. Similar compensatory adjustments in water intake have been previously shown for several other drugs which modulate concurrent alcohol intake (Ericson et al., 1998; Rezvani et al., 2007; Steensland et al., 2007; Suchankova et al., 2013). In addition, we observed that NAcc Shell PAC1R knockdown increased the motivation to drink alcohol in a PR schedule. Under PR reinforcement schedules, ratio requirements increase

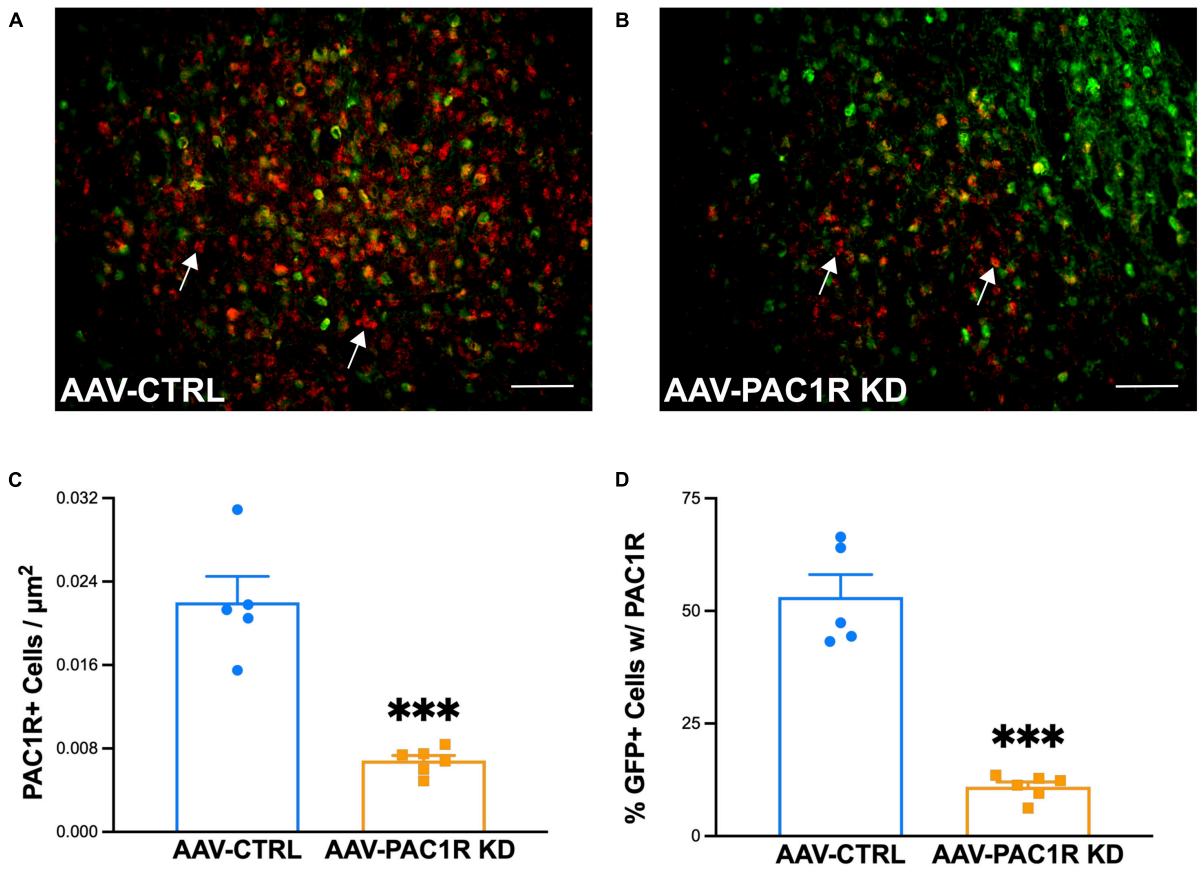

FIGURE 5 | Effect of NAcc Shell PAC1R shRNA knockdown on PAC1R levels in the NAcc Shell of rats (n = 6/group). **(A,B)** Representative images of PAC1R (red) and GFP (green) expression in the NAcc Shell of AAV-CTRL and AAV-PAC1R KD rats **(C)** NAcc Shell PAC1R shRNA knockdown rats ("AAV-PAC1R KD") showed a significant reduction in the number of PAC1R positive cells in the NAcc Shell, compared to AAV controls ("AAV-CTRL"). **(D)** NAcc Shell PAC1R shRNA knockdown also significantly reduced the number of PAC1R positive cells specifically in virally infected cells (GFP-labeled cells). White arrows point to example PAC1R-labeled cells. Scale bars represent 100 µm. Data represent Mean ± SEM. $^{***}p \leq 0.001$.

with subsequent reinforcer deliveries and the influence of local response rates on performance are reduced. NAcc Shell PAC1R knockdown reduced the break point, an objective measure of the effort an animal will expend to obtain a reinforcer that is sensitive both to the subjects' incentive state and to the reinforcer's stimulus properties (Walker et al., 2008).

In contrast to alcohol drinking, NAcc Shell shRNA PAC1R knockdown did not alter self-administration of an equally reinforcing saccharin solution. Saccharin is often used as an alternative reinforcing solution to assess the selectivity of the effects of a manipulation on alcohol intake vs. a more general one on all reinforcing substances (Sabino et al., 2009, 2011, 2013b; Valenza et al., 2016; Torruella-Suárez et al., 2020; Benvenuti et al., 2021; Kwok et al., 2021). An alternative interpretation of our findings could be that NAcc Shell shRNA PAC1R knockdown increases the palatability of alcohol, as prior reports have shown that PACAP administration in the NAcc reduces appetitive orofacial responses to sucrose and hedonic eating, although this finding was unique to the NAcc Core (Hurley et al., 2016, 2019). However, this is unlikely due to the similar saccharin self-administration in AAV-PAC1R KD and AAV-CTRL rats. As saccharin is not caloric, it is possible that the NAcc Shell shRNA PAC1R knockdown drives animals to increase caloric intake, although this seems unlikely in light of the fact that PACAP effects on hunger are mediated by hypothalamic regions (Resch et al., 2011, 2014; Hurley et al., 2019). Further, it has been previously reported that PACAP administration in the NAcc Shell does not affect sucrose intake (Gargiulo et al., 2021), further supporting the notion that the NAcc PACAP/PAC1R system plays a selective role in the effects of alcohol. Future studies may directly address the question of whether NAcc Shell PAC1R knockdown increases caloric intake.

PACAP does not appear to be synthetized within the NAcc (mRNA not found locally), and therefore an upstream source of PACAP onto NAcc PAC1R containing neurons is hypothesized (Köves et al., 1991; Masuo et al., 1993; Zhang et al., 2021). Major inputs to the NAcc originate from the medial prefrontal cortex (mPFC), which in the rat is comprised of dorsal (prelimbic; PrL) and ventral (infralimbic; IL) areas (Ongür and Price, 2000; Heidbreder and Groenewegen, 2003), that project to the NAcc Core and Shell, respectively (Barker et al., 2015; Koob and Volkow, 2016; Ma et al., 2020; Zinsmaier et al., 2021).

These differential glutamatergic inputs are thought to provide functional specialization to striatal sub-regions (Cox and Witten, 2019), and a "Stop and Go" system has been proposed, wherein the PrL to the NAcc Core pathway may mediate drug craving and habit formation, while an opposing pathway from the IL to the NAcc Shell may act as a brake on drug and alcohol intake, by exerting inhibitory control over this Go pathway (Peters et al., 2008; LaLumiere et al., 2012; Meinhardt et al., 2013; Ma et al., 2014; Koob and Volkow, 2016; George and Hope, 2017). In this framework, we might interpret our results as a loss of control over drinking caused by an impaired ability of PACAP projections to the NAcc Shell to inhibit alcohol drinking. This relevant upstream source may be the IL, as PACAP is abundantly expressed there (Zhang et al., 2021), but further studies would be required to confirm this hypothesis.

The exact mechanism whereby functional deletion of PAC1R in the NAcc Shell leads to an increase in alcohol intake is unknown. The PACAP/PAC1R has been shown to affect glutamatergic and GABAergic signaling in other brain areas, through pre- or post-synaptic mechanisms (Macdonald et al., 2005; Costa et al., 2009; Varodayan et al., 2020). PACAP is also capable of exerting effects on neuronal excitability in the absence of glutamatergic or GABAergic input (May et al., 2021). This highlights the need for follow-up electrophysiological studies to further understand the specific actions of PAC1R activation in the NAcc Shell.

PACAP has two isoforms, PACAP-38, which represents the vast majority of PACAP in the brain, and PACAP-27 (Miyata et al., 1989; Piggins et al., 1996). Our data are in agreement with a recent report showing that administration of the peptide agonist PACAP-27 in the NAcc Shell decreases ethanol drinking in Long-Evans rats, while PACAP(6–27) antagonism of PAC1R acutely increases ethanol drinking (Gargiulo et al., 2021). Interestingly though, in that study, PACAP-38 and PACAP(6–38) had no effect when infused in the NAcc Shell, suggesting that the increase in alcohol drinking following knockdown of PAC1R we observed here may reflect a PACAP-27 dependent mechanism, although PACAP-38 is known to represent over 90% of the total PACAP present in the brain (Piggins et al., 1996). Further, this previously published study has suggested that the sub-region of the NAcc Shell along the rostro-caudal axis targeted is important for the effects of PACAP, as PACAP administration in the rostral shell decreased ethanol drinking, while in the caudal shell it increased drinking (Gargiulo et al., 2021). In our study, the AAV-PAC1R KD virus was targeted to the rostral portion of the NAcc, therefore our findings are in line with this previous report. We add to this previous study by showing a PAC1R-dependent mechanism, as the antagonists used above [PACAP(6–27), PACAP(6–38)] have been shown not to be selective for PAC1R, as they also bind VPAC2R (Dickinson et al., 1997). Indeed, RNA interference is a very useful technique for long term tissue-specific knockdown of precise targets, and it also has potential for translational use in the treatment of human disease (Shan, 2010; Takizawa et al., 2010). Importantly, the AAV-PAC1R-KD virus used in this study leads to a knockdown of all PAC1R isoforms (Blechman and Levkowitz, 2013), as different isoform expression has been proposed to fluctuate over the course of alcohol use (Gargiulo et al., 2020). Future studies focused on the expression of specific PAC1R variants in alcohol-preferring and wildtype animals prior to and during ethanol exposure are of great interest.

One limitation of this study is that the experiments were performed solely on male rats. Interestingly, it has been shown that there are no differences in lever-pressing between male and female sP rats (Lorrai et al., 2019) and no sex differences in the effect of PACAP administration in the NAcc Shell on home cage alcohol drinking in Long-Evans rats (Gargiulo et al., 2021). However, future studies will have to be performed in female animals, considering the sex differences seen in NAcc physiology and morphology, as well as those in alcohol drinking (Kovanen et al., 2010; Dragan et al., 2017; Liu et al., 2020; Radke et al., 2021; Townsley et al., 2021).

Overall, this study provides evidence that the loss of function of PAC1R in the NAcc Shell leads to excessive drinking and heightened motivation to drink, and therefore suggests that the PACAP/PAC1R system in this area may work as a "brake" on excessive alcohol drinking.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee of the Boston University Medical Campus.

AUTHOR CONTRIBUTIONS

MM, VS, and PC designed the experiments. MM, TP, and ME performed the experiments. MM and VS analyzed the data. MM wrote a first draft of the manuscript. All authors edited the manuscript and approved the final version.

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The Role of Beta-Endorphin in Cocaine-Induced Conditioned Place Preference, Its Extinction, and Reinstatement in Male and Female Mice

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Endogenous opioids have been implicated in cocaine reward. However, the role of each opioid peptide in this regard is unknown. Notably, the role of each peptide in extinction and reinstatement is not fully characterized. Thus, we assessed whether cocaine-induced conditioned place preference (CPP) and its extinction and reinstatement would be altered in the absence of beta-endorphin. We also examined if sex-related differences would exist in these processes. Male and female mice lacking beta-endorphin and their respective controls were tested for baseline place preference on day 1. On day 2, mice were treated with saline/cocaine (15 mg/kg) and confined to the vehicle- or drug-paired chamber for 30 min, respectively. In the afternoon, mice were treated with the alternate treatment and confined to the opposite chamber. Mice were then tested for CPP on day 3. Mice then received additional conditioning on this day as well as on day 4. Mice were then tested for CPP on day 5. Mice then received extinction training on day 9. On day 10, mice were tested for extinction and then reinstatement of CPP following a priming dose of cocaine (7.5 mg/kg). Male and female mice lacking beta-endorphin did not exhibit CPP following single conditioning with cocaine. On the other hand, only male mice lacking beta-endorphin failed to show CPP after repeated conditioning. Nonetheless, reinstatement of CPP was blunted in both male and female mice lacking beta-endorphin compared to controls. The present results suggest that beta-endorphin plays a functional role in cocaine-induced CPP and its reinstatement, and sex-related differences exist in the regulatory action of beta-endorphin on the acquisition but not reinstatement of cocaine CPP.

Keywords: cocaine, beta-endorphin, conditioned place preference, extinction, reinstatement, sex

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INTRODUCTION

Cocaine addiction is a serious public health and socioeconomic challenge in the United States and many other countries around the globe. In 2001, it was estimated that roughly 1.2 million people in the United States consumed cocaine for the very first time (O'Brien and Anthony, 2005). In addition, in 2007, over 2 million people over the age of 12 were current cocaine users, and females

were found to be more susceptible to the addictive properties of cocaine (Kasperski et al., 2011). The prevalence of cocaine addiction in Europe and Latin America and other countries including the United States is of a major concern (Castells et al., 2016). Yet, there is no medication to effectively treat cocaine addiction (Castells et al., 2016; Pierce et al., 2018).

Opioid peptides (beta-endorphin, enkephalin, and dynorphin) and receptors (mu, delta, and kappa opioid receptors) are expressed in the central nervous system and are known to play a functional role in motivated behaviors, natural reward, and most importantly, drug reward (Shippenberg and Herz, 1986, 1987; Spanagel et al., 1990, 1992; Skoubis et al., 2001, 2005). For example, opioid receptor agonists increase the rewarding and reinforcing actions of psychostimulants, which have been demonstrated using self-administration and conditioned place preference (CPP) paradigms (Le Merrer et al., 2009). In contrast, opioid receptor antagonists have been shown to decrease the rewarding and reinforcing actions of cocaine (Houdi et al., 1989; Ramsey and van Ree, 1991; Suzuki et al., 1992; Gerrits et al., 1995; Kim et al., 1997; Rademacher and Steinpreis, 2002; Hummel et al., 2006), suggesting that endogenous opioid peptides may regulate these actions of cocaine. However, the role of each opioid peptide in the rewarding and reinforcing action of cocaine is not fully characterized.

Several studies have shown that beta-endorphin plays a functional role in the reinforcing actions of cocaine. For example, cocaine has been shown to cause the release of beta-endorphin in the nucleus accumbens (NAc; Olive et al., 2001; Roth-Deri et al., 2003, 2008), a response shown to be involved in cocaine self-administration (Roth-Deri et al., 2003, 2008). Endogenous beta-endorphin may also be involved in the rewarding action of cocaine. For instance, we have previously shown that the rewarding action of cocaine, using a single conditioning paradigm, was reduced in mice lacking beta-endorphin compared to their wild-type controls (Marquez et al., 2008; Nguyen et al., 2012), suggesting beta-endorphin may play a functional role in the rewarding action of acute cocaine. However, whether beta-endorphin plays a functional role in the rewarding action of cocaine after repeated administration of the drug is unclear.

Preclinical studies demonstrated that female rats develop cocaine-induced conditioned place preference (CPP) faster than male rats (for a review, see Becker and Koob, 2016). Female rats also acquire CPP at lower doses of cocaine (5, 10 mg/kg versus 20 mg/kg) than male rats (Russo et al., 2003). Sex-related differences have also been reported following intermittent- and long-term cocaine self-administration in adult rats (Algallal et al., 2020). This sex-related difference is also observed in adolescent (PND34) rats (Zakharova et al., 2009). However, the underlying mechanism of this sex-related difference in cocaine reward remains mostly unknown. Therefore, we also determined if sex-related differences exist in the rewarding action of cocaine and if endogenous beta-endorphin plays a functional role in cocaine reward after its acute or repeated administration.

Relapse represents a serious issue in the treatment of drug addiction. Craving can be easily triggered by any drug-associated cues, external environmental cues, or re-exposure to the drug itself (Bossert et al., 2013). Thus, understanding the underlying mechanisms of craving and relapse may aid in the development of medications to manage addiction. There is some evidence showing beta-endorphin is involved in extinction and drug seeking behaviors (del Cerro and Borrell, 1987; Roth-Deri et al., 2004; Simmons and Self, 2009). Thus, in the present study, we also determined the role of beta-endorphin in extinction and reinstatement. Considering the place conditioning paradigm is used as a measure of reward, extinction, and reinstatement (Bardo and Bevins, 2000; Mueller and Stewart, 2000; Botreau et al., 2006; Carey et al., 2007; Jackson et al., 2013), we used this paradigm to characterize the role of endogenous beta-endorphin in cocaine-induced CPP and its extinction and reinstatement. We also assessed if sex-related differences would exist in the regulatory action of beta-endorphin in these processes.

MATERIALS AND METHODS

Subjects

A total of 14 male and 16 female mice lacking beta-endorphin (Rubinstein et al., 1996) and their wild-type littermates/age-matched controls (n = 6–8 mice per genotype of each sex) bred in-house were used for all the experiments. A number of studies showed that there was no compensatory changes in the diurnal corticosterone levels, and other component of the hypothalamic-pituitary-adrenal axis (Rubinstein et al., 1996). Furthermore, the expression of opioid receptors in different brain regions and spinal cord (Mogil et al., 2000) or the total brain levels of mu opioid receptors (Slugg et al., 2000) were unchanged in these mice compared to their wild-type controls. The latter study also showed normal coupling of the mu opioid receptors to the potassium channels (Slugg et al., 2000). Moreover, the distribution and level of opioid peptides enkephalins and dynorphins seemed to be unchanged in mice lacking beta-endorphin compared to their wild-type controls (Rubinstein et al., 1996). The breeding pairs were originally obtained from Jackson Laboratories (Bar Harbor, ME, United States). Pups were weaned at the age of 21 days and genotyped a few days later, as described in our earlier report (Tseng et al., 2013). Mice were housed 2–4 per cage with the same-sex littermate. Subjects were maintained in a temperature-controlled environment (22 ± 1°C) under a 12-h light/dark cycle (6 am – 6 pm) and had free access to food and water in their home cages. All behavioral experiments were conducted during the light cycle between 9 am and 4 pm. All experimental procedures were according to the NIH guidelines for the care and use of animals in research and approved by the Institutional Animal Care and Use Committee at Western University of Health Sciences (Pomona, CA, United States).

Drugs

Cocaine hydrochloride purchased from Sigma-Aldrich (St. Louis, MO, United States) was dissolved in saline and administered intraperitoneally.

Experimental Procedures

To Determine the Role of Beta-Endorphine and Sex in Cocaine-Induced Conditioned Place Preference and Its Extinction and Reinstatement

The place conditioning paradigm has been used as a behavioral assay in preclinical studies to assess the rewarding actions of cocaine and other addictive drugs (Bardo and Bevins, 2000). This paradigm has also been used as an animal model of extinction and reinstatement because, like any other conditioned response, CPP can be extinguished and reinstated (Sanchez and Sorg, 2001; Itzhak and Martin, 2002; Kelley et al., 2007). We used a three-chambered place conditioning apparatus (ENV-3013, Med Associates Inc., Saint Albans, VT, United States) and an unbiased paradigm to examine the role of sex and beta-endorphin in cocaine-induced CPP and its extinction and reinstatement. The details of the procedure and apparatus have been provided elsewhere (Nguyen et al., 2012; Tseng et al., 2013). The place conditioning protocol consisted of three phases and was conducted over 10 days, as depicted in diagram below: the acquisition phase, extinction phase, and reinstatement phase.

Pre-conditioning	Conditioning AM: S or C (30min) PM: S or C (30min)			Post-conditioning	Left untouched in home cages		Forced Extinction AM: S (30min) PM: S (30min)	Reinstatement C (7.5mg/kg)
D1 TEST	D2	D3 Acute TEST	D4	D5 TEST	D6	D7	D9 TEST	D10 TEST
(15min)		(15min)		(15min)			(15min)	(15min)

S- Saline
C- Cocaine (15mg/kg)

The Role of Beta-Endorphin and Sex in the Acquisition of Conditioned Place Preference Following Single Conditioning With Cocaine

We first used single conditioning with cocaine to assess the role of beta-endorphin in the rewarding action of acute cocaine. To this end, mice (6–8 mice of each sex per genotype) were tested for preconditioning (or baseline) place preference on day 1 (D1). On this day, each mouse was placed in the central neutral chamber and allowed to roam the conditioning and neutral central chambers freely for 15 min. The amount of time, that mice spent in the three chambers, was recorded via MED-PC IV (Med Associates, Inc.). On day 2, animals were treated with cocaine (15 mg/kg, i.p.) or saline and confined to the drug-paired chamber (DPCh) or vehicle-paired chamber (VPCh) for 30 min. In the afternoon, mice received the alternate treatment and were confined in the opposite chamber for 30 min. Mice were then tested for place preference on day 3 (D3), as described for day 1.

The Role of Beta-Endorphin and Sex in the Acquisition, Extinction and Reinstatement of Conditioned Place Preference Induced by Repeated Conditioning With Cocaine

To determine the role of beta-endorphin in cocaine reward after repeated conditioning, shortly after the test, mice received their respective twice-daily conditioning on this day as well as on day 4. On day 5 (D5), animals were tested again for place preference after repeated conditioning with cocaine. After that, mice were left undisturbed in their home cages on days 6 and 7 and then tested for natural extinction on day 8 (data not shown). To determine the role of beta-endorphin in extinction, animals received forced extinction training on day 9, in which mice were conditioned with saline in both conditioning chambers. On day 10, mice were tested for extinction, as described on day 1, in the morning (between 10 and 11 AM). Animals were considered to express extinction when there was no significant difference between the amount of time that mice spent in the drug-paired (DPCh) versus vehicle-paired (VPCh) chamber. In the afternoon (between 2 and 3 PM), mice were tested for the reinstatement of cocaine CPP immediately following a single cocaine injection (7.5 mg/kg, i.p.). On each test day, mice were placed in the neutral chamber and allowed to freely explore the three chambers for 15 min. The amount of time that mice spent in each chamber was recorded on each test day.

Data Analysis

The data are presented as means (±SEM) of the amount of time that animals spent in drug-paired (DPCh) versus vehicle-paired chamber (VPCh). A three-way repeated-measure analysis of variance (ANOVA) was performed, followed by the Fisher's LSD *post hoc* test for multiple comparisons. A $P \leq 0.05$ was considered statistically significant.

RESULTS

Acquisition and Reinstatement of CPP Were Blunted in Male Mice Lacking Beta-Endorphin Compared to Their Wild-Type Controls

The amount of time that male beta-endorphin wild-type (left half of the panel) and knockout (right half of the panel) mice spent in the vehicle-paired (VPCh) and drug-paired (DPCh) chambers on the baseline preference test day (day 1; D1) as well as on test days for CPP after single conditioning on day 3 (D3), day 5 (D5) and on extinction and reinstatement (**Figure 1**). A three-way repeated-measure ANOVA revealed a significant effect of genotype [$F_{(1,55)} = 4.22$; $P < 0.05$], a significant effect of context [$F_{(1,55)} = 11.10$; $P < 0.01$] and a significant interaction between the two factors [$F_{(1,55)} = 6.51$; $P < 0.01$]. The *post hoc* test showed a significant difference in the amount of time that wild-type mice (beta-END+/+) spent in the DPCh vs. VPCh on the postconditioning (D3 or D5) as well as on the reinstatement test day ($P < 0.05$) but not before conditioning (D1) or the extinction test day (**Figure 1**, left half of the panel). On the other hand, the beta-endorphin knockout mice did not exhibit any CPP response following either single or repeated conditioning with cocaine (**Figure 1**, right half of the panel). There was no significant ($P > 0.05$) difference between the amount of time that mice spent in the DPCh vs. VPCh following the challenge dose of cocaine on the reinstatement test day in mice lacking beta-endorphin (beta-END−/−). The *post hoc* analyses of the data showed that there was a significant difference in the amount of time that wild-type mice spent in the DPCh on the postconditioning test days as well as on the reinstatement test day ($P < 0.05$). These results show that the acquisition of cocaine-induced CPP after single

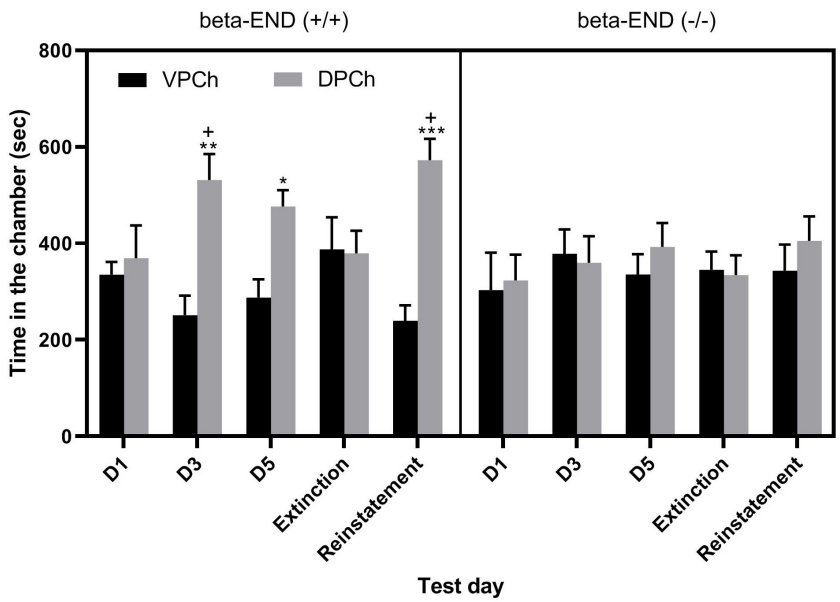

FIGURE 1 | CPP was blunted in male mice lacking beta-endorphin compared to their wild-type controls following single and repeated conditioning with cocaine (15 mg/kg) as well as on the reinstatement test day following a challenge dose of cocaine (7.5 mg/kg, i.p.). Data are mean (±SEM) of the amount of time that animals (n = 6–8 mice per genotype) spent in the drug-paired chamber (DPCh) versus vehicle-paired chamber (VPCh) before (day 1; D1) and after single (day 3; D3) and repeated (day 5; D5) conditioning with cocaine as well as on the extinction and reinstatement test days. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ vs. its respective VPCh; +$P < 0.05$ vs. mice lacking beta-END on that test day.

and repeated conditionings is reduced in the absence of beta-endorphin. Likewise, the reinstatement of CPP was blunted in the absence of the peptide.

Reinstatement of Cocaine-Induced CPP and Its Acquisition Following Single but Not Repeated Conditioning With Cocaine Were Reduced in Female Mice Lacking Beta-Endorphin Compared to Their Wild-Type Controls

Figure 2 shows the amount of time that female wild-type (left half of the panel) and beta-endorphin knockout (right half of the panel) mice spent in the conditioning chambers before (D1) and after single (D3) and repeated (D5) cocaine conditioning as well as on the extinction and reinstatement test days. A three-way repeated-measure ANOVA revealed a significant effect of genotype [$F_{(1,70)} = 3.86$; $P = 0.05$], a significant effect of context [$F_{(1,70)} = 12.85$; $P < 0.001$], but there was no significant interaction between the two factors [$F_{(1,70)} = 1.06$; $P > 0.05$]. The *post hoc* test showed a significant ($P < 0.05$) increase in the amount of time that female wild-type (beta-END+/+) mice spent in the DPCh vs. VPCh on the postconditioning test days (D3 and D5) as well as on the reinstatement test day (**Figure 2**, left half of the panel). On the other hand, mice lacking beta-endorphin (beta-END−/−) failed to exhibit any CPP after the single conditioning with cocaine (**Figure 2**, D3, right half of the panel). Interestingly, these mice showed a significant CPP after repeated conditioning with cocaine. Yet, they failed to show a significant reinstatement (**Figure 2**, D5, right half panel). These results indicate that female wild-type mice exhibited a significant CPP following single and repeated cocaine conditioning. On the other hand, beta-endorphin knockout mice showed a significant CPP response only after repeated cocaine conditioning. Furthermore, the reinstatement of CPP was reduced in mice lacking beta-endorphin compared to their wild-type controls.

DISCUSSION

The main findings of the present study are that the rewarding action of acute cocaine was reduced in both male and female mice lacking beta-endorphin. Likewise, the CPP response induced by repeated cocaine conditioning was attenuated in male, but not female mice lacking beta-endorphin. In mice undergone extinction training, cocaine was able to reinstate the CPP response in both male and female wild-type but not knockout mice. Together, these results suggest that beta-endorphin plays a functional role in the rewarding action of cocaine following single and repeated conditioning but there seem to be some sex-related differences in this response. However, beta-endorphin plays an essential role in the processes leading to the reinstatement of cocaine CPP regardless of the sex of mice.

Cocaine has been shown to cause the release of beta-endorphin in the NAc (Olive et al., 2001; Roth-Deri et al., 2003),

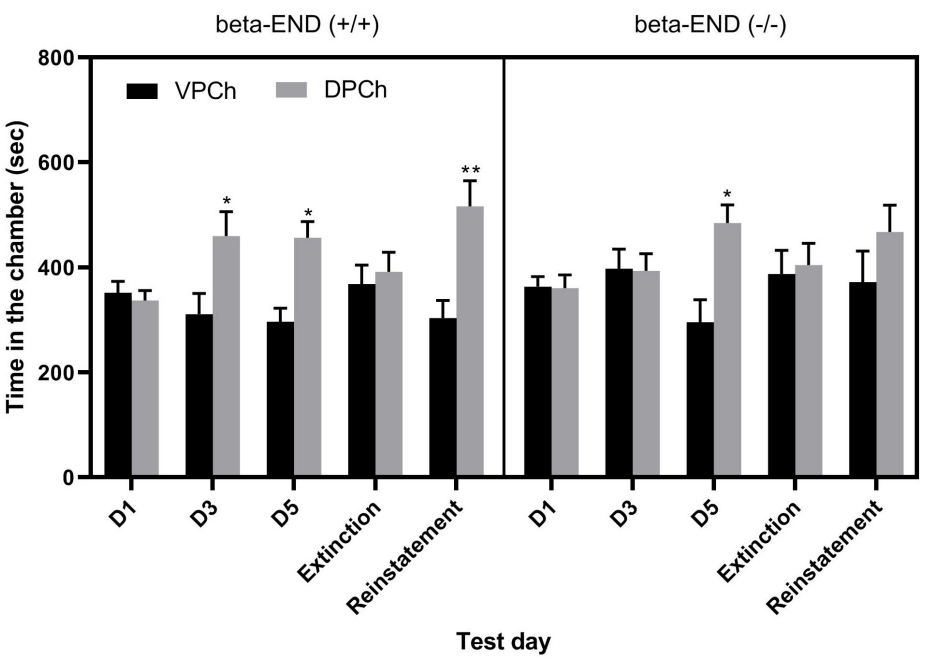

FIGURE 2 | The CPP response was blunted in female mice lacking beta-endorphin compared to their wild-type controls following single but not repeated conditioning with cocaine (15 mg/kg) as well as on the reinstatement test day. Data are mean (±SEM) of the amount of time that mice (n = 8 mice per genotype) spent in the drug-paired chamber (DPCh) versus vehicle-paired chamber (VPCh) on day 1 (D1) and after single (day 3; D3) and repeated (day 5, D5) conditioning with cocaine as well as on the extinction and reinstatement test days; $^{*}P < 0.05$; $^{**}P < 0.01$ vs. their respective VPCh on that day.

a response that may be important in the acquisition of cocaine self-administration (Roth-Deri et al., 2004, 2006, 2008) as well as in incubation processes (Dikshtein et al., 2013). Interestingly, we have previously shown that the rewarding action of acute cocaine was reduced in male mice lacking beta-endorphin compared to their wild-type controls (Marquez et al., 2008; Nguyen et al., 2012). In the present study, we wanted to extend these findings to female mice as well as assessed the role of beta-endorphin in CPP induced by repeated cocaine conditioning in both male and female mice. Previous studies have demonstrated that there are sex-related differences in the acquisition of cocaine CPP (Hilderbrand and Lasek, 2014). For example, females have been demonstrated to acquire cocaine CPP with fewer conditioning sessions and at lower doses than males (Russo et al., 2003). On the contrary, reports are showing no sex-related differences in cocaine reward using a wide range of doses (3–25 mg/kg) of cocaine (Bobzean et al., 2010). Consistent with the latter study, we found no difference in cocaine-induced CPP between male and female wild-type mice using either single or repeated conditioning.

While wild-type mice exhibited CPP following both single and repeated cocaine conditioning, we observed no significant CPP response in male or female mice lacking beta-endorphin following single cocaine conditioning, showing that the rewarding action of acute cocaine is abolished in the absence of beta-endorphin. We found that beta-endorphin is essential for the acquisition of CPP after repeated conditioning in male mice as well. Interestingly, female mice lacking beta-endorphin showed a robust CPP response after repeated conditioning with cocaine, indicating that endogenous beta-endorphin differentially contributes to the rewarding action of cocaine after its repeated administration. Although the underlying mechanism of this male/female difference regarding the role of beta-endorphin in cocaine reward after repeated cocaine conditioning is unknown at present, there may be some interactions between beta-endorphin and sex hormones to regulate the CPP response. Considering that the mesolimbic dopaminergic neurons have been implicated in the rewarding action of cocaine and that the activity of these neurons is regulated by sex hormones (Festa et al., 2004; Festa and Quinones-Jenab, 2004; Nazarian et al., 2004; Tobiansky et al., 2016) and beta-endorphin (Di Chiara and Imperato, 1988; Johnson and North, 1992), it is tempting to propose that neuronal inputs to the mesolimbic dopaminergic neurons containing beta-endorphin and sex hormones may have overlapping functions to facilitate neurotransmission along these neurons and one can compensate for the lack of the other. This explains why we observed a robust CPP response in female but not male mice lacking beta-endorphin since sex hormones, most likely estrogen, may have compensated for the lack of beta-endorphin in female wild-type mice. However, this convergent becomes functional only after repeated cocaine conditioning because the rewarding action of acute cocaine was blunted in both male and female mice lacking beta-endorphin. Thus, further studies are needed to delineate the underlying

mechanism of the interaction between beta-endorphin and sex hormones in regulating cocaine reward following single versus repeated cocaine conditioning.

Drug craving and particularly relapse represents a serious issue in the treatment of drug addiction. Relapse can be easily triggered by any drug-associated cues, external environmental cues, or re-exposure to the drug itself (Bossert et al., 2013). A common reinstatement model in rodents uses either priming injections of the drug, exposure to different stressors, or contextual cues to test if the previously extinguished response can be reinstated (O'Brien et al., 1992). In the present study, we used a priming injection of cocaine (7.5 mg/kg) to determine if there was any difference in the reinstatement of cocaine-induced CPP between female and male mice and whether beta-endorphin would play a functional role in this response. We found that both male and female wild-type mice exhibited a significant CPP response following the priming dose of cocaine; yet, there was no difference in the magnitude of the CPP response between male and female mice on the reinstatement test day. Interestingly, however, cocaine failed to reinstate a CPP response in male or female mice lacking beta-endorphin. One may argue that male mice lacking beta-endorphin did not exhibit CPP, and thus one should not expect reinstatement of CPP in those mice. While this argument may be valid in some cases, the lack of reinstatement in knockout mice cannot be explained by the blunted CPP response in knockout mice because female mice lacking beta-endorphin expressed a robust CPP response after repeated conditioning with cocaine yet failed to exhibit a significant CPP response on the reinstatement test day. Thus, the present result provides the first evidence that endogenous beta-endorphin plays an essential role in processes leading to the reinstatement of cocaine CPP at least in C57BL/6 mice.

Research from Dr. Kreek's laboratory has shown that animals exhibiting cocaine-induced CPP but not cocaine administration alone had elevated POMC mRNA levels in the hypothalamus (Zhou et al., 2012), raising the possibility that beta-endorphin may be needed for the acquisition and reinstatement of cocaine CPP. However, presently, it is unclear how beta-endorphin contributes to the processes leading to reinstatement. It is noteworthy to state that cocaine causes the release of beta-endorphin in the NAc (Amalric et al., 1987; Olive et al., 2001; Roth-Deri et al., 2003, 2004, 2006), a brain region where direct injection of beta-endorphin has been shown to induce CPP (Amalric et al., 1987). Furthermore, direct injection of beta-endorphin in the NAc elicited cocaine-seeking behaviors (Simmons and Self, 2009), raising the possibility that NAc may be the primary target where beta-endorphin exerts its regulatory actions on cocaine reward and reinstatement. Nevertheless, further research is needed to define how cocaine impacts the endogenous beta-endorphin and how endogenous beta-endorphin regulates the reinstatement of cocaine-induced CPP. Similarly, further research is needed to identify the brain region(s)/circuit(s) involved in these processes and define how endogenous beta-endorphin interacts with sex hormones to regulate cocaine-induced CPP and its reinstatement.

In summary, our results indicate that beta-endorphin is involved in the rewarding actions of acute cocaine in both male and female mice. However, there was a sex-related difference in this response in mice lacking beta-endorphin, in which female but not male mice lacking beta-endorphin exhibited CPP following repeated conditioning with cocaine. Nonetheless, re-exposure to cocaine failed to reinstate the CPP response in mice lacking beta-endorphin regardless of the sex of mice. Collectively, the present results indicate that there is an interaction between endogenous opioid peptide beta-endorphin and sex of mice in the acquisition of CPP after repeated conditioning with cocaine but not reinstatement of cocaine CPP.

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 Animal Care and Use Committee at Western University of Health Sciences (Pomona, CA, United States).

AUTHOR CONTRIBUTIONS

PS conducted the experiments and prepared the first draft of the manuscript. KL designed the project, analyzed the data, and finalized the manuscript. Both authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Self-Administration of Entactogen Psychostimulants Dysregulates Gamma-Aminobutyric Acid (GABA) and Kappa Opioid Receptor Signaling in the Central Nucleus of the Amygdala of Female Wistar Rats

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Male rats escalate intravenous self-administration of entactogen psychostimulants, 3,4-methylenedioxymethcathinone (methylone) and 3,4-methylenedioxymethamphetamine (MDMA) under extended access conditions, as with typical psychostimulants. Here, we investigated whether female rats escalate self-administration of methylone, 3,4-methylenedioxypentedrone (pentylone), and MDMA and then studied consequences of MDMA and pentylone self-administration on $GABA_A$ receptor and kappa opioid receptor (KOR) signaling in the central nucleus of the amygdala (CeA), a brain area critically dysregulated by extended access self-administration of alcohol or cocaine. Adult female Wistar rats were trained to self-administer methylone, pentylone, MDMA (0.5 mg/kg/infusion), or saline-vehicle using a fixed-ratio 1 response contingency in 6-h sessions (long-access: LgA) followed by progressive ratio (PR) dose-response testing. The effects of pentylone-LgA, MDMA-LgA and saline on basal GABAergic transmission (miniature post-synaptic inhibitory currents, mIPSCs) and the modulatory role of KOR at CeA GABAergic synapses were determined in acute brain slices using whole-cell patch-clamp. Methylone-LgA and pentylone-LgA rats similarly escalated their drug intake (both obtained more infusions compared to MDMA-LgA rats), however, pentylone-LgA rats reached higher breakpoints in *PR* tests. At the cellular level, baseline CeA GABA transmission was markedly elevated in pentylone-LgA and MDMA-LgA rats compared to saline-vehicle. Specifically, pentylone-LgA was associated with increased CeA mIPSC frequency (GABA release) and amplitude (post-synaptic GABAA receptor function), while mIPSC amplitudes (but not frequency) was larger in MDMA-LgA rats compared to saline rats. In addition, pentylone-LgA and MDMA-LgA profoundly disrupted CeA KOR signaling such as both KOR agonism (1 mM U50488) and KOR antagonism (200 nM nor-binaltorphimine) decreased mIPSC frequency suggesting recruitment of non-canonical KOR signaling pathways. This study confirms escalated self-administration of entactogen psychostimulants under LgA conditions in female

rats which is accompanied by increased CeA GABAergic inhibition and altered KOR signaling. Collectively, our study suggests that CeA GABA and KOR mechanisms play a critical role in entactogen self-administration like those observed with escalation of alcohol or cocaine self-administration.

Keywords: intravenous self-administration, MDMA, pentylone, methylone, central amygdala, inhibitory synaptic transmission, electrophysiology

INTRODUCTION

The entactogen psychostimulant drugs 3,4-methylenedioxy methamphetamine (MDMA), 3,4-methylenedioxymethcathinone (Methylone) and 3,4-methylenedioxypentedrone (Pentylone) are commonly abused substances. MDMA, Methylone and Pentylone are monoamine transporter inhibitors and substrates with increased selectivity for serotonin over dopamine or norepinephrine transporters (Baumann et al., 2012; Simmler et al., 2013, 2014a). Importantly, MDMA, Methylone and Pentylone are structurally closely related such that MDMA differs from Methylone by only the ketone on the beta carbon, while Methylone differs from Pentylone with respect to the length of the α-alkyl chain (Simmler et al., 2014b). Previous intravenous self-administration (IVSA) studies in male rats indicated that MDMA exhibits low efficacy as a reinforcer, leading to low overall drug intake and high inter-subject variability compared with, e.g., cocaine or methamphetamine (Dalley et al., 2007; Bradbury et al., 2014; Creehan et al., 2015). This has long been assumed to be a consequence of the pharmacological selectivity of MDMA for serotonin transporter inhibition and efflux, compared with the closely related methamphetamine. However, previous studies demonstrated that MDMA is a more effective reinforcer when animals are initially trained to self-administer mephedrone (Creehan et al., 2015), or under higher ambient temperature conditions (Cornish et al., 2003, 2008; Aarde et al., 2017). Furthermore, male rats will obtain more infusions of MDMA when trained under daily extended or long-access sessions (6-h) compared to short access (2-h) sessions (Vandewater et al., 2015). Finally, despite 4-methylmethcathinone exhibiting preferential serotonin release (Kehr et al., 2011; Wright et al., 2012), similar to MDMA, it is a robust reinforcer in rat IVSA models (Hadlock et al., 2011; Creehan et al., 2015; Nguyen et al., 2017a; Marusich et al., 2021). Thus there is evidence that under some circumstances, the serotonin transporter selective entactogen class stimulants can produce compulsive drug seeking behavior in rodent IVSA.

Within the class of entactogen stimulants, the propensity to support robust self-administration may vary. The dose-substitution paradigm has been commonly used to robustly test the relative potential for abuse of reinforcing drugs in animals with prior self-administration experience, analogous to human abuse liability testing. For example, Pentylone appears to be more efficacious as a reinforcer than Methylone in a dose-substitution comparison in male and female rats originally trained to self-administer methamphetamine and α-pyrrolidinopentiophenone, respectively (Dolan et al., 2018; Javadi-Paydar et al., 2018). This may be because Pentylone exhibits reduced efficacy as a monoamine transporter substrate compared to MDMA or Methylone (Dolan et al., 2018), and displays less serotonin selectivity as a monamine transporter inhibitor relative to MDMA (Baumann et al., 2012; Simmler et al., 2014a, 2016). This pharmacological profile suggests that Pentylone would be a highly efficacious reinforcer in rat IVSA procedures but it has not been well characterized apart from the two above-mentioned dose substitutions studies in animals trained on other drugs. Importantly, there are only limited data available that elucidate the abuse liability of entactogen stimulants in female subjects. These data show that, at least under 2-h access conditions, the IVSA of 4-methylmethcathinone (Mephedrone), Methylone and MDMA do not differ dramatically between male and female rats (Creehan et al., 2015; Vandewater et al., 2015; Javadi-Paydar et al., 2018). While Methylone IVSA is similar to MDMA IVSA when male rats are permitted 2 h daily sessions, Methylone appears to be much more effective than MDMA under 6 h daily access conditions (Vandewater et al., 2015; Nguyen et al., 2017a). Therefore, subtle differences in IVSA methods may either reveal or obscure differences in abuse liability. This may be critical for the accuracy of inferences made about two or more closely related entactogen psychomotor stimulants.

Thus, one major goal of this study was to determine if long-access to IVSA of three entactogens leads to escalating drug intake in female rats, as it does in males. As has been reviewed, it is increasingly recognized as important to confirm similarities and differences that may obtain between the sexes in a range of biomedical and neuroscience investigations (Clayton and Collins, 2014; Shansky and Murphy, 2021). A second goal was to test the hypothesis that extended access sessions would lead to increased IVSA of methylone relative to MDMA, as predicted by the indirect comparison of male long-access IVSA data (Vandewater et al., 2015; Nguyen et al., 2017a). Lastly, we aimed to investigate neuroadaptations in synaptic transmission in the central nucleus of the amygdala (CeA) given its key role in the acute reinforcing actions of drugs of abuse as well the negative emotional state associated with drug withdrawal (Koob and Volkow, 2016). The CeA is composed primarily of GABAergic neurons and represents the major output area of the larger amygdaloid complex (Gilpin et al., 2015; Roberto et al., 2020). Chronic administration of drugs of abuse including ethanol (Roberto et al., 2010, 2004; Gilpin et al., 2015; Kirson et al., 2021), cocaine (Kallupi et al., 2013; Schmeichel et al., 2017; Sun and Yuill, 2020), methamphetamine (Li et al., 2015), or opioids (Bajo et al., 2011, 2014; Nguyen et al., 2017b; Kallupi et al., 2020) enhance CeA GABA transmission representing a key molecular mechanism underlying maladaptive behaviors associated with addiction. Importantly, the CeA expresses several pro- and anti-stress promoting systems regulating its neuronal activity including the dynorphin/kappa opioid receptor (KOR) system, and chronic

administration of drugs of abuse recruits these CeA stress systems (Koob and Schulkin, 2019; Koob, 2021). Specifically, cocaine-LgA is associated with a profound recruitment of CeA dynorphin/KOR signaling such as blockade of CeA KOR signaling reduces anxiety-like behaviors and cocaine-induced locomotor sensitization. Interestingly, cocaine-LgA also lead to a profound dysregulation of the CeA dynorphin/KOR system at the molecular level such as the KOR agonist U50488 increased CeA GABA release while the KOR antagonist nor-binaltorphimine decreased it (Kallupi et al., 2013). However, it has not yet been investigated whether or how MDMA-LgA or Pentylone-LgA affect CeA neuronal activity including GABAergic transmission and its regulation by the dynorphin/KOR system.

Thus, here we used acquisition of self-administration under long-access (6-h) conditions, and post-acquisition dose substitutions under a Progressive Ratio schedule of reinforcement to assess potential differences in behavioral patterns in entactogen self-administration. For example, steeper escalation during LgA acquisition, or upward shifts in dose-response functions, are often inferred to represent meaningful differences in "addictiveness." However, a difference in training-dose can appear to show differential "escalation" of IVSA of the same drug, such as with methamphetamine (Kitamura et al., 2006), and animals trained on a more-efficacious drug will respond for more of a less-efficacious drug, compared with those trained on the latter (Creehan et al., 2015; Vandewater et al., 2015). To determine if similar neuroadaptations are produced by long-access self-administration of drugs which produced different behavioral patterns, we performed *ex vivo* slice electrophysiology to assess changes in CeA GABA transmission and its regulation by the dynorphin/KOR system in female MDMA-LgA and Pentylone-LgA rats.

MATERIALS AND METHODS

Animals

Female (N = 72) Wistar rats (Charles River, New York, NY, United States) entered the laboratory at 10 weeks of age and were housed in humidity and temperature-controlled (23 ± 1°C) vivaria on 12:12 h light:dark cycles. Animals had *ad libitum* access to food and water in their home cages. All experimental procedures took place in scotophase and were conducted under protocols approved by the Institutional Care and Use Committees of The Scripps Research Institute and in a manner consistent with the Guide for the Care and Use of Laboratory Animals (National Research Council [U.S.] Committee for the Update of the Guide for the Care and Use of Laboratory Animals, Institute for Laboratory Animal Research [U.S.], and National Academies Press [U.S.], 2011).

Drugs

Pentylone·HCl and Methylone·HCl were obtained from Cayman Chemical. 3,4-methylenedioxymethamphetamine (MDMA) HCl was obtained from NIDA Drug Supply. The MDMA analog was obtained from Fox Chase Chemical Diversity Center (Doylestown, PA, United States). Drugs were dissolved in physiological saline for the i.v. routes of administration. Dosing is expressed as the salt.

We purchased tetrodotoxin (TTX) from Biotium (Hayward, CA, United States), and AP-5, CGP55845A and DNQX, U-50488, and nor-binaltorphimine from Tocris (Bristol, United Kingdom) for the electrophysiological recordings. Stock solutions of the drugs were prepared in either distilled water or dimethyl sulfoxide (DMSO) and added to the bath solution to achieve the desired concentration.

INTRAVENOUS CATHETERIZATION

Rats were anesthetized with an isoflurane/oxygen vapor mixture (isoflurane 5% induction, 1–3% maintenance) and prepared with chronic intravenous catheters as described previously (Nguyen et al., 2017a, 2018). Briefly, the catheters consisted of a 14-cm length polyurethane-based tubing (MicroRenathane®, Braintree Scientific, Inc., Braintree, MA, United States) fitted to a guide cannula (Plastics one, Roanoke, VA, United States) curved at an angle and encased in dental cement anchored to an ∼3-cm circle of durable mesh. Catheter tubing was passed subcutaneously from the animal's back to the right jugular vein. Catheter tubing was inserted into the vein and secured gently with suture thread. A liquid tissue adhesive was used to close the incisions (3M™ Vetbond™ Tissue Adhesive; 1469S B). A minimum of 4 days was allowed for surgical recovery prior to starting an experiment. For the first 3 days of the recovery period, an antibiotic (cephazolin) and an analgesic (flunixin) were administered daily. During testing and training, intravenous catheters were flushed with ∼0.2–0.3 ml heparinized (32.3 USP/ml) saline before sessions and ∼0.2–0.3 ml heparinized saline containing cefazolin (100 mg/ml) after sessions. Catheter patency was assessed once a week, beginning in the third week of training, *via* administration through the catheter of ∼0.2 ml (10 mg/ml) of the ultra-short-acting barbiturate anesthetic, Brevital sodium (1% methohexital sodium; Eli Lilly, Indianapolis, IN, United States). Animals with patent catheters exhibit prominent signs of anesthesia (pronounced loss of muscle tone) within 3 s after infusion. Animals that failed to display these signs were considered to have faulty catheters and were discontinued from the study. Data that were collected after the previous passing of the test were excluded from analysis.

SELF-ADMINISTRATION PROCEDURE

Experiment 1 Acquisition

Following recovery from catheter implantation, rats were trained to self-administer MDMA (0.5 mg/kg per infusion; N = 14), methylone (0.5 mg/kg per infusion; N = 12), pentylone (0.5 mg/kg per infusion; N = 15), or saline vehicle (N = 8) using a fixed-ratio 1 (FR1) response contingency in 6-h sessions. One individual in the MDMA group, 2 individuals in the methylone group and 2 individuals in the Pentylone group were lost due to non-patent catheters. One individual in the Pentylone group was lost due to the catheter being chewed off by the cage

mate. Operant conditioning chambers (Med Associates; Med-PC IV software) enclosed in sound-attenuating cubicles were used for self-administration studies as previously described (Nguyen et al., 2017a,b, 2018). A pump pulse calculated to clear non-drug saline through the catheter started the session to ensure the first reinforcer delivery was not diluted, and a single priming infusion was delivered non-contingently if no response was made in the first 30 min of the session. In Experiment 1, rats that received at least one priming infusion during the first week (i.e., Sessions 1–5) included 1 saline, 4 MDMA, 6 Methylone, and 9 Pentylone rats. Rats that received at least one priming infusion during the second week (i.e., Sessions 6–10) included 3 saline, 2 MDMA, 4 Methylone, and 3 Pentylone rats. Acquisition training was conducted for 14–15 sessions depending on the group so only the first 14 sessions are analyzed for the comparison. 7 MDMA rats from Experiment 1 were trained on a variable number of sessions (14–34 total including acquisition) awaiting euthanasia for electrophysiological recordings.

Experiment 1 Progressive-Ratio Dose-Response Testing

Rats in active drug groups were next subjected to dose substitution with the respective training drug (0.125, 0.5, 1.0, 2.5 mg/kg/infusion), followed by dose substitution with methamphetamine (0.01, 0.05, 0.1, 0.5 mg/kg/infusion), in a randomized order under a Progressive Ratio (PR) response contingency. One individual in the Pentylone group was lost due to the catheter being chewed off by the cage mate. The saline group completed five sequential *PR* sessions but again, only vehicle was available. For the PR, the sequence of response ratios started with one response then progressed thru ratios determined by the following equation (rounded to the nearest integer): Response Ratio = 5e^(injection number × j) – 5 (Richardson and Roberts, 1996). The value of "*j*" was 0.2 and was chosen so as to observe a "breakpoint" within ∼3 h. The last ratio completed before the end of the session (1 h after the last response up to a maximum of 3 h sessions) was operationally defined as the breakpoint. Following assessment with the training drug, groups were permitted to self-administer methamphetamine doses (0.01, 0.05, 0.1, 0.5 mg/kg/infusion) in a randomized order under the same *PR* schedule of reinforcement.

Experiment 2 Acquisition

Following recovery from catheter implantation, rats were trained to self-administer MDMA (0.5 mg/kg per infusion; $N = 8$), pentylone (0.5 mg/kg per infusion; $N = 11$), or saline vehicle ($N = 4$), using a fixed-ratio 1 (FR1). One individual in the MDMA group was euthanized for illness. Acquisition training was conducted for 11–14 sessions depending on the group so only the first 11 sessions are analyzed for the comparison. In Experiment 2, rats that received at least one priming infusion during the first week (i.e., Sessions 1–5) included 2 saline, 0 MDMA, and 0 Pentylone rats. Rats that received at least one priming infusion during the second week (i.e., Session 6–10) included 1 saline, 0 MDMA, and 0 Pentylone rats. Following acquisition, rats were trained on a variable number of sessions (11–35 total including acquisition) awaiting euthanasia for electrophysiological recordings.

Animals for Electrophysiology

Electrophysiological recordings were performed from a total of 27 chosen rats. Rats were selected based upon higher number of infusions relative to lower-preferring subjects, with the exception of saline control rats, which were selected randomly. Specifically, we recorded from 8 rats from the saline-control group (cells per individual animal: 8-8-2-4-6-2-6-6), 11 rats from the MDMA-LgA group (cells per individual animal: 1-2-3-2-3-2-2-8-2-6-2), and 8 rats from the Pentylone-LgA group (cells per individual animal: 5-11-6-7-5-5-8-5). 2 MDMA (both from Experiment 1), 0 Pentylone and 4 saline rats that received a priming infusion proceeded to electrophysiological experiments. Neither of the MDMA rats received a priming infusion after Session 3. Tissue for electrophysiology was collected 18 h after the last self-administration session at the time animals would anticipate the next self-administration session. Importantly, rats were allowed to freely cycle during the self-administration process, and estrous cycle stage for each rat was determined upon sacrifice to evaluate its potential impact on CeA physiology. Estrous cycle was assessed based on cytological appearance of vaginal smear after euthanasia as described in McLean et al. (2012). However, rats from both the saline and MDMA-LgA group were mainly in either pro-estrus or estrus, while Pentylone-LgA rats were either in estrus or diestrus. Thus, based on an unequal representation of estrous cycle stages in the different groups, data for GABA signaling and KOR pharmacology were pooled and thus, with the current data set a potential impact of the estrous cycle on CeA GABA transmission as well as KOR function could neither be excluded or confirmed.

