NEUROPEPTIDES AND BEHAVIOUR: FROM MOTIVATION TO PSYCHOPATHOLOGY

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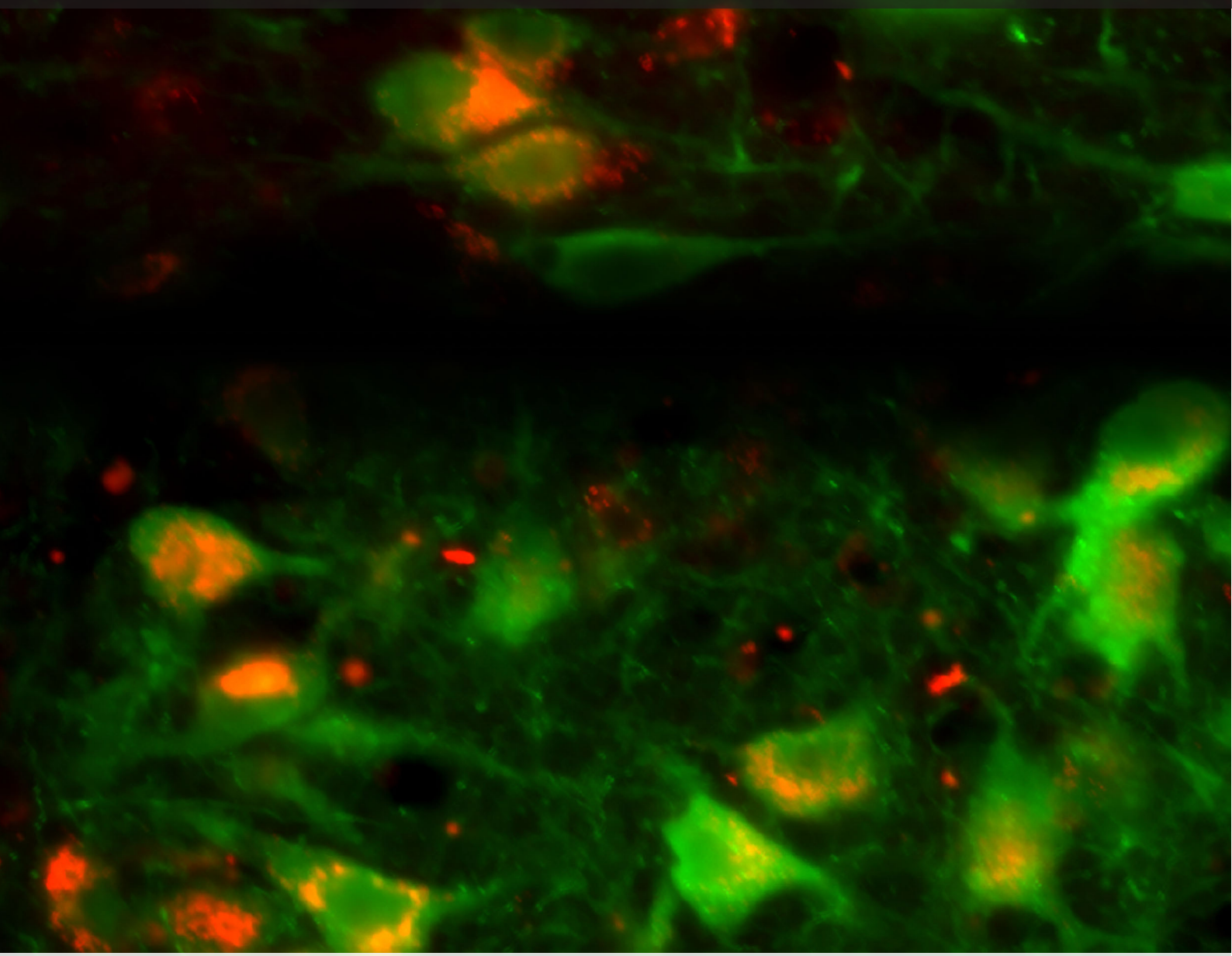

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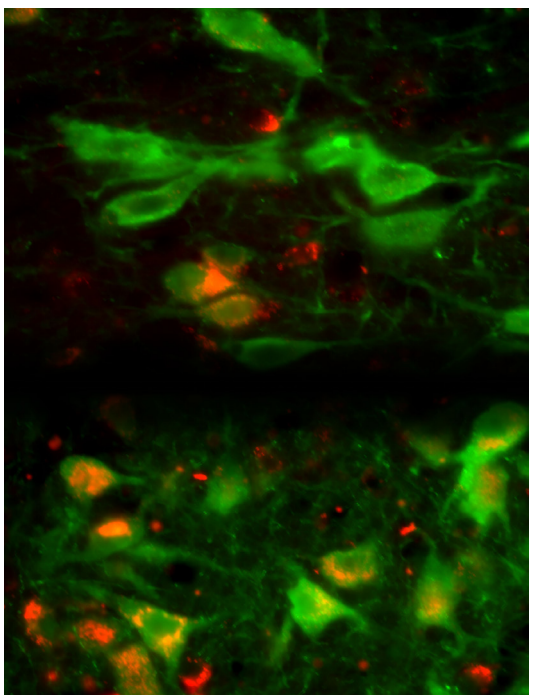
NEUROPEPTIDES AND BEHAVIOUR: FROM MOTIVATION TO PSYCHOPATHOLOGY