Slice Preparation and Electrophysiological Recordings

Preparation of acute brain slices containing the central nucleus of the amygdala (CeA) and electrophysiological recordings were performed as previously described (Varodayan et al., 2018; Suárez et al., 2019; Khom et al., 2020a,b; Steinman et al., 2020). Briefly, deeply anesthetized rats (3–5% isoflurane anesthesia) were quickly decapitated, and their brains placed in an ice-cold oxygenated high-sucrose cutting solution composed of 206 mM sucrose, 2.5 mM KCl, 0.5 mM $CaCl_2$, 7 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 26 mM $NaHCO_3$, 5 mM glucose, and 5 mM HEPES. We cut 300 μm thick coronal slices with the medial subdivision of the CeA using a Leica VT 1000S and incubated them for 30 min in 37°C warm, oxygenated artificial cerebrospinal fluid (aCSF), composed of (in mM) 130 NaCl, 3.5 KCl, 2 $CaCl_2$, 1.25 NaH_2PO_4, 1.5 $MgSO_4$, 24 $NaHCO_3$, and 10 glucose, followed by another 30 min incubation at room temperature. We identified CeA neurons with infrared differential interference contrast optics using a 40× water-immersion objective (Olympus BX51WI), and a CCD camera (EXi Aqua, QImaging). Using whole-cell patch technique, we recorded from 135 neurons pharmacologically isolated, action-potential independent miniature inhibitory post-synaptic currents (mIPSC) by adding the sodium-channel

blocker tetrodotoxin (500 nM, TTX), blockers of glutamate-mediated neurotransmission [6,7-dinitroquinoxaline-2,3-dione, 20 μM (DNQX) and DL-2-amino-5-phosphonovalerate, 30 μM (AP-5)], and the $GABA_B$ receptor antagonist CGP55845A (1 μM) to the bath aCSF solution. All neurons were held –60 mV. We performed recordings in a gap-free acquisition mode with a 20 kHz sampling rate and 10 kHz low-pass filtering using a MultiClamp700B amplifier, Digidata 1440A, and pClamp 10 software (MolecularDevices, San Jose, CA, United States). We pulled patch pipettes from borosilicate glass (3–5 mΩ, King Precision) and filled them with a KCl-based internal solution composed of 145 mM KCl, 5 mM EGTA, 5 mM $MgCl_2$, 10 mM HEPES, 2 mM Mg-ATP, and 0.2 mM Na-GTP; pH was adjusted to 7.2–7.4 using 1N NaOH. We recorded only from neurons with an access resistance (R_A) < 15 MΩ and with a R_A change <20% during the recording, as monitored by frequent 10 mV pulses.

Data Analysis and Statistics

The number of infusions obtained in the IVSA experiments was analyzed by repeated measures *rmANOVA* with Sessions (acquisition only) or Dose as within-subjects factors. Significant main effects from the r*mANOVA* were further analyzed with *post hoc* multiple comparisons analysis using the *Tukey* procedure for multi-level, and the *Dunnett* procedure for two-level factors. Two missing data points (caused by program failure) in the Pentylone-trained rats during Session 11 were interpolated from the values before and after the last Session.

Frequencies, amplitudes, and current kinetics including current rise and decay times of mIPSCs were analyzed using MiniAnalysis software (Synaptosoft, Decatur, GA, United States). Data are given as means ± S.E.M of raw values for mIPSC basal characteristics or from normalized values when assessing the effects of the KOR agonist U-50488 or the KOR antagonist nor-binaltorphimine (norBNI) on mIPSCs. Differences in mIPSC baseline characteristics were determined by one-way *ANOVA* and a *Dunnett post hoc* analysis. *Per se* effects of U-50488 or norBNI on mIPSCs were calculated by *one-sample t-tests*, and differences in drug effects across treatments were then also determined by *one-way ANOVA* with *Dunnett post hoc* analyses. The criterion for significant results for both behavioral and electrophysiological data was set at $P < 0.05$ and all analyses were conducted using Prism 7 for Windows (v. 7.03; GraphPad Software, Inc., San Diego CA).

RESULTS

Female Wistar Rats Escalate Self-Administration of Entactogen Psychostimulants Under Extended Access (6-h) Conditions

The mean number of infusions obtained by rats trained on vehicle saline (N = 8) decreased across sections, whereas infusions obtained by rats trained on pentylone, methylone, or MDMA (for structural formulae, see **Figure 1**) increased across the 14-session acquisition interval with the lowest mean drug-intake observed in the MDMA group and highest in the Pentylone group (**Figure 2A**). Analysis of the saline, MDMA (N = 13), Methylone (N = 10), and Pentylone (N = 12) groups confirmed a main effect of Session [$F(13,481) = 15.2$; $P < 0.0001$], of Group [$F(3,37) = 3.324$; $P = 0.03$] and of the interaction of factors [$F(39,481) = 2.368$; $P < 0.0001$], on infusions obtained. The *post hoc* test confirmed that infusions were significantly increased compared to the first session in the Methylone (Sessions 8–14), Pentylone (Sessions 5–14), and MDMA groups (Sessions 9, 11–14); no significant differences in infusions were confirmed within the Vehicle trained group. Additionally, the Pentylone group was significantly different from Vehicle group during Sessions 5 and 10–14 and from the MDMA group during Sessions 5–6, 13–14. The drug-lever responding (%) was significantly higher compared to responding Session 1 (**Figure 2B**). The *ANOVA* confirmed a significant main effect of Session [$F(13,481) = 5.799$; $P < 0.0001$] but not of Group [$F(3,37) = 1.649$; $P = 0.1948$] or of the interaction of factors [$F(39,481) = 5.799$; $P = 0.4339$]. The *post hoc* test confirmed that drug-lever responding during Sessions 5–14 was significantly different from the first session, collapsed across groups. During the final 5 sessions of acquisition, Pentylone and Methylone groups exhibited >80% drug-associated lever responding. The MDMA group exhibited >80% drug-associated lever responding during the final 2 sessions.

Dose Substitution in Female Wistar Rats Following Escalation of Self-Administration of Entactogen Psychostimulants

The rats trained on Pentylone (N = 9), Methylone (N = 7), or MDMA (N = 10) under long-access conditions exhibited group differences during dose substitution experiments (**Figure 3A**).

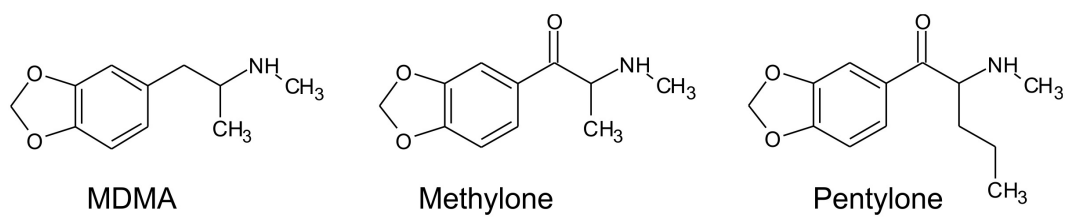

FIGURE 1 | Structural formulae of MDMA, Methylone, and Pentylone.

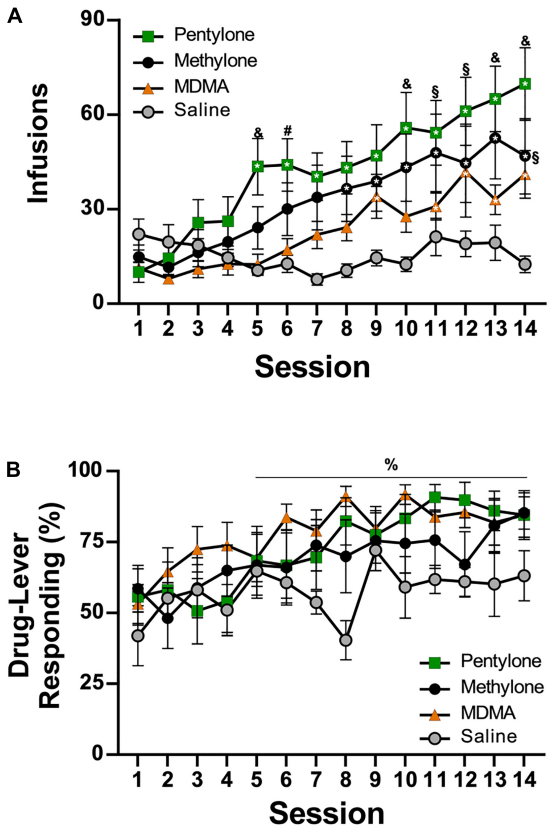

FIGURE 2 | (A) Mean (±S.E.M.) infusions of Pentylone (*N* = 12), Methylone (*N* = 10), MDMA (*N* = 13), and saline (*N* = 8; Vehicle) obtained under extended access conditions. **(B)** Mean (±S.E.M.) percent of responses on the drug-associated lever. A significant difference from the first session, within group, is indicated with *, a significant difference from the first session, collapsed across groups, with%, a difference from the Vehicle and MDMA groups with &, a difference from the Vehicle group with §, and a difference from the MDMA group with #.

Analysis confirmed a main effect of Dose [$F(4,92) = 30.04$; $P < 0.0001$], of Drug [$F(2,23) = 6.067$; $P = 0.0077$] and of the interaction of factors [$F(8,92) = 2.57$; $P < 0.05$], on infusions obtained. Overall, rats trained on Methylone and Pentylone increased their intake to an approximately similar extent and received higher number of infusions compared to rats trained on MDMA. Pentylone-trained rats reached higher breakpoints than Methylone and MDMA-trained groups in *PR* tests. When presented with methamphetamine substitution (**Figure 3B**), Pentylone-LgA rats ($N = 8$) similarly received higher number of infusions compared to rats trained on both Methylone-LgA ($N = 5$) or MDMA-LgA rats. Analysis confirmed a main effect of Dose [$F(4,92) = 30.04$; $P < 0.0001$], of Drug [$F(2,23) = 6.067$; $P = 0.0077$] and of the interaction of factors [$F(8,92) = 2.57$; $P < 0.05$], on infusions obtained. One Pentylone animal that maintained patency was eliminated for exhibiting no dose sensitivity in the MA challenge, and two Methylone animals were eliminated due to failed catheter patency. To further explicate the role of drug training history, the Pentylone-trained group were evaluated on doses of MDMA and the MDMA-trained group on doses of Pentylone, using the *PR* procedure. Pentylone supported higher levels of responding than did MDMA regardless of the training drug (**Figure 4**). Analysis confirmed a main effect of Dose [$F(4,132) = 35.75$; $P < 0.0001$], of Drug [$F(3,33) = 12.47$; $P < 0.0001$] and of the interaction of factors [$F(12,132) = 2.098$; $P < 0.05$], on infusions obtained. The *post hoc* test confirmed that Pentylone-trained rats obtained a significantly higher number of infusions of Pentylone (0.125–2.5 mg/kg/infusion) compared to vehicle, and MDMA-trained rats also obtained more infusions of Pentylone than of vehicle (0.5–2.5 mg/kg/infusion). Similarly, each group obtained significantly more MDMA infusions (0.5 mg/kg/infusion) compared with vehicle. Within each drug, the groups did not differ, and exhibited similar dose-effect functions.

Self-Administration of Methylenedioxymethamphetamine or Pentylone Heightens Central Nucleus of the Amygdala Inhibitory Signaling

Next, we assessed whether intravenous self-administration of MDMA (MDMA-LgA) or Pentylone (Pentylone-LgA) impacts CeA GABA transmission given that the CeA is highly sensitive to drugs of abuse such as alcohol or cocaine (Lesscher and Vanderschuren, 2012; Kallupi et al., 2013; Schmeichel et al., 2017; Roberto et al., 2020). We recorded pharmacologically isolated action-potential independent miniature inhibitory post-synaptic currents (mIPSCs) in 42 neurons from saline-control animals, 33 neurons from MDMA-LgA and 52 neurons from Pentylone-LgA rats (Whereas Pentylone rats that were selected for electrophysiological studies self-administered relatively more infusions, rats Saline or MDMA rats exhibited mean levels of drug-intake that were statistically indistinguishable from all rats that underwent behavioral testing; **Supplementary Figures 1–3**). We found that MDMA-LgA and Pentylone-LgA increased CeA GABAergic transmission. Specifically, a *one-way ANOVA* [$F(2,124) = 4.993$, $P = 0.0082$] with *Dunnett post hoc* analysis revealed that Pentylone-LgA but not MDMA-LgA significantly increased mIPSC frequencies compared to saline-controls (Saline: 0.74 ± 0.09 Hz vs. Pentylone-LgA: 1.12 ± 0.09 Hz, $P = 0.0040$ vs. MDMA-LgA: 0.87 ± 0.09 Hz, $P = 0.4828$) suggesting enhanced vesicular GABA release (see **Figures 5A,C**).

Moreover, both pentylone-LgA and MDMA-LgA significantly increased mIPSC amplitudes [*one-way ANOVA*: $F(2,124) = 7.156$, $P = 0.0011$; *Dunnett post hoc* analysis: Saline: 57.1 ± 2.2 pA vs. MDMA-LgA: 67.9 ± 3.2 pA, $P = 0.0261$ vs. Pentylone-LgA: 70.0 ± 2.5 pA, $P = 0.0006$, **Figures 5B,D**] indicative of heightened post-synaptic $GABA_A$ receptor function. Pentylone-LgA was further associated with faster mIPSC rise times [*one-way ANOVA*: $F(2,124) = 5.461$, $P = 0.0053$] while mIPSC rise times were similar between MDMA-LgA and saline controls (*Dunnett post hoc* analysis: Saline: 2.67 ± 0.04 ms vs. MDMA-LgA: 2.64 ± 0.04 ms, $P = 0.8393$ vs. Pentylone-LgA: 2.50 ± 0.04 ms, $P = 0.0051$, **Figures 5B,E**). Lastly, mIPSC decay times did not significantly differ between experimental

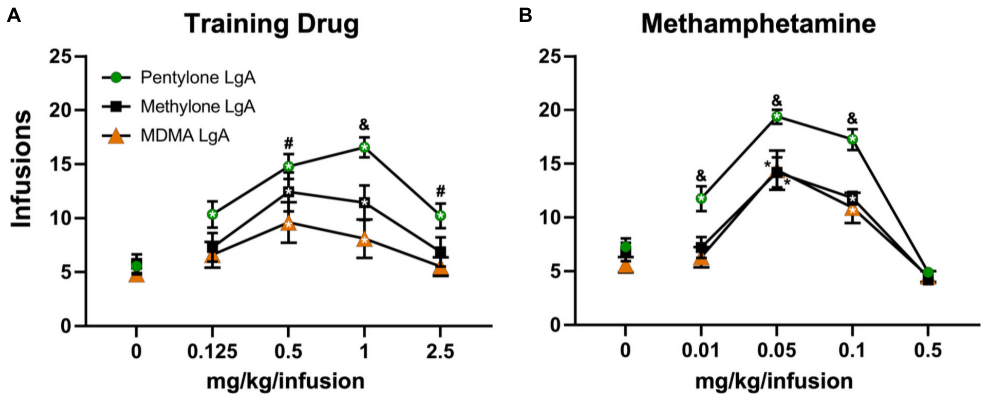

FIGURE 3 | Mean (±S.E.M.) infusions of the respective training drug and of methamphetamine obtained by groups trained in LgA- IVSA of pentylone (*N* = 8–9), methylone (*N* = 5–7), or MDMA (*N* = 10) are illustrated. A significant difference from saline, within group, is indicated with * (both inside and outside symbol), a significant difference from both other groups with &, and a difference from the MDMA LgA group with #.

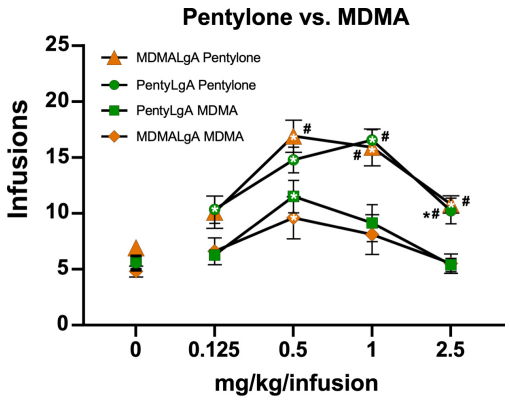

FIGURE 4 | Mean (±S.E.M.) infusions of Pentylone and of MDMA obtained by groups trained in LgA IVSA of pentylone (*N* = 8–9) or MDMA (*N* = 10). A significant difference from saline, within group, is indicated with *, a significant difference from MDMA, within each LgA group, is indicated with #.

groups [$F(2,124) = 1.545$, $P = 0.2173$, Saline: 9.0 ± 0.3 ms vs. MDMA-LgA: 9.1 ± 0.4 ms vs. Pentylone-LgA: 8.3 ± 0.3 ms, see **Figures 5B,F**]. These data indicate that MDMA-LgA and Pentylone-LgA induce profound neuroadaptations to increase CeA GABA signaling which is a characteristic neuroadaptation observed after self-administration of other drugs of abuse.

Methylenedioxymethamphetamine and Pentylone Self-Administration Disrupt Endogenous Kappa Opioid Receptor Signaling

Given that CeA dynorphin/KOR signaling drives behaviors associated with excessive drug consumption including cocaine or alcohol self-administration (Koob, 2008; Kallupi et al., 2013; Anderson et al., 2019; Bloodgood et al., 2020), we lastly tested whether MDMA-LgA or Pentylone-LgA would also alter KOR-mediated regulation of vesicular CeA GABA release. As shown in **Figure 6**, activating KOR by application of the selective agonist U-50488 [1 μM as in Kallupi et al. (2013) and Gilpin et al. (2014)] in saline-controls significantly decreased mIPSC frequency (63.1 ± 3.6%, $t = 10.13$, $df = 10$, $P < 0.0001$, *one-sample t-test*) without affecting any post-synaptic measures indicating that KOR agonism reduces CeA pre-synaptic GABA release (**Figures 6A,B**). Conversely, application of the KOR antagonist nor-binaltorphimine [norBNI, 200 nM, as in Kallupi et al. (2013) and Gilpin et al. (2014)] increased mIPSC frequency (119.1 ± 7.9%, $t = 2.414$, $df = 9$, $P = 0.039$) in saline-controls indicative of a tonic endogenous dynorphin/KOR signaling regulating GABA signaling under physiological conditions also in female rats (**Figures 6A,C**). Moreover, norBNI did not alter post-synaptic properties of mIPSCs in saline-controls as has been previously reported (Kallupi et al., 2013).

Application of U-50488 (1 μM) similarly decreased mIPSC frequency in MDMA-LgA (62.7 ± 8.8%, $t = 4.243$, $df = 10$, $P = 0.0017$, *one-sample t-test,* **Figures 7A,B**) and Pentylone-LgA (50.5 ± 6.4%, $t = 7.747$, $df = 11$, $P < 0.0001$, *one-sample t-test,* **Figures 8A,B**) rats. A *one-way ANOVA* analysis further confirmed that the effects of U-50488 on mIPSC frequency did not differ between saline, MDMA-LgA and Pentylone-LgA rats [$F(2,31) = 1.196$, $P = 0.3161$]. U-50488 did not alter mIPSC amplitudes and current kinetics in MDMA-LgA rats (see **Figure 7B**), but it significantly decreased mIPSC amplitudes in Pentylone-LgA rats without affecting mIPSC rise and decay times indicating that KOR-activation after Pentylone-self-administration also decreases post-synaptic $GABA_A$ receptor function presumably leading to reduced neuronal inhibition (see **Figure 8B**). Moreover, a *one-way ANOVA* analysis confirmed highly significant differences in the effects of the KOR-antagonist norBNI on CeA vesicular GABA release in MDMA-LgA and Pentylone-LgA rats compared to saline-controls [$F(2,33) = 13.13$, $P < 0.0001$]. Specifically, unlike to the control group (**Figure 6C**) where we found that application of norBNI (200 nM) increased mIPSC frequency, in both MDMA-LgA (79.0 ± 7.5%, $t = 2.682$, $df = 12$, $P = 0.02$, *one-sample t-test*, see **Figure 7C**) and Pentylone-LgA rats (65.6 ± 5.5%, $t = 6.249$, $df = 11$, $P < 0.0001$, *one-sample*

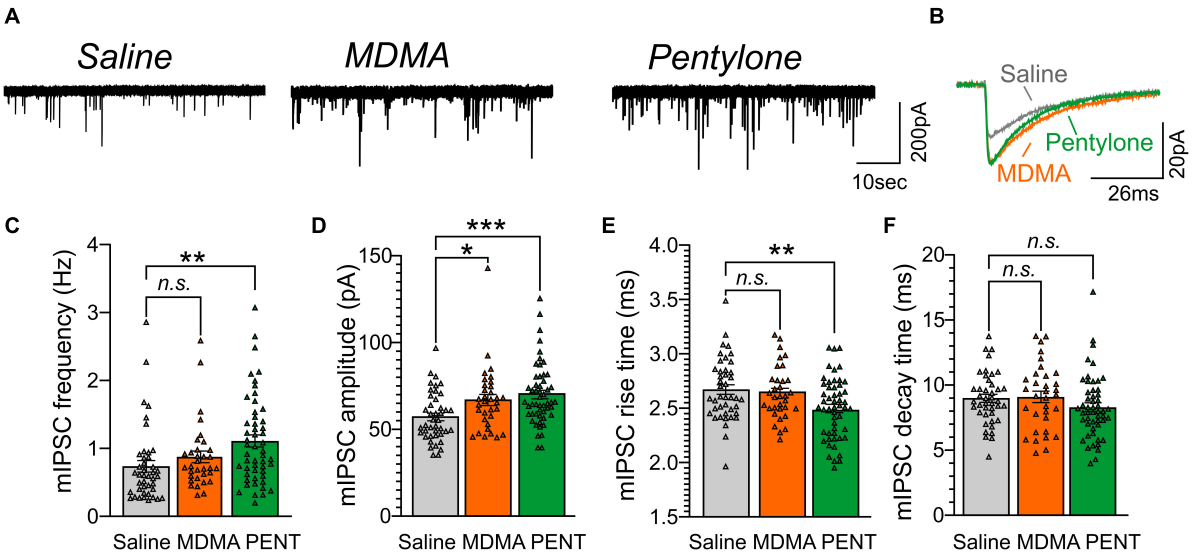

FIGURE 5 | (A) Representative mIPSC recordings from CeA neurons from female Wistar rats self-administering Saline (left panel), MDMA (middle panel), or Pentylone (abbreviated PENT, right panel). **(B)** Scaled mIPSC averages illustrating the effects of MDMA-LgA and Pentylone-LgA on mIPSC amplitudes and kinetics. Bars in represent means ± S.E.M. of mIPSC **(C)** frequencies, **(D)** amplitudes, **(E)** rise, and **(F)** decay times. Differences between groups were calculated using a one-way ANOVA with Dunnet *post hoc* analyses. (*) = $P < 0.05$, (**) = $P < 0.01$, (***) = $P < 0.001$.

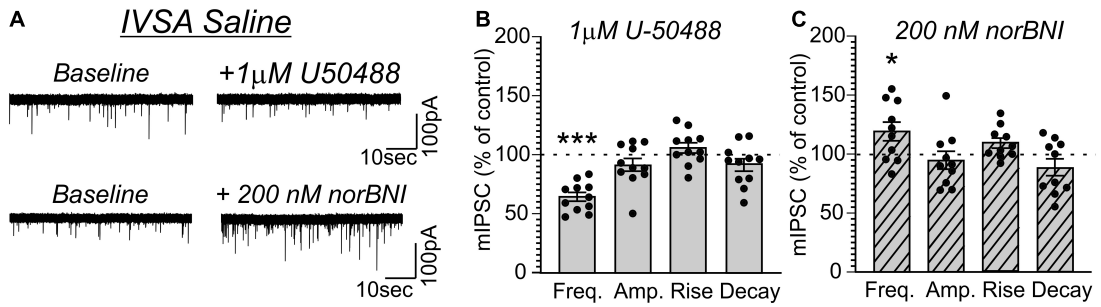

FIGURE 6 | (A) Representative mIPSCs from CeA neurons during control and during superfusion with the KOR-agonist U-50488 (1 μM, upper panel) or the KOR-antagonist norBNI (200 nM, lower panel) are shown. Bars represent means ± S.E.M of the normalized effects either **(B)** U-50488 or **(C)** norBNI at the indicated concentration on mIPSC characteristics. Statistically significant differences to baseline control were calculated using a one-sample *t*-test. (*) = $P < 0.05$, (***) = $P < 0.001$.

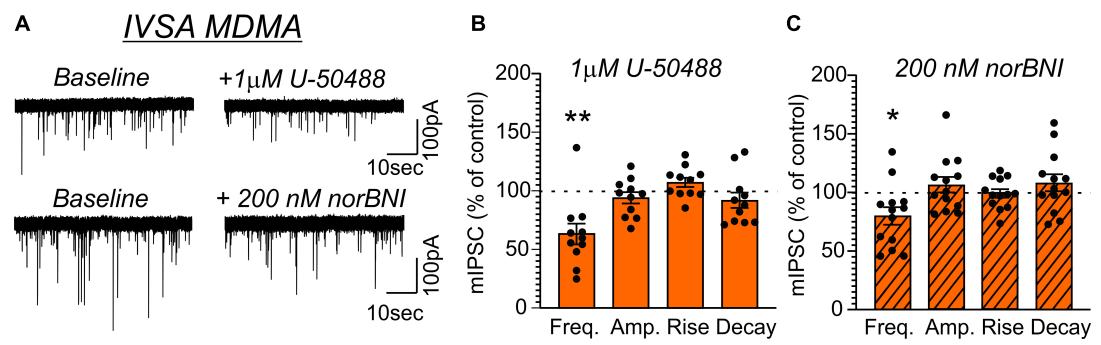

FIGURE 7 | (A) Representative mIPSCs from CeA neurons during control and during superfusion with the KOR-agonist U-50488 (1 μM, upper panel) or the KOR-antagonist norBNI (200 nM, lower panel) are shown. Bars represent means ± S.E.M of the normalized effects of **(B)** U-50488 or **(C)** norBNI on mIPSC characteristics. Statistically significant differences to baseline control were calculated using a one-sample *t*-test. (*) = $P < 0.05$, (**) = $P < 0.01$.

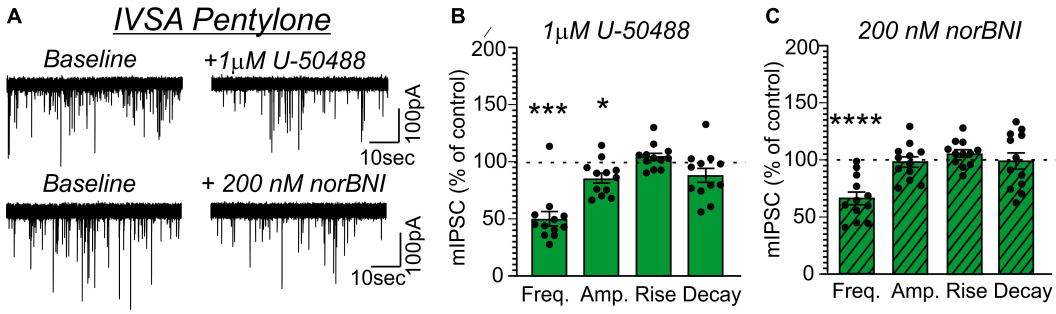

FIGURE 8 | (A) Representative mIPSCs from CeA neurons during control and during superfusion with the KOR-agonist U-50488 (1 μM, upper panel) or the KOR-antagonist norBNI (200 nM, lower panel) are shown. Bars represent means ± S.E.M of the normalized effects either **(B)** U-50488 or **(C)** norBNI at the indicated concentration on mIPSC characteristics. Statistically significant differences to baseline control were calculated using a one-sample *t*-test. (*) = $P < 0.05$, (***) = $P < 0.001$, (****) = $P < 0.0001$.

t-test, see **Figure 8C**) norBNI decreased GABA release. Overall, this switch in the tonic role of KOR in modulating GABA release combined with the evidence that antagonist and agonist display a similar pharmacological profile suggests that both excessive MDMA and Pentylone self-administration under long-access conditions induce significant neuroadaptations of KOR receptor signaling. Lastly, norBNI did not significantly alter any post-synaptic mIPSC characteristics including amplitude, rise, or decay times in either MDMA-LgA (**Figure 7C**) or Pentylone-LgA rats (**Figure 8C**).

DISCUSSION

This study shows that female rats readily acquire the self-administration of methylone, pentylone, and MDMA under 6-h long-access (LgA) daily training conditions. The groups trained on Methylone and Pentylone increased their intake to an approximately similar extent, with MDMA-trained animals increasing to a slightly lower extent, when considered as a population. This represents the first replication of extended-access IVSA intake of entactogen cathinones and MDMA that was previously reported for male rats trained to self-administer Methylone, Mephedrone, or MDMA (Vandewater et al., 2015; Nguyen et al., 2017a). Moreover, this is the first study to demonstrate a profound dysregulation of CeA neuronal activity in response to self-administration of entactogens in female rats. Together these results confirm that there is nothing qualitatively protective about the entactogens relative to other drugs of abuse, e.g., methamphetamine, cocaine or alcohol, and apparent differences in behavioral responding in intravenous self-administration procedures may be a function of the duration of action of a training dose [akin to what has been reported for methamphetamine (Kitamura et al., 2006)]. The post-acquisition dose-effect curves further emphasize that that in some cases the training history may (methamphetamine) or may not (Pentylone/MDMA) interact with the available drug to determine self-administration rate. However, systematic dose functions for highly effective reinforcers such as Pentylone and methamphetamine illustrated that all groups, regardless of training history, exhibited motivated drug-seeking behavior. One unexpected outcome was the self-administration of the MDMA analog, since it was constructed to be the amphetamine analog of Pentylone (**Supplementary Figure 4**). In the between groups analysis of the *PR* dose-substitution, Pentylone was more efficacious in comparison with Methylone (i.e., the cathinone analog of MDMA). The MDMA analog compound exhibited, if anything, reduced potency and similar efficacy relative to MDMA, represented by a rightward shift of the dose-response curve. This is a further caution against simplistic structure-activity inferences about *in vivo* activity in the intravenous self-administration procedure.

The drug substitution experiments show that inferences that MDMA is less addictive based on lower rates of intravenous self-administration during acquisition or in a dose-substitution procedure may be misleading. This was further confirmed by the electrophysiological experiments. The disruption of CeA synaptic transmission that has been associated with escalated self-administration of a range of drugs also occurred in the MDMA-LgA group in this study. Effects were similar in entactogen trained groups that exhibited differences in behavioral drug intake. Specifically, we found that both MDMA-LgA and Pentylone-LgA exhibited markedly elevated CeA GABA transmission leading to enhanced local inhibition by either increasing pre-synaptic GABA release (Pentylone-LgA) and/or enhancing post-synaptic $GABA_A$ receptor function (MDMA-LgA and Pentylone-LgA). Importantly, elevated inhibitory CeA signaling is a key molecular mechanism driving behaviors associated with drug abuse including escalation of drug intake in response to the emergence of the negative emotional state (Koob, 2021). Thus, our electrophysiological data indicate that Pentylone-LgA increased GABA transmission at both pre- and post-synaptic sites including potential changes in $GABA_A$ receptor subunit composition leading to a presumably stronger CeA neuronal inhibition, while MDMA-LgA only elevated post-synaptic $GABA_A$ receptor function but did not affect CeA GABA release.

Our study revealed that regulation of CeA synaptic GABA transmission by the dynorphin/KOR system in female rats does not differ from that in male rats (Gilpin et al., 2014;

Kallupi et al., 2020); that is, activation of KOR in female rats also decreases CeA GABA release, while KOR antagonism increases CeA GABA transmission supporting a tonic role of KOR in the basal CeA GABA activity. Furthermore, both MDMA and Pentylone self-administration under long-access conditions disrupted CeA regulation by the dynorphin/KOR system. Specifically, we found that KOR activation with U-50488 decreased CeA GABA transmission in both MDMA-LgA and Pentylone-LgA rats mainly *via* reducing pre-synaptic GABA release. Moreover, the KOR antagonist norBNI did not *increase* CeA GABA transmission (as in saline controls) but *decreased* it in both MDMA-LgA and Pentylone-LgA rats. Interestingly, Pentylone-LgA animals escalated their drug intake significantly more than MDMA-LgA rats suggesting that distinct neuroadaptations within CeA GABAergic synapses, associated with more pronounced local inhibition, may potentially account for the observed differences in drug escalation. KOR activation with U-50488 decreased post-synaptic $GABA_A$ receptor function only in Pentylone-LgA rats suggesting larger inhibitory effects of KOR activation on CeA GABAergic synapses after Pentylone-LgA.

Similar paradoxical effects of norBNI on CeA GABA signaling (i.e., norBNI decreasing CeA GABA transmission instead of increasing it) have been previously reported after cocaine-LgA (Kallupi et al., 2013). However, while after cocaine-LgA the effect of the KOR agonist on CeA GABA release had also changed directionality, i.e., KOR activation led to increased instead of decreased GABA signaling, in our study the KOR agonist U-50488 decreased CeA GABA release. This indicates some distinctions of the neuroadaptations at GABAergic synapses in response to cocaine-LgA vs. MDMA-LgA or Pentylone-LgA. Potentially, the fact that cocaine, MDMA and pentylone exhibit different mechanisms of action with respect to their activities at different monoamine transporters (Steinkellner et al., 2011; Baumann and Volkow, 2016; Sandtner et al., 2016; Simmler et al., 2014b, 2016; Saha et al., 2019; Glatfelter et al., 2021) may account for distinct neuroadaptations at CeA GABAergic synapses. Interestingly, the fact that norBNI did not increase CeA GABA release after Pentylone and MDMA self-administration may suggest a loss of tonic dynorphin signaling in the CeA at first sight, but it could also stem from alternative or non-canonical KOR signaling cascades resulting from repeated drug exposure (Nguyen et al., 2017b, 2021). Indeed, KOR signaling has been shown to be highly sensitive to stressful events (Polter et al., 2017; Estave et al., 2020; Przybysz et al., 2021) and recent studies show constitutive activity of KOR at GABAergic synapses in the ventral tegmental area in response to brief cold-water swim stress leading to altered effects of KOR antagonism (Polter et al., 2017). Moreover, KOR activation is coupled to several distinct intracellular signaling cascades involving G-protein coupled Receptor Kinases (GRK) and members of the mitogen-activated protein kinase (MAPK) family or β-arrestin-dependent pathways, amongst other classical G-protein mediated mechanisms (Bruchas and Chavkin, 2010; Lovell et al., 2015; Ho et al., 2018; Uprety et al., 2021). Alternatively, there is evidence that norBNI is not just a KOR antagonist but can act under specific circumstances as a biased agonist capable of activating, e.g., c-Jun N-terminal kinase (Bruchas et al., 2007) which also might explain the paradoxical effect of norNI in the present study. Thus, we hypothesize that the observed norBNI effects stem rather from changes in KOR signaling than a loss of CeA dynorphin, however, future studies utilizing different KOR antagonists will facilitate more insights into this phenomenon. Lastly, the observed neuroadaptations might also represent a combination of different signaling mechanisms activated by repeated drug exposure as well as subsequent drug withdrawal.

Overall, this study represents the first replication of extended-access IVSA intake of entactogen cathinones and MDMA in female rats, similar to that previously reported for male rats. The *in vivo* efficacy of cathinone compounds as reinforcers may not be supported by simplistic structure-activity inferences, nor by simplistic analysis of response rates in the acquisition of IVSA. Comparison of training groups across IVSA of the same compounds indicates a more similar motivational state. Furthermore, our studies also reveal similar profound neuroadaptations of CeA GABA transmission, and its regulation by the dynorphin/KOR system, in both MDMA-LgA and Pentylone-LgA groups, despite behavioral differences in the acquisition phase. Heightened GABA signaling associated with increased local inhibition in the CeA might represent a consistent, key mechanism underlying the escalation of drug self-administration.

AUTHOR'S NOTE

This paper adheres to the principles for transparent reporting and scientific rigor of preclinical research recommended by funding agencies, publishers, and other organizations engaged with supporting research.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Care and Use Committees of The Scripps Research Institute.

AUTHOR CONTRIBUTIONS

SK, JN, MR, and MT designed the studies, conducted statistical analysis of data, created figures, and wrote the manuscript. SK, JN, YG, and SV performed the research and conducted initial data analysis. All authors approved the submitted version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2021.780500/full#supplementary-material

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Anatomical and Functional Characterization of Central Amygdala Glucagon-Like Peptide 1 Receptor Expressing Neurons

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Glucagon-like peptide 1 receptors (GLP-1Rs) are highly expressed in the brain and are responsible for mediating the acute anorexigenic actions of widely prescribed GLP-1R agonists. Neurobiological efforts to localize the hypophagic effects of GLP-1R agonists in the brain have mainly focused on the hypothalamus and hindbrain. In this study, we performed a deep anatomical and neurophysiological characterization of GLP-1Rs in the central nucleus of the amygdala (CeA). At an mRNA level, we found that *Glp1r* is diffusely coexpressed in known CeA subpopulations like protein kinase c δ (*Prkcd*), somatostatin (*Sst*), or tachykinin2 (*Tac2*). At a cellular level, we used *Glp1r*-Cre mice and viral Cre-dependent tracing to map the anatomical positions of GLP-1R cells across the rostral-caudal axis of the CeA and in CeA subdivisions. We found that $Glp1r^{CeA}$ cells are highly enriched in the medial subdivision of the CeA (CeM). Using whole cell patch clamp electrophysiology, we found that $Glp1r^{CeA}$ neurons are characterized by the presence of I_h-like currents and resemble a low threshold bursting neuronal subtype in response to hyperpolarizing and depolarizing current injections. We observed sex differences in the magnitude of I_h-like currents and membrane capacitance. At rest, we observed that nearly half of $Glp1r^{CeA}$ neurons are spontaneously active. We observed that active and inactive neurons display significant differences in excitability even when normalized to an identical holding potential. Our data are the first to deeply characterize the pattern of *Glp1r* in the CeA and study the neurophysiological characteristics of CeA neurons expressing *Glp1r*. Future studies leveraging these data will be important to understanding the impact of GLP-1R agonists on feeding and motivation.

Keywords: central amygdala (CeA), anatomy, neural circuit, electrophysiology, glucagon-like peptide 1 receptor (GLP-1R), glucagon-like peptide 1 (GLP-1)

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INTRODUCTION

Obesity is a growing health problem for developed nations. In the United States alone, the CDC estimated that obesity prevalence among adults increased from 30.5 to 42.4% over the last 18 years indicating more than a third of adults in the United States are obese (BMI > 30). With obesity comes an increased risk for chronic diseases including cardiovascular disease, cancer, and type II diabetes. Many individuals with type II diabetes are prescribed drugs that target the glucagon-like

peptide 1 receptor (GLP-1R) due to the clinical benefit of enhancing the release of insulin while also decreasing appetite (Müller et al., 2019). Despite their wide use, a complete understanding of the full biological and behavioral impacts of GLP-1R agonism is lacking.

When administered *via* intracerebroventricular (icv) injection, GLP-1R agonists elicit hypophagia indicating that neuronal GLP-1Rs are required for this hypophagic effect. Thus, the anorexigenic effect of GLP-1R agonists can be attributed to the activation of neuronal not peripheral GLP-1Rs (Burmeister et al., 2013, 2017; Secher et al., 2014; Sisley et al., 2014; Adams et al., 2018). Attempts to localize and attribute the actions of GLP-1R signaling on feeding to specific brain nuclei have focused mainly on hypothalamic and hindbrain GLP-1Rs known to control hunger states or meal termination (Hayes et al., 2009; Kanoski et al., 2011; Alhadeff et al., 2014). However, the function of GLP-1Rs in limbic sites—like the lateral septum (LS), bed nucleus of the stria terminalis (BNST), and the central amygdala (CeA)—likely plays a key motivational role in feeding. Of relevance, recent work has shown that BNST GLP-1Rs and GLP-1R-expressing cells may in part drive stress-induced hypophagia and that LS GLP-1Rs functionally regulate feeding and motivation (Terrill et al., 2016; Williams et al., 2018). In the CeA, however, very little is known about how GLP-1Rs regulate either feeding or motivation.

The CeA is a complex heterogenous structure that contains both orexigenic and anorexigenic subpopulations of neurons. These neuronal subpopulations regulate emotional and survival behaviors including responses to predators and noxious stimuli (Haubensak et al., 2010; Li et al., 2013; Janak and Tye, 2015; Douglass et al., 2017; Hardaway et al., 2019; Ip et al., 2019; Steinberg et al., 2020; Weera et al., 2021). Previous studies using mice and rats have identified the CeA as a site for GLP-1R expression; however, the pattern of GLP-1R expression within a framework of known CeA populations has been unclear. In this study, we performed a deep anatomical characterization of CeA GLP-1Rs and GLP-1R-expressing neurons and utilized slice electrophysiology to characterize the membrane and neurophysiological properties of $Glp1r^{CeA}$ neurons.

MATERIALS AND METHODS

Animals

We used 8–16 week old C57BL6/J (Jackson Labs, Bar Harbor, ME) or *Glp1r*-Cre (MGI ID: 5776617) male and female mice for this study (Williams et al., 2016). For all experiments, animals were group housed 3–5/cage. Mice were maintained on a standard 12:12 light cycle with lights on at 7 a.m. Unless otherwise indicated, food and water were provided *ad libitum*. All procedures were performed according to an approved animal protocol by the University of Alabama at Birmingham Institutional Animal Care and Use Committee.

Stereotaxic Surgery

Survival surgeries were performed on 6–8 week old *Glp1r*-Cre mice. On the day of the surgery, animals received 0.1 mg/kg Buprenorphine SR (sc) and 5 mg/kg Meloxicam SR (sc). Under isoflurane inhalation (0.5–5%), mice were placed in a stereotaxic frame. The scalp was depilated and sterilized using rotating 70% EtOH and betadine application. Topical lidocaine (4%) and triple antibiotic were then applied to the scalp in preparation for incision. After ensuring a deep plane of anesthesia *via* loss of toe pinch reflex, a midline incision was made to expose the skull surface. A craniotomy was then performed at the injection site above the CeA. AAV5-hSyn-DIO-mCherry (300 nl, Addgene - lot# v63478) was injected at −1.30 mm posterior, ±2.90 mm lateral, and −4.60 mm ventral of bregma. Mice remained in the colony for 3–4 weeks following surgery for post-operative recovery and to allow accumulation of mCherry expression in the CeA prior to perfusion or electrophysiological recordings.

Histology

Glp1r-Cre mice were injected with a terminal dose of tribromoethanol (250 mg/kg). After reaching a deep plane of anesthesia confirmed *via* loss of toe pinch reflex, animals were transcardially perfused with 0.01 M PBS and then 50 ml of 4% paraformaldehyde in 0.01 M PBS. Brains were extracted and post-fixed in 4% paraformaldehyde/PBS overnight then transferred to 30% sucrose for cryoprotection for 36–48 h prior to cryosectioning. Free floating sections were mounted to Superfrost plus microscope slides (Fisher Scientific; Waltham, MA) and coverslipped with mounting media containing DAPI (Vector Laboratories).

Fluorescence *in situ* Hybridization

Fresh tissue was harvested from 8 to 16 week old C57BL6J or *Glp1r*-Cre male and female mice and flash frozen on dry ice then stored at −80°C. Brains were cryosectioned at 20 μM and directly mounted onto Superfrost Plus slides (Fisher Scientific; Waltham, MA) then stored in a sealed container at −80°C. The single molecule RNAscope fluorescent multiplex assay was performed according to the manufacturer's protocol (Advanced Cell Diagnostics; Newark, CA). The target probe for *Glp1r* was applied in tandem with the appropriate probe for *Prkcd*, *Sst*, *Tac2*, or *Cre*. For each RNAscope multiplex florescent assay, the target probe for *Glp1r* was assigned to 647 nm excitation while target probes for *Prkcd*, *Sst*, *Tac2*, and *Cre* were assigned to 550 nm excitation using the appropriate amplification buffers. Appropriate negative controls from the manufacturer were included for each assay for verification of probe specificity. The following target probes were used for this manuscript:

446391-Mm-*Tac2*	Accession #: NM_009312.2
404631-Mm-*Sst*	Accession #: NM_009215.1
441791-Mm-*Prkcd*	Accession #: NM_011103.3
418851-C3-Mm-*Glp1r*	Accession #: NM_021332.2
312281-*Cre*	Accession #: KC845567.1

Imaging and Image Analysis

Imaging was performed on a Keyence BZ-X810 under 20X magnification. *Glp1*r was imaged with 647 nM to maximize sensitivity of detection. For tissue sections obtained from C57BL6/J mice, tiled z-stacks of the CeA and surrounding region were captured using optical sectioning. For tissue sections obtained from *Glp1r*-Cre mice, tiled z-stack images of the

CeA and surrounding regions were captured using widefield florescence imaging. Raw images were then stitched and a maximum intensity projection made in Keyence Analyzer. Stitched raw images were obtained for individual channels including DAPI, 647, 550, and 488 nm. Prior to creating composite images, non-specific background signal was removed through image subtraction of images obtained on 488 nm from those obtained on 647 and 550 nm. No probes were developed using 488 nm fluorophores. Composite images were then created and processed for quantitative analysis. Cell counts and area measurements were performed in ImageJ (Fiji). Final images were assembled in Adobe Illustrator 25.2.3.

For quantitative analysis of *Glp1r* expressing neurons across the rostral-caudal axis, three subsections were defined with respect to bregma coordinates as determined using The Mouse Brain in Stereotaxic Coordinates, 4th ed. (Franklin and Paxinos, 2019). The range for anterior, middle, and posterior CeA were defined as follows: the anterior CeA falls within 0.71–1.03 mm posterior of bregma, middle CeA falls within 1.03–1.43 mm posterior of bregma, and posterior CeA falls within 1.43–1.79 mm posterior of bregma. Following acquisition, images were categorized as either anterior, middle, or posterior for quantitative analysis. Classification was determined using anatomical landmarks including the overall size of the CeA, the size and width of the basolateral amygdala, the position of the intercalated cells, and the presence and shape of the stria terminalis. Count data for this analysis was obtained from 6 to 8 images per mouse (2 male and 2 female) with 3–4 images of sections ∼100 μM apart with respect to bregma acquired per hemisphere per mouse.

Patch Clamp Electrophysiology

Slice Preparation

For electrophysiology, animals were removed from their cage and brought to the lab for brain slice preparation. The animal rested in a quiet chamber for 30–45 min prior to slice preparation to dissipate stress associated with animal transport from the vivarium. Mice were treated with a lethal dose of tribromoethanol (250 mg/kg, i.p.), and, after a deep plane of anesthesia was reached, animals were transcardially perfused with cold, sodium free N-methyl-D-glucamine (NMDG) artificial cerebrospinal fluid (aCSF) [(in mM) 93 N-methyl-D-glucamine, 2.5 KCl, 1.2 NaH_2PO_4, 30 $NaHCO_3$, 20 HEPES, 25 Glucose, 5 L-ascorbic acid, 2 Thiourea, 3 sodium pyruvate, 10 $MgSO_4$ X $7H_2O$, 0.5 $CaCl_2$ X $2H_2O$]. All solutions were saturated with 95% CO_2 and 5% O_2. The brain was rapidly dissected and coronal 300 μM sections prepared in ice cold, oxygenated NMDG aCSF using a Leica VT1200S at 0.07 mm/s. Slices were immediately transferred to 32°C NMDG aCSF for 10 min, and then normal 32°C aCSF [(in mM): 124 NaCl, 4.4 KCl, 2 $CaCl_2$, 1.2 $MgSO_4$, 1 NaH_2PO_4, 10.0 glucose, and 26.0 $NaHCO_3$]. Slices rested in normal aCSF for at least 30 min prior to recordings.

Recording

Whole cell patch clamp recordings were performed in the CeA guided by DIC microscopy and mCherry fluorescence. Slices were then transferred to a recording chamber (Warner Instruments), submerged in normal, oxygenated aCSF and maintained at 32°C with a flow rate of 2 ml/min. We patched mCherry-expressing neurons in a balanced fashion in all CeA subdivisions in which they were present. For all recordings we used a potassium gluconate internal solution [(in mM): 135 $C_6H_{11}KO_7$, 5 NaCl, 2 $MgCl_2$, 20 HEPES, 0.6 EGTA, 4 Na_2ATP, 0.4 Na_2GTP at a final osmolarity of 290 mOsm at a pH of 7.3]. For voltage clamp recordings, neurons were voltage clamped at −70 mV using a Multiclamp 700B and currents were digitized with an Axon 1550B digitizer (Molecular Devices, Fremont, CA).

Data Analysis

Data were analyzed in Clampfit 11.1 (Molecular Devices, San Jose, CA). Membrane capacitance and resistance were determined online using a −10 mV square pulse. We did not correct for liquid junction potential. Resting membrane potential was determined using a 2' gap free current clamp recording where we took the average following a stabilizing period (usually 30"). For active neurons, we counted the number of action potentials during this short recording to determine the average spiking frequency. For rheobase, we identified the injected current at the peak of the first action potential. For recordings at −70 mV, we injected a steady variable amount of negative current using the amplifier while recording gap-free in current clamp until the cell reached −70 mV. For determination of spiking subtype, we identified whether the cells had rebound spikes following a negative current injection, the latency to first spike, and if a cell had spike frequency adaptation. Late spiking neurons had a latency to first spike of >100 ms. Regular spiking neurons had a latency < 100 ms and minimal spike frequency adaptation. Low threshold bursting neurons had a short latency to the first spike, reduced spike frequency following the first 1–3 action potentials, and had a characteristic rebound spike. For detection of I_h-like currents, we measured the difference in cell current immediately following a negative voltage step to the point of stable current (at least 250 ms into a 500 ms voltage step). We measured the difference at each voltage step. To determine the proportion of neurons that demonstrated I_h-like currents we used a threshold of −20 pA at the most hyperpolarized voltage step (−120 mV). Any cell with < −20 pA at −120 mV was empirically categorized as lacking I_h-like current.

Statistics

All statistical analyses were performed using Graphpad Prism version 9.0.0. For electrophysiology data, original data were analyzed in Clampfit 11.1. Figures were assembled in Adobe Illustrator.

RESULTS

Previous murine studies have demonstrated the presence of GLP-1Rs in the CeA (Cork et al., 2015; Graham et al., 2020); however, we sought to characterize the pattern of GLP-1R expression within a framework of known genetic markers of the CeA. To that end, we used fluorescence *in situ* hybridization in the CeA to characterize *Glp1r* mRNA. In our first experiment, we found that *Glp1r*-containing cell bodies did not coexpress *Prkcd*, a member

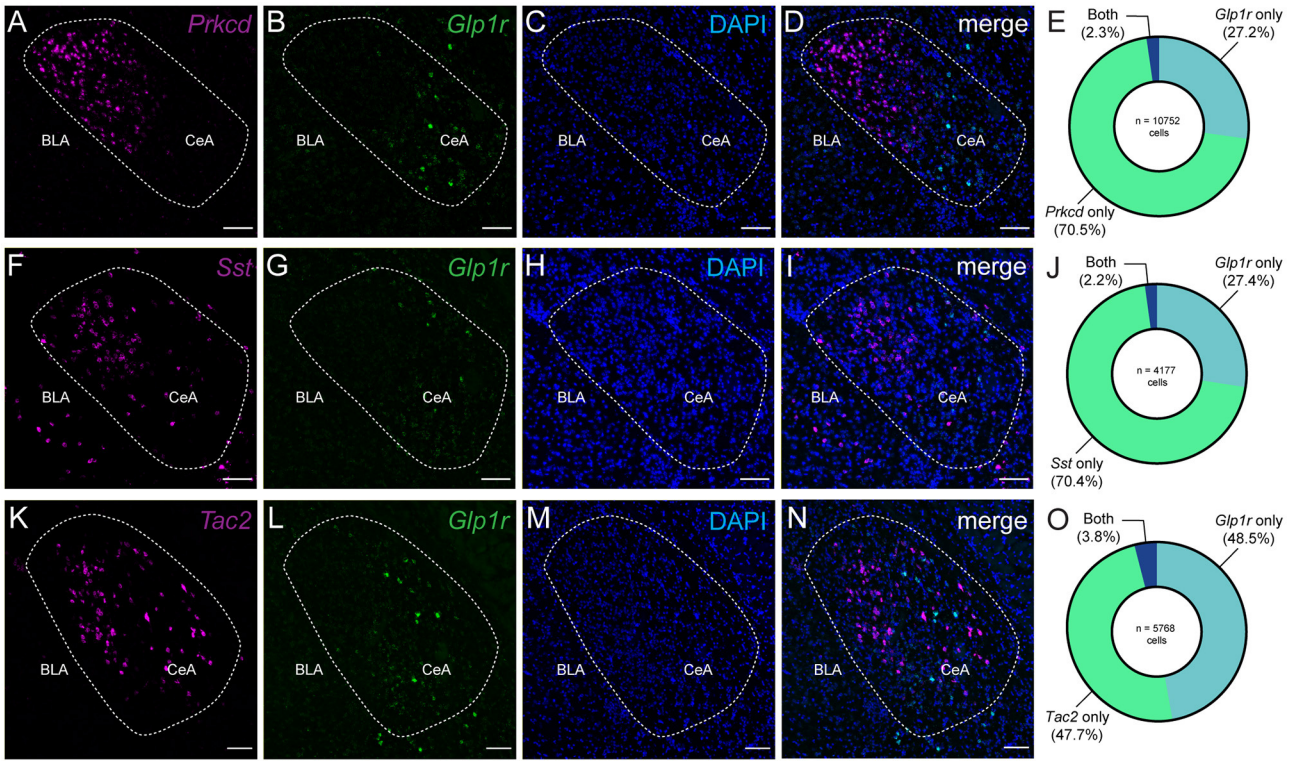

FIGURE 1 | The Glucagon-like peptide 1 receptor is expressed diffusely amongst known genetic markers of the central amygdala. **(A–D)** Example single channel and merged images of *Prkcd*, *Glp1r,* and DAPI mRNA in the CeA of wild type mice. **(E)** Donut plot depicting percentages of *Prkcd, Glp1r*, and double-labeled cells. **(F–I)** Example single channel and merged images of *Sst*, *Glp1r,* and DAPI mRNA in the CeA of wild type mice. **(J)** Donut plot depicting percentages of *Sst, Glp1r*, and double-labeled cells. **(K–N)** Example single channel and merged images of *Tac2* and *Glp1r* mRNA, and DAPI in the CeA of wild type mice. **(O)** Donut plot depicting percentages of *Tac2, Glp1r*, and double-labeled cells. For **(E,J,O)**, count data were obtained from 6 to 8 images acquired from 4 wildtype C57BL6/J mice (2 male and 2 female) with 3–4 images per hemisphere per mouse. **(E)** A total of 10,752 cells were counted with 2,688 ± 558 (s.e.) cells counted per animal. **(J)** A total of 4,177 cells were counted with 1,044 ± 82.2 (s.e.) cells counted per animal. **(O)** A total of 5,788 cells were counted with 1,442 ± 229 (s.e.) cells counted per animal. Scale bar = 100 μm for all images.

of the protein kinase c family that is highly enriched in the lateral and central subdivisions of the CeA (**Figures 1A–E**) (Haubensak et al., 2010). Of the total neurons counted ($n = 10{,}752$ cells, 2,688 ± 558 cells per mouse, 4 mice), only 2.3% expressed both *Prkcd* and *Glp1r*. Notably, the CeL also has an abundant cell group that express the neuropeptide somatostatin (*Sst*) (Li et al., 2013), and these neurons do not coexpress *Prkcd*. Together, *Sst* and *Prkcd* make up the dominant, nearly exclusive cell types in the CeL (McCullough et al., 2018b). As with *Prkcd*, we observed only a small population of *Glp1r*+ neurons coexpress *Sst* (2.2%) with respect to the total number of cells counted ($n = 4{,}177$ cells, 1,044 ± 82.2 cells per mouse, 4 mice) (**Figures 1F–J**). In both experiments, we observed that *Glp1r* transcripts were enriched in cell bodies of the medial subnucleus of the CeA (CeM); therefore, we selected *Tac2*, a neuropeptide whose expression is more enriched in the CeM. Interestingly, *Glp1r* was not robustly coexpressed with cells that express *Tac2* with only 3.8% of total neurons counted ($n = 5{,}768$ cells, 1,442 ± 229 cells per mouse, 4 mice) showing coexpression (*Glp1r*+/*Tac2*+) (**Figures 1K–O**) (Andero et al., 2014). These data are consistent with a diffuse coexpression pattern of *Glp1r* within known genetically-defined populations of the CeA.

While amplification-based *in situ* hybridization is an effective strategy for mRNA detection, we used an alternative cell-labeling approach to quantify the pattern of *Glp1r* cell distribution in the CeA. In order to validate the *Glp1r*-Cre mouse line, we employed florescence *in situ* hybridization techniques to assess the penetrance and fidelity of *Cre* expression through colocalization analysis of *Glp1r* and *Cre* mRNA (**Figures 2A–G**). Of the *Glp1r*+ cells counted ($n = 6{,}595$ cells, 824.4 ± 125.6 s.e. cells per mouse), 77.6% expressed *Cre* demonstrating high penetrance of this transgenic mouse strain. Additionally, 95.6% of total *Cre*+ cells counted ($n = 5{,}351$ cells, 668.9 ± 101.3 s.e. cells per mouse) coexpressed *Glp1r* confirming high fidelity. Together, these data verify the validity of the *Glp1r*-Cre mouse. Using *Glp1r*-Cre mice, we stereotaxically injected AAV-DIO-mCherry into the CeA (**Figure 2I**) which produced an expression pattern of mCherry that resembled native *Glp1r* mRNA. Using these mice, we serially mounted and counted $Glp1r^{CeA}$ cells across the rostral-caudal axis and observed $Glp1r^{CeA}$ cells throughout the entire range of the CeA and outside, but proximal to the CeA (**Figure 2M**). In response to this finding, we performed careful cell counts across three equal A-P segments and observed a higher density of $Glp1r^{CeA}$ cells in the middle third of the CeA;

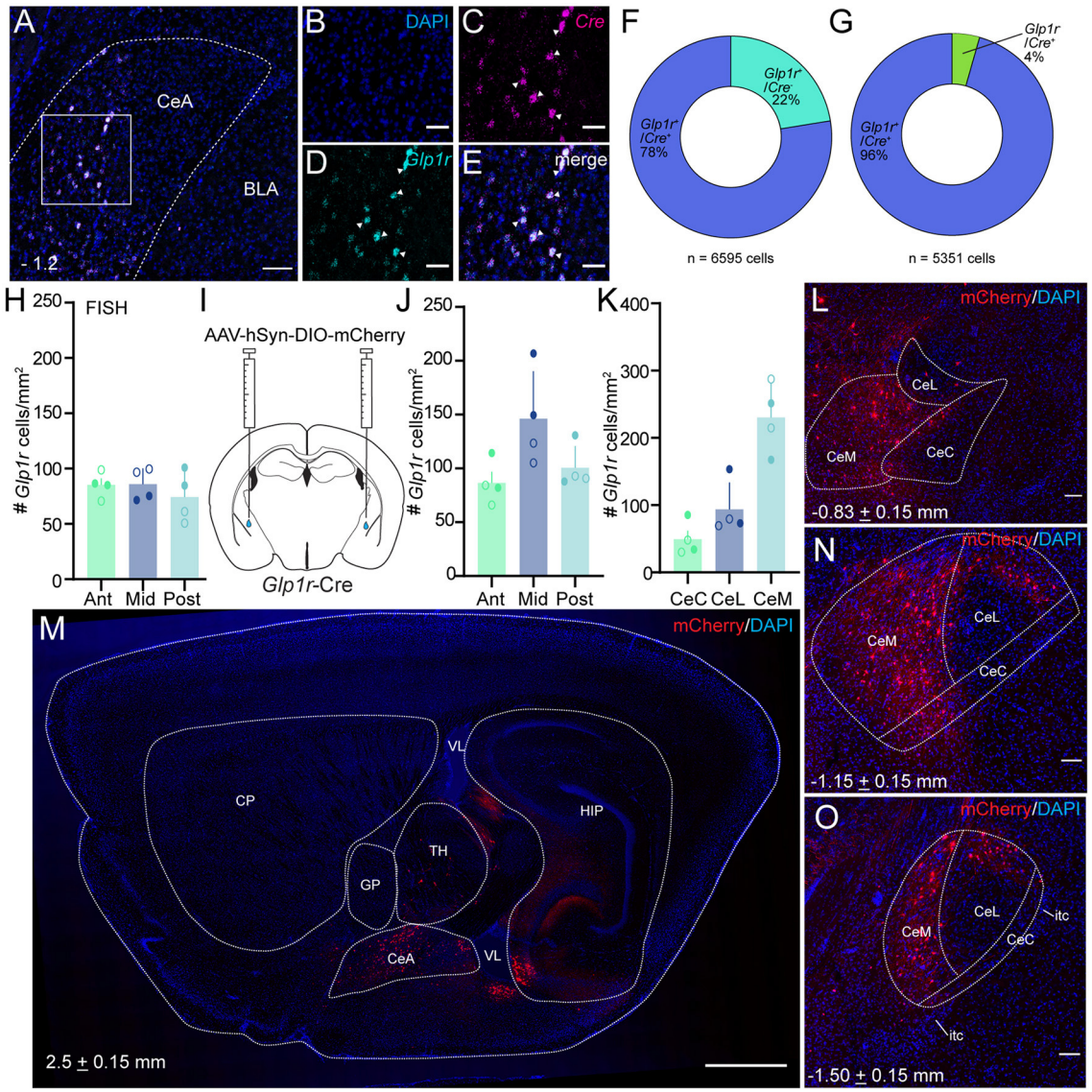

FIGURE 2 | *Glp1r*^{CeA} neurons are distributed across the rostral-caudal axis and enriched in the CeM. (**A–G**) *Glp1r*-Cre line validation using dual florescence *in situ* hybridization. **(A)** Wide field merged view of CeA with *Glp1r* in cyan and *Cre* in magenta. Square represents enlarged, single channel view shown in **(B–D)** and enlarged merged view in **(E)**. **(F)** Quantification of *Cre* mRNA expression within *Glp1r*+ neurons (n = 6,595 cells, 824.4 ± 125.6 s.e. cells per mouse). **(G)** Quantification of *Glp1r* mRNA expression within *Cre*+ neurons (n = 5,351 cells, 668.9 ± 101.3 s.e. cells per mouse). Data shown in **(F,G)** was obtained from 8 images taken from 4 mice. **(H)** Schematic of CeA injection of AAV-hSyn-DIO-mCherry in *Glp1r*-Cre mice. **(I)** Quantification of FISH labeled *Glp1r*^{CeA} neurons across the rostral-caudal axis (counts from 3 to 4 sections per subdivisions per mouse). **(J)** Quantification of virally tagged *Glp1r*^{CeA} neurons across the rostral-caudal axis [n = 4, including two male and two female wild type mice; Ordinary one-way ANOVA with Tukey's multiple comparisons test; f = 4.190, p = 0.0520 (Ant vs. Mid), 0.7997 (Ant vs. Post) and 0.1402 (Mid vs. Post)]. **(K)** Quantification of *Glp1r*^{CeA} neurons at different subdivisions of CeA [n = 4, including two male and two female wild type mice; Ordinary one-way ANOVA with Tukey's multiple comparisons test; f = 21.89, p = 0.3125 (CeC vs. CeL), 0.0004 (CeC vs. CeM) and 0.0025 (CeL vs. CeM)]. For **(H–K)**, open circles represent data obtained from female mice and closed circles represent data obtained from male mice. **(L,N,O)** Example coronal images of *Glp1r*^{CeA} distribution in anterior **(L)**, middle **(N)** and posterior **(O)** segments of the CeA. **(M)** Example sagittal image of *Glp1r*^{CeA} distribution. Note some viral tagging of putative *Glp1r* neurons outside the CeA. Scale bars represent 100 μm **(A,M)** or 50 μm **(B–E)**. For all experiments, *p < 0.05, **p < 0.01, and ***p < 0.001. CP, caudoputamen; GP, globus pallidus; TH, thalamus; VL, lateral ventricle; HIP, hippocampal region.

however, this did not reach statistical significance (**Figure 2J**). Similarly, quantification of *Glp1r*-expressing cells at the mRNA level across the three segments of the CeA revealed no difference in the density of *Glp1r*-expressing cells (**Figure 2H**). We then used anatomical landmarks and atlases to count *Glp1r*+ cells in the lateral, capsular, and medial subdivisions of the CeA and identified a significant enrichment in the density of *Glp1r*^{CeA} cells in the CeM (**Figures 2K,L,N,O**). Thus, *Glp1r*^{CeA} cells are expressed throughout the rostral-caudal axis of the CeA and enriched in the output subnucleus of the CeA - the CeM.

To gain insights into the functional nature of *Glp1r*^{CeA} cells, we used *ex vivo* brain slices combined with whole cell patch clamp electrophysiology. We injected male and female *Glp1r*-Cre mice with AAV-DIO-mCherry and waited 3–4 weeks prior to preparing fresh brain slices (**Figures 3A,B**). *Glp1r*^{CeA} neurons were identified using DIC and epifluorescence (**Figure 3C**). On average, *Glp1r*^{CeA} neurons had a membrane capacitance (Cm) of 45.75 ± 3.03 pF and a membrane resistance (Rm) of 309 ± 26.9 mΩ (**Figures 3E,F**). In gap free current clamp mode, we measured the average resting membrane potential (RMP) and excitability of *Glp1r*^{CeA} neurons. Interestingly, we observed that 42% of *Glp1r*^{CeA} neurons are spontaneously active at rest (**Figure 3D**) and had an average RMP of −51.93 ± 1.59 mV (**Figure 3G**). Spontaneously active *Glp1r*^{CeA} neurons displayed an average firing frequency of 0.47 Hz. To understand if the activity state of *Glp1r*^{CeA} neurons is associated with neurophysiological characteristics, we analyzed the Cm, Rm, and RMP within both active and inactive *Glp1r*^{CeA} neuron subgroups. Consistent with their activity states, active neurons had a higher RMP than inactive neurons (active = −43.48 ± 1.217 mV vs. inactive = −57.97 ± 1.878 mV), but we observed no differences in Cm (active = 40.66 ± 5.039 pF vs. inactive = 49.39 ± 3.681 pF) or Rm (active = 360.5 ± 53.98 mΩ vs. inactive = 272.3 ± 23.99 mΩ) (**Figures 3E–G**). We also quantified differences in basal neuronal properties in male vs. female *Glp1r*^{CeA} neuron subgroups. Surprisingly, we observed a sex difference in the Cm of *Glp1r*^{CeA} neurons, where *Glp1r*^{CeA} neurons from female mice (39.56 ± 4.584 pF) had a significantly lower Cm than male from male mice (51.95 ± 3.646 pF) (**Figure 3E**). We observed no statistically significant differences in the Rm (male = 260.1 ± 26.51 mΩ vs. female = 358 ± 45.25 mΩ) or RMP (male = −53.53 ± 2.691 mV vs. female = −50.34 ± 1.679 mV) between male and female mice (**Figures 3E,G**).

Using whole cell patch clamp electrophysiology, we probed other neurophysiological phenomena that typify CeA neurons. On average, *Glp1r*^{CeA} neurons show I_h-like currents (**Figures 3K,L,Q**) though we observed that 20% of *Glp1r*^{CeA} neurons showed no detectable I_h-like currents (inset in **Figure 3K**). We quantified I_h-like currents in both active and inactive neurons and observed a statistically significant reduction in I_h-like currents in active neurons that was most evident at a hyperpolarized potential of −120 mV (**Figure 3K**). Similarly, we observed a significant reduction in I_h-like current in *Glp1r*^{CeA} neurons from female mice relative to male mice that was evident at multiple holding potentials (**Figure 3L**).

In response to ramping positive current injections in current clamp mode, *Glp1r*^{CeA} neurons demonstrated action potentials with an average threshold of −32.8 ± 0.800 mV (**Figure 3H**). We observed no statistical differences between active (−33.08 ± 1.292 mV) and inactive (−32.64 ± 1.034 mV) or between male (−32.77 ± 1.108 mV) and female (−32.88 ± 1.179 mV) *Glp1r*^{CeA} neurons. As nearly half of *Glp1r*^{CeA} neurons were spontaneously active, we measured the current needed to produce an action potential (rheobase) at rest and while holding the cell at −70 mV. At rest, the rheobase for *Glp1r*^{CeA} neurons was 64.26 ± 6.45 pA whereas at −70 mV the rheobase was 118.8 ± 10.74 pA (**Figures 3I,J**). At rest, we found that active neurons (39.22 ± 8.305 pA) demonstrate a significantly lower rheobase than inactive neurons (82.15 ± 7.814 pA) (**Figure 3I**). Interestingly, active neurons (76.99 ± 9.579 pA) displayed a significantly lower rheobase than inactive neurons (140.9 ± 16.24 pA) even when held at −70 mV (**Figure 3J**). We observed no statistically significant differences in *Glp1r*^{CeA} neuron rheobase between male and female mice at rest (males: 69.97 ± 8.487 pA vs. females: 58.56 ± 9.756 pA) (**Figure 3I**) or at −70 mV (males: 118.3 ± 14.66 pA vs. females: 115.6 ± 18.79 pA) (**Figure 3J**).

To further characterize *Glp1r*^{CeA} neuronal excitability, we measured changes in membrane voltage and action potentials in response to increasingly positive square current steps in *Glp1r*^{CeA} neurons held at their resting membrane potential and at −70 mV. In response to current injections, we found that active *Glp1r*^{CeA} neurons display significantly more action potentials than inactive *Glp1r*^{CeA} neurons at rest (**Figure 3M**) or at −70 mV (**Figure 3O**) and the increase in the number of action potentials was most pronounced at the highest positive current injections. We observed no difference in current injection-induced action potential firing between male and female *Glp1r*^{CeA} neurons either at rest (**Figure 3N**) or held at −70 mV (**Figure 3P**). Forty-one percent of *Glp1r*^{CeA} neurons showed spike frequency adaptation at depolarizing current steps (data not shown). Using these data we also classified each neuron according to spike characteristics (Dumont et al., 2002; Chieng et al., 2006; Herman et al., 2013; Li and Sheets, 2018). The majority (73%) of *Glp1r*^{CeA} neurons were a low threshold bursting (LTB) subtype with a characteristic rebound action potential that occurs following cessation of a hyperpolarizing current step and low latency action potentials in response to depolarizing current steps (**Figure 3R**). LTB *Glp1r*^{CeA} neurons were equally distributed between active and inactive neurons (inset pie chart in **Figure 3R**). Moreover, we did not observe a significant difference in the overall distribution of spike types amongst active/inactive or male/female *Glp1r*^{CeA} neurons (**Figure 3S**). The sites of these recordings are presented in **Figure 3T**.

DISCUSSION

In this study, we performed the first deep anatomical characterization of *Glp1r* expression and neurophysiology study of *Glp1r*-expressing cells in the CeA of C57BL6/J mice. At an anatomical level, a previous study using a *Glp1r*-Cre mouse strain identified *Glp1r*-expressing cells throughout the brain including the CeA (Cork et al., 2015). Their observations are mirrored by a study from Jensen and colleagues who used a novel GLP-1R antibody and observed GLP-1R protein expression in the CeA (Jensen et al., 2018). In a recent study using a transgenic mouse with an mApple-tagged GLP-1R, the authors rigorously mapped sites of GLP-1R expression throughout the brain and made similar observations, including robust expression in the CeA (Graham et al., 2020). Consistent with their findings using a transgenic mouse, the authors used fluorescence *in situ* hybridization as a convergent method to characterize native *Glp1r* mRNA. They showed that *Glp1r* mRNA is present in the CeA and medial amygdala, and, in the CeA, *Glp1r* is coexpressed

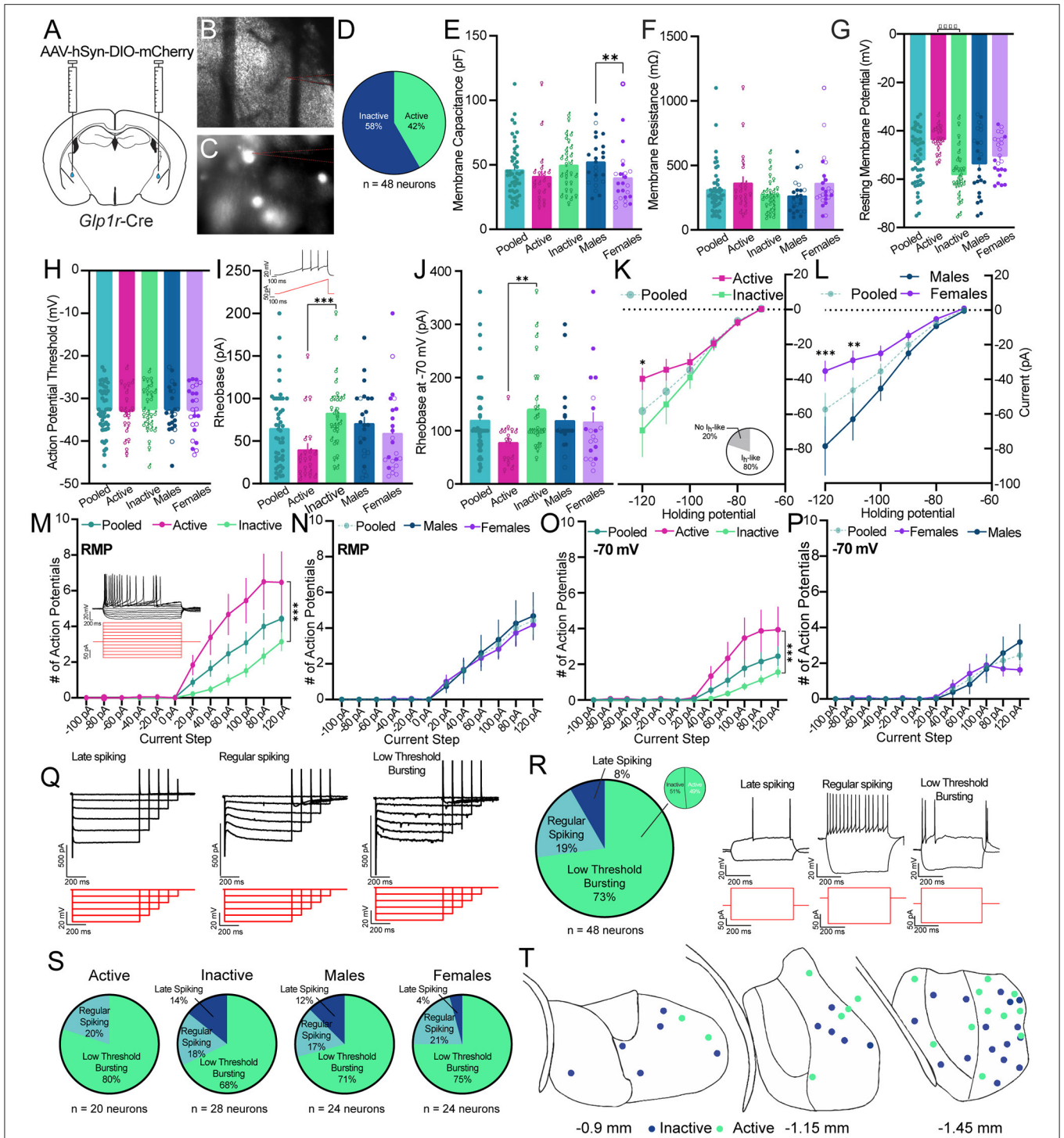

FIGURE 3 | *Ex vivo* patch clamp electrophysiological characterization of *Glp1r*^{CeA} neurons. **(A)** Schematic of CeA injection of pAAV-hSyn-DIO-mCherry in *Glp1r*-Cre mice. **(B,C)** Example images of live brain slices and fluorescently labeled cells in the CeA during electrophysiological recordings from *Glp1r*-Cre mice. **(D)** Pie chart illustrating the number of *Glp1r*^{CeA} neurons that were active/inactive. **(E)** Average membrane capacitance (Cm) of *Glp1r*^{CeA} neurons in all cells (pooled) (teal), in neurons empirically determined to be active (pink) or inactive (green), or in neurons recorded from male (blue) or female (purple) mice. Within the active/inactive data sets the sex of the mouse from which the neuron was recorded is depicted by use of male and female symbols. Within the male/female data sets the activity state of the neuron is indicated by a closed (inactive) or open (active) symbol. Data were analyzed using a Mann-Whitney *U* test between active/inactive ($p = 0.0736$) and male/female ($p = 0.0063$) subgroups. **(F)** Average membrane resistance (Rm) of *Glp1r*^{CeA} neurons in all cells (pooled) or active, inactive, male, or female subgroups. The symbol conventions used are identical to those used in **(E)**. Data were analyzed using a Mann-Whitney *U* test between active/inactive ($p = 0.2296$) and

(Continued)

FIGURE 3 | male/female ($p = 0.0609$) subgroups. **(G)** Average resting membrane potential (Vrest) of all *Glp1r*^{CeA} neurons (pooled), or active, inactive, male, or female subgroups. The symbol conventions used are identical to those used in **(E)**. Data were analyzed using a Mann Whitney *U* test between active/inactive ($p < 0.0001$) and male/female ($p = 0.4677$) subgroups. **(H)** Average action potential threshold of all *Glp1r*^{CeA} neurons (pooled), or active, inactive, male, or female subgroups. The symbol conventions used are identical to those used in **(E)**. Data were analyzed using a Mann-Whitney *U* test between active/inactive ($p = 0.8319$) and male/female ($p = 0.9796$) subgroups. **(I)** Average rheobase of all *Glp1r*^{CeA} neurons (pooled), or active, inactive, male, or female subgroups at rest. The symbol conventions used are identical to those used in **(E)**. Data were analyzed using a Mann-Whitney *U* test between active/inactive ($p = 0.0001$) and male/female ($p = 0.2276$) subgroups. Inset: representative cell response in current clamp mode to gradually increasing depolarizing current injection (rheobase). Bottom: Stimulation waveform **(J)** Average rheobase current of all *Glp1r*^{CeA} neurons (pooled), or active, inactive, male, or female subgroups at −70 mV. The symbol conventions used are identical to those used in **(E)**. Data were analyzed using a Mann-Whitney *U* test between active/inactive ($p = 0.0058$) and male/female ($p = 0.4877$) subgroups. **(K)** Average I_h-like current at hyperpolarizing membrane potentials in all, active, or inactive *Glp1r*^{CeA} neurons. Active and inactive subgroups were compared using 2-way repeated measures ANOVA with Sidak's multiple comparisons test at each holding potential; Holding potential X Activity state: $F_{(5,215)} = 2.413$, $p = 0.0373$; Holding potential: $F_{(5,215)} = 28.95$, $p < 0.0001$; Activity state: $F_{(1,43)} = 1.467$, $p = 0.2324$. The inset pie chart displays the proportion of neurons that demonstrate significant I_h-like current using a threshold of −20 pA for the −120-mV voltage step. **(L)** Average I_h-like current at hyperpolarizing membrane potentials in all, male, or female *Glp1r*^{CeA} neurons. Male and female subgroups were compared using 2-way repeated measures ANOVA with Sidak's multiple comparisons test at each holding potential; Holding potential X Sex: $F_{(5,215)} = 4.976$, $p = 0.0002$; Holding potential: $F_{(5,215)} = 34.80$, $p < 0.0001$; Sex: $F_{(1,43)} = 6.394$, $p = 0.0152$. **(M)** Action potentials in response to depolarizing current steps at rest in all, active, or inactive *Glp1r*^{CeA} neurons. Active and inactive subgroups were compared using 2-way repeated measures ANOVA with Sidak's multiple comparisons test at each current injection; Current Injected X Activity state: $F_{(11,473)} = 7.881$, $p < 0.0001$; Current Injected: $F_{(11,473)} = 33.93$, $p < 0.0001$; Activity state: $F_{(1,43)} = 11.83$, $p = 0.0013$. Top: representative cell response in current clamp mode to increasing current steps. **(N)** Action potentials in response to depolarizing current steps at rest in all, male, or female *Glp1r*^{CeA} neurons. Male and female subgroups were compared using 2-way repeated measures ANOVA with Sidak's multiple comparisons test at each current injection; Current Injected X Sex: $F_{(11,473)} = 0.2256$, $p = 0.9958$; Current Injected: $F_{(11,473)} = 24.96$, $p < 0.0001$; Sex: $F_{(1,43)} = 0.07738$, $p = 0.7822$. **(O)** Action potentials in response to depolarizing current steps at −70 mV in all, active, or inactive *Glp1r*^{CeA} neurons. Active and inactive subgroups were compared using 2-way repeated measures ANOVA with Sidak's multiple comparisons test at each current injection; Current Injected X Activity state: $F_{(11,418)} = 5.822$, $p < 0.0001$; Current Injected: $F_{(11,473)} = 19.79$, $p < 0.0001$; Activity state: $F_{(1,38)} = 8.353$, $p = 0.0063$. **(P)** Action potentials in response to depolarizing current steps at −70 mV in all, male, or female *Glp1r*^{CeA} neurons. Male and female subgroups were compared using 2-way repeated measures ANOVA with Sidak's multiple comparisons test at each current injection; Current Injected X Sex: $F_{(11,418)} = 1.358$, $p = 0.1903$; Current Injected: $F_{(11,418)} = 14.02$, $p < 0.0001$; Sex: $F_{(1,38)} = 0.05488$, $p = 0.8160$. **(Q)** Example I_h-like currents across three observed spiking subtypes for *Glp1r*^{CeA} neurons. The capacitive currents have been cropped for visualization purposes. Voltage step waveforms are shown below each cell. **(R)** Pie chart illustrating the distribution of spiking subtypes (late spiking, regular spiking, or low-threshold bursting) for *Glp1r*^{CeA} neurons in response to hyperpolarizing or depolarizing current steps. Example late spiking, regular spiking and low-threshold bursting *Glp1r*^{CeA} neurons in response to depolarizing current steps are shown to the right. **(S)** Pie charts illustrating the distribution of spiking subtypes among inactive, active, male, or female subgroups. The distributions of active/inactive and male/female subgroups were compared using a Chi-squared test for trend ($p = 0.1511$ for active/inactive and $p = 0.4911$ for male/female). **(T)** Schematic of distribution of recorded *Glp1r*^{CeA} neurons. Blue dots represent inactive and green dots represent active neurons. $N = 48$ neurons from male ($n = 10$) and female ($n = 7$) mice with 24 neurons per sex.

with the GABAergic neuronal biosynthetic enzyme *Gad1*. Since *Gad1* is only expressed in neurons, we infer that the *Glp1r* cells we characterized in our study are primarily neuronal.

We observed that *Glp1r*^{CeA} neurons are not defined by the sole coexpression with either *Prkcd*, *Sst,* or *Tac2*. *Prkcd* is a gene that marks a population of lateral and capsular CeA neurons that are activated by aversive unconditioned stimuli, inhibited by aversive conditioned stimuli, and inhibit food intake when activated (Haubensak et al., 2010; Cai et al., 2014; Cui et al., 2017; McCullough et al., 2018a). *Sst* is a neuropeptidergic gene that marks a population of lateral and medial CeA neurons almost completely distinct from *Prkcd* that are activated by aversive conditioned stimuli, modulate passive and active defensive responses, and promote freezing and behavioral cessation when activated using optogenetics (Li et al., 2013; Yu et al., 2016). *Tac2* is a neuropeptidergic gene enriched in medial and some lateral CeA neurons and modulates the expression of conditioned fear (Andero et al., 2014, 2016). A recent study identified that ∼50% of *Glp1r*^{CeA} neurons coexpress the cytokine interleukin-6 (IL-6) and that direct administration of IL-6 to the CeA could produce a subtle decrease in feeding, but the functional impact of IL-6 in combination with CeA GLP-1Rs is unclear (Anesten et al., 2019). The limited overlap we observed between *Prkcd*/*Sst*/*Tac2* and *Glp1r* in the CeA suggests that the anorexigenic actions of peripherally-applied or intracerebral GLP-1R agonists are likely not due to the direct activation of these neurons, but do not preclude their activation through indirect network effects (Gabery et al., 2020). Further experiments using *in vivo* recordings are necessary to test direct and indirect models of action of GLP-1R agonists on these genetically defined CeA neurons.

Using Cre-dependent viral tracing in combination with *Glp1r*-Cre mice, we performed cell counts of *Glp1r* cells across the rostral-caudal axis and in CeA subnuclei. *Glp1r*^{CeA} neurons are located throughout the rostral-caudal axis with a non-significant increase in the middle third of the CeA and are more densely distributed in the CeM. Coincidently, the CeA also receives innervation from Preproglucagon (PPG, the precursor gene of GLP-1)-expressing neurons in the nucleus of the solitary tract and this innervation is most dense in the CeM (Llewellyn-Smith et al., 2011; Williams et al., 2018; Anesten et al., 2019). The functional and behavioral role of PPG inputs to the CeA are unknown.

Using brain slice electrophysiology, we performed a neurophysiological characterization of *Glp1r*^{CeA} neurons in male and female mice. Surprisingly, we found that nearly half of these neurons are active at rest. We analyzed neurophysiological characteristics associated with inherent neuronal activity states. Not surprisingly, we found that active *Glp1r*^{CeA} neurons have a higher resting membrane potential than inactive neurons and are more sensitive to current injections. Interestingly, the increased sensitivity of active *Glp1r*^{CeA} neurons to current injection-induced action potentials persists even when held at an identical polarized membrane potential of −70 mV. As seen in other CeA neuronal physiology studies, *Glp1r*^{CeA} neurons on average show hyperpolarization-activated (I_h) currents; however, we did

observe some cells for which no substantial I_h-like current was detected (20%). Interestingly, we found reduced I_h-like currents in *Glp1r*^{CeA} neurons recorded from female mice and that, on average, these neurons have a lower membrane capacitance. The functional implications of these sex differences is unclear although they may be related to overall morphometric size differences that have been reported in the amygdala (Hines et al., 1992; Qiu et al., 2018). For *Glp1r*^{CeA} neurons, we characterized their excitability using current injections in current clamp mode and determined their spiking subtype. Overall, we found that *Glp1r*^{CeA} neurons are predominantly a low threshold bursting subtype characterized by rebound spikes from hyperpolarizing current injections. We speculate that cells of the low threshold burst subtype may be particularly sensitive to excitatory input or activation of intracellular signaling pathways that enhance excitability like GLP-1Rs. Several heroic neurophysiological studies of CeA neurons in general or CeL neurons demonstrate a diversity of spiking types (Dumont et al., 2002; Chieng et al., 2006; Hunt et al., 2017; Adke et al., 2021). Only a couple of studies have focused neurophysiological recordings on the CeM, which is more diverse with respect to gene expression and axonal projections (Herman et al., 2013; Li and Sheets, 2018; McCullough et al., 2018b). Interestingly Li and colleagues showed that periaqueductal gray (PAG)-projecting CeA neurons are distributed in the CeL and CeM and did an extensive study of their firing properties and excitability broken down by firing subtype. PAG-projecting CeM neurons are heterogenous and comprised of equal proportions of regular spiking, fast-spiking, and bursting subtype and demonstrate voltage sag in response to hyperpolarizing current injections indicative of I_h-like currents (Li and Sheets, 2018). However, the authors did not observe sex differences in the voltage sag of PAG-projecting CeM neurons. These data suggest that *Glp1r*^{CeA} neurons are likely distinct from CeM-projecting neurons, however further studies using anterograde and tracers are needed to rule out this possibility. CeA neurons that express corticotropin releasing factor receptor 1 (CRFR1 or *Crhr1*) are distributed in the medial and lateral subdivisions of the CeA, and the reported membrane properties, resting membrane potential, and spiking types most closely match those in this study (Herman et al., 2013). CRFR1, like GLP-1R, is a $G_{\alpha s}$-coupled receptor so it will be interesting to know in the future if *Glp1r*^{CeA} neurons coexpress *Crhr1*.

GLP-1R agonists are widely prescribed for the treatment of type II diabetes and they also have the added benefit of suppressing appetite and producing weight loss (Drucker, 2018). Recently the FDA approved the use of semaglutide (a new generation GLP-1R agonist) for the treatment of obesity. Unfortunately, the mechanisms by which these drugs exert their anorexigenic effects in the brain is still unclear. We hypothesize that the CeA, in addition to the hypothalamus and hindbrain, is an important limbic nucleus that mediates some of the anorexigenic effects of these drugs. Because the CeA is a nucleus that receives endogenous GLP-1 through preproglucagon inputs from the NTS, we also speculate that that CeA GLP-1Rs may play a role in feeding, emotional, and motivational processing. One study using non-human primates demonstrated that GLP-1Rs are present in the amygdala (Heppner et al., 2015), but further research using post-mortem tissue from humans is needed to demonstrate that GLP-1Rs are expressed in the human amygdala. In addition, basic research using genetic and neural circuit approaches are needed to characterize a circuit logic for the mode of action of GLP-1R agonists.

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 UAB IACUC.

AUTHOR CONTRIBUTIONS

NZ, EC, CL, SK, and MD collected data for the manuscript. NZ and JH analyzed the data. NZ, SV, and JH wrote the manuscript and assembled the figures. JH designed the study. All authors contributed to the article and approved the submitted version.

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Effects of Glucagon-Like Peptide-1 Receptor Agonist Exendin-4 on the Reinstatement of Cocaine-Mediated Conditioned Place Preference in Mice

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A high percentage of relapse to compulsive cocaine-taking and cocaine-seeking behaviors following abstinence constitutes a major obstacle to the clinical treatment of cocaine addiction. Thus, there is a substantial need to develop effective pharmacotherapies for the prevention of cocaine relapse. The reinstatement paradigm is known as the most commonly used animal model to study relapse in abstinent human addicts. The primary aim of this study is to investigate the potential effects of systemic administration of glucagon-like peptide-1 receptor agonist (GLP-1RA) exendin-4 (Ex4) on the cocaine- and stress-triggered reinstatement of cocaine-induced conditioned place preference (CPP) in male C57BL/6J mice. The biased CPP paradigm was induced by alternating administration of saline and cocaine (20 mg/kg), followed by extinction training and then reinstatement by either a cocaine prime (10 mg/kg) or exposure to swimming on the reinstatement test day. To examine the effects of Ex4 on the reinstatement, Ex4 was systemically administered 1 h after the daily extinction session. Additionally, we also explored the associated molecular basis of the behavioral effects of Ex4. The expression of nuclear factor κβ (NF-κβ) in the nucleus accumbens (NAc) was detected using Western blotting. As a result, all animals that were treated with cocaine during the conditioning period successfully acquired CPP, and their CPP response was extinguished after 8 extinction sessions. Furthermore, the animals that were exposed to cocaine or swimming on the reinstatement day showed a significant reinstatement of CPP. Interestingly, systemic pretreatment with Ex4 was sufficient to attenuate cocaine- and stress-primed reinstatement of cocaine-induced CPP. Additionally, the expression of NF-κβ, which was upregulated by cocaine, was normalized by Ex4 in the cocaine-experienced mice. Altogether, our study reveals the novel effect of Ex4 on the reinstatement of cocaine-induced CPP and suggests that GLP-1R agonists appear to be highly promising drugs in the treatment of cocaine use disorder.

Keywords: cocaine, reinstatement, exendin-4, pretreatment, nuclear factor κβ, conditioned place preference, swim

INTRODUCTION

Recent epidemiological data from the World Drug Report indicate that substance use disorder is spreading widely and rapidly, causing about 585,000 deaths worldwide per year (UNODC, 2019). Cocaine is considered one of the most commonly consumed illicit psychostimulants (Butler et al., 2017). Additionally, cocaine abuse is characterized by a high rate of relapse following a long period of detoxification. In abstinent human addicts, stressful life events and reexposure to the psychostimulants themselves are well known as two major stimuli for relapse to cocaine-associated behavior (Jaffe et al., 1989; O'Brien, 1997; Sinha et al., 1999). In fact, to date, the high relapse rate remains a major obstacle to the clinical treatment of cocaine-addicted individuals (O'Brien, 1997). Unfortunately, despite extensive efforts over the past decades to search for medications to treat cocaine addiction and reduce relapse, the effectiveness of available modulators has been reported to be limited. Thus, there is a substantial need for identifying more effective medications to treat cocaine use disorder and prevent relapse.

The glucagon-like peptide-1 (GLP-1) is traditionally thought to be a neuropeptide and circulating hormone found both in the nucleus tractus solitarius (NTS) neurons of the hindbrain (Alvarez et al., 1996) and in L cells of the intestines (Novak et al., 1987). While GLP-1 has a short duration of action, GLP-1 receptor (GLP-1R) agonists including exendin-4 (Ex4) display a longer half-life (Gallwitz, 2004) compared with GLP-1 and easily cross the blood-brain barrier (Holscher, 2010). Additionally, GLP-1R agonists can effectively regulate food intake (Hayes et al., 2008) as well as glucose-dependent insulin secretion (Holst and Seino, 2009), gastric emptying, and glucagon secretion (Matsuyama et al., 1988). Given these biological properties, Ex4 is clinically used for the treatment of type II diabetes mellitus and obesity (Andersen et al., 2018). Besides, Ex4 has also received increasing attention in recent years because of the suppressive actions on the maladaptive behavior in rodents including cocaine-induced CPP as well as locomotor activity and self-administration (Hernandez and Schmidt, 2019). However, few studies, thus far, have explored the potential effects of Ex4 on the cocaine- and stress-triggered reinstatement of cocaine-induced CPP and the expression of nuclear factor κβ (NF-κβ) in the nucleus accumbens (NAc).

Nuclear factor κβ is generally referred to as an important transcription factor due to its involvement in the transcription of many genes, such as those heavily implicated in immune responses and inflammation (Chen and Greene, 2004; Lin and Karin, 2007). These neuroinflammatory reactions are believed to play a pivotal role in neural adaptations after repeated consumption of psychostimulants (Kohno et al., 2019). Meanwhile, it is interesting to note that the anti-inflammatory agents appear highly promising in the treatment of substance use disorders (Kohno et al., 2019). For instance, previous studies indicated that inhibition of NF-κβ can block the rewarding effects of cocaine and the ability of previous cocaine exposure to augment the place preference of an animal for the cocaine-paired environment (Russo et al., 2009). Furthermore, the underlying benefits of some pharmacological agents including garcinol (Dunbar and Taylor, 2017; Monsey et al., 2017) and cannabidiol (de Carvalho and Takahashi, 2017) on cocaine-seeking behavior could also be attributed, at least partially, to an inhibition of neuroinflammation (e.g., direct and indirect inhibition of NF-κβ activation) and antioxidant (e.g., inhibition of iNOS) properties (Padhye et al., 2009; Kozela et al., 2017). Accordingly, modulation of NF-κβ signaling may be used as a promising strategy for addiction treatment. Importantly, other GLP-1R agonists have been confirmed to reduce the activation of the NF-κβ signaling by inhibiting the p65 subunit of the NF-κβ complex (Kozela et al., 2017; Ma et al., 2018). Therefore, it is conceivable that GLP-1R agonist Ex4 may attenuate cocaine- and stress-primed reinstatement of cocaine-mediated CPP and contribute to neuroplastic alternations in the NF-κβ-p65 expression in the cocaine-experienced mice.

In this study, we first evaluated the behavioral effects of the clinically available GLP-1R agonist Ex4 on the cocaine- and stress-triggered reinstatement of cocaine-induced conditioned place preference (CPP) in male C57BL/6J mice. In addition, the associated changes in the accumbal expression of NF-κβ-p65 were explored as well.

MATERIALS AND METHODS

Animals

Male C57BL/6J mice (aged: 8–10 weeks, weighing 18–24 g) were obtained from the Experimental Animal Center of Ningxia Medical University (Yinchuan, China). They were maintained in specific pathogen-free animal cages on a reversed light cycle with lights on at 07:00 p.m. and lights off at 07:00 a.m. They were housed in groups (4 per cage) in a temperature-controlled room (23 ± 3°C), and standard rodent chow and tap water were available *ad libitum*. Behavioral testing was conducted during the light cycle of the mice. Mice were habituated for 1 week before any experimentation. To reduce the psychological stress provoked by an unfamiliar room, each mouse was habituated in the testing room for 30 min before the beginning of each behavioral testing. In this study, all experimental procedures involving animals and equipment were conducted according to the associated laws and regulations and were authorized by the Animal Research Ethics Committee of Ningxia Medical University.

Reagents and Antibodies

The testing drug cocaine-hydrochloride (cocaine-HCl) was provided by China National Medicines Corporation Limited (Beijing, China). Cocaine-HCl was dissolved in 0.9% physiological saline solution (0.9% NaCl) to a concentration of 4 mg/ml and freshly prepared for daily injection. The volume of injection was 0.1 ml [(0.4 mg × 1 ml)/4 mg]. Saline was injected as a vehicle solution in this study. Ex4 was purchased from MedChemExpress (MCE, United States) and dissolved in 0.9% NaCl solution at a concentration of 0.04 μg/ml. It was administered at 2.0 μg/kg body weight and was prepared prior to daily injection. Ex4 and cocaine-HCl were alternatively administered on the left or right side of the peritoneum.

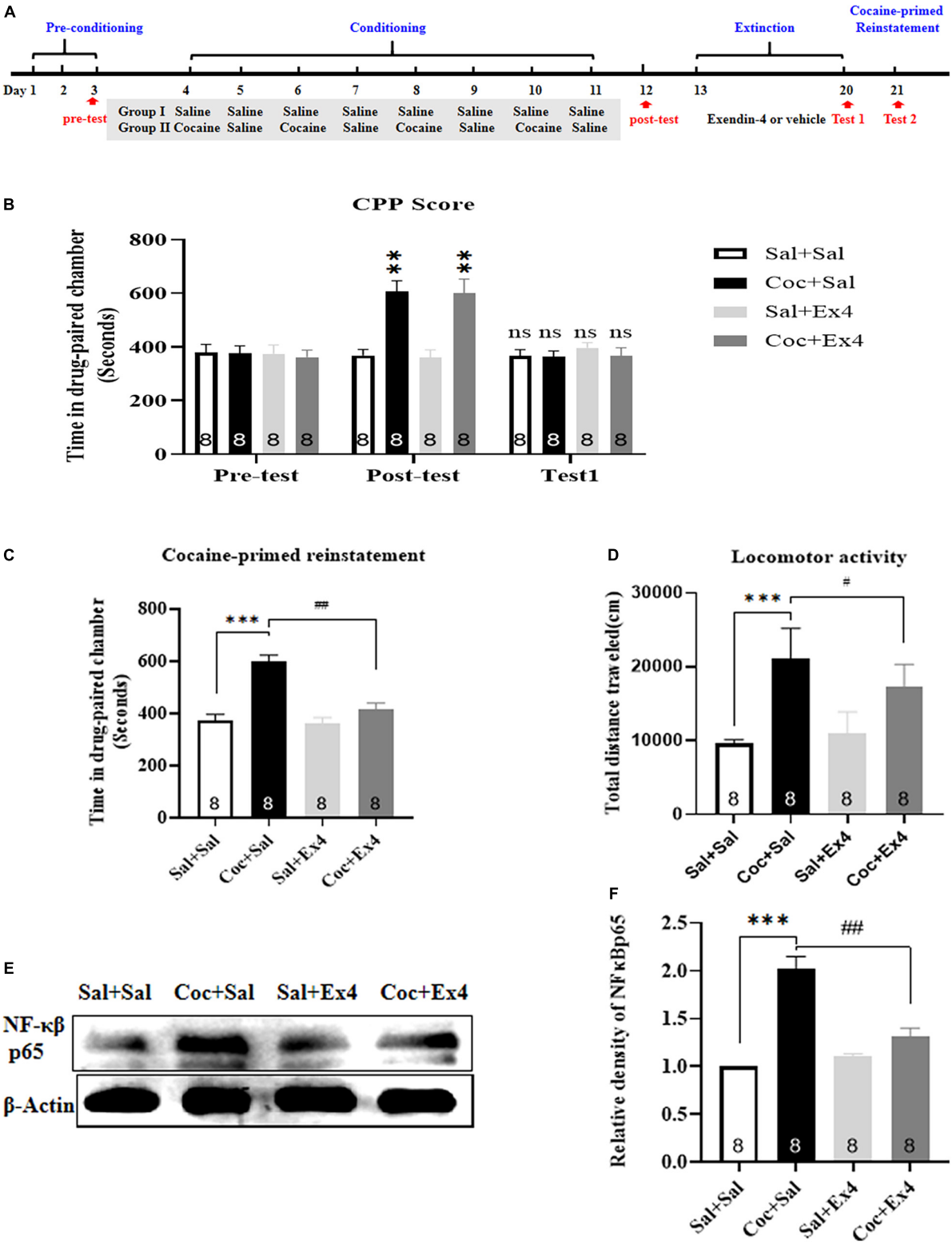

FIGURE 1 | The systemic pretreatment with exendin-4 prevented cocaine-primed reinstatement of cocaine-induced CPP. **(A)** The experimental schedule for saline as well as cocaine and exendin-4 treatments. **(B)** After cocaine conditioning, animals displayed a significant place preference for the cocaine-paired compartment. ** represents $p < 0.01$ vs Sal + Sal group, $n = 8$ each group, two-way rmANOVA. Additionally, CPP scores after vehicle or exendin-4 administration suggested that the cocaine-induced CPP was completely extinguished after 8 days extinction training. ns represents no significant, difference, $n = 8$ each group, two-way rmANOVA followed by Dunnett's multiple comparisons test. **(C)** The systemic pretreatment with exendin-4 significantly prevented cocaine-primed reinstatement of

(Continued)

FIGURE 1 | cocaine-induced CPP. Half dose of cocaine on the reinstatement day induced a significant reinstatement CPP that was attenuated by The systemic pretreatment with exendin-4 after each extinction. *** represents $p < 0.001$ vs Sal + Sal, ## represent $p < 0.01$ vs Coc + Sal, $n = 8$ each group, two-way ANOVA followed by a Tukey's *post hoc* test. **(D)** The systemic pretreatment with exendin-4 significantly attenuated locomotor activity. Half dose of cocaine on the reinstatement day induced a significant increase in locomotion that was attenuated by systemic pretreatment with exendin-4 after each extinction. *** represents $p < 0.001$ vs Sal + Sal, # represent $p < 0.05$ vs Coc + Sal, $n = 8$ each group, two-way ANOVA followed by a Tukey's *post hoc* test. **(E)** Representative image of NF-κβ p65 proteins in different groups detected by Western blot analysis at the end of CPP test of reinstatement ($n = 8$ in each group). **(F)** Semiquantitative analysis of the relative levels of NF-κβ p65 by densitometric analysis in different groups. Priming induced by cocaine up-regulated the expression of NF-κβ p65, and this effect was alleviated by systemic pretreatment with exendin-4 after each extinction. *** represents $p < 0.001$ vs Sal + Sal, ## represent $p < 0.01$ vs Coc + Sal, two-way ANOVA followed by a Tukey's *post hoc* test. All data are presented as the mean ± SEM.

Antibody for phospho-NF-κβ subunit p65 was purchased from Abcam (San Francisco, CA, United States). During the whole period of experiments, all antibodies and drugs were stored in refrigerators at −20 and 4°C, respectively.

Conditioning Apparatus

The general procedure of this study was performed as depicted previously with slight modifications (Meye et al., 2016). In brief, the place-conditioning chambers consisted of two larger distinct compartments (24 cm × 14 cm × 30 cm) divided by a smaller intermediate compartment (7.0 cm × 7.0 cm × 30 cm) with two removable guillotine doors. On each test day, guillotine doors were raised, so the animals had access to move freely throughout the entire place-conditioning chambers during the habituation period and on the test days. However, the removable guillotine doors were closed during the conditioning and extinction training. One of the larger compartments was composed of four black walls and a smooth black floor, and the other compartment was composed of four white walls and a rough white floor covered with blue sandpaper. The smaller central compartment included two gray walls and a smooth floor leading into the corresponding compartment. Briefly, these compartments with different colors and floor textures provide animals with different visual and tactile cues when they receive cocaine or saline injections. Tracking of the mice in the apparatus was performed using an infrared video camera suspended about 1 m above the conditioning apparatus. The overhead infrared camera and computer were used to record the real-time positions of the mice and their movement throughout the three compartments. The time spent and total distance traveled in each chamber were recorded using a computerized video tracking system (behavior analysis software Smart 3.0; Panlab, Spain; supported by RWD Life Science Co. Ltd., China). The biased CPP protocol was used in this study.

Preconditioning Stage (Days 1–3)

On days 1–2, the guillotine doors were raised, and all mice were placed in the smaller central compartment to freely move throughout the three compartments for 20 min prior to the commencement of the pre-test. On day 3, mice were placed in the middle area of the conditioning apparatus with the guillotine doors open, and their time spent in each compartment was recorded for 20 min to determine the baseline preference. As previously described in the literature, the white compartment (non-preferred compartment) was assigned as the cocaine-paired compartment, and the black one (preferred compartment) was assigned as the saline-paired compartment in our study (Meye et al., 2016). The CPP score was designed as a significant increase in the amount of time that mice spent in the cocaine-paired chamber after the conditioning or on the reinstatement test day compared with control mice (Sal–Sal group).

Acquisition of Conditioned Place Preference (Days 4–12)

After completing the baseline preference, animals were randomly divided into two groups (saline group and drug group) for the CPP training. In detail, for the saline group (Group I), animals were given a daily injection of saline no matter which compartment the animals were placed in. For the treated group (Group II), mice were conditioned with saline or cocaine (20 mg/kg) for 20 min on alternate days. The place preference testing took place on day 12. On the drug-free test day, the removable guillotine doors were lifted, and mice were positioned in the center of the conditioning apparatus and allowed to freely explore for 20 min. After the mice completed the testing, they were returned to their cages. The CPP score is expressed as the time spent in the drug-paired chamber.

Extinction of Conditioned Place Preference (Days 13–20)

Following the CPP test, animals were randomly separated into two groups (vehicle group and Ex4 group that was treated with Ex4 or saline 1 h after each extinction training, respectively), resulting in four independent groups: Sal + Sal, Coc + Sal, Sal + Ex4, and Coc + Ex4. During this time, half of the group of mice received daily administration of Ex4, and the other half received a daily injection of saline 1 h after they were confined in the previously designated cocaine-paired compartment for 20 min. Place preference for the cocaine-paired compartment was reexamined on day 20 to evaluate whether CPP was successfully extinguished after 8 extinction sessions. In this study, extinction criteria were considered to be achieved when there was no significant difference in the CPP scores in comparison with the Sal + Sal group.

Reinstatement of Conditioned Place Preference (Day 21)

Following the extinction test, mice were reinstated by a swim protocol or a lower dose of cocaine (10 mg/kg, intraperitoneal [i.p.]). On the reinstatement test day, the guillotine doors were raised, and animals were provided free access to the entire conditioning apparatus for 20 min. The aim of the reinstatement test was to evaluate the effects of Ex4 pretreatment during extinction on the reinstatement of the cocaine-induced CPP.

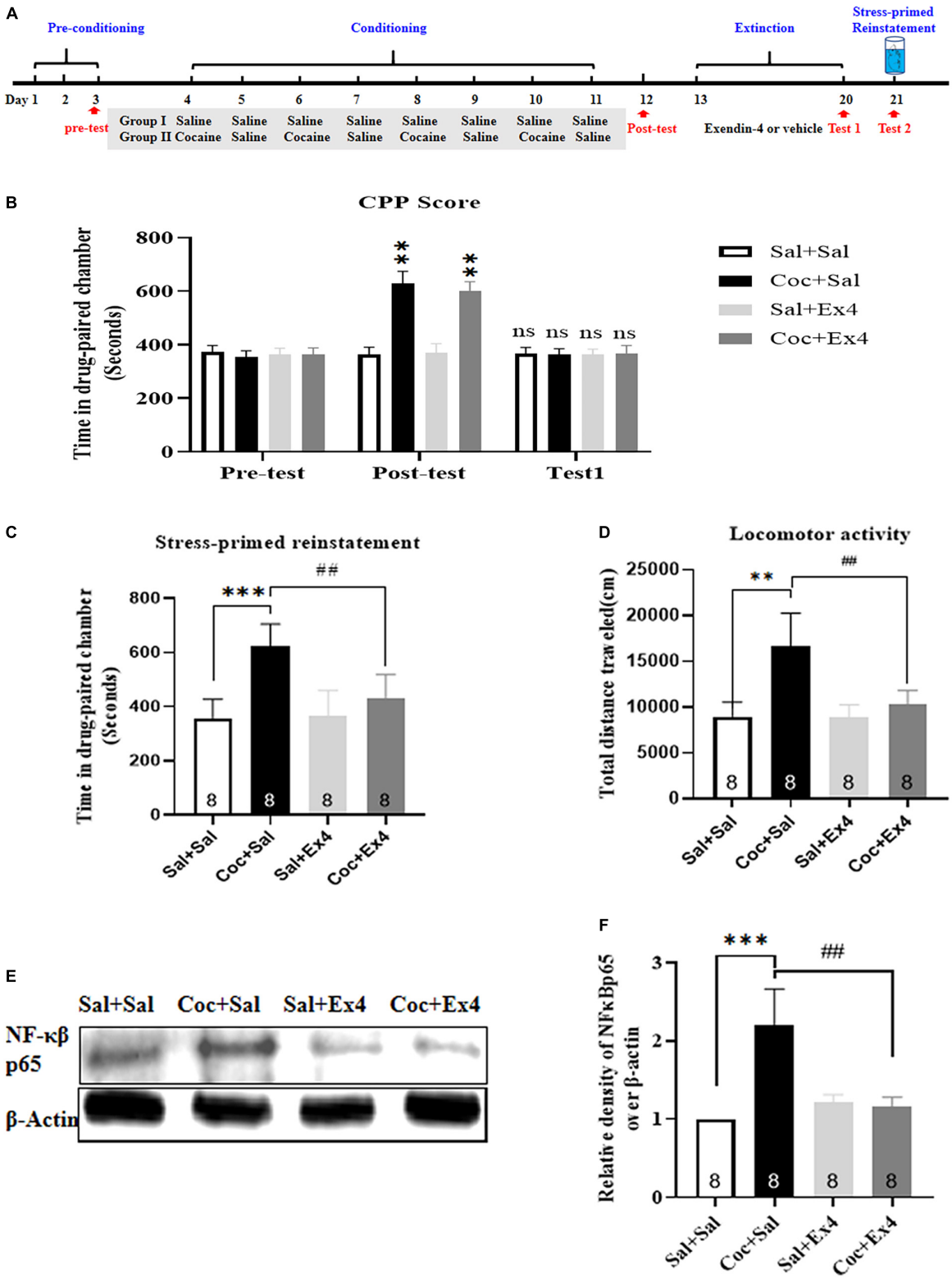

FIGURE 2 | The systemic pretreatment with exendin-4 blocked stress-induced reinstatement of cocaine-induced CPP. **(A)** The experimental schedule for Swim, saline as well as cocaine and exendin-4 treatments. **(B)** After cocaine conditioning, animals developed a robust room preference for the cocaine-paired compartment. ** represents $p < 0.01$ vs Sal + Sal group, $n = 8$ each group, two-way rmANOVA. Additionally, CPP scores after vehicle or exendin-4 administration suggested that the cocaine-induced CPP was completely extinguished after 8 days extinction training. ns represents no significant difference, $n = 8$ each group, two-way rmANOVA followed by Dunnett's multiple comparisons test. **(C)** The systemic pretreatment of exendin-4 significantly blocked stress-induced reinstatement

(Continued)

FIGURE 2 | of cocaine-induced CPP. Swim on the reinstatement day induced a strong reinstatement CPP that was attenuated by systemic pretreatment with exendin-4 after each extinction. *** represents $p < 0.001$ vs Sal + Sal, ## represent $p < 0.01$ vs Coc + Sal, $n = 8$ each group, two-way ANOVA followed by a Tukey's *post hoc* test. **(D)** The systemic pretreatment with exendin-4 significantly suppressed locomotor activity. Swim on the reinstatement day produced a clear increase in locomotion that was suppressed by systemic pretreatment with exendin-4 after each extinction. *** represents $p < 0.001$ vs Sal + Sal, # represent $p < 0.05$ vs Coc + Sal, $n = 8$ each group, two-way ANOVA followed by a Tukey's *post hoc* test. **(E)** Representative image of NF-κβ p65 proteins in different groups detected by Western blot analysis at the end of CPP test of reinstatement ($n = 8$ in each group). **(F)** Semiquantitative analysis of the relative levels of NF-κβ p65 by 1 densitometric analysis in different groups. Stress induced by Swim up-regulated the expression of NF-κβ p65, and this effect was alleviated by systemic pretreatment with exendin-4 after each extinction. *** represents $p < 0.001$ vs Sal + Sal, ## represent $p < 0.01$ vs Coc + Sal, two-way ANOVA followed by a Tukey's *post hoc* test. All data are presented as the mean ± SEM.

Experiment 1

For the cocaine-primed reinstatement, mice in the saline-conditioned control group were primed with saline (2.5 ml/kg, i.p.), while animals in the cocaine-conditioned group were primed with a half dose of cocaine (10 mg/kg, i.p.). The time spent in the three compartments was recorded to assess the effects of Ex4 on the cocaine-primed reinstatement CPP. At the end of the CPP test of reinstatement, all mice were sacrificed using rapid decapitation for Western blotting. The NAc was extracted from the brain, snap-frozen in liquid nitrogen, and stored at −80°C until the initiation of Western blot. The timeline is shown in **Figure 1A**.