Topic Editors:
Deborah Suchecki, Universidade Federal de São Paulo, Brazil
Carol F. Elias, University of Michigan, Ann Arbor, United States



MCH conjugated with rhodamine is internalized in the dorsal raphe neurons of the cat. The photomicrographs show that neurons with serotonin immunoreactivity (green) internalized MCH conjugated with rhodamine (red). Also, it is readily observed that MCH-rhodamine is present in small-sized non-serotonergic neurons. This data strongly suggest that MCH receptors are present in serotonergic and non-serotonergic neurons

Image adapted from: Torterolo P, Scorza C, Lagos P, Urbanavicius J, Benedetto L, Pascovich C, López-Hill X, Chase MH and Monti JM (2015) Melanin-Concentrating Hormone (MCH): Role in REM Sleep and Depression. Front. Neurosci. 9:475. doi: 10.3389/fnins.2015.00475

The discovery of the involvement of neuropeptides with behaviours other than regulatory motivated ones took place in the midst of 1960's with David de Wied's first report on the influence of pituitary peptides on memory. This major scientific breakthrough opened a new frontier of studies in Endocrinology and its related fields, Neuroendocrinology and Psychoneuroendocrinology.

Neuropeptides were initially thought to be involved in homeostatic regulation and secreted only from neurons located in the hypothalamus; they are now recognized neurotransmitters, produced in and secreted from distinct brain areas, associated with a myriad of, not only, motivated, but also psychopathological behaviours. Motivated behaviours are determinant for individual and species survival, but their expression in a large spectrum and deviations from average may give rise to a number of psychiatric conditions.

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EDITORIAL
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Editorial: Neuropeptides and Behavior: From Motivation to Psychopathology

Deborah Suchecki[1]* *and Carol F. Elias*[2]

[1] *Departamento de Psicobiologia, Escola Paulista de Medicina, Universidade Federal de São Paulo, São Paulo, Brazil,*
[2] *Department of Molecular and Integrative Physiology, University of Michigan, Ann Arbor, MI, United States*

Keywords: neuropeptides, behavior, psychiatric disorders, sex differences, neuronal plasticity

Editorial on the Research Topic

Neuropeptides and Behavior: From Motivation to Psychopathology

The discovery of the involvement of neuropeptides with behaviors other than regulatory motivated ones took place in the mid-1960s with David de Wied's first report on the influence of stress hormones, such as ACTH and arginine vasopressin (AVP) infused intra-cerebroventricularly, on memory (1). This major scientific breakthrough opened a new frontier of studies in Endocrinology and its related fields, Neuroendocrinology and Psychoneuroendocrinology.

Neuropeptides were thought to be involved in homeostatic regulation and secreted only from hypothalamic neurons; they are now recognized neurotransmitters, produced in, and secreted from distinct brain areas, associated with a myriad of, not only motivated but also psychopathological behaviors (2). Motivated behaviors are determinant for individual and species survival, but their expression in a large spectrum and deviations from average may give rise to a number of psychiatric conditions.

This research topic brings together 3 original studies and 10 review papers on the regulatory role of neuropeptides on sleep, feeding-, maternal-, and social behaviors. In addition, the implication of neuropeptides on both ends of this spectrum—lack of or excessive motivation—will also be discussed, as more and more, changes in neuropeptide production such as neuropeptide Y (NPY), oxytocin (OT), corticotropin-releasing hormone/factor (CRH/CRF), orexin, melanin-concentrating hormone (MCH), prolactin, and opioids have been associated with depression, anxiety, drug addiction, eating, and social disorders.

Pomrenze and colleagues used a transgenic rat that expresses CRH immunoreactive neurons, and they were able to show that this rat is a useful tool to study CRH projections, mainly from the central nucleus of the amygdala and the bed nucleus of the *stria terminalis*. CRH is the primary mediator of the hypothalamic–pituitary–adrenal axis stress response, subjected to plasticity and adaptations in cases of chronic stress exposure. Herman and Tasker presented a review of the mechanisms involved in the adaptations of the stress response at the level of the paraventricular nucleus of the hypothalamus, including the enhancement of CRH and AVP production, reduced glucocorticoid negative feedback activity, and increased excitatory neurotransmission at the CRH neurons. These converging alterations lead to increased CRH activity, characteristic of a number of stress-related psychiatric disorders.

In recent years, dysregulation of extra-hypothalamic CRF systems has gained attention in the context of alcohol use disorders. Quadros and colleagues presented a comprehensive review on the role of CRF and urocortin systems (and their related elements), in alcohol-induced escalated

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Hubert Vaudry,
University of Rouen, France

***Correspondence:**
Deborah Suchecki
deborah.suchecki@gmail.com

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aggression, and in the transition from alcohol use to dependence, due to the direct modulation of CRF on the brain reward system.