Experiment 2

The animal model of stress-induced reinstatement was established using a swim protocol as previously described in mice (Kreibich and Blendy, 2004; Carey et al., 2007; Redila and Chavkin, 2008; Mantsch et al., 2010; Meye et al., 2016). In brief, mice were required to swim in a 30 cm × 20 cm cylindrical polypropylene pool filled with water at a controlled temperature of 20–25°C for 6 min. The depth of the water was chosen based on the ability to touch the bottom of the pool with their hind limbs. Subsequently, mice were dried and recovered in their home cage for 20 min. The testing of stress-induced reinstatement was initiated by placing the animals in the middle area of the conditioning apparatus, where they were allowed to freely explore for 20 min. The time spent in the three compartments was recorded. At the end of the CPP test of reinstatement, the mice were sacrificed using rapid decapitation. The NAc tissue was removed from the brain, snap-frozen in liquid nitrogen, and stored at −80°C for Western blot. The timeline of experimental protocols is depicted in **Figure 2A**.

Locomotor Activity

Locomotor activity is traditionally thought to be one of the most useful measurements to evaluate the behavioral effect of psychostimulants (Steketee and Kalivas, 2011). Locomotor activity, assessed by total distance, is automatically recorded to further evaluate the effects of systemic pretreatment with Ex4 on the cocaine-primed and stress-induced reinstatement when animals are subject to the 20-min reinstatement test in the conditioning compartments.

Western Blotting Analysis

After completing all behavioral experiments, mice were sacrificed using rapid decapitation for Western blotting, and it was initiated to detect the expression level of NF-κβ-p65 proteins in the NAc. The NAc was chosen because it is known as a critical brain reward region implicated in cocaine reward (Hernandez et al., 2019). The general procedure of dissection of NAc tissue was conducted as previously described (Shin et al., 2003). Coronal sections of mouse brains were performed at prefrontal cortex (PFC) level and NAc/dorsal striatum level in a cooled 0.5 mm mouse brain matrix (Braintree, United States). Over ice glass, the PFC and dorsal striatum were carefully removed using forceps, whereas the NAc were dissected using a cooled 18-G micropunch, respectively. These NAc tissue samples were instantly frozen on dry ice and then stored at −80°C. The samples were homogenized on ice. Total protein was extracted using RIPA lysis buffer containing phenyl methane sulphonyl fluoride (PMSF), phosphatase inhibitors, and protease inhibitors (cat. no. KGP2100; KeyGEN Biotechnology Co., Ltd., Jiangsu, China). The protein sample was centrifuged for 5 min at 12,000 × *g* at 4°C. The protein concentration of the supernatants was measured using the BCA Protein Quantitation Assay Kit (KGPBCA; KeyGEN Biotechnology Co. Ltd., Jiangsu, China). Equal amounts of protein (50 μg per lane) from the Sal + Sal, Coc + Sal, Sal + Ex4, and Coc + Ex4 groups were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; cat. no. KGP113; KeyGEN Biotechnology Co. Ltd., Jiangsu, China) containing 30% acrylamide-methylene bisacrylamide, 1.0 mol/L Tris–HCl (*PH* = 6.8), 1.5 mol/L Tris–HCl (*PH* = 8.8), 10% ammonium persulfate, 10% SDS, TEMED, and then transferred to polyvinylidene difluoride membranes (Millipore, United States). After blocking with 5% skim milk powder for 2 h, the membranes were incubated in primary antibodies targeting NF-κβ-p65 (1:1,000; Abcam) and β-actin (1:2,000; Abcam) at 4°C overnight. After washing three times with TBST for 15 min, the membranes were incubated with the corresponding secondary antibody (1:1,000) for 2 h at room temperature. Immunoreactivity was visualized using the ECL Western blotting detection reagents and then analyzed through scanning densitometry. The proteins were detected using an enhanced chemiluminescence reagent, and the ratio of the gray value of the target protein band to the gray level of the β-actin band is used to quantify the relative expression level of the target protein.

Statistical Analysis

All data are presented as means ± standard errors of the means. GraphPad Prism 8.4.0 software (GraphPad Software, La Jolla, CA, United States) was used to analyze all statistics in this study. The CPP score from the post-test and test 1 was analyzed

using two-way repeated-measures analysis of variance (two-way rmANOVA) when compared with Sal + Sal group. The effects of systemic administration of Ex4 on the priming- and stress-induced reinstatement of cocaine-related CPP, locomotor activity, and the expression level of NF-κβ-p65 in the NAc were assessed using two-way analysis of variance (two-way ANOVA). *Post hoc* comparisons in the two-way rmANOVA were performed using Dunnett's multiple comparisons test or Tukey's multiple comparisons test. Differences with p values less than 0.05 were considered statistically significant.

RESULTS

The Systemic Pretreatment With Exendin-4 During Extinction Blocked Cocaine-Primed Reinstatement of Cocaine-Induced Conditioned Place Preference and Nuclear Factor-κβ-p65 Expression in the Nucleus Accumbens

We first examined the effect of systemic pretreatment with Ex4 after each extinction session on the cocaine-primed reinstatement and NF-κβ-p65 expression in the NAc. A total of 35 mice were used in this experiment, with three mice excluded due to initial room bias or extinction training failure. As shown in **Figure 1B**, after completing 8 days of cocaine-mediated CPP training, there was a statistical significance in CPP scores among these groups [treatment: $F_{(3,28)} = 6.061$, $p = 0.0027$; test: $F_{(2,28)} = 11.69$, $p = 0.0004$; interaction $F_{(6,28)} = 8.462$, $p < 0.0001$; two-way rmANOVA], and Dunnett's multiple comparison *post hoc* analysis showed that mice in the drug group that were treated with cocaine on alternating days displayed a significant increase in CPP scores compared with the Sal + Sal group ($p = 0.0023$ for Coc + Sal group, and $p = 0.0085$ for Coc + Ex4 group), indicating that cocaine-induced a robust preference for the cocaine-paired chamber and that a biased CPP paradigm was successfully established. Subsequently, animals from the saline and drug groups were randomly assigned to four independent groups: Sal + Sal, Coc + Sal, Sal + Ex4, and Coc + Ex4. All of them were required to undergo daily injection of Ex4 and extinction for 8 days. A two-way rmANOVA followed by Dunnett's multiple comparison test showed that there was no significant difference in CPP scores compared with the Sal + Sal group ($p > 0.05$; $n = 8$ per group; **Figure 1B**), indicating that the cocaine-induced CPP response was completely extinguished. Afterward, these extinguished animals were reinstated with a half dose of cocaine (10 mg/kg) prior to the beginning of the reinstatement test. As expected, two-way ANOVA suggested a significant difference in the time spent in the cocaine-paired chamber [$F_{(3,28)} = 21.82$, $p < 0.001$; **Figure 1C**], and Tukey's multiple comparison *post hoc* analysis showed that the Coc + Sal group developed a robust reinstatement of cocaine-induced CPP compared with the Sal + Sal group ($p < 0.001$) that was significantly attenuated by Ex4 ($p < 0.001$). Aside from CPP, two-way ANOVA also revealed a statistical difference in the locomotor activity [$F_{(3,28)} = 33.56$, $p < 0.001$; **Figure 1D**], and Tukey's multiple comparison *post hoc* analysis showed that the Coc + Sal group presented a significant increase in locomotion compared with the Sal + Sal group ($p < 0.001$) that was significantly attenuated by Ex4 ($p < 0.001$). Overall, these findings showed that systemic pretreatment with Ex4 during extinction was sufficient to block cocaine-primed reinstatement of cocaine-induced CPP and hyperlocomotion.

At the end of the priming test, the NAc of animals was harvested to complete Western blotting. The Western blotting showed that the Sal + Sal, Coc + Sal, Sal + Ex4, and Coc + Ex4 groups had different relative protein contents of NF-κβ-p65 [$F_{(3,28)} = 33.56$, $p < 0.001$, two-way ANOVA; **Figures 1E,F**], and Tukey's multiple comparison *post hoc* analysis showed that the Coc + Sal group had a significant increase in NF-κβ-p65 expression compared with that in the Sal + Sal group ($p < 0.001$), which was significantly reduced by Ex4 ($p < 0.001$). These results revealed that Ex4 normalized the abnormal expression of NF-κβ-p65 in the NAc.

The Systemic Pretreatment With Exendin-4 During Extinction Inhibited Stress-Primed Reinstatement of Cocaine-Induced Conditioned Place Preference and Nuclear Factor-κβ-p65 Expression in the Nucleus Accumbens

We further explored the effect of systemic pretreatment with Ex4 after each extinction session on stress-induced reinstatement and NF-κβ-p65 expression in the NAc. A total of 37 mice were used in this experiment, with 3 and 2 mice excluded due to initial preference or extinction training failure, respectively. As shown in **Figure 2B**, following daily CPP training of cocaine for 8 days, there was a statistical significance in CPP scores among these groups [treatment: $F_{(3,28)} = 6.931$, $p = 0.0006$; test: $F_{(2,28)} = 36.14$, $p < 0.001$; interaction $F_{(6,28)} = 7.716$, $p < 0.0001$; two-way rmANOVA], and Dunnett's multiple comparison *post hoc* analysis showed that animals in the drug group that were administered cocaine or saline on alternate days developed a significant increase in CPP score when compared with the Sal + Sal group ($p = 0.0040$ for Coc + Sal group, and $p = 0.0028$ for Coc + Ex4 group), indicating that the preclinical model of biased CPP was successfully induced. Next, we observed the effect of systemic pretreatment with Ex4 in the stress-induced reinstatement of CPP. Animals from the saline and drug groups were randomly split into four groups (Sal + Sal, Coc + Sal, Sal + Ex4, and Coc + Ex4) to experience daily administration of Ex4 and 8 extinction sessions. A two-way rmANOVA followed by Dunnett's multiple comparison test showed that there was no significant difference in CPP scores compared with the Sal + Sal group ($p > 0.05$; $n = 8$ per group; **Figure 2B**), suggesting that the CPP induced by cocaine during conditioning period was fully extinguished. Subsequently, the extinguished animals were required to swim in a specific pool for 6 min and recover in their home cages for 20 min before the reinstatement test. As hypothesized, two-way ANOVA

suggested a significant difference in the time spent in the cocaine-paired chamber [$F_{(3,28)} = 14.77$, $p < 0.001$; **Figure 2C**], and Tukey's multiple comparison *post hoc* analysis showed that the Coc + Sal group developed a strong reinstatement of cocaine-induced CPP compared with the Sal + Sal group ($p < 0.001$) that was significantly attenuated by Ex4 ($p = 0.0021$), indicating that stress caused by swimming was able to produce a strong reinstatement CPP and this response was clearly reversed by systemic pretreatment with Ex4. Similar to the CPP responses, two-way ANOVA indicated an important difference in the locomotion [$F_{(3,28)} = 26.82$, $p < 0.001$; **Figure 2D**], and Tukey's multiple comparison *post hoc* analysis suggested that the Coc + Sal group produced a significant increase in locomotion compared with the Sal + Sal group ($p < 0.001$) that was clearly reversed by Ex4 ($p < 0.001$). Altogether, these data showed that pretreatment with Ex4 during extinction was competent to inhibit stress-induced reinstatement of cocaine-induced CPP and locomotion.

Following the completion of the reinstatement test, these mice were sacrificed by cervical dislocation, and the NAc was collected for Western blotting. There was a different relative protein content of NF-κβ-p65 among the Sal + Sal, Coc + Sal, Sal + Ex4, and Coc + Ex4 groups [$F_{(3,28)} = 41.08$, $p < 0.001$, two-way ANOVA; **Figures 2E,F**], and then Tukey's multiple comparison *post hoc* analysis suggested that the Coc + Sal group had a significant increase in NF-κβ-p65 expression compared with the Sal + Sal group ($p = 0.0007$) that was significantly diminished by Ex4 ($p = 0.0015$). These results revealed that Ex4 weakened the aberrant expression of NF-κβ-p65 in the NAc.

DISCUSSION

Reinstatement is the most commonly used measure to study the relapse in addicts (Vorel et al., 2001). Cocaine (Wit and Stewart, 1981) and stress (Erb et al., 1996) are identified as common relapse triggers. In this study, we, therefore, reveal the important effect of GLP-1R agonist Ex4 in the reinstatement evoked by cocaine and stress. Specifically, we demonstrate that systemic administration of Ex4 after each extinction training attenuates the cocaine- and stress-primed reinstatement of CPP. In addition, similar to reexposure to half dose of cocaine, we also observe that swimming following extinction induces a significant reinstatement of cocaine-mediated CPP. Moreover, animals experiencing cocaine- and stress-reinstatement show a significant increase in the accumbal expression of NF-κβ, which is also normalized by Ex4. Taken together, these findings suggest that GLP-1R agonist Ex4 has suppressing effects on the reinstatement of cocaine-induced CPP, and its pharmacological mechanism might be associated with the NF-κβ signaling inhibition.

Glucagon-Like Peptide-1 Receptor Agonist Exendin-4 and Cocaine-Priming Reinstatement

One of the main findings of this study is the demonstration of the inhibitory actions of Ex4 on the reinstatement of cocaine-induced CPP. We show the biased CPP paradigm was successfully established using cocaine conditioning (20 mg/kg, i.p.), and then the CPP response is extinguished after the cocaine-free extinction sessions (8 days). Additionally, the significant reinstatement of cocaine-induced CPP is induced after reexposure to a half dose of cocaine (10 mg/kg, i.p.). These results are in line with our previous study showing that 20 and 10 mg/kg were sufficient to produce CPP and reinstatement, respectively (Zhu et al., 2021). Furthermore, this study also suggests that the systemic pretreatment with Ex4 after each extinction session significantly prevents the cocaine-priming reinstatement of the conditioned behavior in the study. These are in accordance with numerous findings indicating that these behavioral changes induced by cocaine consumption could be abolished *via* peripheral administration of Ex4 (Skibicka, 2013; Engel and Jerlhag, 2014; Hayes and Schmidt, 2016; Hernandez et al., 2019).

Besides, we also observe the possible effects of Ex4 on the locomotor activity, which is traditionally considered as another important paradigm used to measure the behavioral effects of psychostimulants (Steketee and Kalivas, 2011). In this study, reexposure to an injection of cocaine on the reinstatement test day markedly increases locomotion in mice, in accordance with previous evidence (Steketee and Kalivas, 2011). Importantly, systemic pretreatment with Ex4 induces a significant reduction in locomotion. Altogether, systemic administration of Ex4 after each extinction could exert a profound influence on the reinstatement of maladaptive behavior elicited by repeated cocaine use such as reinstatement CPP and locomotion. From this, our study indicates a crucial effect of GLP-1R agonist Ex4 in reducing reinstatement. However, less is known about the underlying impacts of Ex4 on the stress-induced reinstatement of cocaine CPP and the expression of plasticity-linked proteins including NF-κβ in the NAc.

Glucagon-Like Peptide-1 Receptor Agonist Exendin-4 and Stress-Induced Reinstatement

Another major finding of our work is the evidence of inhibitory actions of Ex4 on the stress-induced reinstatement of cocaine CPP. In this experiment, animals also displayed a strong room preference that was extinguished after the 8 confined extinction sessions. In addition to reexposure to cocaine, numerous clinical studies showed that relapse to addictive behavior was often triggered by stressful life events (Khantzian, 1985; Shiffman and Wills, 1985; Kosten et al., 1986). Similarly, the stressful condition was commonly referred to as another important contributing factor of relapse to substance use disorder in abstinent addicts (Shaham et al., 2000). Furthermore, in preclinical studies, stress caused by swimming was also believed to enhance the rewarding effects of psychostimulant drugs (Piazza et al., 1990). In support of these studies, the CPP paradigm and swimming were widely used as stressors by previous studies (Vaughn et al., 2012; Tung et al., 2016). Accordingly, our data also suggested that exposure to swimming for 6 min prior to the commencement of the reinstatement test was

sufficient to produce the significant reinstatement of CPP in the Coc + Sal group, which was congruent with a previous study (Kreibich and Blendy, 2004; Carey et al., 2007; Redila and Chavkin, 2008; Mantsch et al., 2010; Meye et al., 2016). Intriguingly, the stress-induced reinstatement of previously extinguished cocaine CPP was clearly attenuated by systemic pretreatment with Ex4 after each extinction training in the Coc + Ex4 group.

In addition, this study also demonstrates that exposure to swimming can markedly increase locomotion in animals, in accordance with a previous study (Meye et al., 2016). Similarly, Ex4 also induces a significant reduction in locomotor activity in the stress-induced reinstatement paradigm. From these, the stress-induced reinstatement does not only develop a significant CPP but also increases locomotion, and this response is attenuated by Ex4 pretreatment.

Taken together, the results of this work were provocative and revealed the inhibitory effect of GLP-1R agonist Ex4 in the two reinstatement paradigms of cocaine-induced CPP. However, the associated mechanisms of pharmacological actions of Ex4 on inhibiting stress-induced reinstatement are still unknown to date.

Neuroinflammation and Reinstatement

In recent years, neuroinflammation has received considerable attraction due to its implication in many brain diseases (Block and Hong, 2005; Tansey et al., 2007). Furthermore, exposure to stressors contributes to a series of changes in neuroimmune response (Frank et al., 2019). In addition, cocaine is reported to enhance the activation of NF-κβ signaling in the reward area of the brain including NAc, and the signaling pathway can exert profound impacts on controlling the morphology of NAc neurons as well as attenuating the rewarding effects of cocaine (Russo et al., 2009). NF-κβ is an important transcription factor due to its involvement in inflammation and immune responses (Chen and Greene, 2004; Lin and Karin, 2007). Importantly, the anti-inflammatory agents are reported to act as a novel and effective approach for behavioral treatment of substance use disorders (Kohno et al., 2019). More interestingly, the pharmacological mechanism by which many inhibitors of neuroinflammation significantly attenuate the rewarding effects of cocaine are believed to be closely associated with direct and indirect inhibition of NF-κβ activation (Padhye et al., 2009; Kozela et al., 2017). Considering the significance of NF-κβ signaling in cocaine reward (Russo et al., 2009), we examined the effects of Ex4 on the expression of NF-κβ in cocaine-priming or stress-induced reinstatement animals. Our data show that cocaine exposure is adequate to upregulate NF-κβ signaling that may be normalized by systemic pretreatment with Ex4, in agreement with previous studies suggesting that another GLP-1R agonist liraglutide effectively inhibited the NF-κβ signaling pathways (Liu et al., 2008; Kozela et al., 2017; Ma et al., 2018; Li et al., 2020). Consequently, the behavioral effects of Ex4 on cocaine might be closely associated with the NF-κβ signaling inhibition.

Of note, there is a range of limitations and questions in this study. For example, many neurotransmitters such as dopamine, norepinephrine, and serotonin reportedly had a far-reaching effect on the rewarding and reinforcing properties of cocaine (Ritz et al., 1990). Except for the NAc, a growing body of studies also revealed that systemic administration of Ex4 was also expressed in other brain regions of the reward system such as the ventral tegmental area (VTA) and lateral septum (LS) (Hernandez and Schmidt, 2019). Accordingly, future experimental studies are urgently needed to validate the specific mechanisms underlying cocaine-primed and stress-induced reinstatement of cocaine CPP. Furthermore, to date, the precise mechanisms by which Ex4 exerted suppressive effects on cocaine- and stress-primed reinstatement remain unknown although the CPP score and expression of NF-κβ were decreased in the Coc + Ex4 group. One possible explanation is that the expression of NF-κβ was already enhanced by repeated exposure to cocaine prior to the reinstatement test due to previous studies showing that cocaine was shown to effectively induce an increase in the expression of NF-κβ in the NAc (Russo et al., 2009). And the upregulated expression of NF-κβ was reduced by Ex4 treatment, but this effect may have occurred before the beginning of the reinstatement test. Consequently, other possible factors such as the reinstatement test, drugs, and CPP should be included in future research to precisely elucidate the pharmacological mechanism underlying the behavioral effects of Ex4 on the cocaine- and stress-induced reinstatement. Moreover, evaluating the ability of Ex4 to affect the reinstatement of cocaine CPP by modifying the activity of specific neuromodulators in areas of the brain reward system is another problem that must be addressed. Additionally, it is not fully understood whether NF-κβ signaling in the NAc is an independent mechanism through which the Ex4 regulates the reinstatement of cocaine CPP as only four groups (Sal + Sal, Sal + Ex4, Coc + Sal, and Coc + Ex4) are included in this study. Furthermore, experimental studies are necessary to evaluate other pharmacological mechanisms of action of Ex4 in the brain for the treatment of cocaine use disorder. Besides, the important factor of sex differences in the efficacy of Ex4 in reducing the rewarding and reinforcing effects of cocaine is fully ignored as only the adult male mice are used in this study. Additional researches are essential to evaluate whether other GLP-1R agonists have a similar potential as Ex4 on other psychostimulant abuse. Finally, although the changes in NF-κβ expression after using Ex4 could be clearly observed in this study, the corresponding changes should be further investigated in human clinical experiments.

In summary, this study shows that reexposure to cocaine and stress induced by swimming on the reinstatement day markedly augmented the time spent in the cocaine-paired chamber, increased locomotor activity, and facilitated the expression of NF-κβ in the NAc. Importantly, these alterations could be prevented by systemic pretreatment with Ex4 after each extinction session. Our findings provide innovative insights into the therapeutic

potential of the clinically available Ex4 for the treatment of cocaine relapse. Therefore, GLP-1R agonists including Ex4 may be a promising approach to treat substance use disorder.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Research Ethics Committee of Ningxia Medical University.

AUTHOR CONTRIBUTIONS

HG, TS, and FW participated in designing the experiments, preparation of the manuscript, analysis of data, and in the revision. CZ designed and performed the experiments, analyzed data, made the figures, performed the statistical analysis, and drafted the manuscript. All authors participated in the study design, data collection, analysis of data, and preparation of the manuscript, took responsibility for the integrity of the data and the accuracy of the data analysis, contributed to the article, read, and approved the final manuscript and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2021.769664/full#supplementary-material

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REVIEW
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Understanding the Role of Orexin Neuropeptides in Drug Addiction: Preclinical Studies and Translational Value

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Orexins (also known as hypocretins) are neuropeptides that participate in the regulation of energy metabolism, homeostasis, sleep, feeding, stress responses, arousal, and reward. Particularly relevant to the scope of the present review is the involvement of the orexin system in brain mechanisms that regulate motivation, especially highly motivated behavior, arousal, and stress, making it an ideal target for studying addiction and discovering treatments. Drug abuse and misuse are thought to induce maladaptive changes in the orexin system, and these changes might promote and maintain uncontrolled drug intake and contribute to relapse. Dysfunctional changes in this neuropeptidergic system that are caused by drug use might also be responsible for alterations of feeding behavior and the sleep-wake cycle that are commonly disrupted in subjects with substance use disorder. Drug addiction has often been associated with an increase in activity of the orexin system, suggesting that orexin receptor antagonists may be a promising pharmacological treatment for substance use disorder. Substantial evidence has shown that single orexin receptor antagonists that are specific to either orexin receptor 1 or 2 can be beneficial against drug intake and relapse. Interest in the efficacy of dual orexin receptor antagonists, which were primarily developed to treat insomnia, has grown in the field of drug addiction. Treatments that target the orexin system may be a promising strategy to reduce drug intake, mitigate relapse vulnerability, and restore "normal" physiological functions, including feeding and sleep. The present review discusses preclinical and clinical evidence of the involvement of orexins in drug addiction and possible beneficial pharmacotherapeutic effects of orexin receptor antagonists to treat substance use disorder.

Keywords: orexin, cocaine, alcohol, opioids, nicotine

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INTRODUCTION

Since their discovery in 1998 (de Lecea et al., 1998; Sakurai et al., 1998a), orexins (Orxs) have received growing research interest. Orexins are neuropeptides that have been shown to regulate a range of physiological and behavioral functions, including energy metabolism, homeostasis, arousal, sleep, and feeding (Sutcliffe and de Lecea, 2000; Mieda and Yanagisawa, 2002). Particularly important for the scope of this review is the role of these neuropeptides in modulating stress

(Berridge et al., 2010) and reward-motivated states (Harris et al., 2005; Martin-Fardon et al., 2010, 2016), components that have a strong impact on drug addiction. Independently discovered in 1998 by two independent groups (de Lecea et al., 1998; Sakurai et al., 1998a), orexin A (OrxA) and orexin B (OrxB), also referred to as hypocretin 1 (Hcrt1) and hypocretin 2 (Hcrt2), are neuropeptides that derive from a common precursor, prepro-orexin, that is produced exclusively in well-defined subregions of the hypothalamus (HYP). An estimated 3,000–6,700 neurons express Orx in the rat brain, and 50,000–80,000 neurons express Orx in the human brain (Thannickal et al., 2000; Fronczek et al., 2005; Modirrousta et al., 2005; de Lecea, 2015; Soya and Sakurai, 2020), distributed in the lateral HYP (LH), dorsomedial HYP (DMH), and perifornical area (PFA; de Lecea et al., 1998; Sakurai et al., 1998a).

Two isoforms of G protein-coupled receptors have been identified as molecular targets for Orxs, orexin receptor 1 (OrxR1 [Hcrt-r1]) and orexin receptor 2 (OrxR2 [Hcrt-r2]; Sakurai et al., 1998a). OrxR1 binds OrxA with 20–30 nM affinity but has much lower affinity (10- to 1,000-fold lower) for OrxB, whereas OrxR2 binds both peptides with similar affinity (in the 40 nM range; Sakurai et al., 1998b; Scammell and Winrow, 2011). Both OrxRs are G protein-coupled receptors. OrxR1 was initially thought to couple exclusively G_q protein, and OrxR2 was thought to couple G_q and $G_{i/o}$ proteins (Sakurai et al., 1998b; Smart et al., 1999; Lund et al., 2000; Holmqvist et al., 2002), but more recent evidence suggests that OrxR signaling is significantly more diverse. In fact, OrxRs are able to couple members of at least three G-protein families and other proteins, through which they regulate non-selective cation channels, phospholipases, adenylyl cyclase, and protein and lipid kinases (e.g., Kukkonen and Leonard, 2014). These two OrxRs are extensively distributed throughout the brain (Marcus et al., 2001), which can explain intricate participation of the Orx system in regulating several physiological functions. In the brain, OrxR1 and OrxR2 exhibit mostly distinctive expression patterns, with some overlap (Marcus et al., 2001).

One important consideration is that the Orx system plays a pivotal role in several addiction-related behaviors (for review, see Aston-Jones et al., 2010; Mahler et al., 2012; Matzeu and Martin-Fardon, 2020b). The first evidence of the involvement of the Orx system in drug addiction was reported almost two decades ago. These studies demonstrated the involvement of LH Orx neurons in morphine-related behaviors, including drug seeking, dependence, and withdrawal (Georgescu et al., 2003; Harris et al., 2005), opening new perspectives into investigating neuronal mechanisms that are involved in the etiology of drug addiction. Since then, the body of scientific literature on the involvement of the Orx system in drug addiction has grown continuously, including its involvement in cocaine, alcohol, opioid, and nicotine addiction.

Drug addiction has been associated with alterations of the number of Orx neurons (James et al., 2019; Sharma et al., 2020; Matzeu and Martin-Fardon, 2021) and *Orx* gene expression (Sharma et al., 2020). In humans, chronic substance use has been shown to affect nutritional status and eating habits (Rush et al., 2016; Cusack et al., 2021; Mahboub et al., 2021) and cause sleep disturbances (Brooks et al., 2021; Frers et al., 2021; Miller et al., 2021). Therefore, pharmacological manipulations of the Orx system by targeting OrxRs may be a promising strategy to reduce drug intake, mitigate relapse vulnerability, and normalize feeding and sleep. The present review discusses preclinical studies that used animal models of operant behavior and clinical evidence of the involvement of the Orx neuropeptides in drug addiction and the possible beneficial pharmacotherapeutic effects of OrxR antagonists to treat substance use disorder.

Most studies that examined the role of the Orx system in drug addiction only used male subjects. If both males and females were used, then the studies were simply inclusive of both sexes and did not necessary study sex differences. Importantly, however, studies have revealed sexual dimorphism in Orx activity (for review, see Grafe and Bhatnagar, 2020). Sex differences in *Orx* and *OrxR1/R2* mRNA both peripherally and centrally have been documented in preclinical studies (Taheri et al., 1999; Johren et al., 2001, 2002). Female rats exhibit higher levels of *Orx* mRNA in the HYP and higher levels of OrxA in cerebrospinal fluid compared with males (Johren et al., 2002). Moreover, in female rats, Orx-positive neurons show greater activation compared with males (Grafe et al., 2017). Also, OrxR1 and OrxR2 expression appears to be higher in females compared with males, at least in some brain areas (Johren et al., 2002; Loewen et al., 2017). Although only a few clinical studies are available about sex differences in the Orx system, they suggest that women exhibit higher levels of Orx in the brain compared with men (Wennstrom et al., 2012; Schmidt et al., 2013; Lu et al., 2017). Understanding the interplay between sex, Orx, and addiction needs to be addressed, particularly when considering treatments for substance use disorder.

NEUROADAPTATIONS OF THE OREXIN SYSTEM INDUCED BY DRUGS OF ABUSE

Cocaine

Prolonged cocaine consumption induces significant alterations of the number of Orx neurons in the HYP and it has been shown that chronic cocaine use increases the number of Orx-expressing cells (James et al., 2019; Matzeu and Martin-Fardon, 2021). These studies used two different models to induce addiction-like behavior: extended access to cocaine self-administration for 6 h/day for 21 days (Matzeu and Martin-Fardon, 2021) and intermittent access to cocaine self-administration for 5 min, followed by 25 min of cocaine non-availability (repeated 12 times/day for 14 days; James et al., 2019). The addiction-like behavioral phenotype that is induced by these animal models was accompanied by persistent increases in the number of Orx-immunoreactive hypothalamic neurons starting at 14–21 days and up to 150 days following the last cocaine exposure (James et al., 2019; Matzeu and Martin-Fardon, 2021). Importantly, this increase in expression was observed exclusively for Orx and not for other neuropeptides, such as melanin-concentrating hormone, in the same hypothalamic region (James et al., 2019). A study investigated the participation of Orx transmission

in the posterior paraventricular nucleus of the thalamus (pPVT) in cocaine-related behaviors, showing that exposure to cocaine under extended access conditions increased OrxR2 immunoreactivity at 14–21 days of abstinence, which returned to basal levels after 35 days (Matzeu and Martin-Fardon, 2021). This observation was particularly important because it confirmed the participation of OrxR2 signaling in the pPVT in mediating the priming effect of intra-pPVT OrxA-induced cocaine-seeking behavior (Matzeu et al., 2016; Matzeu and Martin-Fardon, 2021). The persistent upregulation of Orx peptide during abstinence from cocaine, together with alterations of OrxR2 in the pPVT, suggests that cocaine compromises the Orx system, and this effect persists into abstinence and most likely affects brain regions beyond the pPVT that receive HYP Orx projections.

Alcohol

Similar to cocaine, alcohol consumption has been shown to dysregulate the Orx system, but chronic alcohol intake data have been somewhat controversial. For example, chronic home cage alcohol drinking was shown to reduce *Orx* mRNA expression in the HYP, whereas acute oral alcohol administration by gavage was associated with an increase in *Orx* expression (Morganstern et al., 2010). Moreover, alcohol dependence downregulated *Orx* mRNA expression at 12 h of withdrawal in rats that were made dependent *via* repeated intragastric (i.e., gavage) alcohol administration (Sharma et al., 2020). Consistent with these data, a clinical study reported that blood OrxA levels in patients with alcohol use disorder were inversely related to the severity of withdrawal symptoms (i.e., stronger withdrawal symptoms were associated with lower OrxA expression; Bayerlein et al., 2011). This observation might partially account for the exacerbation of lethargy during the day that is experienced by subjects who suffer from alcohol use disorder during alcohol withdrawal (for review, see Miller et al., 2021).

Opposing results were recently found in our study that investigated the participation of the pPVT in alcohol seeking. We found a significant increase in *Orx* mRNA expression in the HYP and *OrxR1* and *OrxR2* mRNA expression in the pPVT following extinction at 8 h of withdrawal in rats that were made alcohol dependent by chronic intermittent alcohol vapor exposure (Matzeu and Martin-Fardon, 2020a). Consistent with our findings, the Orx system was reported to be upregulated in adult rats following binge-like patterns of alcohol intoxication during adolescence (Amodeo et al., 2020) and in rats that exhibited high novelty-induced locomotor activity, a predictor of high alcohol consumption (Barson et al., 2013). Interestingly, pre-fertilization maternal alcohol consumption in zebrafish significantly increased the number of Orx neurons and alcohol consumption in offspring (Collier et al., 2020). The dependence induction procedure, time point of withdrawal, age of the animals, species, and other experimental manipulations might account for discrepancies in these preclinical studies.

Opioids

The dysregulation of Orx neuropeptides has also been demonstrated following opioid abuse. An increase in the number of Orx-producing neurons was found in postmortem brains from individuals with heroin addiction (Thannickal et al., 2018). Similar increases in Orx-producing cells were induced in wildtype mice by long-term morphine administration that lasted up to 8 weeks after morphine cessation (Thannickal et al., 2018). Moreover, intermittent access to fentanyl was associated with an increase in the number Orx cells in the HYP (Fragale et al., 2021), suggesting that a common effect of chronic opioid use is the upregulation of Orx-producing cells.

Other Drugs of Abuse

The downregulation of *Orx* gene expression in blood was reported in nicotine-dependent cigarette smokers and subjects with Δ^9-tetrahydrocannabinol dependence (Rotter et al., 2012), suggesting that Orx system dysregulation is a common factor across several classes of drugs of abuse.

ROLE OF OREXIN NEUROPEPTIDES IN DRUG INTAKE

Cocaine

Orexin neuropeptides facilitate drug-directed behavior, especially under motivationally salient, high-effort conditions (Borgland et al., 2009; Mahler et al., 2014). Particularly significant is a study that used a short-hairpin RNA-encoding adeno-associated viral vector to knock down *Orx* expression throughout the HYP in adult rats and found lower motivation for cocaine intake under both progressive-ratio (PR) and fixed-ratio (FR) schedules in rats that were given extended access to cocaine self-administration. Interestingly, knocking down *Orx* also reduced the intake of a palatable food reward (i.e., sweetened condensed milk). Importantly, *Orx* silencing did not affect food or water consumption and had no effect on general measures of arousal or stress reactivity (Schmeichel et al., 2018). These findings support the hypothesis that Orx neuropeptides promote operant responding for both drug and non-drug rewards, specifically under conditions that require a high degree of motivation (e.g., PR schedules, extended access to cocaine, and a highly palatable food reward).

Much evidence indicates that OrxR1 is critical for driving highly motivated responding for cocaine, particularly in rats with high motivation for the drug. The blockade of OrxR1 by systemic SB334867 administration decreased cocaine self-administration in rats under limited access conditions under an FR5 timeout 20 s (TO20 s) schedule of reinforcement (Hollander et al., 2012) or PR schedule of reinforcement (Hollander et al., 2012; Prince et al., 2015) but did not alter food self-administration under an FR5 TO20 s schedule of reinforcement (Hollander et al., 2012). OrxR1 knockout mice self-administered significantly less cocaine than wildtype mice (Hollander et al., 2012). Another study described the lack of an effect of peripheral OrxR1 and OrxR2 antagonist administration on reducing cocaine self-administration under an FR1 TO20 s schedule in rats with limited access to cocaine (Smith et al., 2009). These findings suggest that Orx signaling is unnecessary for already established cocaine self-administration under low-effort conditions (e.g., low FR combined with

short cocaine self-administration sessions). The blockade of OrxR1 preferentially reduced cocaine self-administration under high-effort conditions (e.g., low cocaine dose and PR schedule; Brodnik et al., 2015), in rats with extended access to cocaine (Schmeichel et al., 2017), and in rats that were given intermittent access to cocaine and exhibited an addiction-like phenotype (James et al., 2019). These data demonstrate that OrxR1 plays an important role in regulating the reinforcing and reward-enhancing properties of cocaine and that Orx transmission is important for establishing and maintaining cocaine intake under high motivation conditions.

Brain regions need to be identified where Orx neuropeptides can mediate drug-directed behavior. For example, Orx inputs to the ventral tegmental area (VTA) are particularly interesting. The majority of Orx axons in the VTA are passing fibers, with only a small portion of Orx fibers in direct synaptic contact on VTA dopamine and γ-aminobutyric acid neurons (Balcita-Pedicino and Sesack, 2007). Intra-VTA OrxA increases the firing rate of VTA dopamine neurons (Korotkova et al., 2003; Muschamp et al., 2007) and increases extracellular dopamine levels in the prefrontal cortex (PFC) and nucleus accumbens shell (NAcSh; Narita et al., 2006, 2007; Vittoz and Berridge, 2006; Vittoz et al., 2008), key brain structures of the neurocircuitry of addiction. The administration of OrxA directly in the VTA increased the motivation to self-administration cocaine, reflected by an increase in cocaine self-administration in discrete trials and under PR schedules of reinforcement but not under an FR schedule of reinforcement (Espana et al., 2011) as previously reported following intracerebroventricular OrxA administration (Boutrel et al., 2005). Altogether, these data confirm that the Orx system may not influence cocaine self-administration when conditions to obtain cocaine require low effort. Orexin inputs to the VTA appear to play a pivotal role in controlling cocaine intake when conditions to obtain the drug require a high level of motivation. Additional studies confirmed the importance of Orx transmission in the VTA during cocaine self-administration. Intra-VTA administration of the OrxR1 antagonist SB334867 reduced cocaine self-administration (Muschamp et al., 2014). These findings were subsequently extended to the central nucleus of the amygdala (CeA), showing that intra-CeA SB338467 administration decreased cocaine self-administration in rats under extended access conditions (Schmeichel et al., 2017; **Figure 1**).

Alcohol

Similar to preclinical research on cocaine, there is extensive interest in investigating the effects of OrxR1 antagonism on alcohol intake. SB334867 has been shown to reduce alcohol intake under conditions that are characterized by high impulsivity (i.e., binge-like consumption; Olney et al., 2015). SB334867 decreased alcohol consumption and preference exclusively in rats that had high preference or motivation to drink alcohol (Moorman and Aston-Jones, 2009; Lei et al., 2016a; Moorman et al., 2017) and in genetically alcohol-preferring rats (Lawrence et al., 2006; Jupp et al., 2011). Moreover, OrxR1 blockade selectively decreased the escalation of alcohol drinking in mice that were alcohol-dependent, without altering lower levels of alcohol intake in non-dependent mice (Lopez et al., 2016).

In contrast, the blockade of OrxR2 with TCSOX229 did not alter alcohol intake (Lei et al., 2016a). However, other studies showed that OrxR2 blockade with subcutaneous JNJ10397049 administration dose-dependently reduced alcohol self-administration in rats (Shoblock et al., 2011). Intraperitoneal administration of the OrxR2 antagonist LSN2424100 lowered breakpoints on a PR schedule and reduced alcohol consumption in alcohol-preferring rats (Anderson et al., 2014). Corroborating these data, intracerebroventricular TCSOX229 administration reduced alcohol self-administration (Brown et al., 2013), confirming that OrxR2 signaling mediates some aspects of alcohol drinking. Other evidence suggests that the involvement of OrxR1 and OrxR2 activity in alcohol intake may be attributable to a general effect that is not specific to alcohol consumption. In fact, it was shown that blockade of both OrxRs reduced water intake (Anderson et al., 2014). Nevertheless, these data indicate that Orx neuropeptides *via* OrxR1 and OrxR2 signaling regulate the motivation for alcohol, and targeting OrxRs might be one approach to prevent compulsive, highly motivated behaviors that are directed toward alcohol drinking that is characteristic of alcohol use disorder.

Studies of central effects of Orx transmission on alcohol drinking have identified the involvement of the NAcSh, medial PFC (mPFC), CeA, and VTA (Lei et al., 2016b, 2019; Olney et al., 2017). OrxR1 antagonism in the NAcSh was particularly effective in reducing high levels of alcohol drinking in mice, with no significant effect in mice that exhibited only a moderate level of drinking (Lei et al., 2019, 2020; Kwok et al., 2021). The blockade of OrxR2 with TCSOX229 in the VTA and CeA did not alter alcohol drinking (Olney et al., 2017), but TCSOX229 administration in the nucleus accumbens core (NAcC) decreased alcohol intake (Brown et al., 2013; **Figure 1**). Barson et al. (2015) reported that OrxA and OrxB administration in the anterior PVT (aPVT) but not pPVT increased alcohol intake, an effect that was reversed by the Orx2R antagonist TCSOX229 (Barson et al., 2015, 2017), supporting the hypothesis that the aPVT plays a role in mediating alcohol drinking *via* OrxR2 signaling (**Figure 1**). Overall, alcohol consumption data implicate Orx neuropeptides in the pathological motivation for alcohol drinking and demonstrate that both OrxR1 and OrxR2 play an important role in controlling voluntary alcohol consumption.

Opioids

Several studies have investigated the involvement of OrxR antagonists in animal models of opioid use disorder. In the last two decades, the abuse of prescription opioids (e.g., oxycodone, remifentanil, and fentanyl) has received increasing attention because of the increase in the misuse of these medications and increase in overdoses (Seth et al., 2018). One of the first studies that tested the effects of OrxR antagonists on opioid self-administration showed that systemic SB334867 administration reduced heroin intake under both FR and PR schedules of reinforcement (Smith and Aston-Jones, 2012). OrxR2 antagonism with NBI80713 was subsequently shown to decrease heroin self-administration in rats that were given

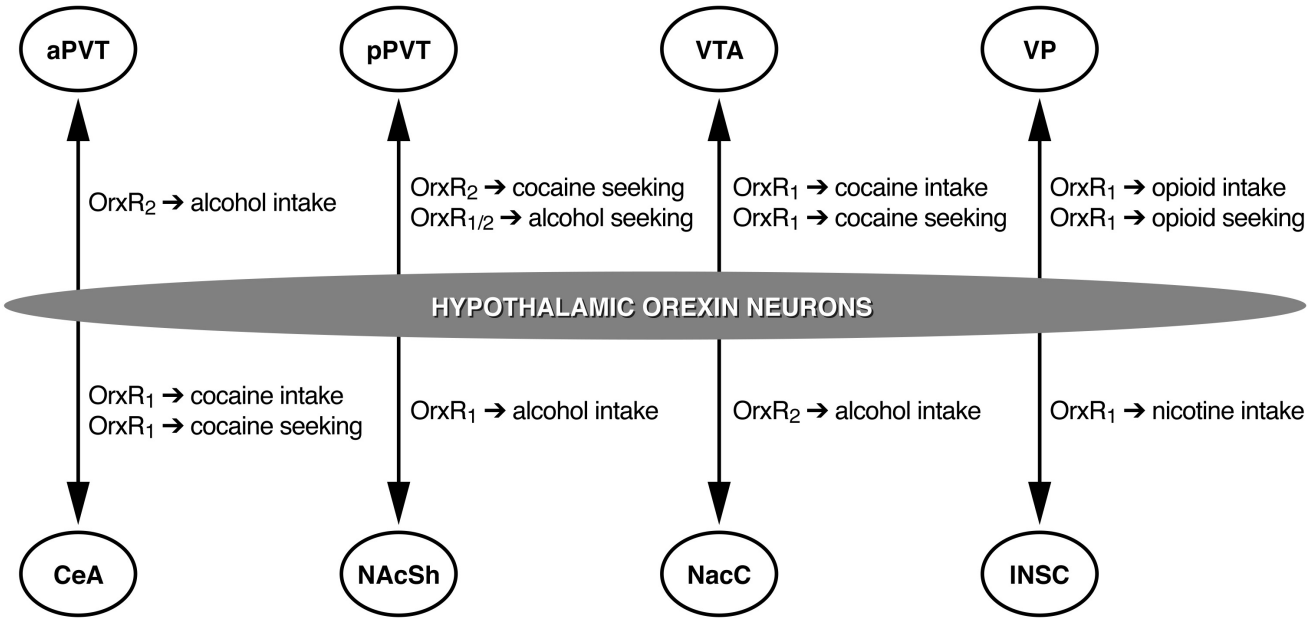

FIGURE 1 | Schematic representation of the roles of different OrxR subtypes in different brain regions. aPVT, anterior paraventricular nucleus of the thalamus; pPVT, posterior paraventricular nucleus of the thalamus; VTA, ventral tegmental area; VP, ventral pallidum; CeA, central nucleus of the amygdala; NAcSh, nucleus accumbens shell; NAcC, nucleus accumbens core; INSC, insular cortex. Arrows represent Orx projections.

extended access to heroin (Schmeichel et al., 2015). However, when the effects of a different OrxR2 antagonist, TCSOX229, on oxycodone self-administration were tested under extended access conditions, no effect was observed (Matzeu and Martin-Fardon, 2020c). Systemic SB334867 administration significantly reduced oxycodone self-administration. Consistent with the efficacy of SB334867 in reducing oxycodone intake, other studies showed that this compound reduced the consumption of two other prescription opioids, remifentanil (Porter-Stransky et al., 2017) and fentanyl (Mohammadkhani et al., 2019, 2020). Interestingly, the efficacy of SB334867 in reducing remifentanil self-administration was preserved when the compound was injected in the ventral pallidum (VP; Mohammadkhani et al., 2019, 2020; **Figure 1**). These findings add to the growing body of evidence that targeting the Orx system may be beneficial for the treatment of opioid use disorder.

Nicotine

Only limited data are available on the involvement of Orx neuropeptides in nicotine addiction. For example, Hollander et al. (2008) showed that systemic SB334867 administration or a site-specific injection of SB334867 in the insular cortex reduced nicotine self-administration (**Figure 1**) and abolished the stimulatory effects of nicotine on brain reward systems, reflected by reversal of the nicotine-induced lowering of intracranial self-stimulation thresholds. The blockade of OrxR1 appears to effectively reduce nicotine intake. Uslaner et al. (2014) reported that OrxR2 blockade had no effect on nicotine self-administration. Moreover, somatic signs of nicotine withdrawal were attenuated by the OrxR1 antagonist SB334867 but not by the OrxR2 antagonist TCSOX229 in mice and were also attenuated in *Orx* knockout mice (Plaza-Zabala et al., 2012). Additionally, local SB334867 infusion in the paraventricular nucleus of the hypothalamus decreased the expression of nicotine withdrawal (Plaza-Zabala et al., 2012). Overall, these data demonstrate that OrxR1 signaling plays a role in the reinforcing effects of nicotine and expression of nicotine withdrawal.

ROLE OF OREXIN NEUROPEPTIDES IN DRUG SEEKING

Cocaine

For nearly two decades, our group and others have contributed significantly to characterizing the role of Orx neuropeptides in cocaine seeking. Intracerebroventricular OrxA administration led to the dose-dependent reinstatement of cocaine seeking without altering cocaine intake in rats, an effect that depended on corticotropin-releasing factor, suggesting that Orx and stress systems may closely interact to regulate cocaine-seeking behavior (Boutrel et al., 2005). Intracerebroventricular OrxA administration elevated intracranial self-stimulation thresholds, suggesting that OrxA negatively regulates the activity of brain reward circuitry. Furthermore, the selective OrxR1 receptor antagonist SB334867 blocked the stress-induced reinstatement of extinguished cocaine-seeking behavior (Boutrel et al., 2005; Zhou et al., 2012), confirming a role for Orx neuropeptides in driving drug seeking through the activation of stress pathways in the brain. When injected systemically, SB334867 but not the OrxR2 antagonist 4PT also reversed conditioned reinstatement

that was induced by cocaine-related stimuli (Smith et al., 2009; Zhou et al., 2012; Martin-Fardon and Weiss, 2014a) but not reinstatement that was triggered by a priming injection of cocaine (Zhou et al., 2012). Another study found that pretreatment with SB334867 significantly attenuated cocaine-seeking behavior that was elicited by a drug-associated context following either extinction or abstinence (Smith et al., 2010), further demonstrating that OrxR1 signaling is critical for conditioned reinstatement. Altogether, these findings suggest that OrxR1 is necessary for cocaine seeking that is elicited by previously drug-paired cues/contexts and stress.

Studies have investigated discrete brain regions that are critically important for cocaine-seeking behavior. The PVT, especially the pPVT, was identified as one such key brain structure (**Figure 1**). A recent electrophysiological study that used a brain slice preparation for cellular recordings found that the superfusion of OrxA onto pPVT neurons increased the frequency of spontaneous excitatory postsynaptic currents (sEPSCs) but had no effect on miniature EPSCs (mEPSCs), suggesting a network-driven effect of OrxA. The amplitudes of s/mEPSCs were unaffected by OrxA, indicating a presynaptic action on glutamate release (Matzeu et al., 2018). Thus, the effect of OrxA in the pPVT appeared to be presynaptic and solely target glutamate release, with no involvement of postsynaptic efficacy. Microinjections of OrxA in the pPVT reinstated extinguished cocaine-seeking behavior, and this reinstatement was abolished by a concomitant injection of the OrxR2 antagonist TCSOX229 (Matzeu et al., 2016; **Figure 1**). Interestingly, this priming effect of OrxA in inducing cocaine-seeking behavior was not long lasting (Matzeu and Martin-Fardon, 2021), implying that the HYP (Orx)↔pPVT circuit undergoes neuroadaptive changes during abstinence, reflected by alterations of the efficacy of OrxA in inducing reinstatement when injected in the pPVT. In addition to the PVT, other brain regions that receive Orx inputs, such as the VTA and CeA, have been investigated because of their known role in cocaine-seeking behavior. Similar to observations in the pPVT, local injections of OrxA in the VTA reinstated cocaine-seeking behavior (Wang et al., 2009). However, in contrast to the pPVT, this priming effect of OrxA was reversed by a selective OrxR1 antagonist but not by an OrxR2 antagonist (Wang et al., 2009). Supporting the importance of OrxR1 signaling in the VTA during cocaine-seeking behavior (**Figure 1**), another study showed that SB334867 administration in the VTA attenuated the conditioned reinstatement of cocaine-seeking behavior, an effect that was not observed when the same OrxR1 antagonist was injected in the PVT (James et al., 2011). Finally, intra-CeA SB334867 administration significantly reduced the stress-induced reinstatement of cocaine-seeking behavior (Schmeichel et al., 2017). Together with findings in the VTA and pPVT, these results suggest the brain region-dependent involvement of OrxR1 vs. OrxR2 in cocaine-seeking behavior (e.g., OrxR1 in the VTA and CeA and OrxR2 in the pPVT; **Figure 1**).

Alcohol

Several studies have shown that the Orx system is strongly engaged during alcohol-seeking behavior (e.g., Millan et al., 2010). OrxR1 blockade reduced alcohol cue-induced reinstatement (Lawrence et al., 2006; Martin-Fardon and Weiss, 2014b; Moorman et al., 2017), supporting the hypothesis that Orx neuropeptide transmission at OrxR1 specifically regulates high levels of motivation for alcohol. SB334867 has also been reported to effectively reduce the stress-induced reinstatement of alcohol-seeking behavior, but this effect was not specific for alcohol and instead generalized to the stress-induced reinstatement of sucrose seeking (Richards et al., 2008).

Investigations of specific brain regions where Orx neuropeptides are implicated in the control of alcohol-seeking behavior revealed that the pPVT plays a pivotal role in the stress-induced reinstatement of alcohol-seeking behavior (Matzeu and Martin-Fardon, 2020a; **Figure 1**). An intra-pPVT injection of the dual OrxR antagonist TCS1102 prevented stress-induced reinstatement selectively in alcohol-dependent rats (Matzeu and Martin-Fardon, 2020a). Another study showed that an injection of an OrxR2 antagonist in the NAcC did not affect alcohol seeking that was triggered by cue presentation (Brown et al., 2013). Similar to cocaine, these findings suggest that the involvement of OrxR1 vs. OrxR2 in alcohol-seeking behavior is brain region-dependent.

Opioids

The pharmacological manipulation of OrxR1 as a potential treatment to prevent opioid relapse has received much research attention. SB334867 attenuated the reinstatement of heroin-seeking behavior that was elicited by discrete cues but not reinstatement that was elicited by a heroin priming injection (Smith and Aston-Jones, 2012). Several studies corroborated the efficacy of SB334867 in preventing prescription opioid seeking. For example, a recent study from our group found that systemic administration of the OrxR1 antagonist SB334867 but not OrxR2 TCSOX229 prevented the conditioned reinstatement of oxycodone-seeking behavior (Matzeu and Martin-Fardon, 2020c). Consistent with these findings, other studies of different opioids showed that OrxR1 blockade reduced the cue-induced reinstatement of remifentanil seeking (Porter-Stransky et al., 2017) and fentanyl seeking (Fragale et al., 2019). Studies that investigated the neurobiological basis of remifentanil seeking found that the VP is a key brain region where Orx neuropeptides that act through OrxR1 (**Figure 1**) can influence the cue-induced reinstatement of remifentanil seeking (Mohammadkhani et al., 2019, 2020).

Nicotine

Limited and somehow controversial data are available on the effects of OrxR antagonists on nicotine-seeking behavior. Plaza-Zabala et al. (2013) showed that pretreatment with the OrxR1 antagonist SB334867 but not OrxR2 antagonist TCSOX229 decreased the cue-induced reinstatement of nicotine seeking. In contrast, Uslaner et al. (2014) showed that OrxR2 blockade significantly prevented nicotine seeking that was triggered by environmental cues. Specifically, the selective OrxR2 antagonist 2-SORA 18 reduced the cue-induced reinstatement of nicotine-seeking behavior but not drug-induced reinstatement (Uslaner et al., 2014). Although in apparent disagreement, these two

studies demonstrate the importance of OrxR signaling for nicotine relapse.

RESTORING NORMAL CIRCADIAN ACTIVITY WITH OREXIN RECEPTOR ANTAGONISTS IN INDIVIDUALS WITH SUBSTANCE USE DISORDER

An estimated 5% of the world's population uses drugs regularly, and nearly 0.6% suffer from a substance use disorder as defined by the *Diagnostic and Statistical Manual of Mental Disorders*, 5th edition (Merz, 2017). Drug abuse elicits detrimental consequences on a person's overall well-being at the psychological, emotional, and social levels (Degenhardt and Hall, 2012). Sleep disturbances, including alterations of sleep architecture and the development of insomnia, are extremely common among people who suffer from substance use disorder (Wang and Teichtahl, 2007; Koob and Colrain, 2020; Brooks et al., 2021; Frers et al., 2021; Miller et al., 2021). Sleep disturbances during withdrawal and the early treatment of substance use disorder are a major problem across all classes of drugs of abuse and have been related to a failure of completing substance use disorder treatment (Wilkerson et al., 2021). The high incidence of insomnia symptoms and poor sleep quality, together with a lack of treatment completion, demonstrate that severe sleep disturbances are a major risk factor for relapse (Brower and Perron, 2010; Wilkerson et al., 2019).

Substance use disorder can also compromise a drug user's nutrition and deleteriously affect dietary habits (Nabipour et al., 2014; Rush et al., 2016; Cusack et al., 2021; Mahboub et al., 2021). Individuals with drug addiction generally exhibit "unhealthy" lifestyles that consequently affect food intake, eventually leading to malnutrition or undernutrition (Wandler, 2003). Such individuals usually have irregular eating patterns, with unbalanced nutrient intake (Mahboub et al., 2021). During detoxification, they also have poor dietary habits, with low food consumption that is often caused by negative symptoms of abstinence from the drug (Mahboub et al., 2021). Notably, the frequency and duration of drug use can differentially influence the nutritional status of drug users (Santolaria-Fernandez et al., 1995), but further discussions of this aspect of drug use are beyond the scope of the present review.

Eating and the sleep/wake cycle are closely related to each another. Preclinical studies have shown that during food deprivation, animals exhibit shorter episodes of sleep, the frequency of which decreases as the length of deprivation increases (Borbely, 1977; Dewasmes et al., 1989), concomitant with an increase in locomotor activity (Moskowitz, 1959; Mabry and Campbell, 1975; Jones et al., 1990) that may be attributable to an increase in feeding behavior. Considering the crucial role of the Orx system in regulating feeding and sleeping, unsurprising is that dysfunction of the Orx system is related to disturbances in food intake and sleep. For example, individuals with narcolepsy who have low levels of Orx neuropeptides exhibit a concomitant increase in palatable food intake (van Holst et al., 2016), with a high incidence of binge eating (Dimitrova et al., 2011), suggesting that Orx peptides play a more intricate role than simply being "feeding peptides."

To date, the literature indicates a role for Orx neuropeptides in mediating feeding behavior and sleep and regulating the motivation for drugs of abuse. As proposed for binge eating disorders (Mehr et al., 2021), we identify the Orx system as a potential neurobiological link between drugs of abuse and co-occurring eating and sleep dysregulations. We propose that pharmacotherapies that normalize Orx signaling, such as with OrxR antagonists, may effectively decrease the motivation for drugs of abuse and ameliorate sleep disturbances and unbalanced nutritional states that afflict individuals with substance use disorder. Overall, the preclinical studies discussed above demonstrate that OrxR1 may be a viable treatment target to selectively reduce craving and the motivation for drug intake. One common limitation of these studies, however, is that the efficacy of OrxR2 antagonism has not always been tested. Indeed, studies indicate that some aspects of addiction-related behaviors are also linked to OrxR2 signaling (e.g., Schmeichel et al., 2015; Matzeu et al., 2016), suggesting that OrxR2 might also play an important role in substance use disorder. Based on the assumption that both OrxR1 and OrxR2 play a role in drug addiction, the use of compounds that target both receptors might be beneficial for the treatment of substance use disorder.

The dual orexin receptor antagonist suvorexant (Belsomra^{TM}) is currently available for the clinical treatment of insomnia in the United States, Canada, and Japan. It is also gaining interest as a potential pharmacological treatment for addiction (Campbell et al., 2020a; James et al., 2020). The comorbidity of substance use disorder, sleep disruption, and malnutrition has been widely documented, and these conditions have been characterized by an imbalance in the Orx system. Thus, the use of suvorexant may be promising for restoring "normal" balance in the Orx system, mitigate relapse vulnerability, and reestablish "normal" physiological functions, including feeding and sleep. A clinical study of non-treatment-seeking subjects with cocaine use disorder who were treated with suvorexant reported promising results. Suvorexant improved sleep, reduced the response to acute stress, and reduced cocaine craving (Suchting et al., 2020). Another ongoing clinical study (Campbell et al., 2020b) is currently examining the efficacy of suvorexant for the treatment of comorbid alcohol use disorder and insomnia. These studies represent an important step toward the potential use of suvorexant for substance use disorder treatment and will provide valuable data to direct future clinical trials with other substances of abuse.

As mentioned above, drug addiction is often associated with dysregulations of eating, most commonly undernutrition. One concern could be that the use of OrxR antagonists that block Orx signaling might further suppress food intake, thus worsening undernutrition. Several studies have shown that OrxR1 blockade significantly decreased food intake and motivation toward food (e.g., Piccoli et al., 2012; Wiskerke et al., 2020; Freeman et al., 2021). However, other studies from our group showed that suppressing OrxR signaling had a minimal effect on food-directed behavior compared with drug-directed

behavior (Martin-Fardon and Weiss, 2014a,b; Martin-Fardon et al., 2018; Schmeichel et al., 2018), suggesting that the use of an OrxR antagonist could have a minimal impact on food-directed behavior and be selective for the treatment of drug-directed behavior. Preclinical studies that examine the impact of OrxR antagonists on food-directed behavior in drug-dependent animals are lacking. Thus, the effect of manipulating the Orx system on an already compromised nutritional state in dependent subjects remains to be addressed.

CONCLUSION AND FUTURE DIRECTIONS

Orexin receptor antagonists are promising anti-addiction medications. Based on the considerable amount of data that have been generated by preclinical and clinical studies of the role of Orx neuropeptides in drug addiction, the National Institute on Drug Abuse, Division of Therapeutics and Medical Consequences, has recognized OrxR antagonists among medication development priorities for the treatment for addiction (Rasmussen et al., 2019). Although the data are very promising, several questions remain unanswered. For example, parallel findings on blood Orx levels in humans with substance use disorder (e.g., alcohol, Δ^9-tetrahydrocannabinol, and nicotine) and preclinical studies of Orx in the brain remain counterintuitive. If the majority of preclinical studies show that OrxR antagonists that decrease Orx transmission can attenuate drug intake and drug-seeking behavior, human data show that stronger withdrawal symptoms are associated with lower blood levels of Orx, which are opposite to expectations (i.e., higher Orx levels rather than lower Orx levels). This suggests that brain and blood Orx levels might not follow the same dynamics. This issue remains unresolved and needs further investigation. Another issue is which OrxR antagonists (e.g., specific OrxR1 or OrxR2 antagonists or dual orexin receptor antagonists, such as suvorexant) are more effective as potential treatments for drug addiction. Additional unresolved issues are whether these compounds are effective across different classes of drugs and whether they are better suited in specific stages of the addiction cycle (Koob and Volkow, 2010; Koob, 2021). Knowing that the Orx system shows sexual dimorphism (Taheri et al., 1999; Johren et al., 2001), also unknown is whether the effects of these OrxR antagonists could be different depending on sex. Furthermore, studies need to characterize the bioavailability of these compounds, their toxicity, and the safety and efficacy of long-term treatment when used to treat comorbid substance use disorder and abnormal physiological functions, such as sleeping and feeding. The majority of preclinical studies have investigated only the acute effects of OrxR antagonists. The safety and efficacy of long-term treatment remain an important issue that needs to be addressed in preclinical studies. Lastly, investigations need to be extended to polysubstance use.

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Leptin Protects Against the Development and Expression of Cocaine Addiction-Like Behavior in Heterogeneous Stock Rats

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Cocaine affects food intake, metabolism and bodyweight. It has been hypothesized that feeding hormones like leptin play a role in this process. Preclinical studies have shown a mutually inhibitory relationship between leptin and cocaine, with leptin also decreasing the rewarding effects of cocaine intake. But prior studies have used relatively small sample sizes and did not investigate individual differences in genetically heterogeneous populations. Here, we examined whether the role of individual differences in bodyweight and blood leptin level are associated with high or low vulnerability to addiction-like behaviors using data from 306 heterogeneous stock rats given extended access to intravenous self-administration of cocaine and 120 blood samples from 60 of these animals, that were stored in the Cocaine Biobank. Finally, we tested a separate cohort to evaluate the causal effect of exogenous leptin administration on cocaine seeking. Bodyweight was reduced due to cocaine self-administration in males during withdrawal and abstinence, but was increased in females during abstinence. However, bodyweight was not correlated with addiction-like behavior vulnerability. Blood leptin levels after ∼6 weeks of cocaine self-administration did not correlate with addiction-like behaviors, however, baseline blood leptin levels before any access to cocaine negatively predicted addiction-like behaviors 6 weeks later. Finally, leptin administration in a separate cohort of 59 animals reduced cocaine seeking in acute withdrawal and after 7 weeks of protracted abstinence. These results demonstrate that high blood leptin level before access to cocaine may be a protective factor against the development of cocaine addiction-like behavior and that exogenous leptin reduces the motivation to take and seek cocaine. On the other hand, these results also show that blood leptin level and bodyweight changes in current users are not relevant biomarkers for addiction-like behaviors.

Keywords: psychostimulant, outbred strains, substance-related disorders, hormones, bodyweight

INTRODUCTION

Cocaine is used primarily for its pleasurable effects by young adults. Overall, 5.5 million Americans reported using cocaine in 2019, which includes 1 million with cocaine use disorders (Samhsa, 2020). While most research on cocaine focuses on its rewarding effect, cocaine also has effects on food intake (Mitchell and Roseberry, 2019) and metabolism (Billing and Ersche, 2015). These results suggest that cocaine use affects bodyweight control and feeding hormones, such as the satiety hormone leptin (Schwartz et al., 1996; Friedman, 2014). This is of therapeutic interest, as increased weight gain during cocaine abstinence can hinder recovery (Cochrane et al., 1998; Billing and Ersche, 2015; Bruening et al., 2018) and feeding hormones can affect rewarding effects of drugs in addition to food. However, the exact role of cocaine self-administration on bodyweight and leptin level, and the effect of leptin on cocaine-seeking is unclear, partly due to the lack of longitudinal studies and experiments with low sample size or identical inbred animals.

Converging lines of evidence suggest that cocaine may alter leptin levels and that leptin may modulate cocaine addiction-like behaviors. Both cocaine and leptin modulate the dopaminergic system and interact during cocaine and food self-administration (Carroll et al., 1989; Figlewicz et al., 2003; Fulton et al., 2006; Hommel et al., 2006). Cocaine has transient anorexigenic effects (Balopole et al., 1979), followed by a compensatory increase in the consumption of fat and carbohydrates (Bane et al., 1993) associated with increased metabolism (Billing and Ersche, 2015) and reduced leptin levels (You et al., 2019). On the other hand, leptin signaling attenuates cocaine-induced locomotor activity (Lee et al., 2018), decreases cocaine reward in a conditioned place preference test (Shen et al., 2016; You et al., 2016) and accelerates extinction from cocaine seeking (You et al., 2016) in rodents. Similar results of the effect of cocaine on feeding have been found in clinical studies (Castro et al., 1987; Weddington et al., 1990; Ersche et al., 2013), but the relationship with leptin is less clear. Reduced leptin levels were reported for crack cocaine users (Ersche et al., 2013; Levandowski et al., 2013; Escobar et al., 2018), but a controlled cocaine-administration study found no effect on leptin levels (Bouhlal et al., 2017). Moreover, in contrast to the results in rats, increased leptin levels were reported to lead to higher cocaine craving in humans (Martinotti et al., 2017), leaving the role of leptin levels on cocaine-seeking unclear.

To address this gap in the literature, we tested the hypothesis that cocaine self-administration leads to increased bodyweight during abstinence through reduced blood leptin levels that contribute to increased cocaine-seeking. A sub-hypothesis is that leptin may be a protective factor against the development of cocaine addiction-like behavior. To this end, we used an animal model of extended access (12 h per day for ∼3 weeks) to intravenous cocaine self-administration in outbred heterogeneous stock (HS) rats (Hansen and Spuhler, 1984; Baud et al., 2013), which mimic the diversity of the human population (Woods and Mott, 2017). Cocaine intake (fixed ratio), motivation to seek cocaine [progressive ratio (PR)], resistance to punishment (contingent footshock) and withdrawal-induced irritable-like behavior (bottle brush) were evaluated and used to calculate an addiction index, which is a composite *Z*-score of the different addiction-like behaviors that is assigned to each rat as a measure of the addiction-like phenotype. To evaluate the impact of cocaine self-administration on bodyweight and blood leptin levels, we used phenotypic data from 306 animals and 120 blood samples from a subset of 60 of these rats from the Cocaine Biobank (Carrette et al., 2021b). We also used a separate cohort of 59 HS rats to test the causal effect of exogenous leptin administration on cocaine-seeking.

MATERIALS AND METHODS

Animals

Male and female radio-frequency identification (RFID) tagged HS rats (Rat Genome Database NMcwiWFsm:HS #13673907, sometimes referred to as N/NIH:HS) were provided by Dr. Leah Solberg Woods. Rats were shipped at 3–4 weeks of age, quarantined, and then pair-housed on a 12 h/12 h normal light/dark cycle in a temperature (20–22°C) and humidity (45–55%) controlled vivarium with *ad libitum* access to tap water and food pellets (PJ Noyes Company, Lancaster, NH, United States). All procedures were conducted in strict adherence to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committees of The Scripps Research Institute and UC San Diego. Experiments were performed in cohorts of 48–60 rats. In the first experiment, we used behavioral data from 306 animals from the Cocaine Biobank[1] (Carrette et al., 2021b) that had survived until the end of the experiment and maintained catheter patency. Next, blood samples from a subset of these animals were selected from the biobank for 30 males (1 of 30 males was excluded as there was insufficient baseline blood sample) and 30 females (1 of 30 females was excluded as the animal had lost catheter patency) with variable addiction index at two time points (baseline and withdrawal). Finally, we used a new separate cohort of 30 males and 29 females to test the effect of leptin on cocaine seeking. During the self-administration paradigm, 4 females and 1 male died and 3 females and 1 male lost catheter patency, these animals were excluded from all subsequent leptin testing. During abstinence in the homecage, another 5 females and 4 males died, and 1 female and 5 males lost catheter patency, these animals were excluded from the extinction testing with leptin. For homecage feeding/plasma collection during abstinence, the animals that died were excluded, but not the ones that lost catheter patency during abstinence. One more male died before final plasma leptin collection. At the end of the experiments (18 weeks after catheter surgery), 24 rats either died of either unknown causes or had failed catheter patency testing using a short-acting anesthetic (brevital), leaving 18 males and 16 females.

Drugs

Cocaine HCl (National Institute on Drug Abuse, Bethesda, MD, United States) was dissolved in 0.9% sterile saline and administered intravenously at a dose of 0.5 mg/kg/0.1 ml

[1]https://www.cocainebiobank.org

per infusion. Leptin (R&D systems, Minneapolis, MN, United States) was dissolved in 0.9% sterile saline and administered intravenously at a dose of 0.6 mg/kg/infusion.

Timeline

The timeline of the experiment is illustrated in **Figure 1A**. Animals were implanted with an intravenous (*i.v.*) catheter and given access to cocaine self-administration for 10 short access (ShA) and 14 long access (LgA) sessions. Withdrawal was tested by comparing irritability-like behavior at baseline and 18 h into withdrawal using the bottle-brush test. Compulsive-like behavior was tested using 1 session with contingent (30%) footshock as an aversive consequence to lever pressing. The motivation for cocaine was tested using a PR schedule

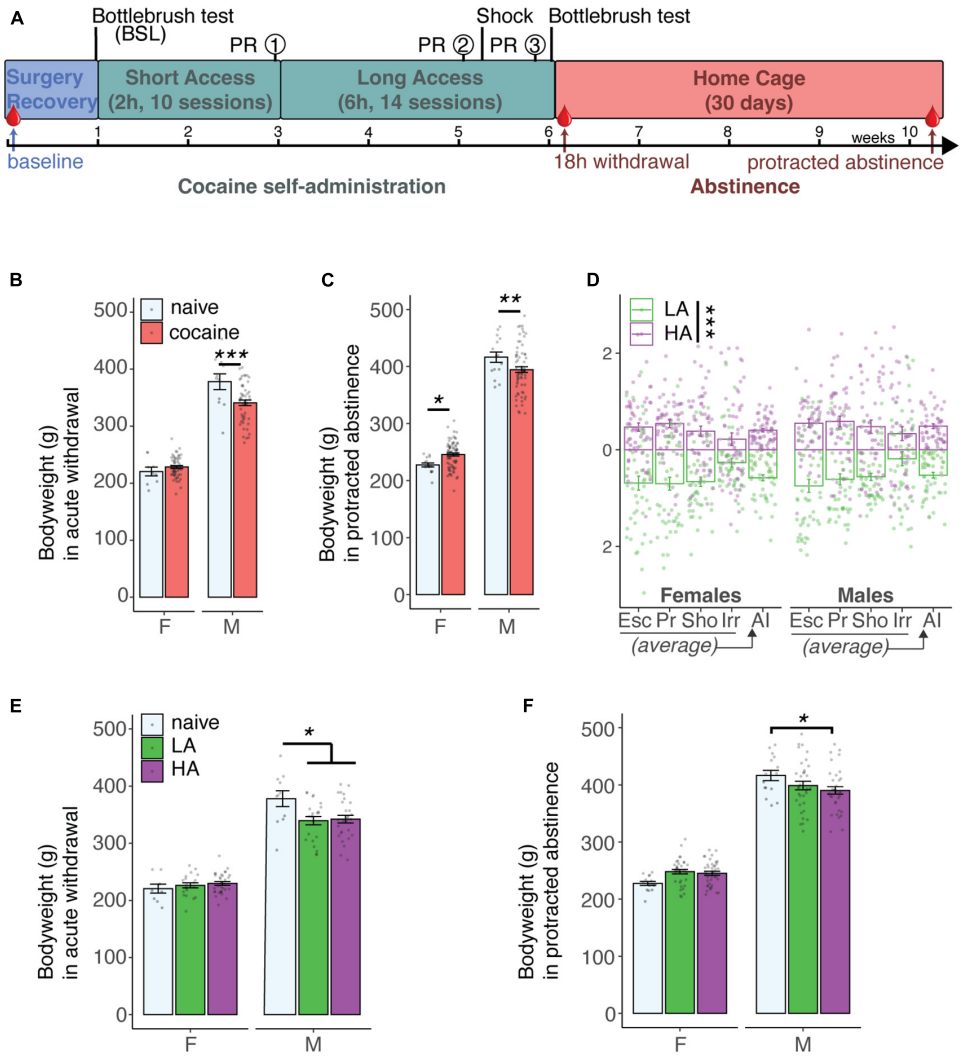

FIGURE 1 | Bodyweights rats. **(A)** Cocaine self-administration behavioral timeline. The red droplet indicates blood collection timepoints. **(B)** Body weight per sex in acute withdrawal following cocaine self-administration compared to naive littermates shows a significant difference between males with ***$p = 0.00025$ [two-way ANOVA with sex and group as between variables: Group × Sex $F(1,120) = 9.62$ with $p = 0.0024$, main effects of Group $F(1,120) = 10.67$ with $p = 0.0014$ and Sex $F(1,120) = 507.71$ with $p < 0.0001$] N: F = 9 naive + 53 cocaine, *M* = 11 naive + 51 cocaine; 3M + 1F cocaine were removed as outliers. **(C)** Body weight per sex in protracted abstinence following cocaine self-administration compared to naive littermates shows a significant difference between males with **$p = 0.018$ and females *$p = 0.045$ [two-way ANOVA with sex and group as between variables: Group × Sex $F(1,170) = 9.75$ with $p = 0.0021$ and main effects of Sex $F(1,170) = 507.71$ with $p < 0.0001$] N: F = 15 naive + 76 cocaine, *M* = 15 naive + 68 cocaine; 1M naive + 3M cocaine were removed as outliers. **(D)** Individual escalation (Esc), motivation (Pr), compulsivity (Sho) *Z*-scores and addiction index show the distinction of the animals with high (HA) and low (LA) addiction-like behaviors. Bonferroni corrected pairwise *t*-tests were significant (***$p < 0.0001$) for each score in males and females. N: F = 74 HA + 56 LA, *M* = 65 HA + 60 LA. **(E)** Body weight per sex further split per group in acute withdrawal following cocaine self-administration showed a similar significant reduction in bodyweight for both HA (*$p = 0.027$) and LA (*$p = 0.020$) males compared to naive males [two-way ANOVA with sex and group as between variables: Group × Sex $F(2,118) = 4.69$ with $p = 0.011$] N: F = 9 naive + 20 LA + 33 HA, *M* = 11 naive + 23 LA + 28 HA; 1F + 3M cocaine were removed as outliers. **(F)** Body weight per sex further split per group in protracted abstinence following cocaine self-administration showed a significant reduction in bodyweight for the HA males (*$p = 0.039$) compared to naive males [two-way ANOVA with sex and group as between variables: Group × Sex $F(2,167) = 5.35$ with $p = 0.0056$] N: F = 15 naive + 35 LA + 40 HA, *M* = 15 naive + 34 LA + 34 HA; 1M naive, 1F + 3M cocaine were removed as outliers.

of reinforcement after ShA, LgA, and shock sessions. This phenotyping protocol has been described in more detail previously (Carrette et al., 2021b).

Jugular Vein Catheterization

This surgery inserts a catheter into the jugular vein to allow for intravenous cocaine self-administration. Rats were anesthetized with vaporized isoflurane (1–5%). Intravenous catheters were aseptically inserted into the right jugular vein using the procedure. Catheters consisted of micro-renathane tubing (18 cm, 0.023-inch inner diameter, 0.037-inch outer diameter; Braintree Scientific, Braintree, MA, United States) attached to a 90-degree angle bend guide cannula (Plastics One, Roanoke, VA, United States), embedded in dental acrylic, and anchored with mesh (1 mm thick, 2 cm diameter). Tubing was inserted into the vein following a needle puncture (22G) and secured with a suture. The guide cannula was punctured through a small incision on the back. The outer part of the cannula was closed off with a plastic seal and metal cover cap, which allowed for sterility and protection of the catheter base. Flunixin (2.5 mg/kg, *s.c.*) was administered as analgesic, and cefazolin (330 mg/kg, *i.m.*) as antibiotic. Rats were allowed 5 days for recovery prior to self-administration. They were monitored and flushed daily with heparinized saline (10 U/ml of heparin sodium; American Pharmaceutical Partners, Schaumberg, IL, United States) in 0.9% bacteriostatic sodium chloride (Hospira, Lake Forest, IL, United States) that contained 52.4 mg/0.2 ml of cefazolin. Catheter patency was tested throughout and at the end of LgA sessions using a short-acting anesthetic (brevital), any animal that failed the test was excluded from the study.

Behavioral Phenotyping

The rats were subjected to only one behavioral procedure per day, during their dark cycle. Detailed protocols can be found in the George lab protocol repository on protocols.io.[2]

- *Operant self-administration.* The rats were allowed to self-administer cocaine individually in operant chambers (29 cm × 24 cm × 19.5 cm; Med Associates, St. Albans, VT, United States) that were enclosed in lit, sound-attenuating, ventilated environmental cubicles and computer-controlled. Each chamber was equipped with two retractable levers that would only be extended for the duration of the test. A press on the right lever activated an infusion pump on a fixed ratio (FR1) schedule to deliver the drug infusion through plastic catheter tubing over 5 s, followed by a 20 time out during which pressing the lever had no scheduled consequences. Right lever presses also activated a cue light that remained illuminated during the time-out. Pressing the left, inactive lever had no scheduled consequences. The rats went through 10 ShA sessions of 2 h and 14 LgA sessions of 6 h. Five sessions were performed per week, with a break over the weekend.

- *Progressive Ratio (PR) responding* was tested to assess motivation. Number of lever presses required to receive a drug infusion increase progressively (according to the following schedule: 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95, 118, 145, 178, . . .). When the rat did not achieve the required number of presses for the next infusion in the hour following the last infusion, the session was terminated with the breakpoint defined as the last achieved ratio. PR tests were performed after ShA, after LgA, and after foot shock tests. For one cohort, two additional PR tests were performed with intravenous (*i.v.*) leptin treatment (0.6 mg/kg in saline) (You et al., 2016) or saline vehicle, 30 min before the session using a Latin square design.

- *Compulsive-like responding using contingent foot shock.* In this self-administration session, 30% of active lever presses were randomly associated with a foot shock (0.3 mA for 0.5 s), delivered through the floor grid.

- *Withdrawal-induced irritable-like behavior:* Rats were placed in the back of a clean cage, irritated by rotating a bottlebrush near their whiskers for 5 s followed by rotating and slowly withdrawing for 5 s, whilst being scored for defensive (escape, jumping, vocalization, grooming, digging) and aggressive (exploration, boxing, biting, following, latency biting) irritable-like behavior. The test was repeated 10 times per animal, with 10 s intervals and scored by 3 observers. The test was performed before ShA to cocaine as a measurement of baseline behavior and 18 h in withdrawal following LgA to cocaine.

- *Extinction responding.* The subset of animals that were tested for cocaine-seeking with leptin treatment were, after 7 weeks of forced abstinence, re-introduced in the SA chambers for 2 h with access to the levers. The right lever press still activated a cue light, but did not result in drug delivery. Three sessions were performed, separated by 1 week of abstinence. Session 1 and session 3 animals received *i.v.* saline, session 2 animals received *i.v.* leptin (0.6 mg/kg in saline), 30 min before the start of the session.

Blood Collection and Analysis

- *Baseline blood (before cocaine):* Retroorbital bleeds were performed under anesthesia with the eye numbed by a topical ophthalmic anesthetic (proparacaine hydrochloride).

- *Terminal (acute cocaine withdrawal: euthanized 18 h in withdrawal after a LgA session or protracted cocaine abstinence: euthanized∼3–4 weeks after the last cocaine intake during SA sessions):* Blood was collected through cardiac puncture after CO_2 inhalation.

Fresh blood was collected in EDTA coated tubes to avoid coagulation and was centrifuged at 2,000 g at room temperature (RT) for 10 min. The supernatant plasma was immediately transferred per 500 μl aliquots into fresh tubes, scored for quality on a scale from 0 to 6, snap-frozen on dry ice, and stored at –80°C. Frozen aliquots were thawed on ice and analyzed using a Leptin Rat ELISA kit (Invitrogen, Waltham, MA, United States).

Data Analysis

Data were analyzed with RStudio, using the rstatix package (for aggregated cohorts in **Figure 1**) and Graphpad Prism (for single cohorts in **Figures 2–4**). Values above the third quartile (Q3) + 1.5× interquartile range (IQR) or below Q1 - 1.5× IQR were considered as outliers and removed from the data. Statistical tests were two-way ANOVA with or without repeated measures as needed and described. For the leptin level

[2]https://www.protocols.io/workspaces/george-lab

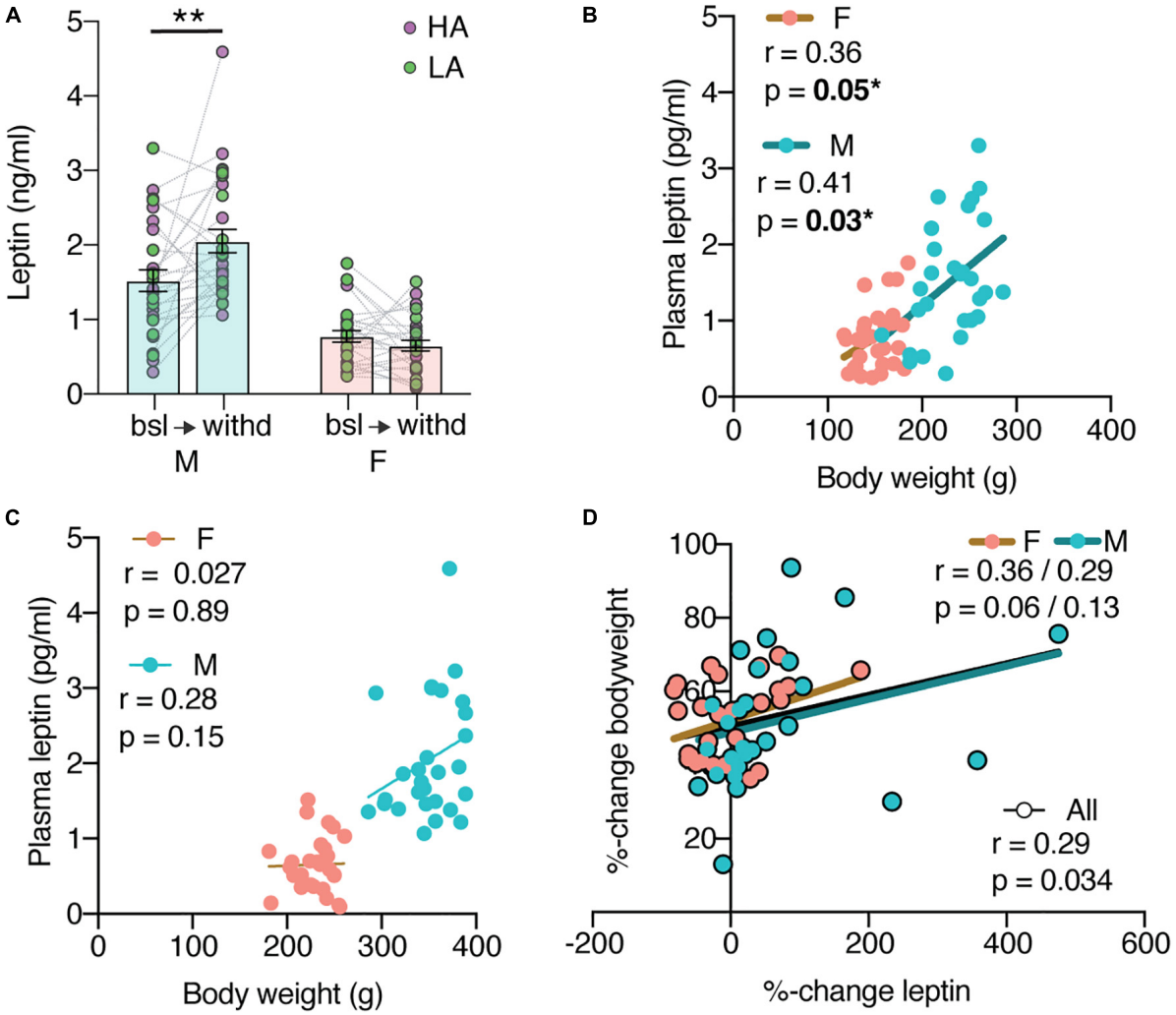

FIGURE 2 | Plasma leptin levels before and after cocaine self-administration. **(A)** Male leptin levels significantly increase from baseline (bsl) to withdrawal (withd) (**p = 0.0012) [mixed effects analysis with sex as between and time as within variables: Timepoint × Sex $F(1,52)$ = 10.33 with p = 0.0022, main effect of sex $F(1,56)$ = 62.64 with $p < 0.0001$]. N: F = 29 bsl + 27 withd, M = 28 bsl + 28 withd; 1M bsl, 2F + 1M withd values were excluded as outliers. **(B)** Baseline leptin levels positively correlate to bodyweight (M: r = 0.41, *p = 0.03; F: r = 0.36, *p = 0.05). **(C)** Withdrawal leptin levels do not correlate to bodyweight (M: r = 0.28, p = 0.15; F: r = 0.027, p = 0.89). **(D)** Percent-change bodyweight does not correlate with the percent-change leptin (M: r = 0.29, p = 0.13; F: r = 0.36, p = 0.064).

change (**Figure 2A**) and food intake analysis (**Figure 3C**), data values were missing at different timepoints due to outliers, so a mixed-effects model was applied instead of ANOVA, using the maximum likelihood-method. Pairwise differences following a significant main effect were calculated with Bonferroni correction. Weight-normalization was performed as indicated by dividing the measurement (plasma leptin level or food intake) by the individual bodyweight at the same timepoint. An addiction index for each rat was calculated as described before (Carrette et al., 2021b) using the average of the Z-scores [(individual value-group average)/group standard deviation] per cohort and per sex for intake, motivation, compulsivity, and irritability as illustrated in **Figure 1D**. A daily escalation index (E_i) can be calculated for each session i [E_i = (rewards in LgA session i – average rewards in LgA session 1)/rewards standard deviation in LgA session 1]. The overall escalation index (Esc) was obtained by taking the Z-score of the average daily escalation indexes for the last 3 days. The motivation index (Pr) was obtained by taking the Z-score of the breakpoint after LgA. The compulsivity index (Sho) was obtained by taking the Z-score of the number of rewards obtained in the shock session. The irritability index (Irr) was obtained by taking the Z-score of the difference in aggressive score in withdrawal after LgA compared to baseline. The addiction index was initially calculated using the average of the escalation, motivation, compulsivity, and irritability indices. However, because the irritability-like behavior was missing for some of the animals and given that we found no correlation between the irritability index and the escalation, motivation, and compulsivity indices we also calculated an addiction index without the irritability index (**Figures 1**–**3**). Overall, the results

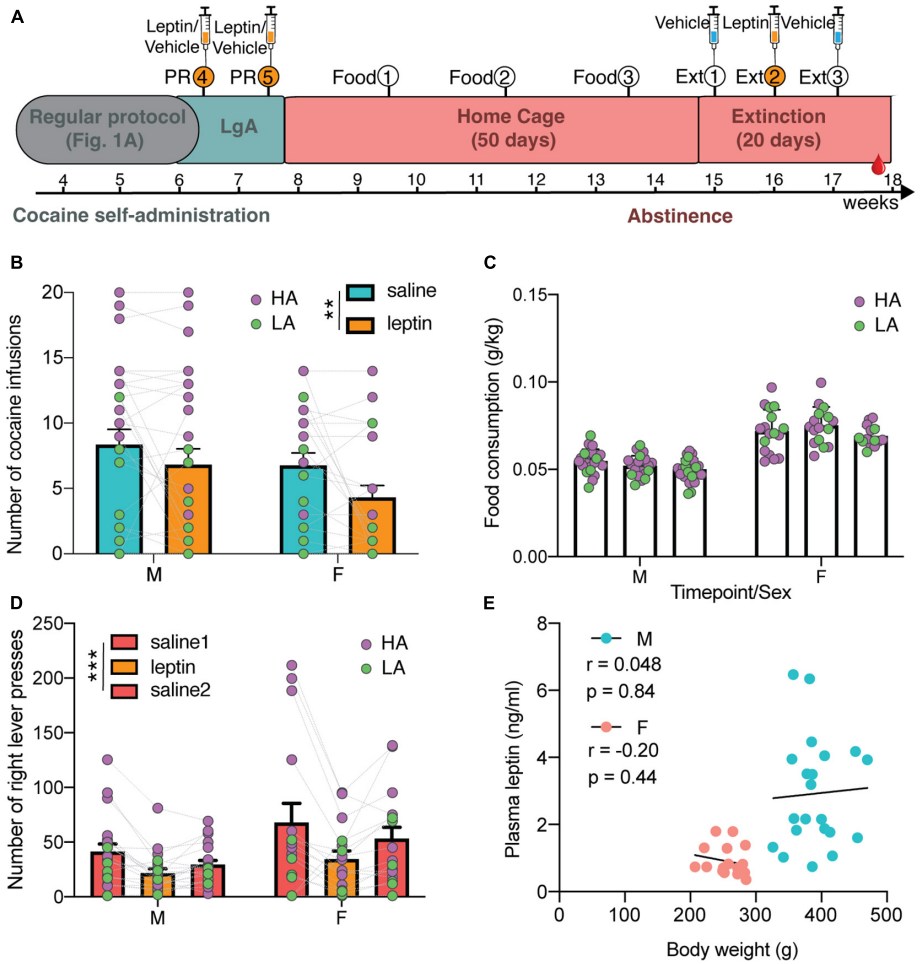

FIGURE 3 | Additional testing in one cohort of animals for leptin and food intake. **(A)** Timeline of additional experiments for characterizing the effect of exogenous leptin on cocaine seeking (syringes indicate i.v. treatment with leptin and/or vehicle), and the effect of cocaine abstinence on food intake and weight. At the end of LgA in the protocol as depicted in **Figure 1A**, 2 PR sessions were added, separated by a LgA session. During a 7-week abstinence, food intake (Food) was measured every 2 weeks. After 7 weeks, animals were returned to the self-administration chambers for three extinction sessions (Ext), each separated by a week. **(B)** Both males and females significantly reduced their cocaine intake after leptin treatment [two-way ANOVA: Treatment × Sex $F(1,48) = 0.46$ with $p = 0.50$, Treatment $F(1,48) = 8.64$ with $^{**}p = 0.0051$, and Sex $F(1,48) = 1.92$ with $p = 0.17$] N: F = 22, $M = 28$. **(C)** Females had a higher weight-normalized food intake than males, but there was no change over time [mixed effects analysis with time as within and sex as between variables: Timepoint × Sex $F(2,70) = 1.42$ with $p = 0.25$, simple main effect of Sex $F(1,39) = 139.7$ with $p < 0.0001$ and Timepoint $F(1.786,62.52) = 3.09$ with $p = 0.058$] N: F = 17, $M = 24$; 3M at time 1, 1F and 2M at time 2, and 2F at time 3 were removed as outliers. **(D)** Both males and females pressed significantly less during extinction after leptin treatment [mixed two-way ANOVA with treatment as within and sex as between variables: Treatment × Sex $F(2,66) = 0.89$ with $p = 0.42$ and simple main effect of Treatment $F(1.429, 47.14) = 11.69$ with $^{***}p = 0.0004$ and Sex $F(1,33) = 3.30$ with $p = 0.078$] N: F = 16, $M = 19$. **(E)** Abstinence leptin levels do not correlate to bodyweight (M: r = 0.048, $p = 0.84$; F: r = –0.20, $p = 0.44$) N: F = 17, $M = 23$; 2M were removed as outlier for weight and/or leptin.

obtained with or without the irritability index were similar (see section "Results").

RESULTS

Cocaine Self-Administration Alters Bodyweight in a Sex-Dependent Manner

Bodyweights at the end of the cocaine self-administration protocol (**Figure 1A**) were found to be significantly different compared to naive littermates in a sex dependent manner, both during acute withdrawal [**Figure 1B**, two-way ANOVA with sex and group as between variables has a significant two-way interaction of Group × Sex $F(1,120) = 9.62$ with $p = 0.0024$, and significant main effects of Group $F(1,120) = 10.67$ with $p = 0.0014$ and Sex $F(1,120) = 507.71$ with $p < 0.0001$; N: F = 9 naive + 53 cocaine, $M = 11$ naive + 51 cocaine; 3M + 1F cocaine were removed as outliers] and protracted abstinence [**Figure 1C**, two-way ANOVA with sex and group as between variables: Group × Sex $F(1,170) = 9.75$ with $p = 0.0021$ and main effect of Sex $F(1,170) = 507.71$ with $p < 0.0001$; N: F = 15 naive + 76 cocaine, $M = 15$ naive + 68 cocaine; 1M naive + 3M cocaine were removed as outliers]. Analysis of simple main effects for group level was performed with a Bonferroni adjustment. There

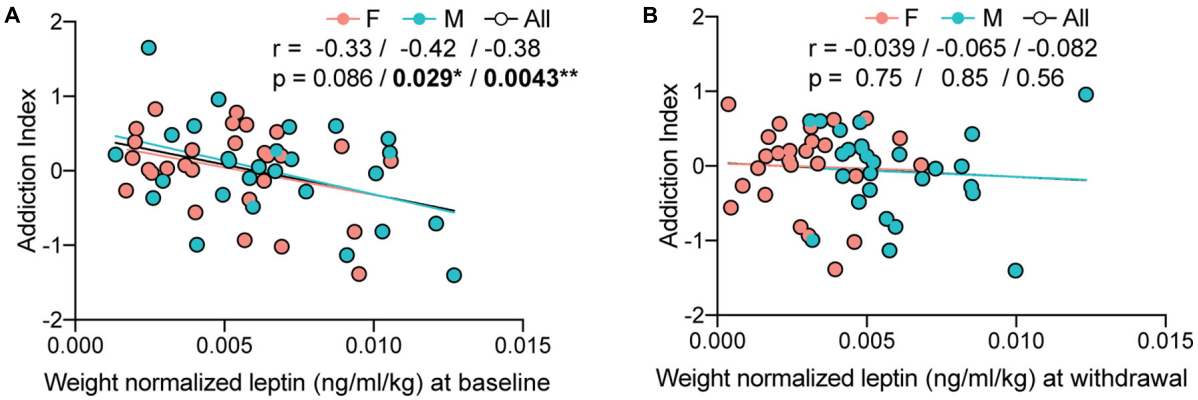

FIGURE 4 | Plasma leptin analysis at baseline. **(A)** The addiction index negatively correlates with weight-normalized baseline leptin levels (M: r = –0.42, *p = 0.029; F: r = –0.33, p = 0.086; all: r = –0.38, **p = 0.0043), N: 28F + 27M; 1M value was excluded as outlier, 1F and 1M were excluded for missing irritability data. **(B)** The addiction index does not correlate with weight-normalized leptin levels in withdrawal (M: r = –0.065 p = 0.85; F: r = –0.039, p = 0.75; all: r = –0.082, p = 0.56), N: 26F + 27M; 2F + 1M values were excluded as outliers, 1F and 1M were excluded for missing irritability data.

was a statistically significant difference in mean bodyweight for both males in withdrawal [$F(1, 120) = 14.2$, $p = 0.00025$], males in abstinence [$F(1, 170) = 5.75$, $p = 0.018$], whose bodyweight decreased compared to naive males, and females in abstinence [$F(1,170) = 4.07$, $p = 0.045$], whose bodyweight increased compared to naive females. The addiction-like behaviors of these animals have been characterized through the self-administration protocol and can be described by the calculated Z-scores of responding during the FR, PR, and shock sessions, or averaged in an overall addiction index (**Figure 1D**). Animals with a positive addiction index were classified as having high addiction-like behaviors (HA), those with a negative addiction index as having low addiction-like behaviors (LA). Bonferroni-corrected pairwise tests for each score in males and females showed significant differences between HA and LA animals ($p < 0.0001$, N: F = 74 HA + 56 LA, M = 65 HA + 60 LA). When looking at the bodyweight differences split further between naive LA and HA groups, the sex difference obviously remains during acute withdrawal [**Figure 1E**, two-way ANOVA with sex and group as between variables: Group × Sex $F(2,118) = 4.69$ with $p = 0.011$; N: F = 9 naive + 20 LA + 33 HA, M = 11 naive + 23 LA + 28 HA; 1F + 3M cocaine were removed as outliers] and protracted abstinence [**Figure 1F**, two-way ANOVA with sex and group as between variables: Group × Sex $F(2,167) = 5.35$ with $p = 0.0056$; N: F = 15 naive + 35 LA + 40 HA, M = 15 naive + 34 LA + 34 HA; 1M naive, 1F + 3M cocaine were removed as outliers]. As above, there was a statistically significant difference in mean bodyweight for both males in withdrawal [$F(2, 118) = 7.07$, $p = 0.001$] and males in abstinence [$F(1, 170) = 5.75$, $p = 0.018$], whose bodyweight decreased after cocaine intake. But no significant main effect was seen for females in abstinence [$F(2,167) = 2.33$, $p = 0.10$]. Bonferroni-corrected pairwise comparisons between the male subgroups showed a similar significant reduction in bodyweight compared to naive males for both HA ($p = 0.027$) and LA ($p = 0.020$) groups in withdrawal and between naive males and the HA group ($p = 0.039$) in abstinence, with a similar trend in the LA group. Note that the results of this analysis are unchanged when using an addiction index that includes irritability-like behavior. Cocaine males also weigh significantly less than naive males in withdrawal [$F(1, 120) = 14.2$, $p = 0.00025$] and abstinence [$F(1, 125) = 7.92$, $p = 0.006$]. Cocaine females have a trend to weigh significantly more than naive females in abstinence, but this did not reach significance with the reduced animal number for which irritability-like behavior was available [$F(1, 125) = 2.18$, $p = 0.143$]. In addition, there were no significant differences between HA and LA males or females in abstinence or withdrawal. Overall, these results indicate that a history of cocaine self-administration differently affects bodyweight in male (decrease) vs. female (increase) but that these effects do not depend on the severity of addiction-like behaviors (HA vs. LA).

Cocaine Self-Administration Increases Endogenous Leptin Levels in Males but Not Females

Since it is documented that cocaine influences leptin, we suspected leptin of being part of the mechanism through which cocaine asserts its effect on bodyweight. We next looked at endogenous leptin levels, within subject, before (as a proxy of naive animals) and after cocaine self-administration. Blood samples were selected from the Cocaine Biobank (Carrette et al., 2021b) from 60 animals, half male and half female. The withdrawal timepoint was chosen as it gave the most significant change in bodyweights. The leptin levels were significantly different between males and females (**Figure 2A**) [main effect of sex $F(1,56) = 62.64$ with $p < 0.0001$; N: F = 29 bsl + 27 withd, M = 28 bsl + 28 withd; 1M bsl, 2F + 1M withd values were excluded as outliers] and changed differently over the cocaine self-administration [mixed effects analysis with sex as between and time as within variables: $F(1,52) = 10.33$ with $p = 0.0022$]. Bonferroni corrected pairwise comparisons showed that male leptin levels significantly increased ($p = 0.0012$). A cocaine-induced leptin level increase could explain the observed decrease in bodyweight. However, cautious interpretation of these results

is required as leptin levels are affected by bodyweight and the animals gained weight over the course of the experiment. At baseline, plasma leptin levels correlate positively to the bodyweights (**Figure 2B**) (M: r = 0.41, p = 0.03; F: r = 0.36, p = 0.05). This correlation is lost after cocaine self-administration, especially in females (**Figure 2C**) (M: r = 0.28, p = 0.15; F: r = 0.027, p = 0.89). Moreover, the percent-change in bodyweight did not correlate with the percent-change in leptin (**Figure 2D**), (M: r = 0.29, p = 0.13; F: r = 0.36, p = 0.064). This suggests that a history of cocaine self-administration affects the relationship between leptin and body weight.

Systemic Leptin Administration Reduces Cocaine Seeking

One cohort of HS animals (N = 29 F + 30 M) that completed the cocaine-self administration protocol (**Figure 1A**) was dedicated to investigate the effect of cocaine on food intake and of leptin on addiction-like behaviors. Specifically the effect of systemic intravenous administration of 0.6 mg/kg leptin, which was shown before to reduce cocaine seeking under extinction conditions (You et al., 2016), was examined on PR responding and reinstatement of cocaine-seeking in a cohort following the earlier described self-administration protocol (**Figure 3A**). Both male and female rats significantly reduced their cocaine intake in a PR schedule after leptin treatment [**Figure 3B**, mixed two-way ANOVA with treatment as within and sex as between subject variables: Treatment × Sex $F(1,48)$ = 0.46 with p = 0.50 and simple main effect of Treatment $F(1,48)$ = 8.64 with p = 0.0051, N: F = 22, M = 28]. There were no sex differences in the test [simple main effect of Sex $F(1,48)$ = 1.92 with p = 0.17]. Per definition, HA and LA animals respond differently to this test [simple main effect of Group $F(1,48)$ = 22.13 with $p < 0.0001$] and both groups were equally affected by leptin treatment [mixed two-way ANOVA with treatment as within and group as between variable has no significant interaction: Treatment × Group $F(1,48)$ = 0.11 with p = 0.74, and a main simple effect of Treatment $F(1,48)$ = 9.37 with p = 0.0036]. Following the test, the animals were kept in their home cage for 7 weeks of forced abstinence. Food intake during the dark cycle was measured every 2 weeks during abstinence (**Figure 3C**). Weight-normalized intake was higher in females [simple main effect of Sex $F(1,39)$ = 139.7 with $p < 0.0001$], and was stable over time in both males and females [mixed effects analysis with time as within and sex as between variables: Timepoint × Sex $F(2,70)$ = 1.42 with p = 0.25, Timepoint $F(1.786,62.52)$ = 3.09 with p = 0.058; N: F = 17, M = 24; 3M at time 1, 1F and 2M at time 2, and 2F at time 3 were removed as outliers]. There was no difference in food intake between HA and LA animals at any of the three timepoints in abstinence [mixed effects analysis: time as within and group as between variables: Timepoint × Group $F(2,70)$ = 0.85 with p = 0.43 and main effect of Group $F(1,39)$ = 0.000027 with p = 0.99]. Next, the effect of leptin on cocaine seeking was examined after 7 weeks of abstinence by three extinction sessions, each performed with a one-week interval, over three consecutive weeks. During these sessions the animals were returned to the SA boxes, where they could lever press, but did not receive cocaine. In week 1, cocaine-seeking was tested with vehicle treatment. Week 2 tested the effect of leptin treatment. Finally, vehicle treatment was repeated in week 3 to confirm that the effect was due to leptin and not only due to an extinction of cocaine-seeking. Both male and female rats responded to the treatment with leptin by significantly reducing lever presses compared to vehicle sessions [mixed two-way ANOVA with treatment as within and sex as between variables: Treatment × Sex $F(2,66)$ = 0.89 with p = 0.42; N: F = 16, M = 19], but there was a simple main effect of Treatment [$F(1.429, 47.14)$ = 11.69 with p = 0.0004] (**Figure 3D**). Here too, HA and LA animals responded differently to the test [simple main effect of Group $F(1,33)$ = 4.85 with p = 0.035]. Moreover, there was a different response to the treatment [mixed two-way ANOVA with treatment as within and group as between variable: Treatment × Group $F(2,66)$ = 4.97 with p = 0.0098], with leptin having a significant effect on HA rats (*post hoc* test $p < 0.001$) but not on LA rats. The latter exhibited significantly less cocaine-seeking than the HA rats. **Figure 3E** shows that the correlation between blood leptin level and bodyweight was still lost (M: r = 0.048, p = 0.84; F: r = –0.20, p = 0.44, N: F = 17, M = 23; 2M were removed as outlier for weight and/or leptin), and that males exhibited higher endogenous blood leptin levels than females in protracted abstinence [simple main effect of Sex $F(1,36)$ = 25.44 with $p < 0.0001$], with levels further increased compared to the levels of the animals at baseline and withdrawal (compare with **Figures 2A–C**). No significant difference was found between HA and LA [two-way ANOVA with sex and group as between variables: Group × Sex $F(1,36)$ = 1.27 with p = 0.27 and main effect of Group $F(1,36)$ = 1.59 with p = 0.22].

Endogenous Leptin Level at Baseline Protects Against the Development of Addiction-Like Behaviors

We then tested if there was a correlation between endogenous weight-normalized leptin levels and the addiction index. At baseline, weight-normalized leptin levels indeed correlated with the addiction index characterized 6 weeks later (**Figure 4A**) (M: r = –0.42, p = 0.029; F: r = –0.33, p = 0.086; all: r = –0.38, p = 0.0043; N: 28F + 27M; 1M value was excluded as outlier, 1F and 1M were excluded for missing irritability data). There was no significant correlation during withdrawal after 6 weeks of cocaine self-administration (**Figure 4B**) (M: r = –0.065, p = 0.85; F: r = –0.039, p = 0.75; all: r = –0.082, p = 0.56; N: 26F + 27M; 2F + 1M values were excluded as outliers, 1F and 1M were excluded for missing irritability data). The results were the same when using raw plasma leptin levels as demonstrated in the supplementary data (**Supplementary Figures 1A,B**) and when calculating the addiction index without irritability-like behavior (r = –0.30 with p = 0.020 at baseline and r = –0.17 with p = 0.22 in withdrawal). Correlating the weight normalized leptin levels to the different components of the addiction index, the escalation index (**Supplementary Figure 1C**), motivation index (**Supplementary Figure 1D**), compulsivity index (**Supplementary Figure 1E**), and irritability index (**Supplementary Figure 1F**) shows that the correlation is mostly driven by motivation.

DISCUSSION

Here we examined the effect of cocaine self-administration on bodyweight and leptin levels, and the effect of leptin on cocaine-seeking in HS rats. Contrary to our working hypothesis, we found reduced bodyweight, and increased leptin in withdrawal and abstinence for males. Plasma leptin levels in withdrawal and abstinence seemed dysregulated by cocaine intake as they lost their typical positive correlation with bodyweight, but no differences between HA vs. LA animals were found. However, when looking at leptin levels in HS rats before the first cocaine self-administration, a negative correlation was found between blood leptin levels and the addiction index 6 weeks later, which indicates that endogenous leptin may be a protective factor against the development of cocaine addiction-like behavior. Finally, administration of leptin decreased cocaine-seeking as measured using a PR schedule of reinforcement during withdrawal and using an extinction session after protracted abstinence. These results demonstrate that leptin provides protective effects against cocaine addiction-like behavior, but that blood leptin levels *per se*, do not predict cocaine-seeking after abstinence.

Leptin is a well-known modulator of bodyweight, through modulation of food intake and food metabolism by acting on the hypothalamic circuitry (Halaas et al., 1995; Farooqi et al., 1999) and by reducing dopamine (DA) signaling in relation to food cues in the mesolimbic circuitry (Hommel et al., 2006; Sadaf Farooqi et al., 2007; Van Der Plasse et al., 2015). Leptin administration, whether systemically or intracranial in the VTA or NAc, has been shown to attenuate cocaine conditioned place preference, cocaine-seeking under extinction conditions and cocaine-induced locomotor activity (You et al., 2016; Lee et al., 2018). In mice, both the injection of the leptin receptor antagonist or deletion of the receptor was shown to enhance cocaine conditioned place preference (Shen et al., 2016). We confirmed and extended these findings by demonstrating that exogenous leptin inhibits cocaine self-administration under a PR schedule in acute withdrawal and under extinction 7 weeks into abstinence. No sex difference was found in the effect of leptin on cocaine-seeking. While leptin reduced PR responding in both HA and LA rats, it reduced cocaine-seeking during extinction only in HA rats, suggesting that HA rats' cocaine-seeking exhibits a long-lasting sensitivity to leptin.

The effect of cocaine use on blood leptin levels in humans is unclear. Indeed, a history of cocaine use has been shown to increase (Levandowski et al., 2013; Martinotti et al., 2017), decrease (Ersche et al., 2013; Escobar et al., 2018) or have no effect (Bouhlal et al., 2017) on blood leptin levels. In rats, cocaine self-administration and craving causes fluctuation of leptin levels (You et al., 2016, 2019). Both cocaine self-administration and the expectancy of access to cocaine decreases leptin levels, an effect which normalizes during abstinence (You et al., 2016). We did not test the direct effect of cocaine on blood leptin levels, however, we found that after self-administration, blood leptin levels were not correlated with bodyweight anymore, suggesting that a history of cocaine self-administration may dysregulate blood leptin levels and that it may not represent a good biomarker of addiction-like behavior during abstinence. We did find that lower blood leptin levels at baseline were associated with higher addiction-like behaviors 6 weeks later using samples from the Cocaine Biobank. This correlation was mainly driven by the motivational component of the addiction index, which is a similar measure to drug-seeking during reinstatement. The Cocaine Biobank (Carrette et al., 2021b) is a unique resource providing phenotypic data and biological samples from HS rats that underwent state-of-the-art extended access to intravenous cocaine self-administration. As a result of their diverse genotypes, HS rats exhibit diverse addiction-like behavior phenotypes, which mimic the diversity in humans (Woods and Mott, 2017). The negative correlation between blood leptin levels at baseline and addiction-like behavior in the biobank samples confirms our findings that leptin decreases cocaine-seeking and that the inhibitory relationship between leptin and cocaine may be bidirectional, as suggested by You et al. (2016). While the predictive effect observed is small ($R^2 \sim 0.13$), it is in line with the protective effect of leptin that we and others observed against cocaine-seeking (You et al., 2016) and reward (Shen et al., 2016; You et al., 2016). The effect of leptin on cocaine-seeking may be related to its inhibitory effect on the brain reward system and the known effect of leptin mutations in mood disorders (Kraus et al., 2001; Atmaca et al., 2002, 2008).

In summary, these results demonstrate that high blood leptin level before access to cocaine may be a protective factor against the development of cocaine addiction-like behavior and that exogenous leptin reduces the motivation to take and seek cocaine. These findings suggest that leptin could be a good target for medication development for the prevention and treatment of cocaine use disorder. However, we show that blood leptin levels fluctuate in withdrawal and abstinence in current users and that it is thus not a relevant biomarker for addiction-like behaviors. Further studies will be required to understand if and how these leptin changes could drive the opposite bodyweight change following cocaine self-administration in males and females.

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 Institutional Animal Care and Use Committees of the Scripps Research Institute and University of California, San Diego.

AUTHOR CONTRIBUTIONS

LC, GdG, and OG contributed to conception and design of the study. LC, CCr, BB, MB, CO, KS, and SS performed experiments. CCo, BB, and LM organized the database. LS and

AP contributed HS rats. LC and OG analyzed the data and wrote the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2022.832899/full#supplementary-material

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REVIEW
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Involvement of the Dorsal Vagal Complex in Alcohol-Related Behaviors

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The neurobiological mechanisms that regulate the development and maintenance of alcohol use disorder (AUD) are complex and involve a wide variety of within and between systems neuroadaptations. While classic reward, preoccupation, and withdrawal neurocircuits have been heavily studied in terms of AUD, viable treatment targets from this established literature have not proven clinically effective as of yet. Therefore, examination of additional neurocircuitries not classically studied in the context of AUD may provide novel therapeutic targets. Recent studies demonstrate that various neuropeptides systems are important modulators of alcohol reward, seeking, and intake behaviors. This includes neurocircuitry within the dorsal vagal complex (DVC), which is involved in the control of the autonomic nervous system, control of intake of natural rewards like food, and acts as a relay of interoceptive sensory information via interactions of numerous gut-brain peptides and neurotransmitter systems with DVC projections to central and peripheral targets. DVC neuron subtypes produce a variety of neuropeptides and transmitters and project to target brain regions critical for reward such as the mesolimbic dopamine system as well as other limbic areas important for the negative reinforcing and aversive properties of alcohol withdrawal such as the extended amygdala. This suggests the DVC may play a role in the modulation of various aspects of AUD. This review summarizes the current literature on neurotransmitters and neuropeptides systems in the DVC (e.g., norepinephrine, glucagon-like peptide 1, neurotensin, cholecystokinin, thyrotropin-releasing hormone), and their potential relevance to alcohol-related behaviors in humans and rodent models for AUD research. A better understanding of the role of the DVC in modulating alcohol related behaviors may lead to the elucidation of novel therapeutic targets for drug development in AUD.