In mice, chronic administration of low doses of ethanol produce a stimulatory action, inducing a phenomenon known as behavioral sensitization, which reflects neurobiological modifications in the reward system. Kawakami and co-workers showed that expression of behavioral sensitization can be blocked by naltrexone, an antagonist of the μ and, to a lesser extent, of the κ opioid receptors, in a sex-dependent manner, with greater efficiency in males than in females.

Keil and colleagues explored the involvement of the cyclic AMP/protein kinase A (cAMP/PKA) signaling pathway in anxiety and depressive disorders, by means of amygdala hyperactivation induced by stressful conditions. PKA is involved in the regulation of a myriad of intracellular signals triggered by distinct neurotransmitter systems related to alertness, mood, and acute and social anxiety. This mechanism of plasticity could be important for the proposition of new therapeutic strategies for conditions that afflict the human population.

Stress recruits numerous neuromodulators and neuropeptides that provide optimal coping conditions and adaptive responses. One such response is increased sleep time, especially of REM sleep, after a stressful event. Machado and Suchecki reviewed the neuropeptidergic and hormonal mechanisms involved in sleep induced by stressful situations. In Wellman and coworker's original article, escapable shock, a model of controllable stress, increased REM sleep, whereas inescapable shock reduces this sleep stage. CRF blocked this positive response to controllable stress, whereas astressin, a CRF receptor 1 antagonist, restored sleep to control levels.

Melanin-concentrating hormone was the focus of Torterolo and colleagues' review with special emphasis on recent electrophysiological recordings and optogenetic stimulation. The authors argued that the greatest firing rate of MCHergic neurons occurred during REM sleep and remote stimulation of those neurons induced sleep. The authors also focused on MCH projections to the dorsal raphe nucleus and the potential role of MCH in the pathophysiology of depression.

Neuropeptide Y and its receptors have a key role in central control of energy homeostasis, sleep, circadian rhythm, memory, and neuronal plasticity. Thorsell and Mathé presented an overview of NPY action on anxiety and stress response, and potentially post-traumatic stress disorder and depression. The authors discussed genetic and epigenetic findings of importance for NPY function and regulation and proposed that the modulation of NPY-ergic activity within the brain constitute potential targets for intervention in affective and alcohol use disorders.

Circulating hormones are also key players in motivation and behavioral adaptations. Among the most versatile of them is prolactin (PRL). Torner highlighted the role of PRL in neurogenesis and stress responses in the male brain. The author showed strong argument supporting the hypothesis that alterations in the PRL system due to stress or exposure to substances or other conditions that reduce neurogenesis may contribute to maladaptive responses and pathological behavioral outcomes. The author discussed the effects of PRL on neurogenesis and neuroprotection and their potential contribution to the onset of psychopathological states such as depression.

Oxytocin is a neuropeptide synthesized primarily by neurons of the paraventricular and supraoptic nuclei of the hypothalamus. These neurons are part of the magnocellular neurosecretory system, releasing OT into the posterior pituitary to promote labor and lactation. However, OT neurons also project to other brain sites and regulate homeostatic processes, social recognition, and fear conditioning. OT also decreases neuroendocrine stress signaling pathway, anxiety, and depression-like behaviors. Acevedo-Rodriguez and colleagues presented data showing that steroid hormones differentially modulated stress responses and altered OT receptor expression. The authors emphasized the role of estrogen receptor β activation and suggested a role for OT in this estrogen receptor β-mediated anxiolytic effect.

The function of neuropeptides and hormones is modulated by neuronal plasticity, synaptic strength, and morphological changes. Cabral and colleagues focused on the importance of mechanisms associated with the permeability of the blood brain barrier (BBB) and the interplay among delicate anatomical interfaces that have the potential to alter physiology and behavior. Authors used ghrelin, a hormone produced by the stomach with important role in energy homeostasis and motivated behaviors. Ghrelin receptors are widespread in the brain, but the accessibility of the hormone is strikingly low. This neuroendocrine issue is discussed in light of the dynamic control of the BBB.

Another aspect discussed in this issue is the crucial role of synaptic strength and interaction in physiology and behavior. Woelfle and colleagues discussed the effect of TCAP (short amino acid sequence found in the distal extracellular tip of teneurin) and the LPHN ligand–receptor system in anxiety, stress, and mood disorders. The teneurin TCAP protein binds and activates the LPHNs, a family of adhesion-associated GPCRs, to regulate numerous neurological and physiological activities. The long-lasting effect of exogenous TCAP and the current molecular model of ligand–receptor interaction suggest a role in neuronal plasticity and behavioral modulation.

Understanding the interplay between neuropeptides and hormones in the control of motivation and behavioral responses is highly relevant for human's physical and mental health. Clearly, research in this field is advancing at a rapid pace. The articles in this eBook highlight novel findings and unanswered questions for future investigation.

AUTHOR CONTRIBUTIONS

DS and CE participated as editor of 5 papers, each, of the 13 published in this Research Topic. They jointly wrote the present editorial.

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METHODS
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A Transgenic Rat for Investigating the Anatomy and Function of Corticotrophin Releasing Factor Circuits

Matthew B. Pomrenze[1†], E. Zayra Millan[2†], F. Woodward Hopf[2], Ronald Keiflin[2], Rajani Maiya[1], Angelo Blasio[1], Jahan Dadgar[1,2], Viktor Kharazia[2], Giordano De Guglielmo[3], Elena Crawford[3], Patricia H. Janak[2], Olivier George[3], Kenner C. Rice[4] and Robert O. Messing[1,2]*

[1] Division of Pharmacology and Toxicology, College of Pharmacy, The University of Texas at Austin, Austin, TX, USA, [2] Department of Neurology, University of California, San Francisco, San Francisco, CA, USA, [3] Committee on The Neurobiology of Addictive Disorders, The Scripps Research Institute, La Jolla, CA, USA, [4] Chemical Biology Research Branch, Drug Design and Synthesis Section, National Institute on Drug Abuse, National Institute on Alcohol Abuse and Alcoholism, Rockville, MD, USA

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Edited by:
Deborah Suchecki, Universidade Federal de São Paulo, Brazil

Reviewed by:
Andreas Stengel, Charité Universitätsmedizin Berlin, Germany
Sherie Ma, The Florey Institute of Neuroscience and Mental Health, Australia

***Correspondence:**
Robert O. Messing
romessing@austin.utexas.edu

† These authors have contributed equally to this work.

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Corticotrophin-releasing factor (CRF) is a 41 amino acid neuropeptide that coordinates adaptive responses to stress. CRF projections from neurons in the central nucleus of the amygdala (CeA) to the brainstem are of particular interest for their role in motivated behavior. To directly examine the anatomy and function of CRF neurons, we generated a BAC transgenic *Crh*-Cre rat in which bacterial Cre recombinase is expressed from the *Crh* promoter. Using Cre-dependent reporters, we found that Cre expressing neurons in these rats are immunoreactive for CRF and are clustered in the lateral CeA (CeL) and the oval nucleus of the BNST. We detected major projections from CeA CRF neurons to parabrachial nuclei and the locus coeruleus, dorsal and ventral BNST, and more minor projections to lateral portions of the substantia nigra, ventral tegmental area, and lateral hypothalamus. Optogenetic stimulation of CeA CRF neurons evoked GABA-ergic responses in 11% of non-CRF neurons in the medial CeA (CeM) and 44% of non-CRF neurons in the CeL. Chemogenetic stimulation of CeA CRF neurons induced Fos in a similar proportion of non-CRF CeM neurons but a smaller proportion of non-CRF CeL neurons. The CRF1 receptor antagonist R121919 reduced this Fos induction by two-thirds in these regions. These results indicate that CeL CRF neurons provide both local inhibitory GABA and excitatory CRF signals to other CeA neurons, and demonstrate the value of the *Crh*-Cre rat as a tool for studying circuit function and physiology of CRF neurons.

Keywords: Cre recombinase, CRF, channelrhodopsin-2, designer receptors exclusively activated by designer drugs, transgenic rat models, central amygdala, Fos, R121919

INTRODUCTION

Corticotrophin-releasing factor (CRF) is a central regulator of endocrine, autonomic, and behavioral responses to stressors (Koob, 2009). Although CRF cell bodies are distributed in several brain regions, they are particularly concentrated in the central amygdala (CeA),

the bed nucleus of the stria terminalis (BNST), and the paraventricular hypothalamic nucleus (PVN; Wang et al., 2011). In the PVN CRF acts as a hormone to regulate the hypothalamic-pituitary-adrenal (HPA) axis and trigger the endocrine stress response (Rivier and Vale, 1983). Outside of the PVN CRF modulates synaptic transmission within specific circuits of the central nervous system (Gallagher et al., 2008). CRF neurons of the CeA are of particular interest, since they contribute to stress-related arousal, conditioned fear, and negative emotional states associated with drug withdrawal (Koob, 2009; Walker et al., 2009; Kravets et al., 2015).

In the rat, the CeA subpopulation that expresses CRF resides in the lateral CeA (CeL) where another, mostly non-overlapping subpopulation expresses enkephalin (Veinante et al., 1997; Day et al., 1999). Approximately 60% of CeL CRF neurons are also immunoreactive for dynorphin (Marchant et al., 2007). Anatomical studies have shown strong projections from the CeL as a whole to the medial CeA (CeM), the brainstem (parabrachial nucleus, reticular formation, locus coeruleus, nucleus of the solitary tract and dorsal vagal complex) and the BNST, with more modest projections to the lateral hypothalamus, lateral one-third of the substantia nigra pars compacta and an adjacent lateral part of the retrorubral field (Petrovich and Swanson, 1997; Zahm et al., 1999; Bourgeais et al., 2001; Dong et al., 2001). For CeL CRF neurons in particular, tract-tracing studies have identified CRF projections from the rat CeA to the locus coeruleus (Van Bockstaele et al., 1998; Reyes et al., 2011), parabrachial nuclei (Moga and Gray, 1985), the midbrain central gray (Gray and Magnuson, 1992), the dorsal vagal complex [including the nucleus tractus solitarius (NTS)] (Gray and Magnuson, 1987), the pontine reticular nucleus (Fendt et al., 1997), the mesencephalic trigeminal nucleus (Sakanaka et al., 1986), and the BNST (Sakanaka et al., 1986). Whether CeL CRF neurons also project locally within the CeA is not clear, and although some CRF immunoreactive fibers have been observed in the CeM (Veening et al., 1984), their source and functional significance are not known. Several recent studies have helped clarify CRF architectures and functions using *Crh*-Cre mouse lines (Gafford et al., 2012, 2014; Wamsteeker Cusulin et al., 2013; McCall et al., 2015), but thorough characterization of CRF circuits across brain structures, and moreover across species, is still lacking.