Keywords: alcohol use disorder, gut-brain axis, interoception, nucleus of the tractus solitarius, vagus nerve

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INTRODUCTION

The dorsal vagal complex (DVC) plays a critical role in relaying interoceptive sensory information to higher order brain regions as well as mediating central output to abdominal viscera via the utilization of various neurotransmitter and neuropeptide systems (Rinaman, 2011; Browning et al., 2017; Browning and Carson, 2021). Many of these same neurotransmitters and neuropeptides have

also been implicated in various aspects of alcohol use disorder (AUD) (Bodnar, 2013; Haass-Koffler et al., 2018; Jerlhag, 2018; Genders et al., 2020; Torruella-Suárez and McElligott, 2020; Ballaz et al., 2021). The DVC, therefore, may be involved in AUD through its various roles in interoceptive functions and signaling systems, but previous research has not fully investigated this hypothesis. Currently, there is a paucity of research directly linking alcohol-related behaviors and alteration in DVC function however, this review will demonstrate that there is substantial evidence which strongly suggests the potential role that the DVC may play in AUD.

The DVC, which contains the nucleus of the tractus solitarius (NTS), dorsal motor nucleus of the vagus (DMV) and area postrema (AP), is localized in the caudal dorsomedial portion of the medulla oblongata and is a crucial component of the autonomic nervous system (Rinaman, 2003b, 2011; Browning et al., 2017; Herman, 2018; Browning and Carson, 2021; Martinez and Kline, 2021). The NTS receives gustatory and visceral information from trigeminal, facial glossopharyngeal, and vagus nerve afferents. Thus, the NTS serves as the initial processing site for many peripheral signals and relays them to various nuclei in the lower brainstem, the limbic system, and hypothalamus. The NTS also sends projections to the DMV, which predominately contains pre-ganglionic parasympathetic motoneurons that innervate the gastrointestinal, pulmonary, and cardiovascular systems and may be a critical locus of alcohol-related end organ dysfunction and peripheral neuropathy. Finally, the AP is a circumventricular organ which is highly vascularized and has access to circulating hormones and other factors such as toxins and cytokines outside of the central nervous system. These circulating chemical messages can be transformed by neurons in the AP to neural input that regulates DVC function. The AP also receives input from the vagus nerve and projects to multiple relay regions such as the NTS, locus coeruleus (LC), parabrachial nucleus (PBN), and ventral lateral medulla (Price et al., 2008). While the AP and the DMV are significant regions of the DVC, this review will primarily focus on NTS connections to corticolimbic and homeostatic areas in the forebrain previously linked to AUD development. Additionally, the NTS contains neuronal subtypes that produce various neurotransmitters and neuropeptides, systems that have previously been shown to be involved in alcohol-related behaviors (**Figure 1**). A better understanding of how the NTS and DVC in general modulate forebrain and peripheral pathways may be critical for potential new conceptual and therapeutic advancement for AUD and other substance use disorders.

NUCLEUS OF THE TRACTUS SOLITARIUS AFFERENTS TO HIGHER ORDER BRAIN REGIONS

The NTS regulates many behavioral responses to internal and environmental stimuli via its projections to forebrain regions. The NTS has been shown to target circuits involved in control of energy homeostasis such as the paraventricular nucleus of the hypothalamus (PVN) (**Figure 2**; Rinaman, 2010). Studies utilizing anterograde tracers microinjected into the caudal NTS as well as glucagon-like peptide 1 (GLP-1) positive immunological staining demonstrate the ascending pathways of these neurons (Rinaman, 1999). The NTS sends direct, stress-sensitive projections to both corticotropin releasing hormone (CRH) neurons and oxytocin neurons within the PVN that regulate hypothalamus-pituitary-adrenal (HPA) axis mediated stress responses (**Figure 3**). Finally, while not a focus of this review, the NTS projects to brainstem areas involved in control of cardiovascular parasympathetic and sympathetic functions including the nucleus ambiguous and the caudal and rostral ventrolateral medulla, respectively.

The NTS also projects to the ventral tegmental area (VTA) and nucleus accumbens (NAc), areas involved in the rewarding properties of food and alcohol as well as other drugs (**Figure 4**; Alhadeff et al., 2012). The NTS also has direct projections to regions such as the prefrontal cortex, central amygdala (CeA), and bed nucleus of the stria terminalis (BNST) which suggests that the NTS has a role in processing cognitive and emotional responses to stress (Schwaber et al., 1982) as well in negative reinforcing properties of alcohol and other substances (Harris and Aston-Jones, 2007; Smith and Aston-Jones, 2008; Koob and Volkow, 2016). In addition to homeostatic stressors, the NTS is sensitive to other stressors including restraint stress, forced swim and immobilization stress, which all have been shown to increase *c-fos* expression (Pezzone et al., 1993; Cullinan et al., 1995). The NTS is also sensitive to chronic variable stress as demonstrated by increased delta FosB immunoreactivity (Flak et al., 2012).

Overall, projections to such higher order regions positions the NTS and the visceral sensory information it relays as a mediator of the rewarding properties of food and alcohol as well as stress sensitivity and negative reinforcement. However, the role that NTS projections play in regulating AUD or substance use disorder development remains to be fully elucidated (Luckman and Lawrence, 2003). Below, we will discuss the roles that multiple neurotransmitters and neuropeptides produced by the DVC play in the modulation of intake and reward behaviors and their possible relevance to alcohol intake behaviors.

NEUROTRANSMITTERS

Norepinephrine

Norepinephrine (NE) is a catecholamine transmitter that is derived from hindbrain nuclei that have projections throughout the brain. It is synthesized in neurons that contain the enzyme dopamine-β-hydroxylase (DβH) which modifies dopamine into NE (Dahlström and Fuxe, 1964). The most well studied source of NE in terms of behavioral functions is the LC. Noradrenergic neurons in the LC play a critical role in regulating behavioral processes under stress conditions as well as cognitive functions including motivation, attention, learning, and memory (Schwarz and Luo, 2015). NE derived from the NTS has classically been examined as a significant signaling component of the sympathetic nervous system, and is involved in life sustaining physiological functions such as cardiovascular regulation and glucose homeostasis. However, NTS-derived NE projections are

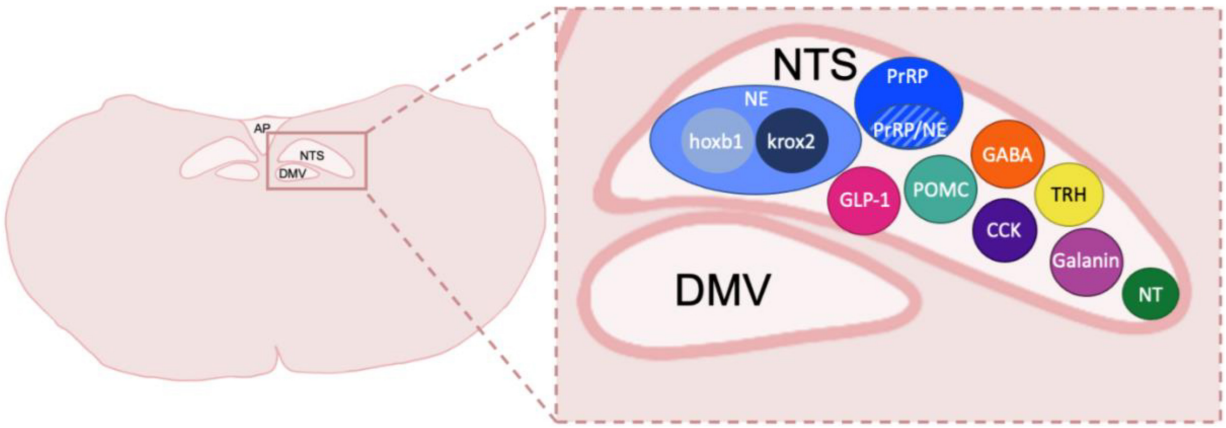

FIGURE 1 | Neuronal types and subtypes in the NTS. Schematic coronal medullary section showing the diversity of cell types located within the NTS. Peptide and transmitter expression includes NE, norepinephrine, which is made up of cellular subtypes hoxb1 and krox2; prolactin-releasing peptide, PrRP which also has a population of neurons that co-express NE; GLP-1, glucagon-like peptide 1; POMC, pro-opiomelanocortin; GABA, gamma-aminobutyric acid; CCK, cholecystokinin; TRH, thyrotropin-releasing hormone; Galanin; NT, neurotensin. Other peptides shown to be in the DVC but not depicted include Met-enkephalin, Dynorphin, Substance P, calcitonin gene related peptide and Neuropeptide Y. NTS, nucleus of the tractus solitarius; DMV, dorsal motor nucleus of the vagus nerve; AP, area postrema. Sizes not to scale or indicative of typical location within a caudal DVC section.

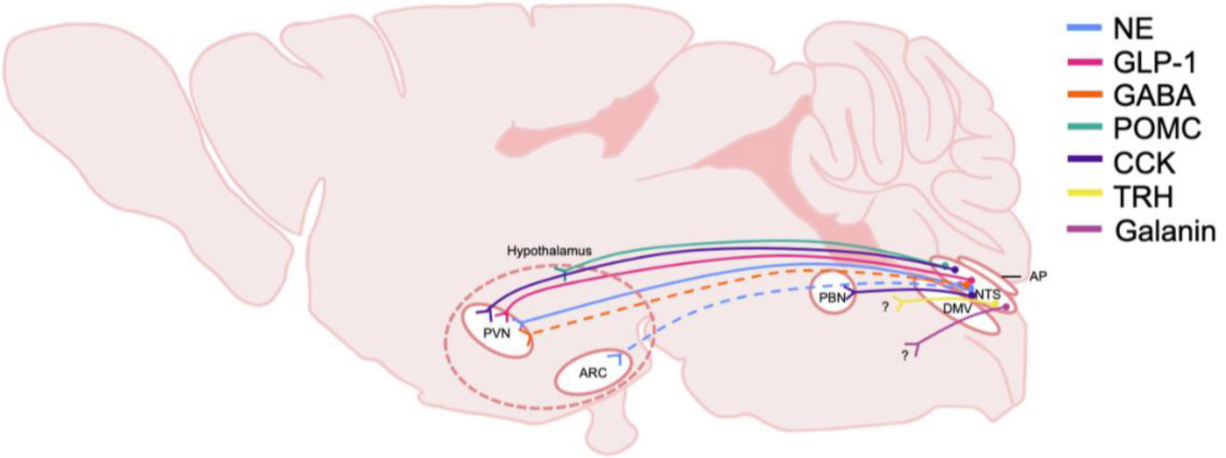

FIGURE 2 | Schematic of DVC projections hypothesized to be involved in central homeostatic systems and AUD. The origin and distribution of central neurotransmitter and peptide pathways participating in consumption behaviors and energy homeostasis and potentially involved in AUD. Peptide and transmitter systems include NE, norepinephrine; GLP-1, glucagon-like peptide 1; GABA, gamma-aminobutyric acid; POMC, pro-opiomelanocortin; CCK, cholecystokinin; TRH, thyrotropin-releasing hormone; Galanin. These projections are derived from the NTS, nucleus of the tractus solitarius; and project to multiple nuclei in the hypothalamus including the PVN, paraventricular nucleus, and the ARC, arcuate. The general area delineating the hypothalamus is denoted with dashed lines. Note that the specific hypothalamic regions that NTS POMC neurons project to is not yet defined, thus nerve endings are shown to terminate in the general hypothalamic region. CCK neurons are shown to project to the PBN, parabrachial nucleus, which also projects heavily to the hypothalamus. Dashed lines indicate that projections have been identified, but functionality and relevance to AUD is not known. Question marks indicate that immunohistochemically, neurotransmitters or peptide are present however, the termination of the projections have not been identified. DMV, dorsal motor nucleus of the vagus nerve; AP, area postrema.

also intertwined in reward and motivational circuits such as those found in the lateral hypothalamus, VTA, NAc, and extended amygdala (Rinaman, 2011). Additional evidence shows the LC receives input from NTS neurons and may help to modulate NE signaling in the cortex, hippocampus, amygdala, thalamus, and cerebellum indirectly through modulation of LC projections to these areas (Rinaman, 2010; Fox et al., 2016). For the scope of this review, we will focus on NE projections arising from the NTS and their involvement in alcohol related behaviors. Noradrenergic cell bodies in the NTS, also called the A2 nucleus, ascend their processes through the ventral noradrenergic bundle (VNAB). These connections from A2 neurons allow for relaying and processing of interoceptive information to cortical and subcortical brain regions which then influence homeostatic and cognitive functioning as well as affective states. The NTS system also has a separate genetic lineage from NE neurons in the

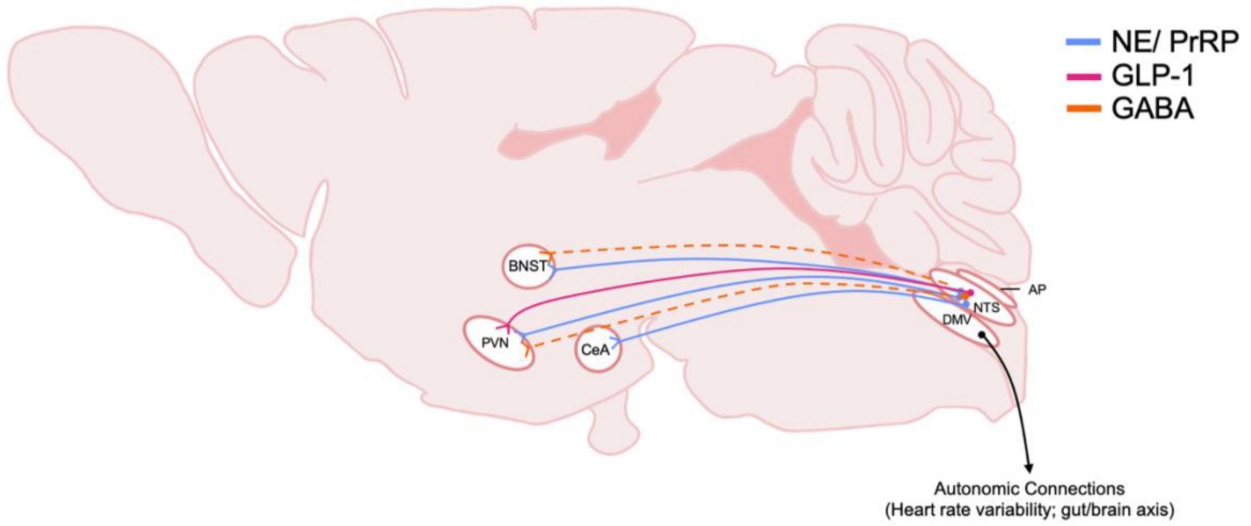

FIGURE 3 | Schematic of DVC projections hypothesized to be involved in central stress systems and AUD. The origin and distribution of DVC neurotransmitter and peptide pathways involved in stress and implicated in AUD. Peptide and transmitter systems include NE, norepinephrine; PrRP, prolactin releasing peptide; GLP-1, glucagon-like peptide 1; GABA, gamma-aminobutyric acid. Dotted lines indicate that projections have been identified, but functionality and relevance to AUD is not known. Note that most of these projections arise from the caudal NTS, nucleus of the tractus solitarius. Also, efferent motor projections from the DMV, dorsal motor nucleus of the vagus nerve, are included to highlight their importance in physiological responses to stress in AUD. BNST, Bed nucleus of the stria terminalis; PVN, paraventricular nucleus of the hypothalamus; CeA, central amygdala; AP, area postrema.

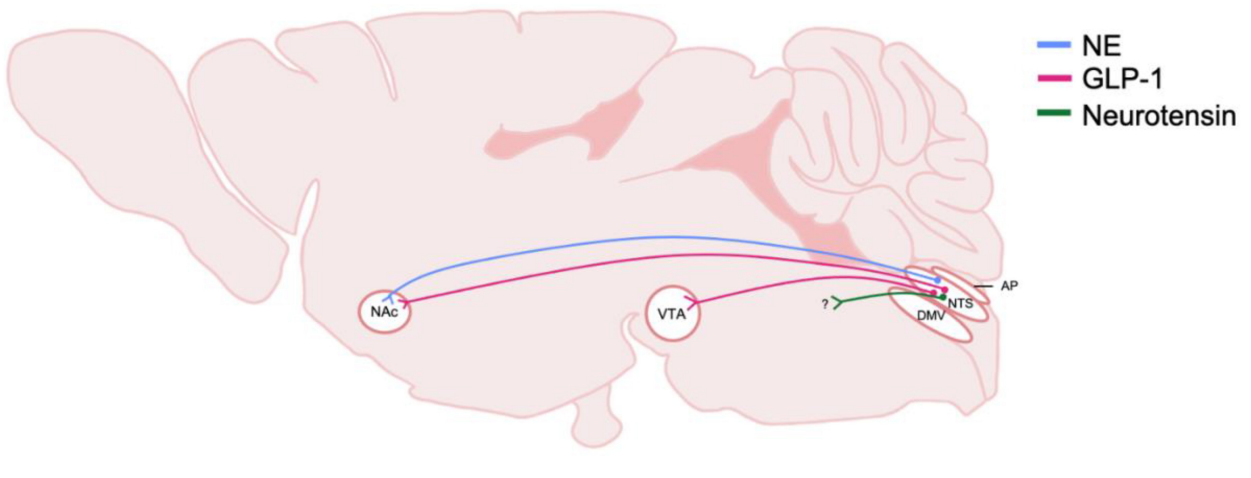

FIGURE 4 | Schematic of DVC projections hypothesized to be involved in central reward systems and AUD. The origin and distribution of central neurotransmitter and peptide pathways hypothesized to be involved in rewarding properties of alcohol. Peptide and transmitter systems include NE, norepinephrine and GLP-1, glucagon-like peptide 1 and neruotensin. These projections are derived from the NTS, nucleus of the tractus solitarius; and project to mesolimbic regions including the NAc, nucleus accumbens, and the VTA, ventral tegmental area. Question marks indicate that immunohistochemically, neurotransmitters or peptide are present, however, the termination of the projections have not been identified. DMV, dorsal motor nucleus of the vagus nerve; AP, area postrema.

LC (Robertson et al., 2013) that may have opposing roles in regulating anxiety-like behaviors. Central NE systems, which are crucial for the stress response, are highly conserved across mammalian species; therefore, understanding this system in preclinical models has significant clinical relevance.

NE modulates neurotransmission pre- and post-synaptically through G-protein coupled adrenergic receptors (ARs) (Strader et al., 1995). In addition to residing in cortical and subcortical brain regions, ARs play a major role within the peripheral nervous system especially in terms of cardiovascular functioning (Reid, 1986; Philipp et al., 2002; Chen and Minneman, 2005). These receptors belong to either α1, α2, or β-ARs families and are modulated by endogenous epinephrine or norepinephrine (Rohrer and Kobilka, 1998). α1-AR are typically G_q coupled; however it has been reported that α1-AR are sometimes coupled to G_i and G_o proteins (Nestler et al., 1996). α2-AR are G_i

coupled and hypothesized to be primarily autoreceptors on presynaptic NE terminals to regulate NE signaling; however some studies indicate a heterosynaptic role of α2-AR, particularly in the extended amygdala (Snyder and Silberman, 2021). Lastly, β-ARs typically utilize G_s signaling and are particularly important in stress/AUD interactions (Gilpin and Koob, 2010; Haass-Koffler et al., 2018; Snyder and Silberman, 2021). Postsynaptic regulation of target circuits by NE varies depending on the brain region and receptor expression. NE neurons in the NTS also have been suggested to co-release other transmitters and peptides such as glutamate (Stornetta et al., 2002), neuropeptide Y (Sawchenko et al., 1985), dynorphin (Ceccatelli et al., 1992), nesfatin-1 (Bonnet et al., 2009), neurotensin (Riche et al., 1990), pituitary adenylate cyclase-activating polypeptide (Das et al., 2007), galanin (Melander et al., 1986), and prolactin-releasing peptide (PrRP) (Chen et al., 1999). In the following sections, we will discuss NTS NE systems and their roles in feeding, reward, motivation, and the stress response, and how these systems may be recruited and modified in AUD.

Nucleus of the Tractus Solitarius Norepinephrine Involvement in Stress and Anxiety-Related Behaviors

The ascending NTS NE system may have a significant role in modulating anxiety-like behaviors. NE has strong regulatory control over limbic circuits, which includes projections from the NTS to the BNST and CeA, regions both associated with modulating behavioral responses to stressful stimuli (Phelix et al., 1992; Park et al., 2009). NTS projections to the limbic brain may be activators of aversive emotional states such as anxiety, fear, and depressive-like behaviors (Itoi and Sugimoto, 2010). These neurons are activated in response to stressful stimuli such as noxious interoceptive stimuli and immune challenge (Gaykema et al., 2007), as well as salient stressful stimuli such as predator odor exposure (Myers and Rinaman, 2005). There are dense projections from NTS NE neurons to the BNST, a functionally heterogeneous region of the extended amygdala implicated in the modification of physiological and behavioral responses to stress (Phelix et al., 1992, 1994; Terenzi and Ingram, 1995; Aston-Jones et al., 1999). LC NE projections to the BNST also exist, however they are much less dense in comparison (Fox et al., 2016). Additionally, the NTS accounts for 5% of NE projections to the CeA (Petrov et al., 1993; Davis et al., 1994; Khoshbouei et al., 2002).

NTS NE neurons selectively target the medial parvocellular (mp) neurons in the PVN (Sawchenko and Swanson, 1981, 1982; Cunningham and Sawchenko, 1988). This brain region contains CRH neurons, which organize and initiate the HPA axis stress response cascade. CRH neurons in the PVN receive direct synaptic input and regulation from NTS NE neurons as shown by light and electron microscope analysis (Liposits et al., 1986a,b). Stressful stimuli have been shown to trigger NE release from NTS neurons thereby recruiting the HPA axis which then increases excitability of CRH neurons (Szafarczyk et al., 1985; Alonso et al., 1986; Rivier and Plotsky, 1986; al-Damluji, 1988; Plotsky et al., 1989; Pacak et al., 1995; Onaka et al., 1996; Onaka, 2004; Wittmann, 2008). These CRH neurons then produce and release CRH in response to excitation from NE neurons. Following CRH secretion, adrenocorticotropin hormone (ACTH) is released from the anterior pituitary into circulation where it activates cells within the adrenal cortex resulting in the synthesis and release of glucocorticoids. These glucocorticoids then provide a negative feedback inhibition on PVN neurons (Herman et al., 2016). NTS NE neurons also innervate PVN oxytocin neurons, which then inhibit PVN CRH neuron activity, potentially as a mechanism to regulate the gain of NE modulation of overall HPA axis function (Smith et al., 2016; Jamieson et al., 2017; Winter and Jurek, 2019). PVN oxytocin neurons reciprocally innervate the DVC as well (Llewellyn-Smith et al., 2012). Oxytocin projections to the NTS have also been shown to have important implications for stress adaptation and may be crucial for GI functionality in response to stress (Jiang et al., 2018), however there does not appear to be oxytocin producing neurons in the DVC.

The NTS NE projections to the hypothalamus appear to be functionally malleable. Altering NE input to mpPVN neurons through lesions or other methods leads to attenuated CRH neuronal responses to interoceptive signaling (Ritter et al., 2003; Bienkowski and Rinaman, 2008). Lesions to the VNAB which affect NTS afferent projections to the PVN result in reductions in NE in the hypothalamus and thus reduced PVN CRH release (Alonso et al., 1986) and subsequent corticosterone release in response to stress (Gaillet et al., 1993). Additionally, NTS NE neurons are sensitive to modulation by glucocorticoids, making these circuits responsive to physiological and behavioral changes during acute and chronic stress (Herman and Cullinan, 1997). Therefore, NE release from NTS neurons into the PVN of the hypothalamus is critical for modulation of the stress response.

Modulation of the PVN by NTS NE seems to be specific to responsiveness of the HPA axis to certain types of stress. This is demonstrated in several studies where selective ablation of DβH-positive projections using saporin toxin impaired responses to stressors such as immune challenge (Li et al., 1996; Bienkowski and Rinaman, 2008), deprivation of glucose (Ritter et al., 2003), but not forced swim stress (Ritter et al., 2003). Microdialysis studies show that NE release in the PVN can occur from various acute stressors as well, where the amount of NE release in the PVN correlates to the amount of ACTH released into circulation (Pacák et al., 1995). Lesions within the VNAB decrease ACTH release and CRH expression in the PVN in response to a foot shock stressor in Sprague Dawley rats (Blandino et al., 2013). Even with the decrease in ACTH release, there was not a subsequent decrease in corticosterone responses (Blandino et al., 2013).

Other studies suggest that NE's role in PVN activation may be more complicated. Chronic stress leads to HPA axis sensitization to signaling from NE inputs (Pardon et al., 2003) as well as increases in the amount of NE inputs to CRH neurons leading to increased HPA axis signaling (Flak et al., 2009). However, it is not known whether increased NE inputs are directly from NTS NE neurons. NE inhibits a population of parvocellular neurons in the PVN via a β-AR mechanism in *ex vivo* slices (Daftary et al., 2000; Han et al., 2002). Also, higher doses of infused NE can reduce release of CRH (Plotsky, 1987) potentially via oxytocin interactions as described above. This evidence suggests that NE's influence on CRH release from the PVN may depend

on the magnitude of NTS activation and which subcircuits are impacted within the PVN. NE release in the PVN may also affect local glutamatergic and GABAergic transmission. In *ex vivo* PVN slices, the excitatory effects of NE on parvocellular PVN neurons could be blocked by glutamate receptor antagonists and tetrodotoxin. This is indicative of NE regulating local glutamate release (Daftary et al., 1998). It is possible that NTS NE neurons co-release glutamate and NE in the PVN due to NTS NE neurons having glutamate transporters (Zhao et al., 2007). NE could also stimulate or inhibit GABAergic neuron activity in parvocellular neurons (Han et al., 2002). Thus, NTS NE possibly influences HPA activity through modulation of local circuitry in the PVN as well as via direct modulation of PVN CRH and oxytocin neurons.

Nucleus of the Tractus Solitarius Norepinephrine in Alcohol and Other Substance Use Disorders

NE transmission is highly responsive to alcohol and is critical for both positive and negative alcohol reinforcement (Koob and Volkow, 2016). In clinical studies, alcohol dependent individuals appear to have higher levels of NE metabolites in cerebral spinal fluid compared to matched controls after acute alcohol administration (Borg et al., 1981). This is also modeled in preclinical studies where there are higher levels of NE metabolites during intoxication and withdrawal in dependent animals that are administered alcohol daily via oral gavage (Karoum et al., 1976). Chronic alcohol administration via IP injection in adult male mice also sensitizes NE terminals to release more NE (Lanteri et al., 2008). These findings are indicative of increased NE transmission resulting from chronic alcohol use and dependency, but whether this increased NE transmission is dependent upon functional alterations in NTS NE neurons is not fully established.

So far, studies have shown an increase of c-Fos (a marker of neuronal activity) in tyrosine hydroxylase (TH) positive neurons in the NTS after IP or intragastric administration of alcohol (Thiele et al., 2000; Lee et al., 2011; Aimino et al., 2018). In the NTS in adult male mice, acute alcohol exposure to *ex vivo* brain slices via bath application enhances GABAergic signaling, likely onto other local circuit GABA neurons, resulting in disinhibition and increased c-Fos expression of NE neurons in the NTS (Aimino et al., 2018). NTS NE neurons have also been shown to be critical for conditioned place preference (CPP) for morphine in mice (Olson et al., 2006). Olson et al. (2006), demonstrated that deficits in CPP in mice with global genetic deletions DβH could be rescued when NTS NE was restored through viral reintroduction of DβH expression. Recovery of DβH expression in the LC however, did not rescue morphine-related CPP behaviors. This strongly suggests that NTS NE may be a common factor in the development of use disorders for numerous substances.

As discussed above, NTS NE projections to the PVN play a strong role in regulating HPA axis function (Herman and Cullinan, 1997). Chronic alcohol consumption is well known to be associated with disruption of the HPA axis and stress response (Koob, 2009; Stephens and Wand, 2012; Koob and Volkow, 2016; Sinha, 2018). A combination of activating HPA and stress circuits with aberrant NTS NE transmission and chronic alcohol exposure may create a vicious feed-forward cycle that exacerbates NE dysfunction (Retson et al., 2015). These alterations create allostatic changes in NE transmission following chronic alcohol exposure and withdrawal. The NTS also sends dense projections to the BNST, where NE is thought to be a critical signal in stress-induced reinstatement to drug seeking behaviors through modulation of local CRH neurons (Silberman and Winder, 2013; Snyder and Silberman, 2021). Interestingly, lesions of VNAB attenuates stress-induced reinstatement of opiates while lesions to LC NE neurons have no effect (Wang et al., 2001), highlighting the importance of NTS NE neurons specifically being involved in the aversiveness of withdrawal and stress-induced reinstatement. Therefore, NTS NE is suggested to be a main driver of the negative affective state that occurs during withdrawal and may be critical in reinstatement to drug seeking behaviors (Koob and Volkow, 2016).

Norepinephrine in Other Consumption Behaviors

NTS NE neurons are involved in suppressing and triggering feeding behaviors in various ways via bidirectional interactions with the hypothalamus. The hypothalamus has regulatory control over the initiation and termination of food intake through projections to caudal brainstem regions, including the NTS (Berthoud, 2002; Woods and D'Alessio, 2008). In turn, NTS projections to the hypothalamus modulate hypothalamic activity to regulate feeding (Ritter et al., 1975; Myers and McCaleb, 1980; Date et al., 2006; Rinaman, 2007). The NTS responds to gut-brain peptides such as ghrelin, which results in the release of NE into the arcuate nucleus of the hypothalamus (Date et al., 2006), a hypothalamic region known to play a critical role in the orexigenic properties of peripheral ghrelin signaling. In one study, selective ablation of NTS NE neurons resulted in elimination of ghrelin-induced consumption (Date et al., 2006). Complementary studies have shown that IP injections of ghrelin did not stimulate feeding in mice with vagotomies (Asakawa et al., 2001). Thus, NTS-mediated NE release into the hypothalamus is important for consumption behaviors regulated by peripheral ghrelin. This system may be targeted to help regulate alcohol consumption since clinical studies with alcohol-dependent individuals have shown that exogenous ghrelin administration increases alcohol craving and self-administration (Leggio et al., 2014; Farokhnia et al., 2018; Deschaine et al., 2021). NTS NE neurons are also important for signaling of anorexic gut-brain peptides. Selective ablation of NTS NE neurons attenuates hypothalamic activation by cholecystokinin (CCK) while CCK-induced anorexia was also attenuated (Rinaman, 2003a). Overall, selectively modulating NTS NE may be a way to influence sensitivity to endogenous and exogenous gut-brain peptides and may be consequential to intake behaviors for food and alcohol.

Additional Considerations of Nucleus of the Tractus Solitarius Norepinephrine Neuron Subtypes

A growing literature indicates that NTS NE neurons arise from distinct subtypes based on developmental origin and expression of distinct transcription factor genes. One of these

neuronal subtypes express Krox20 while the other express Hoxb-1 (**Figure 1**; Robertson et al., 2013). These subtypes appear to be non-overlapping with activation resulting in distinct behavioral output. NTS Hoxb-1 neurons project directly to the insular cortex (Robertson et al., 2013; Chen et al., 2019). It can be inferred these inputs may serve to relay interoceptive signals directly to cortical regions to modulate arousal, affective states, and stress related behaviors (Gogolla, 2017; Chen et al., 2019). While the functional consequences of these projections have not been investigated in the context of alcohol-related behaviors, studies have shown that selective activation of Hoxb-1 neurons using DREADDs reduces anxiety-like behaviors in the context of acute stress through projections to the BNST (Chen et al., 2019). These results are in direct contrast to the known anxiogenic functionality of LC NE neurons (Robertson et al., 2013) and other known NTS NE neuron functions described in previous sections. This is one example of how examining cell type specific differences in neuronal physiology through recently developed genetic manipulating technology could reveal the heterogeneity of neuronal populations beyond their anatomical localization and should be further examined in the context of AUD.

GABAergic PROJECTIONS

In previous studies, GABAergic neurons in the NTS were suggested to be local interneuron or neurons that projected to other adjacent brainstem regions such as the pons, medulla, and spinal targets such as the spinal nucleus of the trigeminal, parvicellular reticular nucleus, and the dorsal column nuclei (Kawai and Senba, 1999; Shi et al., 2021). Utilizing track tracing methods, a recent study demonstrated that NTS GABAergic neurons are also able to project to forebrain areas including the BNST and the PVN (Shi et al., 2021). Identifying these projections from the NTS to the BNST and PVN further suggests the NTS and interoceptive sensory information has a hand in regulating energy homeostasis, the stress response, and affective states of the brain. Given the well characterized interaction between alcohol and the GABAergic system in general, and our initial findings showing a potential for alcohol modulation of NTS GABAergic circuit function (Aimino et al., 2018), further examination of NTS GABA signaling may be a critical for the overall understanding of AUD development, but the functionality of these neurons needs to be further explored.

NEUROPEPTIDES

Neuropeptides are short biomolecules ranging from 3 to 40 amino acids which are utilized for neuroendocrine and synaptic communication to regulate physiological functioning. The ubiquitous use of neuropeptides by various organ systems across different species highlights their significance in biological functioning. This section will review the extensive diversity of neuropeptidergic neurons that reside in the DVC and their potential relevance in the various aspects of AUD.

GLUCAGON-LIKE PEPTIDE 1

Glucagon-like peptide 1 (GLP-1) is a gut-brain peptide produced by enteroendocrine L-cells and neurons in the NTS that decreases food intake, regulates glucose homeostasis by stimulating insulin secretion, inhibits gastric emptying, and modulates glucagon secretion (Novak et al., 1987; Jin et al., 1988). Peripheral GLP-1 release into circulation is dependent upon taste receptor activation, ingestion, and possibly other hormonal signals such as CCK and gastric inhibitory peptide (Rocca and Brubaker, 1999; Hansen and Holst, 2002). While GLP-1 can cross the blood-brain barrier, it is degraded rapidly in circulation with a half-life of around 2 min (Herrmann et al., 1995). The NTS produces the most GLP-1 projections in the brain and is responsible for activating GLP-1 receptors (GLP-1R) centrally (Larsen et al., 1997; Merchenthaler et al., 1999). Since the half-life of and diffusion of circulating GLP-1 in the brain is limited, centrally produced GLP-1 is most likely mediating effects on brain regions non-adjacent to circumventricular organs (Kastin et al., 2002).

Immunological preparations outlining the anatomy of the DVC reveal that GLP-1 does not co-localize with catecholamines (Larsen et al., 1997). GLP-1 neurons have vesicular glutamate transporters suggesting that these neurons may corelease glutamate (Zheng et al., 2015). GLP-1R are conjugated with G_s or G_i proteins and activation of GLP-1R is typically associated with increased GABAergic activity or suppression of glutamatergic activity, such as the activity of GLP-1R found on presynaptic glutamatergic terminals in the cortex and hippocampus (Rebosio et al., 2018; Jerlhag, 2020). Since GLP-1R agonists are already approved for the treatment of type II diabetes and obesity due to its effectiveness in reducing food reward and craving, GLP-1 has become an attractive candidate for the treatment of substance use disorders including AUD (van Bloemendaal et al., 2014). Further mechanistic studies on the role of the GLP-1 system in AUD are needed.

GLP-1 Effects on Reward Circuits Mediating Intake of Food and Alcohol

GLP-1R associated with reward are located in brain regions such as the VTA, NAc, the lateral septum and the paraventricular thalamus (PVT) which may be involved in GLP-1's effects on alcohol-related behaviors (Dossat et al., 2011; Alhadeff et al., 2012; Dickson et al., 2012; Ong et al., 2017). Previous studies demonstrate that administration of GLP-1 results in decreased food intake through centrally mediated GLP-1 signaling (Kanoski et al., 2011), with previous studies focusing on homeostatic centers such as the PVN (Vrang et al., 2007). These studies demonstrate that central GLP-1 release is able to modulate food intake, however, other evidence suggests that central GLP-1 may act as a secondary circuit to induce satiation recruited by stress or large quantities of intake (Holt et al., 2019). More recent studies now point to GLP-1 mediated activity in the mesolimbic reward system as a component in regulating intake of natural rewards and drugs. Direct infusion of GLP-1R agonists into the VTA and NAc result in decreased intake of palatable rewarding

food such as sucrose and high-fat diet, regular chow intake, and body weight in rats that were food-deprived overnight (Alhadeff et al., 2012). This indicates that GLP-1 signaling in the reward system decreases the motivation to feed, especially on highly palatable nutrients possibly through a mechanism of modulating dopamine signaling in VTA and the NAc. This is supported by other neuronal tracing studies showing that GLP-1 producing neurons in the NTS project directly to the brain regions involved in the reward system such as the VTA and the NAc (Alhadeff et al., 2012).

Preclinical studies found that the GLP-1R agonist AC3174 significantly reduced voluntary alcohol consumption in adult male mice. In this study, alcohol intake was assessed using the two-bottle choice paradigm where mice are given the option of drinking either water or alcohol. Voluntary drinking behaviors were measured before and after chronic intermittent ethanol exposure (CIE) where mice were exposed to ethanol vapors for 16 h a day for 4 consecutive days a week. Importantly, the decrease in alcohol consumption continued even after ceasing treatment with the GLP-1R agonist (Suchankova et al., 2015). In another preclinical study, animals treated with a Dipeptidyl-peptidase IV (DPP-IV) inhibitor, which inhibits the enzyme responsible for metabolizing GLP-1, demonstrate a delayed tolerance to alcohol's anxiolytic effects and show decreased anxiety after alcohol withdrawal (Sharma et al., 2015). Since increased anxiety during alcohol withdrawal is a common aspect of relapse, these data suggest that targeting the GLP-1 system may decrease the chances of relapse. While GLP-1R agonists have consistently shown reduction in alcohol intake behaviors, the potential mechanisms involved are not always clear (Marty et al., 2020). The effects of altering endogenous GLP-1 and examining alcohol-mediated behaviors could reveal more insights about how GLP-1 could be used as a therapy. Additional studies are also needed to narrow down the effects of GLP-1R activation to differentiate between effects of peripheral activation versus central activation in attenuating alcohol-directed behaviors.

Highlighting the importance of central GLP-1R in the regulation of alcohol consumption, agonists of GLP-1R reduced alcohol-mediated behaviors in the two-bottle choice paradigm but such effects are lost when GLP-1R are selectively knocked out in the central nervous system. More specifically, GLP-1R activation has been shown to be involved in decreasing alcohol intake behaviors most likely due to the presence of GLP-1R in the mesolimbic reward system (Egecioglu et al., 2013; Shirazi et al., 2013; Suchankova et al., 2015; Vallöf et al., 2019; Marty et al., 2020). However, GLP-1R expression in other brain regions, such as the extended amygdala and PVN (Ghosal et al., 2013), may also play a role in reduction of alcohol intake in preclinical models via modulation of stress-reinstatement pathways, although this has not been directly tested and findings on GLP-1R activation on anxiety-like behaviors have been mixed. Additional evidence suggests that GLP-1R agonist therapies may also be effective in reducing opioid intake and seeking in preclinical models (Douton et al., 2021a,b). Overall, NTS GLP-1 neuron function in the context of AUD and stress could be a line of research parallel to the effects of NTS NE and stress on alcohol mediated behaviors.

Based on promising preclinical studies and relative safety of repurposing an already FDA-approved treatment for a novel indication, a number of clinical studies are underway examining the potential ability of GLP-1R agonists to reduce alcohol intake (Antonsen et al., 2018). While GLP-1 and AUD associations are not well established in human clinical populations, currently published research shows a number of promising findings. One study utilized a *post hoc* analysis on a case-control study found that GLP-1R polymorphisms were associated with AUD in humans and were related to increased intravenous administrations of alcohol as well as increased activation of the globus pallidus during rewarding feedback on a task that measures reward in an fMRI study (Suchankova et al., 2015). While initial clinical findings are generally promising, given the various roles that GLP-1 plays in consumption, reward, stress, and metabolic function, the effects of GLP-1 on multiple systems needs to be accounted for when interpreting effects on alcohol-related behaviors and as a potential therapy for AUD.

PROLACTIN-RELEASING PEPTIDE

Prolactin-releasing peptide (PrRP) is classically known to stimulate prolactin release as the name suggests (Matsumoto et al., 1999). The canonical role of prolactin release is to serve as a nursing stimulus, however, it also influences reproductive behaviors, cognition, homeostasis, and immunological responses (Freeman et al., 2000; Cabrera-Reyes et al., 2017). PrRP functions in roles beyond mediating prolactin release, such as utilizing interoceptive information about the metabolic state of the organism to modulate the behavioral and endocrine stress response (Rinaman, 2007). There are PrRP neurons densely located in the NTS where PrRP is expressed in roughly 80% of catecholaminergic neurons (Maruyama et al., 2001; Maniscalco et al., 2012). Studies also show that PrRP neurons innervate magnocellular and parvocellular neurosecretory cells in the PVN (Matsumoto et al., 2000). There are PrRP receptors that also populate the PVN and participate in the stress-response as local administration of PrRP into the PVN increases ACTH (Roland et al., 1999; Matsumoto et al., 2000). Other studies found that coadministration of subthreshold NE and PrRP doses resulted in increased plasma ACTH, suggesting a critical interaction between PrRP and NE (Maruyama et al., 2001).

NTS PrRP neurons are sensitive to various stressful stimuli as shown by increased c-Fos expression in these neurons following acute restraint stress (Maniscalco et al., 2015), water immersion restraint stress (Maruyama et al., 2001), hemorrhagic stress (Uchida et al., 2010), and acute inflammatory stress (Mera et al., 2006). Chronic stress via repeated restraint was also shown to upregulate PrRP transcription in the NTS (Tóth et al., 2008). The activation of these neurons in response to stress also seems to be modulated through altered metabolic cues. For example, c-Fos expression following stress exposure is decreased in rats that are fasted (Maniscalco et al., 2015; Maniscalco and Rinaman, 2017). This implies that PrRP/NE neurons modulate acute and chronic stress-responses accordingly with consideration to metabolic state. Overall, these data suggest that PrRP release is relevant

for stress modulation where PrRP and NE release may both synergistically fine tune HPA axis activity.

Thus far, how PrRP release into target areas such as the PVN affects alcohol-mediated behaviors has not been explored in depth. The close relationship between alcohol behaviors and stress circuitry, however positions PrRP as potential mediator of both. Thus, targeting the NTS PrRP system in conjunction with the NE system in AUD may lead to better outcomes when treating the negative withdrawal symptoms of AUD associated with anxiety and when addressing stress-induced relapse.

PROOPIOMELANOCORTIN

The melanocortin system is critical for control of energy homeostasis through its sensitivity to circulating nutritional stimuli and neuromodulatory signals. Proopiomelanocortin (POMC) is a precursor to multiple endogenous peptides such as α-melanocyte stimulating hormone (α-MSH), β-MSH, γ-MSH, β-endorphin, and ACTH (Harno et al., 2018). POMC neurons in the arcuate nucleus of the hypothalamus have been a heavy focus of study in a number of fields related to intake behaviors, but POMC neurons are also present in the NTS and their roles in intake behaviors is less well characterized (Joseph et al., 1983; Heisler and Lam, 2017). POMC expression in NTS neurons shows a lack of co-localization with cells that produce CCK, GLP-1, TH, or choline acetyltransferase (ChAT) which demonstrates that they are a distinct population of cells in the NTS (Georgescu et al., 2020). The NTS processes various metabolic cues from neuronal activity to circulating factors which position NTS POMC neurons to influence energy homeostasis. One study demonstrated how NTS POMC neuronal activity curbs food consumption acutely and chronically utilizing chemogenetics which reveals that NTS POMC neurons regulate feeding and energy homeostasis by incorporating peripheral adiposity signals with neurotransmission from the vagus signifying satiation (Zhan et al., 2013).

Alcohol alters signaling of endogenous opioid peptide systems including the POMC system. A 2-week administration of alcohol was shown to increase POMC mRNA expression in the hypothalamus, with expression decreased after 7 weeks of alcohol administration in another study (Angelogianni and Gianoulakis, 1993; Rasmussen et al., 2002). β-endorphin, a mediator of endogenous analgesia, the stress response, reward, cardiorespiratory regulation, and food intake, is also shown to modulate alcohol intake behavior (Lewis et al., 1987; Appleyard et al., 2003; Fields, 2004; Butler and Finn, 2009). Female transgenic mice with knockout of β-endorphin had significantly decreased alcohol consumption compared to control animals. Notably, stress exposure in β-endorphin knockout mice did not increase alcohol intake as it does in wild-type mice while showing no difference in baseline alcohol preference using two-bottle choice (Racz et al., 2008). In addition, many pharmacological studies demonstrate that antagonizing opioid receptors decreases alcohol intake, self-administration, and relapse in rodents (Altshuler et al., 1980; Volpicelli et al., 1986; Heyser et al., 1999). Opioid antagonists have also been shown to decrease drinking, craving, and relapse in AUD in humans as well (O'Malley et al., 1992, 2002; Volpicelli et al., 1992, 1997; Mason et al., 1994, 1999; Doty and de Wit, 1995). POMC neurons in the NTS also produce β-endorphin (Bronstein et al., 1992). These neurons project to regions in the brainstem associated with autonomic processing and pain control where they influence nociceptive processing and cardiorespiratory activity via β-endorphin release (Cerritelli et al., 2016). How NTS POMC neuronal activity affects alcohol-related behaviors has yet to be explored. The role that these neurons play in suppression of consumption along with their analgesic effects may be relevant for mediating alcohol intake and managing the negative reinforcing properties of alcohol withdrawal. POMC neurons in the arcuate nucleus of the hypothalamus and other central regions have been more extensively studied in the context of chronic alcohol exposure, resulting in alterations in β-endorphin, MSH and ACTH function (Navarro, 2017). There are minimal findings suggesting NTS POMC neurons and β-endorphin, MSH and ACTH are similarly changed by chronic alcohol exposures, however this should be investigated further.

NEUROTENSIN

Neurotensin is a neuropeptide that is expressed throughout the body including centrally, peripherally, and in the GI tract. Most notably, neurotensin was discovered to be present in limbic areas of the brain involved in reward such as the VTA, NAc and CeA (Uhl et al., 1979; Servonnet et al., 2017; Torruella-Suárez et al., 2020; Torruella-Suárez and McElligott, 2020). In the NAc, neurotensin has pre and postsynaptic effects on dopaminergic signaling (Kalivas et al., 1984). Thus, agonizing neurotensin receptors may be able to reduce alcohol intake by inhibiting alcohol induced dopamine release in the NAc.

In the CeA, alcohol consumption has been shown to increase *Fos* expression in neurotensin neurons. When these neurons are selectively deleted via cell-type specific viral manipulations in the CeA, alcohol consumed was significantly reduced. This reduction was not due to changes in overall fluid intake, motivation to intake rewarding substances, or aversion to alcohol (Torruella-Suárez et al., 2020). Thus, the neurotensin neurons in the CeA appears to be sufficient as a mechanism for targeted AUD therapies. Some neurotensin positive neurons are found in the DVC (Uhl et al., 1979; Triepel et al., 1984) as well as other autonomic regulatory regions in the medulla oblongata. The caudal NTS is also densely populated with neurotensin receptors (Higgins et al., 1984). While there are studies that look at neurotensin's effects on drug seeking, reward and consumption behaviors in limbic areas of the brain, it is not known where neurotensin neurons in the NTS project to and what their functioning affects (Torruella-Suárez et al., 2020; Torruella-Suárez and McElligott, 2020). However, NTS neurotensin may also have similar roles in modulating alcohol consumption, potentially via projections to the CeA or other limbic regions. Such studies would be interesting to pursue in relation to AUD.

CHOLECYSTOKININ

Cholecystokinin (CCK) is a peptide that is expressed throughout the GI tract and the central nervous system. It is also the most abundant neuropeptide encountered in the brain (Crawley and Corwin, 1994). Signaling by this peptide at CCK1 and CCK2 receptors has various physiological (Crawley and Corwin, 1994) consequences including stimulating gastric and pancreatic secretion, delaying gastric emptying as well as decreasing consumption through inhibiting orexigenic signaling in the hypothalamus (Chandra and Liddle, 2007). In addition to satiety, CCK has been shown to be involved in learning and memory, nociception, reward, and anxiety-related behaviors (Daugé and Léna, 1998; Crespi et al., 2000; Hadjiivanova et al., 2003; Rotzinger and Vaccarino, 2003; Keppel Hesselink, 2020). This neuropeptide modulates various transmitter systems in the brain including dopamine (Rotzinger et al., 2002), opioids (Hebb et al., 2005), glutamate (Nguyen et al., 2020), GABA (Freund and Katona, 2007), and serotonin (Grignaschi et al., 1993). Most notably, CCK is implicated in modulating the various positive and negative features of AUD (Ballaz et al., 2021).

The current studies with a focus on CCK neurons in the caudal NTS are centered around their roles in energy homeostasis. NTS CCK neurons are sensitive to satiation cues and their activation decreases feeding (D'Agostino et al., 2016; Roman et al., 2017). These neurons project to the PBN and PVN and activation of either set of pathways results in decreased food intake. The manner in which these different NTS CCK projections decrease food intake has been suggested to be opposite in valence. Activation of CCK neurons that project to the PBN is aversive while stimulation of CCK neurons that terminate in the PVN leads to pleasant satiation (Roman et al., 2016, 2017). Activation of NTS CCK neurons that terminate in the PBN also induces anxiety-like behaviors (Roman et al., 2016). Based on these initial findings, future studies examining NTS CCK neurons in relation to AUD may be warranted.

THYROTROPIN-RELEASING HORMONE

Thyrotropin-releasing hormone (TRH) is a neuropeptide that inhibits drinking and feeding behaviors (Vijayan and McCann, 1977; Morley and Levine, 1980). There are an abundance of TRH-immunopositive neurons in the DMV while a moderate amount are localized in the NTS (Fodor et al., 1994). Vagotomy attenuates the satiety effects of peripheral administration of TRH (Morley et al., 1982), suggesting the DVC may play a critical role in relaying TRH signaling to brain regions involved in controlling intake behaviors, but this has not been directly tested. TRH or its metabolite histidyl-proline diketopiperazine, may serve as a potential molecule for signaling satiation for alcohol, similar to GLP-1. An analog of TRH was shown to decrease alcohol preference in the alcohol preferring P rat line in a dose dependent manner while not affecting food intake (Rezvani et al., 1992; Mason et al., 1997). Overall, these findings suggest TRH as an additional DVC neuropeptide of potential interest in AUD research.

GALANIN

Galanin is a highly conserved neuropeptide that is found in the central nervous system as well as the peripheral nervous system and endocrine system (Vrontakis, 2002). It is thought to be a component in regulating nutrient intake (Fang et al., 2011) and has been implicated in cognitive functions such as learning and memory and affective states including anxiety (Wrenn and Crawley, 2001; Holmes et al., 2003; Barrera et al., 2006). Galanin has been shown to be highly co-localized with NE neurons in the pons and the LC as well as possibly participating in similar roles in stress in conjunction with NE (Melander et al., 1986; Holets et al., 1988; Hökfelt et al., 1998). There is immunoreactivity for galanin in the NTS, however, the functionality of these neurons and the location of their projections have not been examined (Maley, 1996).

Studies point to galanin acting as a modulator of various neurotransmitters through inhibition. Galanin tends to hyperpolarize neurons and can inhibit the release of NE as well as glutamate and serotonin (Pieribone et al., 1995; Xu et al., 1998; Kehr et al., 2002). Galanin may also have a role in alcohol consumption behaviors. Galanin injection in to the PVN increases dopamine release in the NAc (Rada et al., 1998). Galanin can increase alcohol intake when injected into the PVN. This increased alcohol intake then resulted in increased galanin release which implies that there may be a positive feedback loop between alcohol consumption and galanin release in the PVN (Lewis et al., 2005). Like other neuropeptides, galanin requires stronger levels of neural activation to be released when compared to neurotransmitters which makes activity-dependent modulation of signaling between NE and galanin likely (Barrera et al., 2006) and suggests that the galanin system may be a potential target for future AUD treatment development.

ADDITIONAL NEUROPEPTIDES

The NTS contains neurons that produce a number of other neuropeptides that have previously been investigated in relation to AUD, such as Met-enkephalin, Dynorphin, Substance P, Calcitonin Gene Related Peptide and Neuropeptide Y, but the specific role of NTS neurons that produce these peptides has essentially not been previously investigated. Such studies may inform on the overall mechanisms by which these neuropeptides may regulate alcohol intake behaviors and provide specific context to NTS function in AUD development.

CLINICAL RELEVANCE OF DORSAL VAGAL COMPLEX NEUROTRANSMITTER AND PEPTIDE SYSTEMS IN ALCOHOL USE DISORDER

Although the role of the DVC in AUD is not well characterized, a number of studies suggest that the DVC may be a critical area of interest for future AUD therapies. Since the DVC is a primary input region of the vagus and other peripheral

nerves and regulates autonomic output, the DVC and its various neurotransmitter systems can be examined and/or modulated, either directly or indirectly, in clinical and preclinical studies. This allows for a number of future translational studies that can impact AUD research.

Heartrate variability (HRV), an indirect measure of parasympathetic (vagal) tone to the heart, has been established as a valuable biomarker of autonomic health. Recent evidence shows that AUD patients have altered HRV suggesting that these patients have reduced parasympathetic activity and autonomic dysfunction due to alcohol misuse (Yuksel et al., 2016; Cheng et al., 2019). HRV could also be used for informing AUD prognosis since its measurements are associated with drug craving, risky drinking behaviors and relapse (Milivojevic and Sinha, 2018; Ralevski et al., 2019; Hwang et al., 2021). Thus, the monitoring and modulation of DVC pathways and vagal activity may be a critical and under-explored aspect of AUD research. HRV could serve as an innovative diagnostic tool to detect disruption in central neuronal networks by AUD and other substance use disorders.

Of clinical relevance to the DVC gut-brain peptides discussed above, there has been a recent surge of bariatric surgical procedures (∼200,000 per year) as they represent one of the most effective interventions to achieve significant, long-term weight loss to treat medical comorbidities of obesity (Nguyen and Varela, 2017). However, one common procedure, the Roux-en-Y gastric bypass surgery (RYGB) can lead to increased alcohol use and AUD in humans, even in those individuals without a history of significant alcohol use, with similar findings in rodent models (Hajnal et al., 2012; Thanos et al., 2012; Davis et al., 2013; Polston et al., 2013; Blackburn et al., 2017; King et al., 2017). While the exact underlying mechanism remains unknown, pre-clinical studies of RYGB points to neuronal plasticity occurring in the DVC following removal of gastric vagal afferents (Browning et al., 2013; Browning and Hajnal, 2014; Ballsmider et al., 2015; Minaya et al., 2019). One recent preclinical study focused on damage to the gastric branches of the vagus nerve that is unavoidable in the RYGB procedure and found gastric vagal de-afferentation is sufficient to increase alcohol intake in both obese and lean rats (Orellana et al., 2021b), suggesting vagal afferent dysfunction and resulting alterations in DVC activity may play a critical role in AUD development.

Another potential mechanism is altered ghrelin signaling following RYGB. This notion has been further supported by differential AUD outcomes seen following RYGB and vertical sleeve gastrectomy, the two most common weight loss surgeries that may differentially alter ghrelin production as well as involving vagal and central ghrelin receptor signaling in development of postoperative AUD (Orellana et al., 2018, 2019, 2021a). Lastly, emerging evidence suggests that changes in gut microbiota diversity and composition following bariatric surgery may influence AUD outcomes (Martin et al., 2021). Whether such changes are related to altered diet and/or reduced obesity (Sen et al., 2017; Vaughn et al., 2017; Stephens et al., 2018; Minaya et al., 2020), and the extent of which vagus and DVC play role in mediating gut microbiota-related effects in general (Kim et al., 2020), and in AUD in particular (Fuenzalida et al., 2021; Leclercq et al., 2021; Russell et al., 2021), remain high priority questions for translational research thus warrant future mechanistic studies.

Overall, the above studies suggest that disruption to normal vagus nerve signaling, and related disruptions to DVC function, may be a critical yet under-examined factor driving both AUD development and relapse. Corollary to this, vagus nerve stimulation (VNS) as a way to counteract disrupted vagus nerve signaling may hold promise as a novel therapeutic approach in AUD. VNS is already approved as an adjunctive therapy for patients with epilepsy and depression that are resistant to pharmacological treatment, however the mechanisms involved in these effects remains elusive (Senova et al., 2019; Toffa et al., 2020). Preclinical studies with VNS reveal that activating afferent vagal sensory neurons via electrode stimulation alters central neuronal signaling which may help rectify circuits regulating heroin and cocaine intake and seeking (Liu et al., 2011; Childs et al., 2017). In rodents, VNS led to increases in extracellular concentrations of NE and serotonin in the cortex and hippocampus (Roosevelt et al., 2006; Follesa et al., 2007; Manta et al., 2009) although other neurotransmitter and neuropeptide systems in the DVC and their projections to forebrain and midbrain regions are also likely to be involved. Further examination of the effects of VNS in preclinical models of AUD is needed to elucidate the modality of its influence on AUD pathology.