Here, we describe a transgenic *Crh*-Cre rat that permits genetic access to CRF neurons, thereby allowing direct investigation of their anatomy and roles in physiology and behavior. To examine CRF cell localization and projection targets, we crossed *Crh*-Cre rats with a DsRed2/GFP-reporter rat, or infected the CeA with AAVs that express Cre-dependent mCherry, channelrhodopsin (ChR2)-eYFP, or hM3Dq-mCherry. We found that Cre-expressing CeA neurons are immunoreactive for CRF and project to several brain regions in the brainstem and diencephalon. Using the *Crh*-Cre rat to investigate CeL circuitry, we provide new evidence that CRF-expressing CeL neurons act as local interneurons to provide both inhibitory and excitatory signals to the CeL and CeM.

MATERIALS AND METHODS

Development of *Crh*-Cre Rats

All animal studies were approved by the Institutional Animal Use and Care Committees of the Ernest Gallo Clinic and Research Center at the University of California San Francisco, the Scripps Research Institute and of The University of Texas at Austin, and were performed in adherence with the NIH Guide for Care and Use of Laboratory Animals. Studies utilized male and female *Crh*-Cre rats.

We identified the BAC clone CH230-206D8 from the CHORI-230 Rat (BN/SsNHsd/MCW) BAC library, which was derived from an inbred female brown Norway rat (Osoegawa et al., 2004), as containing the promoter region and exons 1 and 2 of the rat *Crh* gene on chromosome 2. The BAC clone has ∼80 kb 3′ of the *Crh* ATG and ∼143 kb of DNA 5′ of the ATG. BAC recombineering was performed as described (Cotta-de-Almeida et al., 2003) with vectors and bacterial host cell lines kindly provided by Dr. Scott Snapper (Harvard Medical School).

A ∼2.7 Kb modified/enhanced Cre metallothionein-1 polyadenaylation (CREM) fragment was PCR amplified from the plasmid p210 pCMV-CREM (Addgene # 8395; Kaczmarczyk and Green, 2001). This fragment contains a modified human beta-globin intron within the Cre coding sequence to prevent expression of Cre recombinase in prokaryotes, thereby making it suitable for interim work in bacteria with plasmids containing loxP sites. The fragment was sub-cloned into the conditional replicon vector pBSB-171 and confirmed by sequencing. The plasmid pBSB-171 allows the cloning of the fragments of interest and contains a floxed aminoglycoside kinase (aph) gene cassette. A c-myc tag (EQKLISEEDL) was inserted (QuikChange II XL Site Directed Mutagenesis kit, Agilent Technologies) immediately before the Cre "stop" codon, and confirmed by sequencing. The completed construct is referred to as CREM-myc pBSB-171.

We designed a forward PCR primer (P1) containing 58 bp homologous to the rat *Crh* sequence immediately adjacent to the ATG of CRF followed by 31 bp of CREM-myc pBSB-171 vector sequence, and a backward PCR primer (P2) containing 23 bp of CREM-pBSB-171 vector sequence followed by 64 bp homologous to the sequence immediately adjacent to the *Crh* stop codon. We then amplified a fragment containing the CREM-myc-floxed aph cassette with rat Crh homology ends by PCR with CREM-myc pBSB-171 and primers P1, P2. Lambda red-driven recombination between this PCR product and the BAC clone CH230-206D8 generated recombinants in which the endogenous Crh coding sequence was replaced with the CREM-myc-floxed aph fragment.

This recombineered CH230-206D8 BAC was transformed with the bacterial Cre expression plasmid 706-Cre;tet (Gene Bridges Gmbh, Heidelberg, Germany) to remove the floxed aph cassette. The recombineered circular BAC DNA without the aph cassette was purified (Bimboim and Doly, 1979) using a NucleoBond BAC 100 kit (Clontech # 740579). This DNA was sent to The University of Michigan Transgenic Animal Model Core for pronuclear injection into Hdr:W1 ES cells and implantation (Filipiak and Saunders, 2006). Rat-tail DNA from

resulting progeny was purified using DNeasy (Qiagen # 69506), screened by PCR, and confirmed by sequencing to identify a total of three founder transgenic rats. Cre-expressing cells were identified by crossing *Crh*-Cre rats with the reporter rat line W-Tg(CAG-DsRed2/GFP) 15Jms (NBRP-Rat Number 0282), which was obtained from the National BioResource Project-Rat in Kyoto, Japan. The reporter rat has a DsRed coding region flanked by LoxP sites followed by a GFP sequence, all under control of a CAG promoter. Cre recombination leads to excision of the DsRed coding region and expression of GFP.

Surgery and Histology

We microinjected 0.8–1.2 μL/side (100 nL/min) of one of the following: AAV-Ef1α-DIO-eYFP, AAV-Ef1α-DIO-ChR2-eYFP (Zhang et al., 2010), AAV-hSyn-DIO-mCherry (UNC Vector Core, Chapel Hill, NC), AAV-hSyn-DIO-hM3Dq-mCherry, or AAV-hSyn-DIO-hM4Di-mCherry (Krashes et al., 2011). Coordinates for the CeA were AP 2.40, ML ± 4.85, DV −8.40 from the skull in adult rats, or AP −2.0, ML ±4.3, DV −7.9 from the skull in adolescent rats weighing 200–220 g. Coordinates for the BNST were AP +0.00, ML ±3.5, DV −6.8 with a 16° angle in adolescent rats weighing 200–220 g. After injection, we waited 10 min for virus to diffuse into the tissue before retracting the injector needle. We used adolescent rats in several experiments to facilitate transduction down axons for efficient labeling of neuronal projections. After 2–4 months, rats were deeply anesthetized with sodium pentobarbital (100 mg/kg, ip) and perfused transcardially with phosphate buffered saline (PBS) followed by 4% paraformaldehyde in PBS. Brains were immediately removed, placed into the same fixative overnight, and then transferred to a 30% sucrose solution at 4°C before sectioning at 40 μm on a cryostat.

We detected co-localization of eYFP or mCherry fluorescence with CRF, prodynorphin, preproenkephalin, somatostatin, protein kinase C delta (PKCδ), or Fos immunoreactivity using immunofluorescent histochemistry. Sections were washed three times in PBS with 0.2% Triton X-100 (PBST) for 10 min at room temperature and then incubated in blocking solution made of PBST with 3% normal donkey serum (Jackson ImmunoResearch, number 017-000-121) or normal goat serum (Jackson ImmunoResearch, number 005-000-121) for 1 h. Sections were then incubated in one or more of the following primary antibodies: rabbit anti-cFos (1:2000, Santa Cruz Biotechnology, sc-52), goat anti-cFos (1:2000, Santa Cruz Biotechnology, sc-52-G), mouse anti-tyrosine hydroxylase (TH; 1:2000, Immunostar, 22941), mouse anti-tryptophan hydroxylase (TPH; 1:1000, Sigma Aldrich, T0678), goat anti-CRF (1:500-1000, Santa Cruz Biotechnology, sc-1761 Lot# B0315), guinea pig anti-prodynorphin (1:500, Neuromics, GP10110), rabbit anti-preproenkephalin (1:100, Neuromics, RA14124), or rabbit anti-PKCδ (1:2000, Santa Cruz Biotechnology, sc-213) with or without mouse anti-NeuN (1:2000, Millipore, MAB377 clone A60) in blocking solution rotating at 4°C for 18–20 h. After three 10-min washes in PBST, sections were incubated in species-specific secondary antibodies Alexa Fluor 488, 568, or 647 (1:700, Thermo-Fisher Scientific, A-21206, A11067, A-11055, A-21202, A- 21208, A-11073, A-21447, A-31573) in blocking solution for 1 h at room temperature. Finally, sections were washed four times in PBS, then mounted in 0.2% gelatin water onto SuperFrost Plus glass slides (Fisherbrand, 12-550-15) and coverslipped with Fluoromount-G (Southern Biotech, 0100-01). Slides were stored in the dark before microscopy and image acquisition.

For somatostatin staining, sections were pretreated with 50% ethanol twice for 10 min each and washed three times in PBS and then blocked in 10% normal donkey serum at room temperature for 10 min. The sections were then incubated with rat anti-somatostatin antibody (Millipore, MAB354) diluted 1:100 in PBS containing 0.05% Triton-X-100 and incubated for 20 h at 4°C with shaking. Sections were washed for 10 min three times in PBS and then incubated with 2% NDS for 10 min. Primary antibody staining was visualized by incubating with Alexa Fluor 488-conjugated anti-rat secondary antibody (1:700 dilution in PBS) for 2 h. Sections were washed four times in PBS and prepared for imaging as described above.

Peroxidase immunohistochemistry was performed using 3,3′-diaminobenzidine tetrahydrochloride (DAB). Sections were first washed for 30 min each in 0.1 M sodium phosphate buffer, pH 7.2 (PB), followed sequentially by 50% ethanol, 50% ethanol with 3% H_2O_2, and then 5% normal donkey serum (NDS) in PB. Sections were then incubated in mouse antiserum against GFP (1:1500, Invitrogen or 1:1000, Abcam) in PB with 2% NDS and 0.2% Triton X-10 (PBTX; 48 h at 4°C). The sections were then washed with PB and incubated in PBTX containing biotinylated donkey anti-mouse IgG (1:1000, Jackson Immunoresearch Laboratories) for 24 h at 4°C. Finally, sections were washed in PB, incubated with peroxidase-conjugated avidin (ExtrAvidin, Sigma-Aldrich) in PB (1:2500; 2 h at RT), washed again, and then incubated in DAB (ImmPACT DAB, Vector Laboratories). Sections were then mounted with PB containing 1% gelatin, dehydrated, cleared in xylene and coverslipped with DEPEX mounting medium (Electron Microscopy Sciences).