The evidence from recent clinical studies with VNS in substance use disorders are promising. Transcutaneous auricular VNS (taVNS), a non-invasive form of vagal stimulation targeting the auricular branch of the vagus nerve around the ear, has been tested as an adjuvant to oral morphine therapy in infants with neonatal opioid withdrawal syndrome. When taVNS is delivered before morphine delivery, the length of morphine treatment was significantly reduced (Wang et al., 2021). taVNS has also been shown to reduce self-reported anxiety and depression symptoms as well as improve sleep quality in AUD patients after withdrawal (Follesa et al., 2007). Currently there is a clinical trial examining the effects of taVNS on alcohol craving and drinking behaviors, which further examines activation of brain regions associated with craving and functional connectivity of neural networks related to addiction[1]. The concurrent development of VNS therapies in clinical practice along with VNS research in translational addiction models will help to improve understanding of the significance of brain-body communication via vagal/DVC circuitry in the development and treatment of AUD. Overall, these studies suggest that further examination of vagus nerve and DVC function in AUD is a promising avenue for discovery of future therapeutic options.

CONCLUSION

The DVC is an intricate brainstem region with a rich variety of neurotransmitters and neuropeptides. These signaling molecules help to relay endocrine and sensory information from the

[1]https://clinicaltrials.gov/ct2/show/NCT04106739

body to the brain. Visceral vagal afferents in the GI tract, heart, liver, and lungs are affected by alcohol consumption (Siegmund et al., 2003). In addition, neural activity in vagal nuclei in the brainstem are also independently affected by acute and chronic alcohol exposure (Covarrubias et al., 2005; Aimino et al., 2018). In this review, we discussed how the DVC projects to and modulates systems such as the homeostatic control system and cortisol system in the hypothalamus as well as the reward system and stress system in limbic regions. These systems are also disrupted by alcohol consumption and withdrawal. The numerous functions of DVC neuropeptides and neurotransmitters within different systems needs to be kept in mind when interpreting the effects of these molecules as well as their roles in modulating alcohol-related behaviors. Modulating endogenous activity of these various systems with gut brain peptide analogs and vagal activity manipulations provide promising avenues for developing effective clinical therapies for AUD. Further study of DVC neurocircuitry, neurotransmitters and neuropeptides will be useful in understanding how this region influences systems recruited during chronic alcohol consumption and ultimately will be integral for developing targeted AUD treatments.

AUTHOR CONTRIBUTIONS

BK and YS contributed to the conception, design, and drafting of the manuscript. BK, YS, AH, KB, and AA revised and edited the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Neuropeptide System Regulation of Prefrontal Cortex Circuitry: Implications for Neuropsychiatric Disorders

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Neuropeptides, a diverse class of signaling molecules in the nervous system, modulate various biological effects including membrane excitability, synaptic transmission and synaptogenesis, gene expression, and glial cell architecture and function. To date, most of what is known about neuropeptide action is limited to subcortical brain structures and tissue outside of the central nervous system. Thus, there is a knowledge gap in our understanding of neuropeptide function within cortical circuits. In this review, we provide a comprehensive overview of various families of neuropeptides and their cognate receptors that are expressed in the prefrontal cortex (PFC). Specifically, we highlight dynorphin, enkephalin, corticotropin-releasing factor, cholecystokinin, somatostatin, neuropeptide Y, and vasoactive intestinal peptide. Further, we review the implication of neuropeptide signaling in prefrontal cortical circuit function and use as potential therapeutic targets. Together, this review summarizes established knowledge and highlights unknowns of neuropeptide modulation of neural function underlying various biological effects while offering insights for future research. An increased emphasis in this area of study is necessary to elucidate basic principles of the diverse signaling molecules used in cortical circuits beyond fast excitatory and inhibitory transmitters as well as consider components of neuropeptide action in the PFC as a potential therapeutic target for neurological disorders. Therefore, this review not only sheds light on the importance of cortical neuropeptide studies, but also provides a comprehensive overview of neuropeptide action in the PFC to serve as a roadmap for future studies in this field.

Keywords: prefrontal cortex, dynorphin, enkephalin, corticotropin-releasing factor, cholecystokinin, somatostatin, neuropeptide Y, vasoactive intestinal peptide

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INTRODUCTION

Neuropeptides are widely distributed in the central nervous system (CNS) where they regulate various biological effects (Herbert, 1993; van den Pol, 2012; Kash et al., 2015; Nusbaum et al., 2017; Castro and Bruchas, 2019; Brockway and Crowley, 2020; Eiden et al., 2020). Currently, most neuropeptide studies are constrained to subcortical brain structures and tissue outside of

the CNS. With the increase in investigations centered on cortical and limbic circuit function underlying higher-order cognition processes, the gap in knowledge of neuropeptide function in cortical circuits has become increasingly apparent. Thus, an increased emphasis on research examining neuropeptide modulation of cortical circuits is needed, particularly as we seek to identify potential therapeutic targets for psychiatric disorders (Salio et al., 2006; Nassel, 2009; Hoyer and Bartfai, 2012; Crowley and Kash, 2015; Smith et al., 2019). In this review, we highlight established knowledge and unknowns of dynorphin, enkephalin, corticotropin-releasing hormone, cholecystokinin, somatostatin, neuropeptide Y (NPY), and vasoactive intestinal peptide (VIP) action in the prefrontal cortex (PFC). This particular set of neuropeptides was chosen to investigate because of their abundance in discrete cells and critical role in motivational and cognitive behaviors that are relevant to a plethora of psychiatric disorders. In turn, we discuss the role of these peptides in regulating executive function, affective behavior, and the role of cortical neuropeptide dysregulation in neuropsychiatric disorders. Changes in neuropeptide expression and action could play a direct role in cortical dysfunction underlying neuropsychiatric disorders as well as enact indirect and widespread effects that have profound implications for systems dysregulation. Leveraging knowledge of neuropeptide action in cortical circuits with insights to cortical circuit dysfunction can help develop more focused and effective treatments that lead to improved therapy (Poyner et al., 2000; Roth, 2019), irrespective if a specific neuropeptide is key to the etiology and/or maintenance of the disorder. Ultimately, we provide a comprehensive overview of neuropeptide action in the PFC by establishing knowns and unknowns, discussing the potential role of neuropeptides in neuropsychiatric disorders and animal models, and providing insights for future research.

Neuropeptides

Neuropeptides are small proteins, composed of 3–100 amino acid residues, encoded by over 70 genes (Snyder and Innis, 1979; Herbert, 1993; Salio et al., 2006; Burbach, 2011; van den Pol, 2012; Kash et al., 2015; Nusbaum et al., 2017; Castro and Bruchas, 2019; Brockway and Crowley, 2020; Eiden et al., 2020; Fricker et al., 2020). Like amino acid and monoamine neurotransmitters, neuropeptides are signaling molecules used by nerve cells to communicate with other neurons, glial cells, and peripheral cells (Gozes et al., 2001). Relative to other signaling molecules, the neuropeptide class is diverse, and is hypothesized to mediate communication across longer time scales and larger volumes leading to broad, long-lasting modulation of neural processes (Kim et al., 2017). Despite this diversity, all neuropeptides share certain key characteristics that govern their signaling: expression and biosynthesis in neurons and peripheral cells, regulated release, the ability to regulate neural function via actions with receptors and/or ion channels, and processing/degradation upon release to terminate and/or modify signaling (Burbach, 2011). In addition to expression in peripheral cells, gene expression and biosynthesis of neuropeptides is associated with neurons (Baraban and Tallent, 2004). Most excitatory and inhibitory neurons in the cortex show expression of neuropeptides or a neuropeptide binding G-protein coupled receptor (GPCR; Tasic et al., 2018; Smith et al., 2019). A recent single-cell RNA-seq study reporting neuropeptide and neuropeptide-selective GPCR expression patterns in mouse neocortical neurons suggest that neuropeptidergic networks may exist within cortical circuits (Smith et al., 2019). These, and the evolving number of publicly available single-cell RNA-seq and *in situ* hybridization data sets, such as those from the Allen Brain Institute, serve as valuable resources to identify putative peptide-expressing brain regions and/or cell types.

Upon translation, neuropeptide precursor proteins undergo proteolytic processing, an activity dependent process controlled by intracellular calcium, that produces active neuropeptides (Hallberg, 2015; Hook et al., 2018; Fricker et al., 2020; Lee and Fields, 2021). Precursor proteins can yield a single neuropeptide, multiple distinct neuropeptides, and/or multiple copies of a single neuropeptide. A single precursor peptide giving rise to multiple copies of the same or related peptide is energetically advantageous and a means for signal amplification (Salio et al., 2006; Li and Kim, 2008; Hook et al., 2018; Fricker et al., 2020). This translational step results in unique families of related neuropeptides with similar physiological function (Hook et al., 2018) and is the first step toward the regulated secretory pathway, another key characteristic that neuropeptides share.

Neuropeptides are stored and released from dense core vesicles (DCVs) which are larger than the small, clear synaptic vesicles (SVs) and small to intermediate-sized vesicles that store and release amino acid and monoaminergic neurotransmitters, respectively (Martin, 1994; Kim et al., 2006; Russo, 2017). DCVs and SVs are differentially sensitive to stimuli that trigger exocytosis. Specifically, they may recruit different Ca^{2+} sensors, allowing for independent regulation of exocytosis in a temporally- and activity-dependent manner (Zhang et al., 2011; Sudhof, 2012; Kim et al., 2017). Neuropeptide release from DCVs is triggered by small elevations in the Ca^{2+} concentration in the cytoplasm, whereas secretion of amino acids from SVs requires higher elevations, as produced in the vicinity of Ca^{2+} channels near the active zone at synapses (Hokfelt et al., 2000; Tallent, 2008; Nusbaum et al., 2017). These variations in DCV and SV sensitivity to exocytosis triggering stimuli may be important for dictating where neuropeptides are capable of being released. Specifically, SVs aggregate near regions on the presynaptic membrane containing release sites, where DCVs are more randomly distributed throughout the cell (Bean et al., 1994). However, nuanced organization of DCV clustering may exist that has yet to be revealed as studies have shown DCVs containing neuropeptides near dopamine receptors (Svingos et al., 1999), in close proximity to release sites (Song et al., 2020), or away from the active zone (Liguz-Lecznar et al., 2016). This indicates not only that DCVs can be released at multiple sites, but also that various combinations of expression and release patterns exist (Chini et al., 2017). Relative to fast neurotransmitters that bind to their cognate receptors with low affinity, neuropeptides bind with high affinity to GPCRs (Tallent, 2008; Burbach, 2011). It is important to note that following release, regulatory proteases can act on neuropeptides to modify their bioactivity. These modifications can cause inactivation, decrease or increase affinity

for GPCR targets, or even confer selectivity for GPCR or ion channels beyond the initial GPCR targets (Mentlein, 2004; Hook et al., 2018). In summary, both the increase in sensitivity of release and variation in location of DCVs leads to neuropeptide signaling properties that shape circuit activity in a spatially- and temporally- distinct manner relative to fast transmitters.

G-Protein Coupled Receptors

When released from their DCVs, neuropeptides bind to their cognate GPCRs to initiate signaling. These receptors constitute the largest family of transmembrane proteins and mediate cellular responses to hormones, neurotransmitters, and neuropeptides through interaction with their extracellular loop binding pockets (Rosenbaum et al., 2009; Heldin et al., 2016). Some neuropeptides exhibit a high degree of promiscuity across GPCRs and can bind several receptor subtypes (Devi, 2001; Hoyer and Bartfai, 2012; Hook et al., 2018). Importantly, GPCRs are the therapeutic target of 34% of FDA-approved medications, highlighting the importance of neuromodulatory systems (Roth, 2019). Although the GPCRs we discuss in this review bind to endogenous neuropeptides that have been identified and characterized, it is important to note many GPCRs have not yet been linked to endogenous ligands and are designated as orphan GPCRs (Tang et al., 2012). Conversely, many neuropeptides have been described and it is unclear what the repertoire of GPCR activation is for these peptides [i.e., cocaine and amphetamine related transcript peptide (CART)]. Neuropeptides interact with proximal G proteins and other GPCR-associated signaling molecules, except in instances where neuropeptide release sites and receptor location are mismatched or not proximally located (Herkenham, 1987). Nevertheless, neuropeptides can bind distal G-protein and receptor sites via volume transmission (van den Pol, 2012). G proteins are heterotrimeric, specialized proteins comprised of an alpha, beta, and gamma subunit (Weis and Kobilka, 2018; Wootten et al., 2018). Upon agonist-induced activation of the GPCR, GDP is exchanged with GTP on the alpha subunit to initiate G-protein-mediated signaling and the separation of the alpha subunit and beta-gamma dimer (Bruchas and Chavkin, 2010; Burbach, 2011; Weis and Kobilka, 2018; Wootten et al., 2018; Lemos Duarte and Devi, 2020; Zastrow and Sorkin, 2021). Subsequently, each subunit interacts with secondary transducers that amplify signaling cascades or impact the function of regulators of intrinsic excitability or synaptic transmission, such as voltage-gated or ligand-gated ion channels. GPCR function and signaling is not limited to the plasma membrane, however, even after internalization GPCRs can continue to signal from endosomes (Zastrow and Sorkin, 2021).There are four subcategories of GPCRs determined by their alpha-subunit: $G_{i/o}$, $G_{q/11}$, G_s, $G_{12/13}$, and G_{olf} (Rosenbaum et al., 2009). Each subtype of G protein alpha subunit has different modulatory effects and enacts different signaling cascades. A simple but useful oversimplification is that G_q, G_s, and $G_{12/13}$ are stimulatory where $G_{i/o}$ is inhibitory. G_q activates the phospholipase C (PLC) pathway enacting critical second messengers that mediate calcium signaling. G_s activates the formation of cyclic adenosine monophosphate (cAMP) and subsequently activation of the protein kinase A pathway. $G_{i/o}$-coupled GPCRs inhibit cAMP formation and recruit G-protein coupled inwardly rectifying channels (GIRKs) or inhibit voltage-gated Ca^{2+} channels to decrease intrinsic excitability. $G_{i/o}$ and G_s (Cahill et al., 2014; Gangarossa et al., 2019; Jiang et al., 2021) coupled GPCRs also evoke MAPK/ERK dimers through GRK/β-arrestin accessory proteins. MAPK and ERK both regulate targets in the cytosol and translocate to the nucleus where they phosphorylate a variety of transcription factors that regulate gene expression. The resulting signal transduction mechanism through each of the four G-protein subtypes may differ depending on receptor ligand, a phenomenon called functional selectivity or biased agonism (Weis and Kobilka, 2018; Wootten et al., 2018; Faouzi et al., 2020; Wingler and Lefkowitz, 2020). Biased agonists activate an isolated portion of a receptor's potential signaling pathways and either have no effect on or inhibit the other signaling cascades. For example, a G protein-biased agonist may only activate the G-protein dependent signaling but result in minimal to no activation of β-arrestin-dependent cascades. Conversely, GRK/β-arrestin biased agonists preferentially activate MAP kinase signaling cascades while sparing or minimally impacting G-protein signaling. Thus, modulatory effects of neuropeptides through GPCR signaling are dependent on the pathways in which they enact.

The Prefrontal Cortex

A detailed description of prefrontocortical function and architecture is beyond the scope of this review. Therefore, we invite the reader to the following review articles for a more in depth look at cortical architecture and circuit function (Carlen, 2017; Fishell and Kepecs, 2020; Ibrahim et al., 2020; Le Merre et al., 2021; Murray and Fellows, 2021; Tejeda et al., 2021). In this review, we focus on aspects of cortical circuits directly relevant to understanding the role of the neuropeptides in the PFC.

Studies using rodents have established the PFC is subdivided into three categories including the dorsomedial PFC (dmPFC), ventromedial PFC (vmPFC), and ventrolateral PFC (vlPFC) (Le Merre et al., 2021). Each subdivision contains distinct cortical subregions that share connectivity and anatomical features (Carlen, 2017). These subregions include the anterior cingulate and dorsal prelimbic (of the dmPFC), infralimbic and ventral prelimbic PFC (of the vmPFC), and orbital frontal cortex (of the vmPFC and vlPFC). Cortical neurons are diverse and can be categorized according to different characteristics, including morphology, patterns of local and long-range connectivity, intrinsic physiology, type of fast neurotransmitter released, and in some cases the neuropeptides they express. The PFC is the brain region with the most connections to other brain regions (Carlen, 2017; Le Merre et al., 2021). Cortical excitatory projection neurons send efferents to a wide array of target brain regions, including limbic structures such as the amygdala, thalamus, multiple nodes of the basal ganglia including the striatum, hypothalamus, monoaminergic centers of the midbrain, and the periaqueductal gray area. Inputs to the PFC arise from various associative and primary sensory cortices, thalamus, hypothalamus, and monoaminergic centers of the midbrain, hindbrain, as well as limbic regions, including the amygdala and

ventral hippocampus. Prefrontal cortical circuits also contain a plethora of inhibitory neurons, which serve to limit the activity of principal excitatory neuron and/or disinhibit them (Ferguson and Gao, 2018; Fishell and Kepecs, 2020; Wang et al., 2020). Classes of cortical neurons are differentially localized across cortical layers and extensively interconnected (Harris and Shepherd, 2015; Carlen, 2017; D'Souza and Burkhalter, 2017; Adesnik and Naka, 2018). Cortical circuits integrate these intracortical connections with subcortical connections and local circuit motifs embedded in the microcircuits process them (Harris and Shepherd, 2015). The laminar structure of the PFC is elaborate and neuron location within layers underlies function (D'Souza and Burkhalter, 2017). For instance, excitatory outputs of cortical circuits tend to have layer specificity (Adesnik and Naka, 2018). These circuit motifs regulate cortical processes in various ways including, signal amplification via recurrent excitatory connections, lateral inhibition via poly-synaptic inhibition, generation and/or maintenance of circuit-wide oscillatory activity, and mechanisms for signal convergence and divergence. Despite decades of research and significant advances in knowledge of nuanced synaptic connectivity of cortical networks and microcircuits therein, there are still many unknowns regarding how information is processed beyond fast excitatory and inhibitory connections. Therefore, uncovering more about how the cortex is organized is necessary to understand the extent to which neuropeptides modulate circuit function and behavior.

Neuropeptides in the Prefrontal Cortex

In the present review, we propose that neuropeptides, in the PFC, act as specialized modalities of communication that convey cellular and synaptic specificity via integration of neuropeptide-producing neurons, enzymes that degrade them, and cells bearing cognate receptors to their specific neuropeptide or family of neuropeptides (**Figure 1A**). For example, consider communicating by cell phone as compared to listening to National Public Radio (NPR): a cell phone transmits a direct signal from one phone to another whereas a radio tower broadcasts a widespread message that is only detected by radios tuned to a specific channel frequency. Radios tuned to the specific channel frequency (NPR) will receive the message, while non-tuned radios will not. In this analogy, the cell phone represents fast-neurotransmitter communication and the radio represents neuropeptide communication (**Figure 1B**). Virtually every neuron has both ionotropic glutamate and GABAergic receptors localized to post-synaptic densities that receive specific connections from specific neurons arising from local circuits or long-range afferents. Synaptic connections between neurons by fast transmitters are akin to direct calls made via cell phones where every person has the capacity to answer or make direct calls to their neighbors or friends and family. Conversely, neuropeptidergic transmission is like radio communication where messages are broadcast globally (neuropeptide volume transmission), but since not every neuron expresses every neuropeptide receptor (e.g., not all radios are tuned in to the correct frequency) neuropeptide transmission confers specificity in the circuit. This provides cells with specific neuropeptide expression to selectively control circuit elements endowed with complementary cognate receptor.

Other principals by which neuropeptides orchestrate PFC circuit function remain unknown. For example, temporal differences in the timescale of neuropeptide action and signaling contributes to a varied timeframe of circuitry regulation found across different neuropeptides. How this temporal difference specifically impacts the effect of neuropeptide action on the circuit has not been thoroughly investigated. Furthermore, a contributor to the varied timeframe of circuitry regulation seen across neuropeptides is the presence and action of peptidases. Specifically, peptidases limit neuropeptide diffusion and prematurely terminate its action. A small number of studies indicate peptidase regulation of neuropeptide action influences neural circuit activity (Wood and Nusbaum, 2002; Trieu et al., 2022). However, little is still known and investigation of peptidase action on circuit dynamics remains a promising area of study. Moreover, although it has been established that the PFC is reliant on its intricate, layered organization for proper function, much remains unknown regarding the integration of neuropeptides and their cognate receptors into this structure (**Figure 1C**). Identifying layer differences in neuropeptide, receptor, peptidase localization, and effective neuropeptide concentrations across layers will be essential to increase our understanding of where neuropeptide signals originate and how they finally impact PFC circuits. Further, it is also of importance to establish how the aforementioned nuances of neuropeptide systems integrated in PFC circuits resonate with nuanced layering of neuronal arborization location of different neuropeptide and peptide receptor-containing neurons and their physiological properties. For instance, if a neuropeptide is expressed in one layer of the PFC and the receptor in another it gives the cortex layer specific intercommunication capacities. Additionally, if the branching patterns of different neuropeptide expressing neurons differ, the layers in which their arborizations reside may also differ. In turn, this leads to variability in innervation by layer specific inputs. Finally, neuropeptide action is highly intertwined with internal states and experience influenced by context, exteroceptive and interoceptive cues, motivation, arousal, and affect (Kennedy et al., 2014; Zelikowsky et al., 2018). For example, the internal state of an organism significantly regulates neuropeptide transmission through various facets. Specifically, internal state may impact neuropeptide release, peptidase activity, cognate receptor expression and function, or downstream signaling. Additionally, neuropeptides themselves may influence the maintenance or transitions between internal state or be changed as a consequence of internal state (**Figure 1D**). However, further investigation of the dynamic nature of internal state and neuropeptide action is required. For example, the field has yet to fully understand the dependence of PFC neuropeptide expression and function on internal state. As a last example, if a neuropeptide receptor impacts an ion channel that is preferentially utilized in specific sub-populations, then that neuropeptide system will have a cell-selective effects even if the receptor is widely expressed. Further delineating these principals is a promising avenue to further investigate how different neuropeptides differentially fit into cortical circuits.

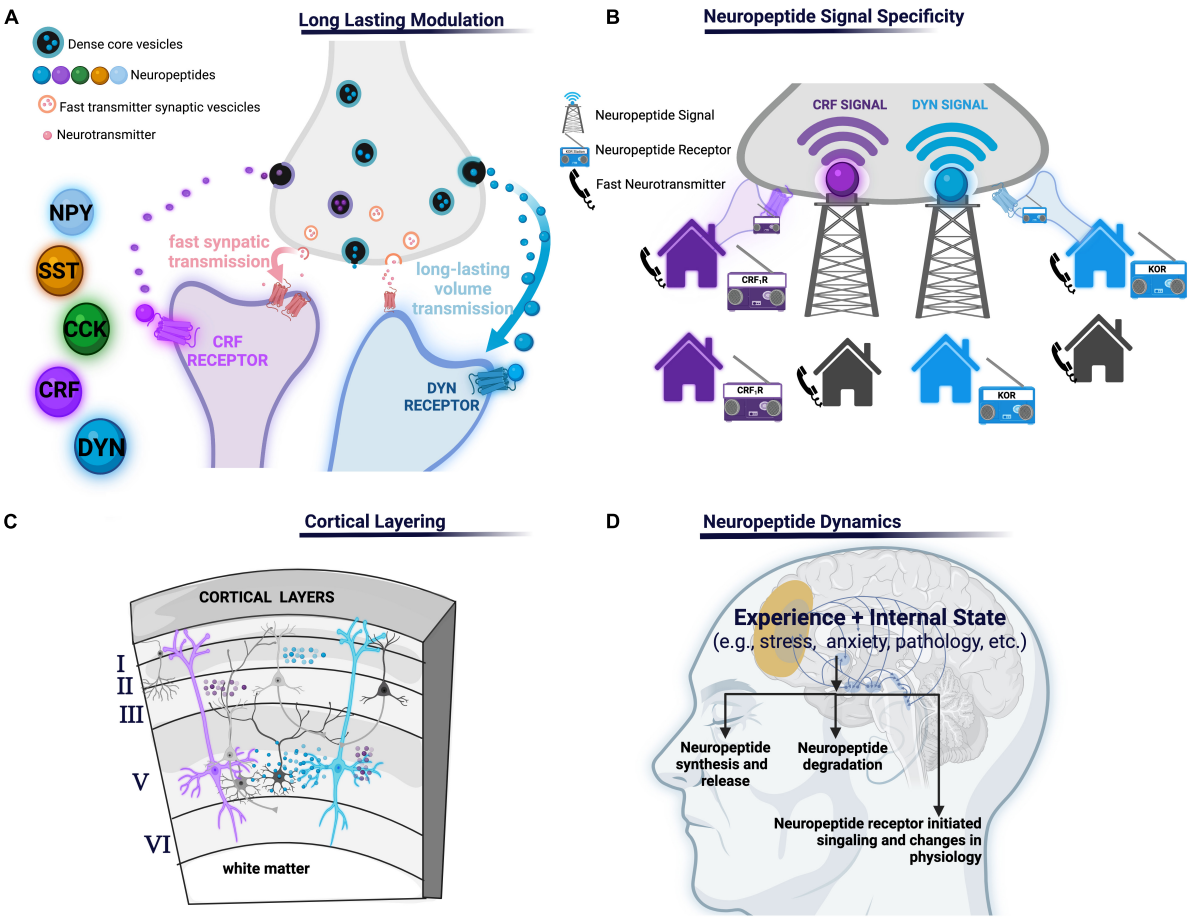

FIGURE 1 | (A) Long-lasting modulation: neuropeptides may mediate communication across longer time scales and larger volumes leading to long-lasting modulation of neural processes relative to shorter volumes and shorter duration of other molecules such as fast amino acid neurotransmitters. **(B)** Neuropeptide signal specificity: neuropeptide transmitters can only be detected if the appropriate neuropeptide receptor is present (like a radio signal the neuropeptide signal can only be received if the radio is tuned to the corresponding radio station, unlike a phone which receives point-to-point calls). **(C)** Cortical layering: different cortical layers may have different peptides or receptors and/or concentrations due to various factors including, but not limited to, differential arborization of PFC neuropeptide- and receptor-expressing cells, concentrations of degrading enzymes, or afferent inputs containing presynaptic receptors. **(D)** Various aspects of neuropeptides transmissions are subject to change, including neuropeptide production and release, degradation by peptidases, or signaling depending on the experience or internal state of an organism. NPY, neuropeptide Y; SST, somatostatin, CCK, cholecystokinin; CRF, corticotropin-releasing factor; KOR, κ-opioid receptor, CRF_1R, corticotropin-releasing factor type 1.

Neuropeptide modulation of cortical circuits modifies PFC processing of sensation, perception, decision-making, cognition, and/or affective behaviors. Dysregulation of the PFC is associated with various psychiatric disorders, including depression (Hare and Duman, 2020), anxiety (Park and Moghaddam, 2017), post-traumatic stress disorder (Koenigs and Grafman, 2009), and substance use disorders (Goldstein and Volkow, 2011). Therefore, the potent regulatory effects of neuropeptides on higher-order cognition processes in the PFC could contribute to symptomology of various psychiatric disorders. To date, there is limited information on how neuropeptides govern cortical circuits and whether there is region-specific regulation of neuropeptide release (Brockway and Crowley, 2020). In turn, throughout this review, to supplement for vast gaps in knowledge of specific neuropeptide function within the PFC, we mention research in other cortical regions or culture systems to make predictions of how neuropeptides may function in the PFC. It is imperative the field further investigations of neuropeptide action within the PFC to uncover these unknowns so ultimately potential therapeutic targets to various psychiatric disorders are revealed.

NEUROPEPTIDE FAMILIES

Dynorphin

Dynorphins (Dyns) are endogenous neuropeptides that play an essential role in regulating nociceptive, cognitive, and affective information. Dyns are synthesized from the precursor prodynorphin (PDyn) (Chavkin et al., 1983), which is cleaved by the enzyme proprotein convertase 2 (PC2) (Day et al., 1998). Cleavage produces three primary forms of Dyn: Dyn A, Dyn B,

and big Dyn, which consists of Dyn A and B. Each form shows a high affinity for the kappa opioid receptor (KOR) (Chavkin et al., 1982; Schwarzer, 2009). The most potent activator of KORs, Dyn A 1–17, is a 17 amino-acid peptide (James et al., 1984). Note that there are less potent forms like Dyn $A_{(1-13)}$ and Dyn $A_{(1-8)}$ (Feuerstein and Faden, 1984). The first five amino acids of Dyn encode the peptide Leu-enkephalin which is essential for binding to KORs (Chavkin and Goldstein, 1981). Dyn B and big Dyn (Fischli et al., 1982) also activate KORs but possess lower KOR binding affinities than Dyn A. In addition to KOR, Dyn A_{1-8}, a truncated form of Dyn A (Minamino et al., 1980), shows moderate affinity to both μ and δ opioid receptors (Toll et al., 1998). Dyn has also been shown to have non-opioid actions at NMDA receptors (Chen et al., 1995; Caudle and Dubner, 1998; Tang et al., 2000). Triggered by membrane depolarization, Dyn is released from DCVs localized to presynaptic or somatodendritic terminals (Molineaux and Cox, 1982; Chavkin et al., 1983; Whitnall et al., 1983; Svingos et al., 1999). Once released, Dyns mainly target KORs (Wagner et al., 1991), which are inhibitory GPCRs that activate $G_{i/o}$ signaling pathways (Taussig et al., 1993). Dyn is hypothesized to mediate inter-cellular communication through volume transmission where the peptide diffuses to binding sites up to 50–100 μm from their release site as measured in the hippocampus (Drake et al., 1994; Castillo et al., 1996; Chavkin, 2000). Although not observed in the PFC, this translates as it suggests that the location of KORs and localization of peptidases that degrade Dyns are essential to understanding the spatiotemporal profile of Dyn/KOR signaling. Together, Dyn signaling through KORs and subsequent downstream effects contribute to sensory and affective information regulation.

Roughly 8% of cortical neurons express PDyn, and 3% express KORs (Smith et al., 2019). Specifically, PDyn is heavily expressed in somatostatin interneurons (Sohn et al., 2014; Smith et al., 2019). PDyn mRNA and Dyn immunoreactivity has been observed across cortical regions in several species (Khachaturian et al., 1985; Peckys and Hurd, 2001; Yakovleva et al., 2006). Still, the relative abundance of this system is over-represented in primates relative to rodents (Hurd, 1996; Merchenthaler et al., 1997; Lin et al., 2006). Thus, the role of cortical PDyn may be more relevant in humans than in rodents and suggests the Dyn/KOR system may play a unique role in humans. In turn, warranting future research. In primary sensory cortices, most PDyn-mRNA expressing neurons are GABAergic somatostatin-containing interneurons (Sohn et al., 2014; Loh et al., 2017b; Smith et al., 2019), whereas, in the PFC, most PDyn-expressing neurons are glutamatergic (Sohn et al., 2014). A recent study using PDyn-iCre mice crossed with tdTomato mice showed strong labeling restricted to superficial layers in the insular cortex (Pina et al., 2020). Rats show temporal shifts in the patterns of cortical PDyn expression at different developmental stages (Alvarez-Bolado et al., 1990) and this dynamic expression pattern is consistent with reported maturational changes in rats' mPFC Dyn/KOR signaling patterns (Sirohi and Walker, 2015). PDyn expression may also change as a function of activity and experience. A recent study reported a transient experience-dependent increase in the percentage of somatostatin interneurons expressing PDyn (Loh et al., 2017a). This is supported by data that show regulation of human cortex PDyn expression by epigenetic changes linked to psychiatric disorders (Taqi et al., 2011; Yuferov et al., 2011; Butelman et al., 2012; Tejeda et al., 2012; Bazov et al., 2018a). These findings suggest Dyn expression can be used as a marker that represents a dynamic functional state of somatostatin (SST) interneurons that are sensitive to developmental and environmental factors underlying psychiatric disorders. However, further research is needed to validate this model.

The abundance of KOR mRNA expression in the cortex suggests that cortical sources of Dyns should affect local circuits (Meng et al., 1993; Svingos and Colago, 2002; Bazov et al., 2018b). KOR and PDyn mRNA expression patterns suggest that different cortical regions show distinct patterns of layer specificity (DePaoli et al., 1994; Peckys and Hurd, 2001; Smith et al., 2019). For example, in humans PDyn mRNA shows a different pattern of expression in the cingulate and dorsolateral PFC. Specifically, PDyn mRNA is widely spread across layers of the cingulate whereas it is tightly confined to limited layers in the dorsolateral PFC. In contrast, KOR is expressed similarly in both regions. Considering abundant expression of KOR and PDyn mRNA in the cortex, it is currently unclear how Dyn producing cells influence incoming KOR-expressing inputs or local-circuit neurons and unknown whether Dyn is released from axon terminals to act homosynaptically or heterosynaptically. It is also not known whether Dyn can be released from PFC cells from somatodendritic compartments to influence incoming inputs to Dyn neurons in a retrograde manner. Future research is necessary to address these unknowns and dissect Dyn/KOR regulation of PFC inputs. Moreover, KORs are differentially expressed in limbic afferent inputs that project preferentially to the PFC relative to primary sensory cortices (Tejeda et al., 2021). KOR mRNA shows increased expression in layer VI pyramidal neurons projecting within the telencephalon (Tasic et al., 2018; Smith et al., 2019). Ultrastructural studies show that prefrontal KOR immunoreactivity is primarily observed in presynaptic terminals with varicosities and presynaptic terminals of symmetric and asymmetric synapses, indicative of excitatory and inhibitory synapses, respectively (Svingos et al., 1999). Consistent with KOR localization on varicosities, KORs are expressed presynaptically in mesocortical dopaminergic terminals in the PFC and inhibit the release of dopamine (Tejeda et al., 2013). However, the expression of KOR in mesocortical neurons is not limited to presynaptic terminals, as KOR activation directly inhibits the activity of PFC-projecting ventral tegmental neurons (Margolis et al., 2006). KORs also regulate excitatory basolateral amygdala, but not ventral hippocampus, inputs to PFC (Tejeda et al., 2015), suggesting that the Dyn/KOR system may filter information coming into the PFC in a pathway-specific manner. KORs also potently inhibit the glutamate-driven enhancement of extracellular GABA levels, suggesting that Dyn may also regulate excitation/inhibition balance (Tejeda et al., 2013). Pathway-specific Dyn/KOR regulation of excitatory synapses and excitation/inhibition balance in different cell types has also been observed in the nucleus accumbens (Tejeda et al., 2017), which would imply that selective filtering of information flow may be a general principle of the Dyn/KOR system. Inhibitory actions

of dynorphin on insular cortex GABA neurons have also been reported (Pina et al., 2020). Collectively, these studies suggest that Dyn/KOR signaling may regulate PFC information processing via multiple mechanisms.

Dysregulation of the Dyn/KOR system has been shown to play crucial roles in drug-seeking, appetitive, and mood disorders. KOR activation promotes aversive behavior in animal models (Mucha and Herz, 1985) and psychotomimetic, anxiogenic, aversive experiences in humans (Pfeiffer et al., 1986). Direct infusion of the KOR antagonist norBNI in either prelimbic or infralimbic cortices diminishes restraint stress-induced increases in arterial pressure and heart rate without affecting stress-induced changes in tail and body temperature (Fassini et al., 2014, 2015). This suggests that KOR signaling in the PFC increases autonomic arousal and may underlie maladaptive stress-related responses, a hallmark of anxiety disorders. Consistent with this notion, mPFC infusion of a KOR antagonist had an anxiolytic effect in an open field (Wall and Messier, 2002; Tejeda et al., 2015). Moreover, systemic or direct injection of KOR agonist in mPFC produces conditioned place aversion (CPA) (Bals-Kubik et al., 1993). Furthermore, CPA induced by systemic KOR agonist is blocked via mPFC infusion of KOR antagonist (Tejeda et al., 2013), indicating that KOR activity in the mPFC is required for KOR-mediated aversion. Other studies have identified a role for the Dyn/KOR system in the infralimbic cortex in reducing anxiety-like behavior. Intra-mPFC administration of the KOR agonist, U69,593, or the KOR antagonist, nor-BNI, reduces and increases anxiety-like behavior, respectively (Wall et al., 2001; Wall and Messier, 2002). Further insight regarding the mechanisms of the Dyn/KOR system's regulation of autonomic, anxiety-like behavior, and aversive responses may benefit future treatments for patients with anxiety and mood disorders.

The Dyn/KOR system may also regulate cognition via regulation of mPFC circuits. A recent study found that KOR activation in the PFC decreased accuracy and responsiveness in a delayed non-match to sample working memory task (Abraham et al., 2021). These effects were recapitulated by optogenetic activation of PDyn expressing PFC neurons and blocked by local infusion of the KOR antagonist norBNI. Conversely, the Dyn/KOR system in the infralimbic cortex may promote working memory as KOR activation and inhibition enhances and decreases putative measures of working memory on the spontaneous alternation memory task (Wall and Messier, 2002; Wall et al., 2001; Wall and Messier, 2002). Collectively, these studies point to a potential role of KOR-PFC signaling in PFC-dependent working memory and this capacity may differ depending on PFC sub-regions.

What is currently unknown is how the many actions of Dyn/KOR signaling impacts the function of PFC circuits to influence behavior. Dopamine transmission and limbic inputs in the PFC are critical for cognitive processing, working memory, and decision-making (Goldman-Rakic et al., 2000; Floresco, 2013; Arnsten, 2015). The Dyn/KOR system is widespread across cortical circuits. However, unique innervation of the PFC by KOR-sensitive afferent inputs, such as the ventral tegmental area and basolateral amygdala, that convey motivationally charged information to promote executive control of behavior inherently confers the PFC Dyn/KOR system the capacity to regulate affective behavior and cognitive control. The inhibitory effect of KOR activity in dopamine and basolateral inputs to the PFC, imparts a specialized function for this system in differentially shaping PFC circuits versus primary and lower-order associative cortical circuits. Moreover, Dyn expression in PFC circuits is not restricted to GABAergic neurons, as is observed in primary sensory cortices (Sohn et al., 2014). This suggest that principal neurons may utilize this peptide to influence incoming afferents and dopaminergic inputs, providing a modulatory signal capable of filtering incoming information and local processing. Differential integration of the Dyn/KOR system into PFC along with unique features of the PFC may be essential contributing factors that position the Dyn/KOR system as a potent regulator of affective, defensive, arousal and executive function.

One function of the PFC Dyn/KOR system may be to regulate dendritic integration in cortical cells by virtue of the diverse actions this system has on PFC circuits. Dopamine transmission in the PFC may drive wide-spread cellular effects that culminate in increased encoding of synaptic information within dendritic compartments within specific ensembles of neurons. By directly inhibiting glutamate release via a presynaptic site of action, KOR may decrease the probability of NMDA receptor activation and subsequent recruitment of active conductances, such as voltage-gated calcium channels, to reduce responsivity of neurons to incoming inputs (Palmer et al., 2014; Augusto and Gambino, 2019). KORs localized within dendritic processes may also influence the way KOR-positive cortical cells process information. $GABA_B$ receptors, $G_{i/o}$-coupled GPCRs similar to KOR, inhibit Ca^{2+} signaling through NMDA receptors (Chalifoux and Carter, 2010), which would be predicted to inhibit Ca^{2+}-regulated processes in somatodendritic compartments essential for shaping input-output transformations. Occasional co-localization of KOR and NMDA receptors in dendrites (Svingos and Colago, 2002) provides an anatomical basis for interaction between KOR and NMDA receptors similar to those with $GABA_B$ receptors. KOR activation also influences the mTOR pathway, which modifies neurite outgrowth, spinogenesis, and synaptic plasticity in cortical neurons (Liu et al., 2019), providing another mechanism by which Dyn may regulate dendritic processing in PFC KOR-containing neurons. Lastly, Dyns can positively or negatively allosterically modulate NMDA receptor activity in a KOR-independent manner (Caudle and Dubner, 1998), influencing excitatory transmission or synaptic integration. Collectively, the concerted efforts of Dyn/KOR signaling is expected to have widespread effects on PFC cortical circuit dynamics and may be important for the selection/deselection of ensembles that encode PFC-dependent behavior.

Enkephalin

Enkephalins (Enks) are endogenous neuropeptides and members of the opioid peptide family that play a significant role in neurotransmission and pain modulation. Enks signal through $G_{i/o}$ opioid receptors, preferentially binding to DOR and MOR (Takahashi, 2016). Enk, DOR, and MOR expression is widely distributed throughout the central, peripheral, and autonomic

nervous systems, multiple organ systems, as well as endocrine tissues and their target organs (Hughes et al., 1975; Tang et al., 1982; Holaday, 1983; Coffield and Miletic, 1987; Duque-Diaz et al., 2017; Smith et al., 2019). There are two structurally different Enk peptides, Met-Enk and Leu-Enk which arise via proteolytic processing from the precursor proteins proenkephalin A and proenkephalin B, referred to as PDyn. Proenkephalin is enriched throughout the brain and gives rise to Met- and Leu-Enk neurons in various regions including the cerebral cortex, basal ganglia, limbic telencephalic nuclei, hypothalamus, and thalamus (Geracioti et al., 2009). Proenkephalin A yields four Met-Enks, one Leu-Enk, one Met-Enk-Arg^6-Phe^7, and one Met-Enk-Arg^6-Gly^7-Leu^8 (Dhawan et al., 1996). In contrast, PDyn yields one Leu-Enk. Leu- and Met-Enks have the highest binding affinity to DOR followed by the MOR (Schafer et al., 1991; Devi, 2001).

Enkephalin and cognate opioid receptor DOR and MOR expression in the cerebral cortex has been thoroughly characterized (Giraud et al., 1983; Taki et al., 2000; Smith et al., 2019). Single-cell RNA sequencing reveals roughly 40% of anterior lateral motor cortex (ALM) and primary visual cortex (VISp) cells contain the pro-enkephalin gene (PENK), 40% contain the gene encoding MOR (Oprm1), and 13% contain the gene encoding DOR (Oprd1). According to single cell sequencing, PENK is primarily expressed in VIP and parvalbumin containing GABAergic neurons, although a small subset of glutamatergic IT neurons also express the gene. Similarly, although Oprm1 is mainly expressed in VIP and somatostatin GABAergic neurons, it is also expressed in a small subset of parvalbumin and layer VI glutamatergic neurons. In contrast, Oprd1 is almost exclusively expressed in somatostatin and PV cells (Smith et al., 2019). Furthermore, immunolabeling methods show neurons expressing MOR are frequently colocalized with proenkephalin, and mainly express on small, non-pyramidal neurons expressing GABA in layers II–IV (Taki et al., 2000). Immunocytochemical detection of opioid peptides in the PFC reveal widespread expression of Enk in layers II and III and V and VI of neocortex in addition to layers II and III of the olfactory cortex (McGinty et al., 1984; McGinty, 1985). Additionally, radioimmunoassays reveal the ratio of Leu-Enk to Met-Enk-Arg^6-Gly^7-Leu^8 is roughly one, which corresponds with the ratio of respective precursor proteins (Giraud et al., 1983). This contrasts with other peptides where the ratio of respective precursor proteins does not correspond with the active peptide, in turn, implying the majority of PENK is converted to active Enk peptide. In summary, Enk and its cognate receptors are expressed across layers of the cortex in both glutamatergic and GABAergic neurons.

Enkephalin impinges on cortical circuitry by regulating synaptic transmission and other cortical neuromodulators. Electrophysiological findings reveal opposing roles of MOR and DOR in synaptic transmission within thalamo-cortico-striatal circuits. Specifically, MOR agonists suppress excitatory thalamic inputs to the ACC, while DOR opioid agonists disinhibit ACC pyramidal neurons by suppressing feed-forward inhibition (Birdsong et al., 2019). Therefore, DOR activation causes hyper-excitable ACC circuits and MOR activation causes inhibition of glutamate release. This suggests Enk action on either MOR or DOR can differentially impact thalamo-cortical-striatal circuitry. Moreover, MOR modulation of cortical circuity is subregion specific. MOR agonists acting on MORs expressed on parvalbumin-interneurons inhibit GABAergic synaptic transmission in the medial orbitofrontal cortex but not lateral orbitofrontal cortex (Lau et al., 2020). MOR antagonism does not reverse this suppression of inhibition which indicates MOR enaction of long-term depression. Ultimately, these regional differences suggest location-dependent differences in MOR coupling to downstream cAMP/PKA signaling cascades or differential expression of functional MORs. Cortical MOR also regulates dopamine action. For example, MOR activation within the PFC elevates local dopamine overflow (Tejeda et al., 2013), which provides a novel mechanism by which MORs may regulate dopamine output beyond disinhibition of dopamine neuron activity within midbrain circuits (Johnson and North, 1992; Matsui and Williams, 2011). Additionally, Enk can modulate cortical circuitry by altering the action of other neuropeptides in the system. D-Ala2-D-Leu5-Enk inhibits K^+-stimulated release of cholecystokinin in the hypothalamus but not the cortex (Micevych et al., 1985). Contrastingly, cortical D-Ala2-D-Leu5-Enk inhibits K^+-stimulated release of VIP in a naloxone dependent manner (Micevychi et al., 1984). Together, these results indicate region-specific, elaborate modulation of cortical circuitry by Enk through MOR and DOR.

Activation of DOR and MOR and their signaling cascades by Enk has been shown to modulate information processing in cortical circuits and associated behaviors. Specifically, studies on Enk modulation of neural circuits as contributors to psychiatric disorders (e.g., addiction, anxiety, depression, and PTSD) and pain regulation are currently active areas of research. Adverse life experiences increase lifetime risk to stress-related psychopathologies, and exposure to stress downregulates endogenous Enk expression in the PFC in rats (Li et al., 2018). However, post-mortem cortical tissue from individuals suffering from substance use disorder showed upregulated PDyn and KOR mRNA, but no significant changes in expression of proenkephalin, MOR, and DOR opioid receptor mRNA was evident. This suggests KOR but not MOR signaling may underlie in part the neurocognitive dysfunctions relevant for addiction and disrupted inhibitory control (Bazov et al., 2013; Nosova et al., 2021).

Prefrontal cortex Enk and MOR signaling has been implicated in modulating reward-seeking and compulsive behaviors. MOR stimulation within the vmPFC induces feeding and hyperactivity (Mena et al., 2011, 2013; Giacomini et al., 2021). Additionally, endogenous cortical Enk transmission is both necessary and sufficient for the expression of impulsive action in a high-arousal appetitive states (Selleck et al., 2015) as well as anticipation and excessive consumption of alcohol (Morganstern et al., 2012). Opioid action in the PFC has been implicated in inhibitory-control deficits associated with addiction and binge-type eating disorders. For example, prenatal ethanol exposure led to increase in Met-Enk in the PFC which is suggested to underlie the facilitation of postnatal ethanol intake (Abate et al., 2017). Furthermore, cocaine self-administration significantly increases MOR and DOR mRNA but not proenkephalin mRNA in

the PFC indicating changes in these targets may underlie cocaine-induced reward and habitual drug-seeking behavior (Sun et al., 2020). Cortical Enk may also regulate compulsive behaviors as models of autism spectrum disorder demonstrate a decrease in cortical endogenous Enk with an increase in repetitive behaviors (Augustine et al., 2020).Together, these studies suggest the role of Enk signaling through MOR and DOR in cortical circuit processing and associated behaviors underlying neurological disorders.

Changes in Enk cognate receptor availability also impacts cortical circuits which regulate reward and motivational processes that underlie psychiatric disorders. Specifically, positron emission tomography (PET) studies show a decrease in MOR opioid peptide binding potential in anterior cingulate for patients with PTSD (Liberzon et al., 2007). Additionally, cortical PET studies have revealed the binding potential of MOR and DOR ligands in patients with chronic neuropathic pain. Individuals experiencing central post-stroke pain had a decrease in [^{11}C]-diprenorphine, a non-selective opioid receptor antagonist, in the insular and lateral prefrontal cortices and anterior cingulate (Willoch et al., 2004; Maarrawi et al., 2007a). Patients with peripheral neuropathic pain had symmetrical bilateral decreases in [^{11}C]-diprenorphine where patients with central post-stroke neuropathic pain primarily had contralateral asymmetrical decreases. This suggests that motor cortex stimulation (MCS) for control of neuropathic pain also decreases [^{11}C]-diprenorphine binding in the anterior cingulate indicating enhanced secretion of endogenous opioids during MCS (Maarrawi et al., 2007b). However, it is unclear from these results whether decrease in opioid receptor availability is due to a loss of opioid-receptor containing neurons, available receptors, and/or increased endogenous opioid peptide release. Ultimately, given that endogenous enkephalin signaling via MOR and DOR regulates excitation-inhibition balance in a circuit specific manner, alterations in opioid receptor availability following stroke may contribute to mal-adaptive behaviors and is suggested to be one of the causes of poststroke pain. In turn, upregulation and action of MOR and DOR in the cortex indicates modulation of cortical circuits underlying pain disorders.

Given the role of Enk underlying psychiatric disorders and suggested modulation of information processing in cortical and subcortical circuits, the use of Enk as a therapeutic has been highly considered. It is important to note that therapeutics pertaining to MOR and DOR are vast, and for the purpose of this review only therapeutic treatments that aim to manipulate Enk as a peptidergic transmitter will be discussed. For a more detailed look at opioid receptor specific therapeutics please consult the following articles (Broom et al., 2002; Spetea et al., 2013; Browne et al., 2020; Grim et al., 2020; Senese et al., 2020). To capitalize on the analgesic effects of endogenous Enks, research has been done to chemically modify Enks so they are more difficult to degrade and analgesic properties of endogenous Enks can be amplified while retaining their ligand specificity for MOR and DOR (Pert et al., 1976; Kropotova et al., 2020). A result of these studies is the development of dual enkephalinase inhibitors (DENKIs) that enhance the analgesic effects of endogenous Enks only at sites of release, avoiding negative side effects including tolerance, respiratory depression, and constipation that derive from widespread MOR and DOR activation (Poras et al., 2014). Despite these advantages, peptidase inhibitors act on many targets and unexpected, negative impacts of their inhibition is a caveat that must be considered. Further, the coupling of Enk to the glycosylation of biopharmaceuticals improved binding to its carbohydrate receptor, pioneering a method that increases the accuracy of therapeutic peptides (Christie et al., 2014). Together, Enk is a promising therapeutic to consider for pain and psychiatric disorders.

Despite the number of studies on the neuromodulatory effects of Enk underlying analgesia and neuropsychiatric-relevant behaviors, there are still many unknowns of Enk function in cortical circuits. For example, although Enks precursor peptides proenkephalin A and Pdyn have been well established, whether there are differential effects of Enk sources arising from proenkephalin A or Pdyn has yet to investigated. Additionally, although Birdsong et al. (2019) established that MOR and DOR regulate the thalamo-cortico-striatal circuit in opposing ways and Lau et al. (2020) determined subregional differences in MOR-coupling downstream cascades, whether MOR and DOR differential regulation of the cortex works independently alongside one another or together is unknown. Furthermore, VIP neurons disinhibit the cortex by inhibiting other cortical interneurons (Millman et al., 2020). Given the colocalization of Enk and VIP expression (Smith et al., 2019; Leroy et al., 2021) and that GABAergic interneuron output at some capacity are inhibited by Enk, Enk release by cortical VIP neurons may cooperate with VIP GABAergic transmission to disinhibit cortical circuits in a spatially- and temporally organized manner. As imbalance in excitatory and inhibitory control of PFC circuits is implicated in various neuropsychiatric disorders, it will be of importance to understand how inhibitory effects of MORs on excitatory inputs versus disinhibition by MOR/DOR may play a role in such excitation/inhibition imbalance. Ultimately, understanding the role of Enk in PFC circuits is necessary to uncover the underpinnings of various psychiatric disorders and further develop therapeutic targets and treatments.

Corticotropin-Releasing Hormone

Corticotrophin releasing factor (CRF) plays a significant role in the integration of endocrine and behavioral responses to stress by acting on the hypothalamic-pituitary-adrenal (HPA) axis (Vale et al., 1981). However, CRF is also expressed in brain regions not associated with the HPA axis such as the cortex. Originating from a 196 amino acid prepropeptide, CRF is synthesized by prohormone convertases at dibasic amino acids (lysine or arginine) to produce a 41-amino acid mature peptide (Hook et al., 2008). CRF is a member of the CRF system comprised of CRF and three additional neuropeptides: Urocortin I (UCNI), Urocortin II (UCNII), and Urocortin III (UCNIII). As there is little known regarding Urocortins in cortical circuits, this review will only cover CRF within the CRF system. CRF has two cognate GPCR receptors, CRF type-1 (CRF1) and CRF type-2 (CRF2) (Dedic et al., 2018), which are fairly similar and share 70% amino acid identity. Despite this similarity, CRF receptors differ in their N-terminal extracellular domain (40% identity)

(Dautzenberg and Hauger, 2002). CRF1 has only one functional splice variant expressed in the brain where CRF2 receptor has three functional splice variants in humans (α, β, and γ) and two in rodents (α and β) (Dedic et al., 2018). CRF2α receptor serves as the major rodent splice variant (Chalmers et al., 1996).

Corticotrophin releasing factor peptides act through CRF receptors with varying affinity (Dedic et al., 2018). Specifically, CRF has a higher affinity for the CRF1 than CRF2 receptor. Although CRF receptors couple primarily to Gs proteins (Millan et al., 1987), they can bind and activate other G proteins as well (Grammatopoulos and Chrousos, 2002; Hillhouse and Grammatopoulos, 2006). This suggests CRF receptors may couple to various signaling pathways. CRF1 activation by CRF or UCNI and CRF2 receptor activation by UCNI, UCNII, or UCNIII can activate ERK1/2 in CHO cells (Brar et al., 2004). Moreover, CRF selectively activates ERK1/2 in different regions of the brain via CRF1 *in vivo* (Refojo et al., 2005). In PFC synaptosomes, UCNIII induces activation of ERK1/2 (Yarur et al., 2020a). Together, these studies indicate CRF receptor signaling pathways are determined by specific G-protein coupling and availability of cellular signaling components in CRF-containing cells.

Corticotrophin releasing factor type-1 and CRF2 are expressed in the olfactory bulb, bed nucleus of the stria terminalis, lateral septum, paraventricular nucleus of the thalamus, dorsal raphe nucleus, and mPFC where expression of CRF1 is greater than CRF2 (Van Pett et al., 2000). In the mPFC, CRF is expressed in neurons across all layers (Yan et al., 1998). CRF is expressed in inhibitory interneurons, primarily in parvalbumin cells and rarely in calbindin or calretinin-expressing cells (Yan et al., 1998). UCNs expression in the cortex is present in sparse fibers in deeper layers (Bittencourt et al., 1999). CRF1 receptor is widely expressed in pyramidal cells of the mPFC (Uribe-Marino et al., 2016). There is also evidence of CVRF1 expression in inhibitory interneurons as the receptor is often co-expressed with other peptides found in various interneuron populations like somatostatin, VIP, and cholecystokinin (Gallopin et al., 2006). To date, there is no conclusive evidence showing the expression of CRF2 receptor within mPFC circuits (Van Pett et al., 2000). However, CRF2 receptor expression on basolateral amygdala inputs to the mPFC have been described (Yarur et al., 2020b). Collectively, these studies suggest that the CRF system is integrated into PFC circuitry and may regulate circuit dynamics.