Confocal Acquisition and 3D Analysis

Three-dimensional stacks of Images were acquired with a 780 Laser Scanning Confocal microscope (Zeiss, Inc.) using either a 20x (1 μm image slice), 40x (0.6 μm image slice), or 63x (0.2 μm image slice) objective. The system is equipped with a stitching stage and Zen software to reintegrate the tiled image stacks. Stitched z series images of the entire CeA were imported into Imaris software (Bitplane-Andor, Inc.) for quantitation. The eYFP (green), and the CRF-A568 fluorescent labels (red) were first three dimensionally traced using the iso-surfacing module to obtain a clean outline of the neuronal cell body and branches, which was then rendered solid using a control based threshold. The isosurfaced eYFP signal was then used to create a new channel to determine how much red signal was present within green iso-surfaced regions. This assay was further corroborated using the colocalization module to confirm the extent and location of overlapping signals. The filament tracer module was used to identify the origin of disjointed axons and outline the neural branches of the same neuron. An alternative analysis of green fluorescent signals within red iso-surfaced neurons was performed for comparison. This use of multiple methods of

analysis allowed us to quantify the location and extent of CRF-like immunoreactivity throughout eYFP positive cell bodies and axons. This approach was used on 1–3 sections per rat from five rats.

Cell Counting

Immunostained sections were imaged on a Zeiss 710 LSM confocal microscope, Zeiss Imager M2 microscope, or a Zeiss Axio Zoom stereomicroscope. Quantification of Fos and co-localization of -mCherry or eYFP with neuropeptides in the CeA were performed on alternate sections from Bregma −1.90 to −3.00 (6–12 sections per rat) using Fiji (Schindelin et al., 2012).

Electrophysiology and Optogenetics

To measure ChR2-evoked GABA IPSCs, we expressed ChR2-eYFP in Cre-expressing neurons and recorded light-evoked IPSCs as in recent work (Seif et al., 2013), with the following exceptions: rats were perfused intracardially with a glycerol-based aCSF (in mM: 252 glycerol, 2.5 KCl, 1.25 NaH_2PO_4, 1 $MgCl_2$, 2 $CaCl_2$, 25 $NaHCO_3$, 1 L-ascorbate, and 11 glucose), and then brain slices were cut in the same solution. A CsCl internal solution (CsCl, 135; HEPES, 10; MgATP, 4; GTP, 0.3; $MgCl_2$, 2; EGTA, 0.5 and QX-314, 5; pH 7.23, Naylor et al., 2010), with DNQX in the bath to block AMPARs, was used for measuring GABA IPSCs.

To distinguish small, evoked IPSCs from spontaneous IPSCs (sIPSCs), we recorded ∼20 traces where a ChR2-eYFP+ terminal was stimulated once with blue light at 111 ms into a 1000 msec sweep. The sIPSC frequency was typically low (0.67 ± 0.13 Hz), and thus the likelihood of observing a spontaneous IPSC exactly at the time of ChR2 stimulation in more than a few of the 20 traces was very low. If an IPSC was observed at the time of ChR2 stimulation in only one or two of the 20 traces in a given cell, we did not consider this a cell responding to ChR2 stimulation. Of note, sIPSCs displayed variability in amplitude within the same cell as reported by others (Delaney and Sah, 2001), making the relative amplitude of evoked vs. spontaneous IPSCs a less reliable measure. For spatial mapping, we used live visualization of the electrode tip and its exact location within the CeA instead of biocytin filling. Had we used biocytin in these experiments, there would have been many neurons filled within the same slice, including neurons where patch-clamping was attempted for several minutes but failed to achieve stable recording.

Chemogenetics and Fos Mapping

Crh-Cre rats were microinjected bilaterally with AAV-hSyn-DIO-hM3Dq-mCherry, AAV-hSyn-DIO-hM4Di-mCherry, or AAV-hSyn-DIO-mCherry into the CeL. After 2–4 months, rats were administered intraperitoneally 2 mg/kg clozapine-N-oxide (CNO; NIMH Chemical Synthesis and Drug Supply Program) and perfused 120 min later for Fos immunohistochemistry. To inhibit CRF1 receptors, we administered 10 mg/kg R121919 (Chen et al., 2004) subcutaneously to rats 30 min before administration of CNO.

Data Analysis

Data are shown as mean ± SEM values and were analyzed by two-tailed *t*-tests or by ANOVA with *post-hoc* Tukey's tests using GraphPad Prism v6.0.

RESULTS

Neurons that Express Cre in *Crh*-Cre Rats are Immunoreactive for CRF

Cre-expressing cells in *Crh*-Cre rats were first identified by crossing *Crh*-Cre rats with W-Tg(AG-DsRed2/GFP)15Jms reporter rats and then immunostaining brain sections from bigenic progeny with anti-GFP antibody. There were clusters of immunoreactive neurons in the CeL and in the dorsolateral BNST (**Figures 1A,B**). We confirmed the presence of Cre activity in mature CeL and BNST neurons by microinjecting AAV-hSyn-DIO-mCherry into the central amygdala or the dorsal BNST of 6 week-old rats and then examining brain slices for the presence of mCherry fluorescence 8 weeks later (**Figures 1C,D**). Surprisingly we did not detect Cre recombination in the ventral BNST or in the paraventricular hypothalamic nucleus, even following microinjection of a large volume (1.2 µl) of Cre-dependent AAV into the hypothalamus.