Studies show CRF modulates PFC synaptic transmission. Iontophoretic application of CRF enhances the activity of neurons in the cortex of Sprague–Dawley rats (Eberly et al., 1983). In addition, CRF bath application increases sEPSC frequency in layer V pyramidal cells from rat PFC slices, an effect that can blocked a by a CRF1 receptor antagonist (Liu et al., 2015). These results suggest that CRF enhances excitatory drive onto PFC principal neurons. Interestingly, lesions of the basolateral amygdala reduced CRF enhancement of sEPSC frequency of ipsilateral layer V pyramidal cells in PFC (Liu et al., 2015), suggesting that basolateral amygdala synapses are a site of action for CRF. Similar results were found in layers II/III and V of adult male C57BL/6J mice (Hwa et al., 2019), where the increases in sEPSC frequency induced by fox odor were ablated by the administration of a systemic CRF1 receptor antagonist. Pretreatment with CP154526, a CRF1 antagonist, suppresses defensive burying and reduces enhanced synaptic transmission elicited by predator exposure. The increase in sEPSC by fox odor exposure occludes the increase of sEPSC induced by CRF bath application, implying that predator odor-related behaviors engages the CRF system in the PFC. Collectively, these studies are consistent with a facilitatory role of CRF on excitatory transmission in the PFC. CRF may also interact with the serotonin system within PFC circuitry. PFC 5-HT signaling has been implicated in anxiety and stress behaviors. 5-HT increases the amplitude and frequency of sIPSCs in the rat PFC (Tan et al., 2004). Co-application of CRF and 5-HT prolong the effects of 5-HT on sIPSC, an effect blocked by astressin, a CRF1 antagonist. Collectively, these studies suggest that CRF regulates synaptic transmission in the mPFC and may be recruited by stressors.

Prior studies reveal dynamic changes in cortical CRF systems in response to stress. Acute restraint stress increases CRF and CRF1 mRNA in the PFC of Sprague–Dawley rats (Meng et al., 2011). On the other hand, chronic social defeat stress decreased CRF mRNA and increased CRF1 mRNA in PFC of Wistar rats (Boutros et al., 2016). In adult males, chronic social defeat stress modified mRNA for CRF receptors only in susceptible animals, but not resilient mice (Guo et al., 2020). In comparison to control and resilient mice susceptible mice, susceptible mice show increased CRF1 mRNA and decreased CRF2 receptor mRNA expression. These results suggest condition and duration of the stressor can differentially regulate CRF to contribute to stress-coping deficits with chronic stressors.

Corticotrophin releasing factor modulation of PFC function and behavior is a rising area of study. Infusion of CRF in the PFC impairs working memory while an infusion of NBI 35965, a CRF1 antagonist, improves working memory (Hupalo and Berridge, 2016). Moreover, PFC CRF neuron activation inhibits working memory, an effect blocked by intra-mPFC CRF1 antagonism (Hupalo et al., 2019). Interestingly, mPFC CRF signaling decreased PFC, and to a lesser extent striatal, neuron task-related encoding. However, intra-mPFC administration of CRF does not modify sustained attention (Hupalo and Berridge, 2016), suggesting that CRF signaling in the PFC may differentially regulate different aspects of PFC-dependent cognition. Interestingly, chemogenetic activation of PFC CRF-expressing neurons impairs working memory, an effect blocked by systemic, but not intra-mPFC, administration of a CRF1 antagonist. The authors concluded that effects of CRF neuron activation is due to release of CRF in PFC terminal regions. This work establishes a role for PFC CRF systems in regulating cognitive function. Further, CRF-containing neurons in the PFC regulate motivated behavior under stress. Specifically, a subset of PFC CRF-containing interneurons is recruited in tail suspension test and ablation or inhibition of these neurons increase immobility time in mice (Chen P. et al., 2020). Interestingly, activation of CRF neurons promotes resilience. These results suggest CRF neurons may become engaged to promote adaptive behaviors to overcome stress rather than driving mal-adaptive behavior. In a mouse model of stress-induced depression, the ablation or antagonism of CRF1 receptors abolishes behavioral despair (Dedic et al., 2018; Deussing and Chen, 2018). Thus, the

PFC CRF may control adaptive and/or mal-adaptive behaviors depending on the severity and/or duration of the stressor. Microinjection of CRF into the PFC increases anxiety-like behavior in the elevated plus-maze (EPM) in both acute and chronically stressed rats (Jaferi and Bhatnagar, 2007). CRF injection in the rat frontal cortex induces anxiogenic actions, but at high doses produces an anxiolytic-like effect (Zieba et al., 2008). Interestingly, the anxiolytic action of may be mediated by engagement of alpha-adrenergic signaling (Smialowska et al., 2021). CRF1 receptor in the PFC has been implicated in the emotional adaptation to stress (Uribe-Marino et al., 2016). Consistent with the CRF-5-HT interactions mentioned above, CRF/CRF1 transmission regulates anxiety-related behaviors through 5-HT2R signaling in the PFC (Magalhaes et al., 2010), indicating that the CRF system interacts in the PFC with other neurotransmitters such serotonin and norepinephrine to regulate anxiety-like behaviors. Collectively, these studies suggest that PFC CRF systems regulate PFC-dependent behaviors, including anxiety-like behavior and cognition.

There is a wide array of evidence that suggests that CRF is a viable target for the treatment of psychiatric disorders. Preclinical and postmortem studies show elevated CRF concentrations in patients diagnosed with major depressive disorder (Nemeroff et al., 1984; Raadsheer et al., 1995; Deussing and Chen, 2018). Furthermore, there is compelling evidence that the progression from recreational to compulsive drug use is driven by a shift in emotional and motivational homeostasis to an allostatic setpoint, resulting in a state of decreased reward function and increased stress responsivity. It is hypothesized that the CRF system plays a significant role in the negative emotional state and habitual drug-seeking in individuals with severe addiction (Silberman et al., 2009; Koob, 2010; Zorrilla et al., 2014). Based on the hypothesis that CRF system dysregulation contributes to negative affect, various clinical trials using CRF1 receptor antagonists have been completed, with conflicting results (Spierling and Zorrilla, 2017). A CRF1 receptor antagonist, NBI 30775/R121919, reduced depression and anxiety scores using patient and clinician ratings without impairing corticotropin and cortisol secretion in patients with MDD (Zobel et al., 2000). Further, a clinical trial in major depressive disorder patients reports that a non-peptidic CRF1 receptor antagonist reduces symptoms of anxiety and depression (Kehne and De Lombaert, 2002). In contrast, the CRF1 receptor antagonist CP-316,311 did not reduce depression score patients with MDD compared with placebo-treated controls (Binneman et al., 2008). The CRF1 receptor antagonist verucerfont (GSK561679) failed to reduce PTSD symptoms compared to placebo (Dunlop et al., 2014). Several factors may contribute to mixed reports, including but not limited to variation in CRF or CRF1 receptor genetic or protein expression, limited target engagement, therapeutic effects that may be only observed in certain conditions (e.g., acute stress and chronic stress), and/or biased signaling associated with different compounds that have been tested in clinical trials. These studies present a challenge to the field to evaluate molecules that modulate CRF signaling to promote therapeutic outcomes in clinical trials.

Although extensive research has been done on the CRF system in the frontal cortex, much remains unknown about its architecture and function in cortical circuits. It is unclear how CRF modulates the activity of the cortical circuitry via regulation of local circuit excitatory and inhibitory neurons and afferent inputs to the cortex. Despite extensive research on the role of the CRF system in regulating stress-related and anxiety-like behavior, little is known about the role of this system in regulating reward processing beyond the context of cognitive tasks. Given that PFC CRF neurons regulate encoding of working memory (Hupalo et al., 2019), it will be of interest to understand how CRF system shapes PFC activity to acute and chronic stressors and during reward processing. It is also unknown how CRF binding protein (CRFBP) influences the CRF system in the PFC. CRFBP was first postulated as a sequester of CRF, effectively reducing CRF concentration and receptor activity (Cortright et al., 1995). Further studies have revealed other actions of CRFBP. Specifically, CRFBP has been shown to have a facilitatory role of CRF-induced potentiation of NMDAR-mediated synaptic transmission in the ventral tegmental area (Ungless et al., 2003), function independently of the CRF receptor (Chan et al., 2000), and act as an escort protein to traffic CRF2a to the cell surface (Slater et al., 2016). CRFBP is expressed in GABAergic cells in the PFC (Ketchesin et al., 2017), whereas CRF and CRF1 receptor expression is primarily observed in glutamatergic cells. This raises the question of how CRF–CRFBP interactions may modulate activity of PFC circuitry during motivationally charged behaviors. Lastly, it is unclear whether urocortins influence prefrontal cortical circuits and behavior given that they differentially activate CRF1 and CRF2 and these receptors may differ in their anatomical location within the cortex.

Cholecystokinin

Cholecystokinin (CCK) is a neuropeptide and gut hormone that belongs to the gastrin family and has various regulatory functions in the brain and gut. In the nervous system, CCK regulates learning and memory, nociception, homeostatic sensation, affective behavior, and drug-seeking behavior (Reisi et al., 2015). CCK peptides evoke downstream signaling pathways via CCK-A and CCK-B receptors, which signal through G_q protein to activate phospholipase Cβ and increase intracellular Ca^{2+} levels (Johnsen, 1998; Williams et al., 2002).

Radio-immune and *in situ* hybridization studies reveal CCK expression is abundant in the cerebral cortex (Beinfeld et al., 1981; Savasta et al., 1988; Ingram et al., 1989; You et al., 1993) and mainly expressed in cortical interneurons (Vanderhaeghen et al., 1981; McDonald, 1982; Hendry et al., 1983; Morino et al., 1994; Gallopin et al., 2006). Single cell reverse transcription polymerase chain reaction experiments indicate CCK mRNA is expressed in approximately 30–40% of GABAergic interneurons in the cortex (Gallopin et al., 2006). Moreover, CCK and CCK mRNA is expressed in both glutamatergic and GABAergic neurons (Radu et al., 2001). CCK-expressing interneurons predominantly display fast-spiking properties, with a smaller subset displaying non-fast-spiking properties (Nguyen et al., 2020). CCK-positive interneuron synaptic transmission is

stronger onto intra-telencephalic (contralateral cortex-projecting PFC neurons) than PAG-projecting pyramidal cells (Liu et al., 2020), indicating that CCK-positive interneurons in the PFC impose inhibitory control of pyramidal output neurons based on output. CCK-positive neurons are also projection neurons. Intersectional genetic approaches reveal CCK-GABAergic cells are more predominant in higher order associative cortices, including both ventral and dorsal aspects of the mPFC, relative to PV-GABAergic cells (Whissell et al., 2015). This suggests there is regional specialization of soma-targeting neurons that utilized CCK as a neuropeptide.

Despite advancements in understanding how CCK-expressing cells are embedded in cortical circuits and use fast inhibitory and excitatory amino acid neurotransmitters to regulate circuit function, there is less known about how these cells use CCK as a peptide transmitter. CCK-immunoreactivity is observed in inhibitory symmetric synapses in the cortex of rodents and non-human primates, suggesting release of CCK along with GABA within the cortex (Hendry et al., 1983). Furthermore, studies delineate that CCK-B receptors are extensively expressed in neocortical pyramidal neurons, and activation of these receptors by their endogenous agonist (CCK) depolarizes and evokes spiking of pyramidal cells (Gallopin et al., 2006). Consistent with this finding, CCK action through CCK-B receptors produce a long-lasting excitation of layer VI neocortical neurons via inhibition of a K^+ leak current (Chung et al., 2009). Since layer VI pyramidal neurons densely innervate thalamic nuclei, these results imply that CCK modulates corticothalamic circuitry. Similar CCK-induced increases in pyramidal cell excitability have also been observed in hippocampal circuits (Dodd and Kelly, 1981; Boden and Hill, 1988). The capacity for CCK to regulate excitability of cells in cortical circuitry, may modify higher level processing and synaptic plasticity. For example, endogenous CCK is released in auditory cortex in response to high frequency stimulation and is necessary for long-term potentiation of excitatory transmission (Chen et al., 2019).

Interestingly, optogenetic inhibition of CCK positive interneurons in the PFC, impaired working memory retrieval in mice (Nguyen et al., 2020). However, it is unclear how the neuropeptide in this cell population may contribute to working memory. Notably, CCK expression and release may be modified by behavioral experiences. For instance, extracellular CCK levels as assessed by micro-dialysis are increased in the frontal cortex of rats in response to restraint stress, the anxiogenic drug yohimbine, and ether (Nevo et al., 1996). Further, rats exposed to a foot-shock stress paradigm show increased CCK-immunoreactivity in the PFC (Siegel et al., 1984). Arousal induced by saline injections is associated with a delayed increase in tissue CCK levels, an effect that is blocked by ketamine pretreatment. A CCK-releasing circuit from the entorhinal cortex to the auditory cortex is critical for associative aversive learning and experience-dependent plasticity (Chen et al., 2019), highlighting the importance of CCK transmission in shaping information processing within cortical circuits and associated behaviors. Additionally, activation of the CCK receptor B by endogenous CCK increases the time mice spend in the open arms of an Elevated Plus Maze behavioral paradigm (Ballaz et al., 2020). This study suggests that CCK and CCK-B receptor drive anxiolytic effects in mice, in addition to aversive learning. Collectively, these studies suggest that CCK, as a peptide transmitter in cortical circuits may be recruited during motivationally charged behaviors to impact circuit function and appropriate PFC-dependent behavior.

Despite the extensive research on CCK and the downstream effects of CCK-A and CCK-B receptor signaling, much remains unknown. It is unclear what the role of CCK originating from GABAergic interneurons versus excitatory neurons is in shaping information processing and behavior. It was previously established that CCK neurons in the amygdala play an important role in modulating fear and anxiety like behaviors, yet the mechanisms of action of CCK in frontal cortical circuits remains unclear (Truitt et al., 2009; Brown et al., 2014; Schmidt et al., 2014). Additionally, CCK has a high degree of homology to gastrin, which shares a common c-terminal sequence (Johnsen, 1998; Baldwin et al., 2010). Like CCK, gastrin signals via CCK-A and CCK-B receptors. In the nervous system, including the cortex, gastrin binds to CCK-B with high affinity (Johnsen, 1998). Immunohistochemistry studies note CCK- and gastrin-positive cells throughout the cortex (Vanderhaeghen et al., 1981), however, it is unclear if gastrin produces analogous effects to CCK, given its high affinity for the CCK-B receptor. Advancements in basic science will be pivotal for determining whether off-label use of CCK ligands in the pipeline for other indications may be considered for treatment of neuropsychiatric disorders. Modulation of CCK has shown anxiolytic effects, enhanced working memory, and influenced motivated behaviors. Thus, CCK serves not only as a promising therapeutic target for psychiatric disorders, but also gives rise to other areas of research.

Somatostatin

The neuropeptide SST was first isolated from hypothalamus and identified as somatotropin-release inhibiting factor (SRIF) (Krulich et al., 1968; Brazeau et al., 1973). There are two active forms of SST derived from the pre-prosomatostatin peptide but differ in amino acid length: SST-14 and SST-28 (Brazeau et al., 1973; Esch et al., 1980; Pradayrol et al., 1980; Schally et al., 1980). Both SST peptides are expressed in the CNS, however the expression and cellular distribution of these two forms varies between cortical and subcortical brain regions (Epelbaum, 1986). SST is stored in large DCVs and released in a calcium-dependent manner (Tapia-Arancibia et al., 1989; Bonanno et al., 1991). SST release from cortical neurons driven by excitatory transmission (Song et al., 2021) and is potentiated by stimulation of other neuropeptides and neuromodulators, such as neurotensin, VIP, and dopamine (Robbins and Landon, 1985; Thal et al., 1986). SST release in in cortical slices has also been documented in response to optogenetic stimulation (Dao et al., 2019). SST release is under the inhibitory control of GABAB receptors, suggesting that SST release is gated by inhibitory neurons as well. Thus, SST release is under bi-directional control in cortical circuits, and is recruited in response to activity and neuromodulation.

Upon release from DCVs, SST binds to its cognate SST receptor (SSTR). Five SSTRs have been cloned and characterized: SSTR1–SSTR5. All SSTRs are $G_{i/o}$-coupled GPCRs and bind both

SST14 and SST-28 with high affinity. SSTR-1–4 exhibit higher binding affinity for SST-14 than SST-28, while SSTR-5 has greater selectivity for SST-28 (Reisine and Bell, 1995; Barnett, 2003). Expression of all five SSTRs has been demonstrated in the cortex, with SSTR1 and SSTR2 as the two most prominent SSTRs in the human and rat cerebral cortices (Dournaud et al., 1996; Bologna and Leroux, 2000). Immunohistochemical analysis of SSTR expression in the somatosensory cortex suggest that SSTRs are differentially localized to different layers (Lukomska et al., 2020). SST activation of SSTRs generally suppresses the release of hormone or neurotransmitter from target neurons by activating a G-protein signaling pathway that inhibits adenylate cyclase and calcium channels (Martel et al., 2012). Cortistatin (CST), a neuropeptide naturally expressed in the cortex, is another endogenous ligand that can bind to SSTR. CST has the same amino acid sequence at the receptor binding site as SST and studies show that CST can active all subtypes of SSTRs with nanomolar affinity to induce similar signaling consequences as SST (de Lecea, 2008; Song et al., 2021). Together, SST and CST act through SSTRs to enact G-protein signaling cascades that regulate neural function.

Somatostatin-expression has been identified in neurons throughout the mammalian brain. In the cerebral cortex, SST is expressed predominantly in a subgroup of GABAergic interneurons. SST-positive GABAergic neurons represent approximately 30% of the total cortical interneuron populations (Rudy et al., 2011; Urban-Ciecko and Barth, 2016). SST interneurons provide dendritic inhibition onto pyramidal neurons to regulate integration of excitatory inputs (Liguz-Lecznar et al., 2016). Further studies reveal SST neurons are heterogenous and show diverse properties in firing pattern, arborization, connectivity, and transcriptome profiles (Fishell and Rudy, 2011; Tasic et al., 2018; Naka et al., 2019) Using a unbiased, large-scale profiling method, Jiang et al. (2015) analyzed that cortical SST neurons consist of three subpopulations: low-threshold or irregular-spiking Martinotti neurons, fast spiking basket cells, and bitufted cells. Thus, multiple types of cells in the cortex have the potential capacity to release SST peptides in addition to GABA.

Electrophysiological studies have demonstrated that SST application results in heterogeneous effects on excitability cortical neurons, with excitation being the most prominent (Olpe et al., 1980; Delfs and Dichter, 1983). Increases in activity in response to SST have also been reported in the hippocampus (Mueller et al., 1986). IPSPs are also inhibited by SST application (Leresche et al., 2000). Recent work has started to dissect how SST may facilitate spiking. SST decreases excitatory synaptic transmission onto PV-expressing interneurons, but not pyramidal neurons, via a presynaptic mechanism (Song et al., 2020). Importantly, this effect was recapitulated by endogenous SST release evoked by optogenetic stimulation of SST neurons, providing a potential mechanism for principal neuron disinhibition. SST-immunoreactivity is observed near GABA release in presynaptic terminals that appose excitatory terminals (Kecskés et al., 2020; Song et al., 2020). This anatomical framework is consistent with a role of SST in regulating excitatory synaptic transmission via a presynaptic site of action. SST may also have direct post-synaptic actions on cortical pyramidal neurons. SST-14 and SST-28 enhance and decrease delayed-rectifier potassium currents in cultured cortical neurons, respectively (Wang et al., 1989). SST signaling via SSTR4 induces hyperpolarization of principal neurons has also been observed in the medial entorhinal cortex (Kecskés et al., 2020), an effect that is more robust in layer III/V than layer II neurons. This is consistent with a role of SSTR4 in retinal ganglion cells in inhibiting L-type Ca^{2+} channels, which enhance intrinsic excitability (Farrell et al., 2014). SST also hyperpolarizes principal neurons in the CA1 of the hippocampus (Mueller et al., 1986). Collectively, these data provide circuit-based mechanisms wherein SST release may regulate information processing in cortical circuits.

Since SST neuropeptide transmission has the potential to influence synaptic transmission and intrinsic excitability in cortical circuits, it is of no surprise that numerous studies have demonstrated that cortical SST interneurons play a critical role in sensory processing, motor control, cognition, and emotion. However, evidence linking SST peptides and their receptor system directly with these cortical functions is limited. Intraventricular (ICV) infusion of SST peptide in rats induced anxiolytic- and antidepressant-like effects in the EPM and forced swim test, respectively (Engin et al., 2008). The anxiolytic effect was recapitulated following ICV infusions of a selective SSTR2 receptor agonist, whereas the antidepressant-like effect was mimicked following infusions of either SSTR2 or SSTR3 agonists (Engin and Treit, 2009). Increasing evidence have shown that heightened brain SST level counteracts stress-induced ACTH, catecholamine, and CRF release, suggesting that SST suppresses stress-induced responses (Brown et al., 1984; Stengel and Tache, 2017). Similar roles of SST neurons in mediating working memory, fear conditioning, and enhancing circuit performance have also been described (Kim et al., 2016; Abbas et al., 2018), yet the role of the of SST neuropeptide in this process is unclear. In the olfactory system, infusion of a SSTR2 agonist into mouse main olfactory bulb (MOB) increases gamma oscillation, synaptic transmission, and enhances odor discrimination performances (Lepousez et al., 2010), suggesting that enhancement of circuit performance may be a feature of SST signaling. Recently, Song et al. (2020) found that SST application in the primary visual cortex (V1) improves visual discrimination in freely moving mice and enhances orientation selectivity of V1 neurons. Further, they demonstrated that SST improves visual perception by enhancing visual gain of V1 neurons via a reduction in excitatory synaptic transmission to PV fast-spiking interneurons but not to regular-spiking neurons (Song et al., 2020). Thus, SST peptide in cortical circuits regulates information processing to shape behaviors subserved by the cortex.

Somatostatin expression in the cortex is impacted in a plethora of neuropsychiatric disorders (Crowley and Kash, 2015; Song et al., 2021). A decrease in SST expression in the cortex is observed in neurodegenerative and psychiatric disorders such as Alzheimer's disease (Davies et al., 1980; Kumar, 2005), Parkinson's disease (Epelbaum, 1986; Iwasawa et al., 2019), Huntington's disease (Rajput et al., 2011), major depressive disorder (Rubinow et al., 1985; Tripp et al., 2011; Lin and Sibille, 2015), bipolar disorder (Fung et al., 2014;

Pantazopoulos et al., 2017), and schizophrenia (Reinikainen et al., 1990; Hoftman et al., 2015). Interestingly, SST neuron activity is necessary for the antidepressant effects of scopolamine (Wohleb et al., 2016), suggesting that decreased activity of SST neurons potentially drives depressive-like behavior. Given preclinical evidence that the brain SST system has profound anxiolytic and anti-depression effects (Crowley and Kash, 2015; Song et al., 2021), this system has therapeutic potential for treating neuropsychiatric disorders. Several synthetic SST analogs have been developed for clinical treatment of endocrine diseases, digestive diseases, and carcinogenic tumor (Gomes-Porras et al., 2020). However, no drugs targeting the SST system are approved or under investigation in clinical trials for the treatment of neurodegenerative and psychiatric disorders, mainly because current SST analogs cannot penetrate the blood–brain barrier (Lamberts et al., 1996). New drug packaging and delivery approaches, as well as gene therapy techniques, will facilitate the development of CNS-targeted SST drugs and genetic treatments.

Despite extensive research on the action of and therapeutic potential of SST and SSTRs, much remains unknown about the role of this system in shaping cortical circuits. Recently, activity dependent cortical SST release via optogenetic stimulation was used providing a potential platform to probe the influence of circuit manipulations and/or behavior on SST release (Dao et al., 2019). Cortical SST neurons are diverse in terms of their molecular profiles, anatomical features, and electrophysiological properties. Considering the distinct firing patterns of SST subpopulations, it is imperative to understand how SST release is fine-tuned by different forms of neuronal activity of diverse types of SST neurons. Furthermore, whether SST acts on different targets and cell compartments differently due to its layer-specific arborization is an unknown. Additionally, other neuron peptides, such as NPY and Dyn, are co-expressed with SST in subpopulations of interneurons (Sohn et al., 2014; Smith et al., 2019) but it is unknown whether SST can be co-released with other peptides from the same site, and how different neuropeptides released from the same neuron shapes cortical signaling processing. Further, CST is also predominantly expressed in cortical GABAergic neurons and often co-expressed with SST (Smith et al., 2019). Additional investigations are needed to understand whether CST and SST peptides that target the same receptors work synergistically, competitively, and/or in parallel in cortex. In summary, the cortical SST system influences cortical information processing. Activation of this system induces anxiolytic and anti-depression effects, and deficits in this system are observed in neurodegenerative and neuropsychiatric disorders. Understanding the nuanced mechanisms by which the cortical SST system shapes cortical information processing and control of behavior will significantly advance the development of new therapeutic treatments for neurological disorders.

Neuropeptide Y

First isolated in 1982 from the porcine hypothalamus, NPY is one of the most widely expressed neuropeptides in the central and peripheral nervous systems (Tatemoto, 1982). NPY consists of 36-amino acid residues and belongs to the family of pancreatic hormone polypeptides (PP). NPY is derived from its 97-amino-acid precursor peptide, pre-pro NPY, into a mature 36 amino-acid NPY1-36, which is cleaved by the enzyme dipeptidyl peptidase 4 to produce NPY3-36 (Aizawa-Abe et al., 2000; Robich et al., 2010; Dvorakova et al., 2014). NPY has widespread cardiovascular, immune, metabolic, and reproductive functions in the peripheral nervous system and regulates neural function underlying feeding, stress-, and addiction-related behaviors (Heilig, 2004; Hirsch and Zukowska, 2012).

Expression of NPY is well conserved across species, including the rat, mouse, and humans across multiple brain regions, such as the hypothalamus, cortical areas, the septum, hippocampus, olfactory bulb, and striatum (Dumont et al., 1992). The widespread effects of NPY are mediated by a family of rhodopsin-like GPCRs: Y1, Y2, Y4, Y5, and Y6. The Y6 receptor subtype is functional in the mouse and rabbit but not in humans and other primates while being absent in the rat (Larhammar and Salaneck, 2004). Notably, there are mixed reports regarding the existence of a Y3 receptor subtype, given that attempts at cloning this receptor have not been successful (Gehlert, 1998; Lee and Miller, 1998; Pedragosa Badia et al., 2013). NPY receptors are predominantly located post-synaptically except for the Y2 receptor, which is pre-synaptically located (Decressac and Barker, 2012). Activating NPY receptors may initiate multiple signaling cascades associated with $G_{i/o}$ proteins (McQuiston and Colmers, 1996; Sah and Geracioti, 2013). Altogether, these studies highlight the need for studying the neuromodulatory role of NPY in regulating cortical physiology and behavior.

Neuropeptide Y is found in layers I, II, V, and VI of the human cortex (Adrian et al., 1983; Chan-Palay et al., 1985; Van Reeth et al., 1987) and highly colocalized with GABA and SST (Sawchenko et al., 1985; Aoki and Pickel, 1989). Although expressed by both pyramidal cells and interneurons, NPY is predominately found in interneurons (Dawbarn et al., 1984; Hendry et al., 1984; Karagiannis et al., 2009) and can be categorized into three main classes: neuroglia form-like neurons, Martinotti-like, and parvalbumin-positive basket cells (Karagiannis et al., 2009). Together, NPY and cognate receptors modulate synaptic excitability and function in cortical areas (Dumont et al., 1992). Specifically, NPY has been shown to reduce the EPSCs and decrease AMPA receptor-mediated glutamatergic neurotransmission onto neocortical pyramidal neurons (McQuiston and Colmers, 1996; Bacci et al., 2002), an effect that has also been observed in other brain regions like the hippocampus (McQuiston and Colmers, 1996; Qian et al., 1997) but not the amygdala (Molosh et al., 2013). Furthermore, NPY also regulates GABAergic neurotransmission onto pyramidal neurons in the neocortex (Bacci et al., 2002), suprachiasmatic nucleus (Chen and Van Den Pol, 1996), thalamus (Sun et al., 2001), but not the hippocampus (Klapstein and Colmers, 1993). Specifically, Bacci et al. (2002) found that NPY produces delayed and long-lasting decreases in EPSCs and increases in IPSCs in pyramidal neocortical neurons, an effect likely due to Ca^{2+}-dependent enhancement of presynaptic GABA release. Furthermore, it was found that NPY also decreased IPSCs in GABAergic interneurons, suggesting NPY may inhibit pyramidal neurons by disinhibition of GABAergic interneurons. Consistent

with the observations that NPY decreases neuronal excitability, functionally, NPY is known to have antiepileptic effects (for a review, see Vezzani and Sperk, 2004) and to provide neurons with protection against cytotoxicity by decreasing microglial reactivity, the release of microglial bioactive factors, NMDA currents and excessive Ca^{2+} entry into neurons (Li et al., 2014). Altogether, these studies suggest that NPY decreases the excitability of cortical circuits.

Neuropeptide Y signaling in the frontal cortex has been linked to affect-related disorders, highlighting the importance of characterizing the neuromodulatory role of NPY for psychiatry. On one hand, reduced NPY activity in cortical circuits has been associated with a plethora psychiatric disorders including anxiety, depression, and PTSD in both preclinical and clinical studies (Zhou et al., 2008; Mickey et al., 2011; Cohen et al., 2012; Melas et al., 2012, 2013; Sah and Geracioti, 2013). Previous work has found low levels of NPY expression in cortical brain regions of humans with a history of depression and death by suicide (Widdowson et al., 1992). Consistent with clinical work, preclinical studies report decreased cortical NPY, and Y1 receptor gene and protein levels expression in animal models of depression (Husum et al., 2001; Jimenez-Vasquez et al., 2007). Indeed, NPY and Y1 receptor agonists have been shown to decrease anxiety-like behavior, fear-suppressed food reinforcement, contextual fear, and social avoidance in rodents (Heilig et al., 1989; Sajdyk et al., 1999; Comeras et al., 2021). However, other reports have shown that activation of the NPY system in the infralimbic cortex impairs the retrieval of extinction in rats (Vollmer et al., 2016). Therefore, NPY-mediated inhibition of cortical circuits may also underlie deficits in fear extinction. Consistent with these findings, a clinical report found a link between impaired recall of extinction memory and reduced vmPFC activation in patients with PTSD (Milad et al., 2009). Together, these studies suggest that NPY modulation of cortical function underlies the etiology affect-related disorders.

Despite a breadth of studies demonstrating that NPY influences alcohol use disorder a few have suggested a link between cortical NPY signaling and alcohol use disorder (Mayfield et al., 2002). Alcohol preferring rats have low levels of NPY in cortical structures (Ehlers et al., 1998) and alcohol withdrawal produces significant reductions in NPY protein in several brain regions, including layers IV and V of the frontal cortex in the rat brain (Roy and Pandey, 2002). Additionally, rats fed an ethanol diet show reduction in NPY-immunoreactivity in the cortex (Bison and Crews, 2003). Previous work suggests that the ability of NPY to decrease alcohol consumption may be in part due to its ability to relieve alcohol withdrawal-induced anxiety (Thorsell and Mathé, 2017). Together, these results suggest that NPY-mediated inhibition of cortical circuits may play a role in the symptomatology observed in mood-and alcohol-related disorders and highlight the potential of NPY-based therapeutics for mental illnesses. Furthermore, the clinical relevance of the NPY system is evident in the clinical interest of NPY-based pharmacotherapies for treating numerous conditions. For example, a recent study suggests that NPY may be an effective treatment for anxiety in patients who have PTSD (Sayed et al., 2018) and intranasally administered NPY may produce rapid antidepressant effects (Mathé et al., 2020). These studies highlight a rich potential in the neuromodulatory role of NPY for the development of pharmacotherapies for treating psychiatric conditions such as anxiety disorders and depression.

In summary, extensive work demonstrates that NPY is a highly conserved neuropeptide involved in disease pathophysiology in the brain. While previous work demonstrates the potential of NPY-based pharmacotherapies for treating psychiatric illnesses, further work is needed to understand the functional effects of NPY signaling and its implications for brain diseases. For example, it is presently known that NPY is expressed within diverse interneuron populations that subserve distinct cortical and behavioral functions (Karagiannis et al., 2009; Smith et al., 2019). However, the role of NPY release from these different interneuron populations on cortical information processing and behavior is unknown. Moreover, NPY is also expressed in glutamatergic neurons of the PFC and it is unclear whether these cell populations release this peptide locally or in distal regions to influence behavior. Lastly, it is unclear whether NPY originating from interneurons and excitatory neurons may cooperate to more efficiently activate all pools of NPY receptor or whether NPY originating from interneurons would bind to sets of NPY receptors that are not accessible to NPY released from excitatory neurons. Understanding how NPY shapes PFC activity will help understand the role of NPY the NPY system in driving behavior under normal and pathological conditions.

Vasoactive Intestinal Peptide

Vasoactive intestinal peptide is a peptide and member of the glucagon/secretin superfamily that signals through VIP receptors 1 and 2 (VPACR 1 and 2). VIP receptors are GPCRs that signal via the G_s signaling pathway. The VIP precursor gene, pre-proVIP, encodes for VIP as well as the peptide histamine isoleucine (PHI) and its human form, peptide histidine methionine (PHM), that both have a lower binding affinity for both VPACRs (Rangon et al., 2005; Igarashi et al., 2011). Different concentrations of VIP may confer differential activation of VPACR 1 and 2. For instance, lower concentrations of VIP (approximately 1 nM) activate VPACR 1/2 and pituitary adenylate cyclase receptor (PAC1-R) in the hippocampus leading to increased NMDA excitatory postsynaptic current amplitudes of CA1 hippocampal neurons (Yang et al., 2009). In contrast, larger concentrations of VIP (100 nM) act solely on the PAC1-R in the hippocampus and are dependent on an increase in Ca^{2+} intracellular levels. Collectively, VIP can regulate cortical function via interactions with VPACRs within cortical circuits.

In the PFC, VIP-positive interneurons are predominately expressed in L1b GABAergic cells expressing the ionotropic serotonin receptor 5HT3a (5HT3aR), as well as cholinergic cells (Tremblay et al., 2016; Anastasiades et al., 2021). Of the 30% of interneurons in the PFC that have 5HT3aR, 40% contain VIP (Tremblay et al., 2016). There are two major subpopulations of VIP interneurons: bipolar and multipolar. Bipolar VIP interneurons are in cortical layers II–VI and heavily concentrated in L2/3 and multipolar VIP cells are populated in the borders of L1/2 and deeper layers (Tremblay et al., 2016). In the cerebral cortex, VIP is radially oriented in bipolar

interneurons with approximately 30% co-localization with GABA and 80% colocalization with acetylcholine (Magistretti, 1990). The mediodorsal thalamus drives VIP-positive interneurons localized in L1b to mediate cortical disinhibition via inhibition of SST-positive cells in L2/3 (Anastasiades et al., 2021). Accordingly, VIP contributes to a disinhibitory microcircuits (Anastasiades et al., 2021). Interestingly, VIP enhances the GABA-mediated inhibition of somatosensory neuron activity (Sessler et al., 1991). It is currently unclear clear how VIP peptide transmission coordinates with GABA-mediated inhibition. Furthermore, although VIP interneurons operate locally in cortical columns within the primary sensory cortex, it is unknown whether this same columnar organization holds true for higher-order cortices, such as the PFC species as functional columnar structures have been delineated in primary sensory cortices and differ across species (Fox, 2018).

Cortical modulation of VIP release is regulated by a variety of ion channels and neurotransmitters. In the cortex, Martin and Magistretti (1989a) revealed that K^+-stimulated VIP release is Ca^{2+}-dependent. In a series of experiments, they discovered that Ni^{2+}, but not Cd^{2+}, nifedipine, diltiazem, or ω-conotoxin, inhibited K^+-evoked release of VIP, implicating T-type, and not L- and N-type, Ca^{2+} channels (Martin and Magistretti, 1989a). These studies suggest that DCVs containing VIP may recruit different pools of voltage-gated calcium channels, and hence different calcium sources, to trigger exocytosis, relative to vesicles containing fast neurotransmitters. Consistent with localization of VIP-positive neurons to superficial layers of the cortex, *in vivo* electrical stimulation of cortical superficial layers evoke VIP release ipsilateral but not contralateral to the stimulation site (Wang et al., 1985). VIP release is under the control of excitation and inhibition. Glutamate and kainic acid, as well as disinhibition with GABA antagonism, increase VIP release in cortical slices (Wang et al., 1986; Magistretti, 1990). Furthermore, VIP release is also sensitive to other neuromodulators known to impact cortical circuit dynamics, including norepinephrine, which decreased basal rates of VIP release (Wang et al., 1986). Conversely, carbachol, a cholinergic agonist increased the spontaneous release of cortical VIP (Magistretti, 1990). This is consistent with studies demonstrating that VIP neurons are recruited by external sources of acetylcholine from regions such as the forebrain (Letzkus et al., 2011). Moreover, since VIP is co-expressed with choline acetyltransferase (ChAT) and non-VIP ChAT neurons are found in cortical layer I, acetylcholine derived for local cortical sources may also regulate release of VIP. The majority of VIP-positive/ChAT-positive interneurons in layer I co-release acetylcholine and GABA. However, in the mPFC, ChAT-containing, VIP-lacking cells also release acetylcholine in L1 (Granger et al., 2020). Contradictory results suggest that endogenous opioid systems may regulate VIP release as topical super fusion of MOR, DOR, and KOR agonists had no effect on the spontaneous release of cortical VIP. Interestingly, naloxone, a competitive opioid antagonist, increased VIP efflux. These results suggest that basal opioid tone may be maximally recruited, occluding agonist effects. Some caveats between the effects of *in vivo* versus *in vitro* administration of DADL, an opioid peptide, on cortical VIP-LI release has been explored. Wang et al., discovered that applying DADL suppressed K^+-stimulated cortical VIP-LI release. In contrast, Micevychi et al. (1984), showed that *in vitro*, cortical application of DADL, had no effect on potassium-evoked VIP-LI release. Lastly, reticular formation stimulation evoked VIP release *in vivo,* while anesthesia suppresses it (Micevychi et al., 1984), suggesting that VIP release may be linked to arousal. Collectively, these studies provide a framework for future studies to dissect the molecular machinery that underlies the release of VIP.

Vasoactive intestinal peptide interneurons within the PFC modulate behavior either through disinhibition or excitation of cortical neurons. Lee et al. (2013), found the primary vibrissa motor cortex (vM1) strongly projects to VIP interneurons that suppress SST cells. This hyperpolarization of the SST cells by the VIP interneurons allows for voluntary whisking behavior (Lee et al., 2013). Further, PFC ChAT VIP interneurons play a role in maintaining attention as ChAT-VIP interneurons directly excite neurons within different layers of the PFC with fast, cholinergic synaptic transmission (Obermayer et al., 2019). VIP-mediated disinhibition that alleviates dendritic inhibition in cortical pyramidal cells and is also associated with fear learning (Letzkus et al., 2011), amplifies visual processing (Keller et al., 2020). It was discovered that VIP disinhibition of ventral hippocampal inputs to the mPFC cause a decrease in prefrontal responses that results in less open-arm avoidance in the elevated plus maze (Lee et al., 2019). Additionally, a decrease in GCaMP signaling during exploration predicted approaches to the open arms and concluded this circuit helped predict exploratory behavior rather than avoidance (Lee et al., 2019). Together, it is evident that VIP regulates various neural functions.

Although, information regarding the function of VIP interneurons in cortical processing and behavior has continued to accrue in recent years, there is still little known about the effects of VIP release in the PFC. There is limited understanding of the effects of VIP on synaptic transmission or intrinsic excitability of distinct cortical cell types. Given that VIP neurons have unique localization and connectivity patterns within cortical circuits, it is of interest to determine how peptidergic VIP transmission may impact inhibitory interneurons that VIP neurons target for disinhibition versus VIP-sensitive excitatory and inhibitory neurons. VIP has also been implicated in mediating glycogenolysis (Martin and Magistretti, 1989b). VIP's action increases cAMP levels to convert glycogen to glucose 1-phosphate, shifting energy homeostasis through interactions with neurons and glia (Magistretti et al., 1986; Magistretti, 1990). Interestingly, PHI/M, have shown a similar effect of cortical glycogenesis, with lower potencies, implying a role for this mechanism within peptides that share significant similar VIP homology (Magistretti et al., 1986; Magistretti, 1990). Overall, these studies suggest VIP regulates energy substrate accessibility within cortical circuits. VIP's contribution to energy homeostasis in the cortex may further be supported by its role in vasodilation. The role of VIP's effects on cortical microvessels has yet to determined. Further, it is unclear if VIP arising cortically released

pools or circulating cerebrospinal fluid near superficial layers where VIP neurons reside contribute to cortical vasodilation (Magistretti, 1990). A recent study demonstrated that optogenetic VIP interneuron stimulation resulted in vasodilation and this effect was not due to release of acetylcholine (Granger et al., 2016, 2020), which raises the possibility that VIP or another substance released from this interneuron population may influence vasodilation. Therefore, studies determining the role of VIP in vasodilation, glycogenolysis, and effects within different cortical layers will provide further knowledge about the neuromodulatory actions of this peptide.

NOVEL APPROACHES TO STUDY NEUROPEPTIDES

Although it is assumed that neuropeptide release modulates PFC processing, the cellular and circuit-based framework by which this occurs is in large part lacking. Many unknowns regarding the consequences of neuropeptide release and subsequent GPCR action in cortical circuits remain. Much of this gap in knowledge stems from limited tools available to dissect the function of neuropeptide transmission in behavior and cortical function. Fortunately, in recent years there has been a rapid expansion of approaches available to study neuropeptide transmission. Here, we address various novel tools that have been developed to facilitate addressing these unknowns (**Figure 2**).

Dissecting Peptide and Receptor Anatomy

To obtain a deep understanding of the role of neuropeptidergic transmission in shaping cortical circuit function, it is imperative to understand how neuropeptide-expressing cells and the incoming inputs and local circuit cells sense this information via neuropeptide receptors. To investigate the anatomy of cells expressing various neuropeptides and cognate receptors, transgenic and viral approaches are increasingly becoming indispensable tools (**Figure 2A**). The ever-increasing availability of Cre-driver lines, that express Cre-recombinases in the presence of a selected neuropeptide or neuropeptide receptor, grant genetic access to cell types that release neuropeptides and contain neuropeptide receptors. Specifically, viruses expressing fluorophores in a Cre-dependent manner can be used in tandem with Cre-driver lines to visualize distribution of cells that putatively express specific neuropeptides or cognate receptors within cortical circuits, as well as their terminals throughout the brain. The advent of intersectional genetic strategies will permit researchers to gain genetic access to sub-populations of neuropeptide and/or GPCR-expressing cells (Fenno et al., 2014, 2020). This is of particular relevance given that neuropeptides and their cognate GPCRs are often expressed on different types of molecularly defined cells. In cases where validated antibodies or radiolabeled/fluorescent ligand exists it is possible to integrate these approaches with aforementioned viral and genetic approaches to dissect how neuropeptides or GPCRs embed themselves into local and long-range circuits. There are various important considerations and potential pitfalls when using genetic and viral approaches that must be addressed with appropriate controls and validation studies, including but not limited to, ectopic expression observed with germline or developmental expression of the recombinase that traces lineage cells that may transiently have neuropeptide or receptor promoter activity, incomplete genetic penetrance to cell types of interest, or Cre-independent transgene expression (Song and Palmiter, 2018; Botterill et al., 2021). Moreover, recombination of Cre or Flpo-dependent transgenes inherently is binary and does not reflect variations in neuromodulator expression levels. This is of importance when working with a new genetic line, virus, or even different brain regions. Additionally, the ever-expanding published single-cell RNA-seq datasets providing transcriptional profiles of individual cortical cell types and the neuropeptides and GPCRs found therein (Tasic et al., 2018; Smith et al., 2019; Krienen et al., 2020), will be useful resources for developing specific hypotheses relating to neuropeptide action in PFC circuits. Spatial transcriptomics including multiplexed *in situ* hybridization, *in situ* sequencing, and spatial barcoding will be able capture the spatial distribution of various cell populations expressing different neuropeptides and cognate receptors and provide an anatomical context to substantiate scRNA-seq studies (Longo et al., 2021). With increased capabilities to look at many neuropeptides and GPCRs, spatial transcriptomics will begin to unravel the "neuropeptidome" of cortical circuits (Smith et al., 2019). It should be noted that expression of mRNA does not confer functional neuropeptide or GPCR protein expression, highlighting caveats of these aforementioned approaches. Moreover, scRNA-seq experiments may also fail to identify neuropeptide or receptors whose mRNA is expressed in low abundance or localized away from the peri-nuclear space/soma, but is still of physiological relevance. Recently, mice expressing fluorescently labeled opioid receptors have been developed, which allows for mapping of receptors within sub-compartments or projections in defined cell types when coupled with anatomical tracing methods or when molecularly defined cells are tagged (Ehrlich et al., 2019; Chen C. et al., 2020). Careful characterization of novel lines with tagged neuropeptide receptors is necessary to ensure that expression or function of the targeted molecule is not impacted. For example, a thorough characterization of KOR-tdTomato fusion protein mice demonstrated that these mice have increase KOR mRNA expression and binding relative to controls (Chen C. et al., 2020), highlighting the importance of validation experiments and associated considerations as was done in the former study. Notwithstanding this approach will be useful for elucidating endogenous neuropeptide receptor localization and trafficking. Taken together, when used in tandem with physiological, biochemical, and functional anatomical approaches, these approaches provide valuable insight to neuropeptide mRNA expression in diverse cell types and anatomical architecture within tissues.

Monitoring Neuropeptide Release

Historically, studies examining peptide release have been limited to acute brain slices or synaptosomal preparations where

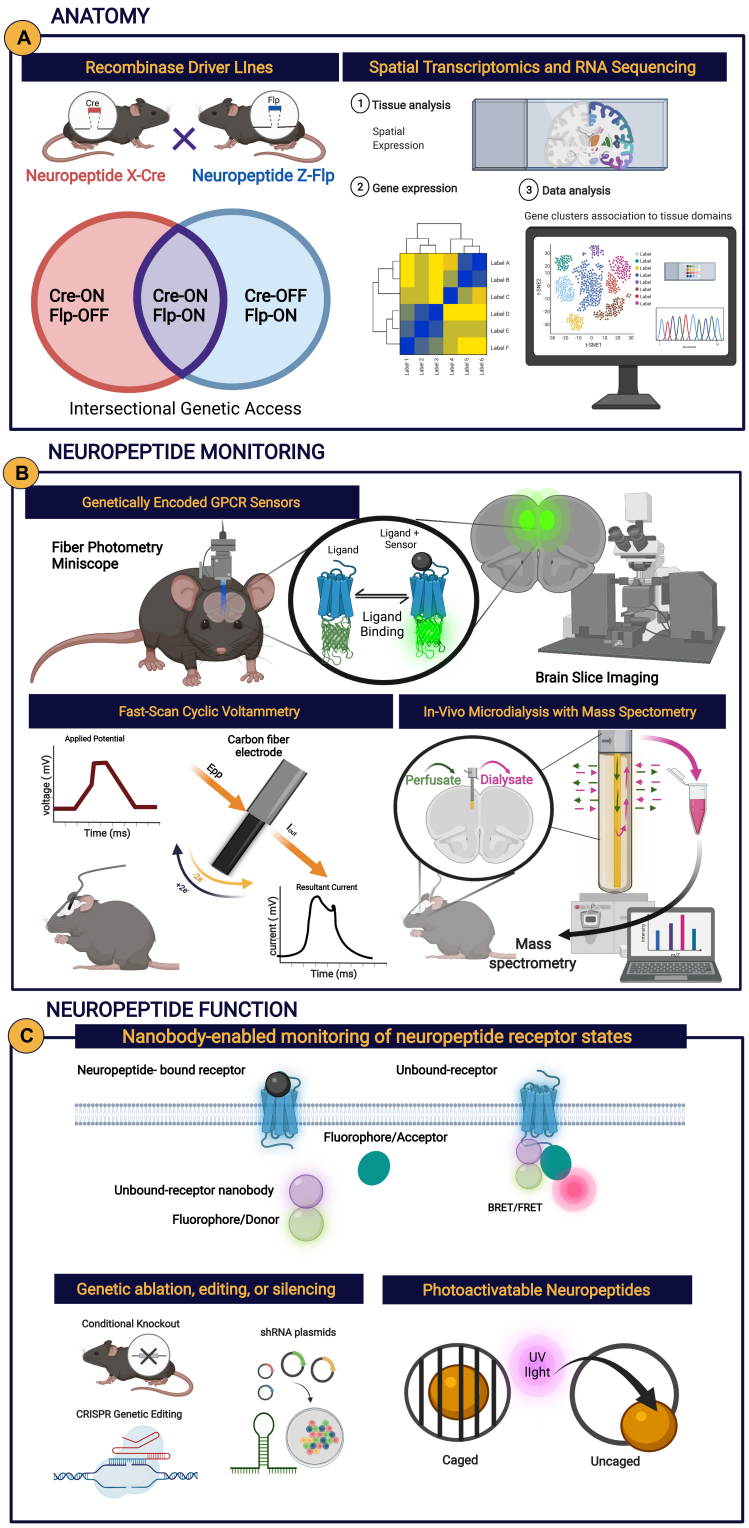

FIGURE 2 | (A) Anatomical methods in the study of neuropeptides include recombinase driver lines used to gain genetic access to neuropeptide or receptor-expressing cells, spatial transcriptomics, and RNA sequencing. **(B)** Neuropeptide monitoring methods include genetically encoded GPCR sensors in conjunction with recording methods (e.g., fiber photometry and miniscope), fast-scan cyclic voltammetry (inspired by Roberts and Sombers, 2018), and *in vivo* microdialysis with mass spectrometry. **(C)** Methods to study neuropeptide function include nanobody enabled monitoring of neuropeptide receptor states (inspired by Che et al., 2020), genetic modifications (e.g., genetic ablation, editing, or slicing), and the use of photoactivatable neuropeptides. Any of the aforementioned novel approaches can be implemented with established methodologies, such as electrophysiology.

radioimmunoassays and ELISAs have been used to monitor peptide levels in the media. At best, microdialysis procedures have been used to measure fluctuations in extracellular neuropeptide levels in behaving animals. Although these techniques have led to significant advancements in the field, they have low temporal and spatial resolution or are restricted to *in vitro* conditions. To address these limitations, novel techniques with increased sampling or analytical sensitivity have arisen (**Figure 2B**). Specifically, fluorescent neuromodulator sensors (Wang et al., 2018; Patriarchi et al., 2019; Peng et al., 2020; Dong et al., 2021; Duffet et al., 2022) provide a means to detect fluctuations in neuropeptide release presence in real time in awake, behaving animals and map sub-cellular sites of neuropeptide release when coupled to high resolution live cell/acute brain slice image. A caveat of genetically encoded fluorescent sensors is that detection of events may be limited to large fluctuations in neuropeptide/receptor activity if the affinity of the neuropeptide for the sensor is not sufficiently high. Additionally, sensors with a very high affinity may display decreased dynamic range or buffer endogenous neuropeptides from acting on their cognate receptors. Lastly, since neuropeptide receptors may bind more than one neuropeptide with high affinity, including unidentified "off-target" actions, it is possible that fluorescent receptor sensor activity may not reflect activity of the neuropeptide of interest. Moreover, electrochemical sensors capitalizing on oxidation/reduction of Tyr in opioid peptides have been employed to monitor putative enkephalin (Roberts and Sombers, 2018). Appropriate controls demonstrating that signals can be detected from endogenously released neuropeptides and the absence of signal upon genetic ablation or inhibition of neuropeptide-expressing neurons will be essential in elucidating the substrates driving fluorescent or electrochemical sensor activity. Neuromodulator receptor-based fluorescent sensors will be useful not only for monitoring neuropeptide transmission in awake-behaving mice but will also be useful for understanding how neuropeptide transmission fits within nuanced architecture and layering of PFC circuits in acute slices and large fields of view *in vivo* with one or two photon imaging. Further, with increasing palettes of genetically encoded calcium and neurotransmitter sensors, future studies will be able to examine how neuropeptide dynamics fluctuate in relation to neuronal activity or other neurotransmitters. This will provide a comprehensive picture of how sensor activity relates to neuropeptide producing cells or receptor expressing cells. *In vivo* microdialysis coupled with mass spectrometry allow for simultaneous detection of various extracellular neuropeptides in addition to fast neurotransmitters and monoamines (DiFeliceantonio et al., 2012; Kennedy, 2013; Al-Hasani et al., 2018). As analytical approaches improve, detection of various species of neuropeptides and their metabolic byproducts will provide insight to the actual molecules present in the extra-cellular space and their metabolism. An added benefit is that microdialysis allows for local drug infusion at the sampling site permitting functional pharmacological or chemogenetic studies to be easily integrated. Ultimately, the development of novel techniques to monitor neuropeptide release will provide a platform to monitor cortical neuropeptide dynamics in freely moving behaving animals and closely link peptide dynamics to discrete aspects of behavior in well-designed studies.

Mapping Receptor Function Within Circuits

Although the presence of GPCRs within cortical circuits could previously be identified with histological and *in situ* hybridization approaches, these approaches do not lend information to GPCR action and kinetics and physiological effects post neuropeptide binding. To identify how neuropeptide GPCRs regulate circuit function, photoactivatable neuropeptides, nanobodies, and advanced pharmacological and immunohistochemical tools are a few of the techniques used to date (**Figure 2C**). Advancements aimed at increasing specificity and efficacy of pharmacological tools, such as agonists and antagonists of neuropeptide targets, will facilitate work aimed at uncovering the role of neuropeptide action in the PFC. Cell-specific pharmacological approaches are rapidly developing that will aid in the dissection of neuropeptide control of cortical circuitry (Yang et al., 2015; Shields et al., 2017). These approaches provide a significant advantage over conventional pharmacological approaches, which do not discriminate between receptors in distinct circuit elements, in that they provide a means to concentrate or permit the selective gating of ligands to molecularly defined neurons or based on connectivity. Photoactivatable neuropeptides provide a means to determine the kinetics of neuropeptide and GPCR signaling as well as map sub-cellular distribution (Yang et al., 2015). When coupled to multi-photon or focal one-photon uncaging they also uncover spatiotemporally precise actions of GPCRs in distinct compartments of neurons. Genetic methods such as knock-in mouse lines expressing GPCRs fused with fluorescent markers reveal internalization of receptors following behavioral or pharmacological paradigms (Ehrlich et al., 2019; Chen C. et al., 2020). Furthermore, conditional knock-out of neuropeptide or receptor genes from cell populations using Cre-LoxP systems (Kim et al., 2018), short hairpin RNA (Moore et al., 2010), or CRISPR-Cas9 (Ran et al., 2013) will allow manipulation of peptide and receptor expression within cortical circuits. Given that neuropeptides are likely embedded into distinct cell types within PFC circuits that may differ based on molecular definition or connectivity, then expanding availability of recombinase driver lines or complementation of viral approaches will permit for dissection of neuropeptides and their receptors in specific cell types or pathways into or out of the PFC. Finally, changes in GPCR confirmational states in response to activation or deactivation can be studied with various techniques. For instance, nanobodies also can reveal GPCR confirmational states. For example, nanobodies that stabilize distinct ligand-delimited GPCR conformations can report real-time ligand-stabilized GPCR states (Che et al., 2020) and genetically encoded biosensors derived from specific nanobodies can provide precise spatial and temporal resolution of GPCR activation and deactivation (Stoeber et al., 2018). It has been shown that agonist binding induces KOR phosphorylation (Appleyard et al., 1997; Li et al., 2002) and immunohistochemistry can be used to report receptor phosphorylation as a measure of

neuropeptide-mediated receptor activation (McLaughlin et al., 2004; Lemos et al., 2011; Abraham et al., 2021).

Collectively, there has been a rapid expansion of tools to dissect the function of neuropeptides. Ideal studies would use the most appropriate tools needed to address specific questions of how neuropeptides and their receptors relate to circuit function and/or behavior, keeping in mind strengths and caveats associated with each approach when designing studies and selecting tools to employ. In practice, dissection of the role of neuropeptides and their receptors in PFC circuits will require the use of multiple converging, complementary approaches, and inclusion of controls and validation studies, where appropriate. When coupled to cutting-edge *ex vivo* and *in vivo* electrophysiological, anatomical, *in vivo* imaging, and well-designed behavioral studies, these ensembles of emerging tools will be crucial for significant advances in our understanding the role of neuropeptides in intercellular communication in cortical circuits.

CONCLUSION

In this review we provide a comprehensive examination and discussion of the literature on neuropeptide modulation of cortical circuitry, with an emphasis on the PFC. To date, knowledge of neuropeptide action is largely limited to subcortical brain structures. Expanding the field's knowledge on neuropeptide action in the PFC and how neuropeptide systems regulate information processing in cortical circuits provides paths to develop therapeutic targets for the treatment of neuropsychiatric disorders. Further, increased knowledge PFC organization and function will provide a greater context for future findings on neuropeptide function. Together, investigating PFC networks and neuropeptide regulation of those circuits while capitalizing on novel approaches will help elucidate contributing factors in neuropsychiatric disorders and novel treatments for these disorders.

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All authors wrote, edited, and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

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