To determine if Cre-expressing neurons also express CRF, we microinjected colchicine (4 µg in 0.8 µL) into the CeA 4 weeks after injection of AAV-Ef1α-DIO-eYFP and 72 h prior to perfusion to allow CRF to accumulate in the cell soma (Merchenthaler, 1984). We found that 94.6 ± 1.2% eYFP+ neurons were immunoreactive for CRF, while 77.1 ± 2.1% of CRF immunoreactive neurons expressed eYFP (**Figure 1E**; $n = 3$ rats, 12 amygdala sections/rat).

We also examined colocalization of ChR2-eYFP and CRF immunoreactivity in *Crh*-Cre rats microinjected with AAV-Ef1α-DIO-ChR2-eYFP and not treated with colchicine. ChR2-eYFP was present in the cell membrane of neuronal cell bodies and processes while CRF immunoreactivity was mainly scattered within neural processes. Because CRF is mainly localized in neural processes, quantification of colocalization in cell bodies using bright-field microscopy at 40x resulted in only a small percentage (16.5 ± 2.7%) of eYFP+ neurons being colocalized with CRF. However, confocal analysis at 63x followed by 3D reconstruction of the neuronal cell bodies and branches using Imaris 3D software revealed that all eYFP+ neurons contained CRF immunoreactivity in the cell soma or branches (**Figure 1F**). Out of 155 neurons analyzed, 100 ± 0% of eYFP + neurons were positive for CRF, while 99.4 ± 0.6% of CRF+ neurons were positive for eYFP.

Using hSyn-DIO-mCherry to identify CRF neurons, we examined co-expression of other neuropeptides in the CeL (**Figure 2**). We found that about 54.2 ± 2.8% of CRF neurons were immunoreactive for dynorphin while there was almost no colocalization with enkephalin, as described previously (Veinante et al., 1997; Day et al., 1999; Marchant et al., 2007). A population of neurons in the CeL expresses somatostatin (SOM),

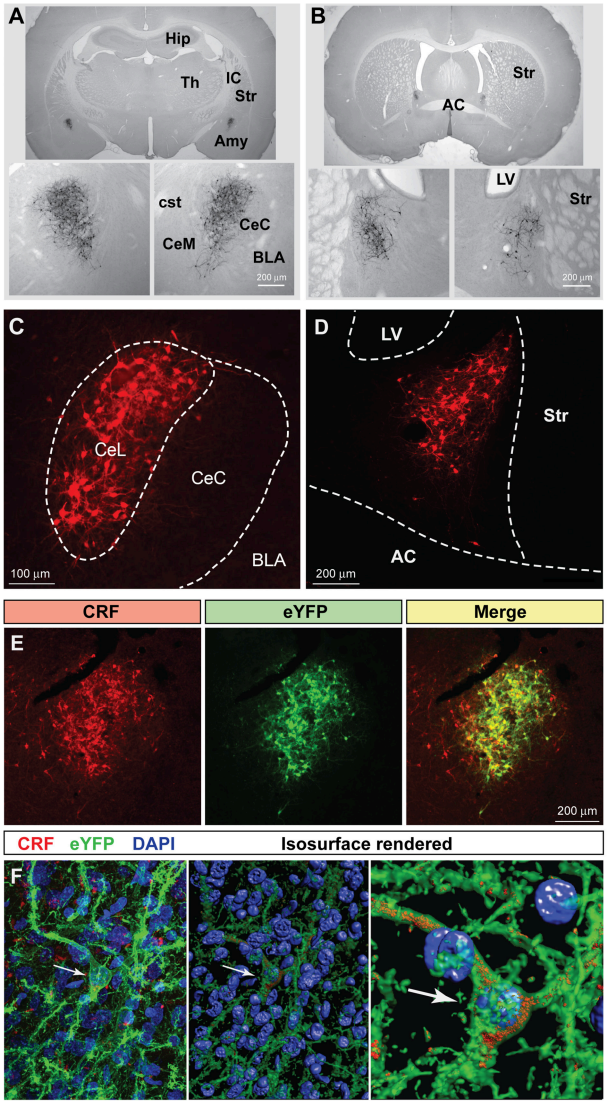

FIGURE 1 | *Crh*-Cre rats express Cre recombinase activity in the CeL and dlBNST. (A,B) Bigenic progeny of a *Crh*-Cre X DsRed2/GFP cross display robust GFP labeling in the CeL and dlBNST. Scale bars, 200 μm. **(C,D)** Cre-dependent mCherry expression in Cre-expressing neurons of the CeL and dlBNST. Scale bars, 100 μm in panel **(C)**; 200 μm in panel **(D)**. **(E)** Cre-dependent eYFP co-localizes with CRF immunoreactivity in the CeL. Scale bar, 200 μm. **(F)** Rendered isosurface analysis demonstrates co-localization of CRF immunoreactivity within CeL neurons that also express Cre-dependent eYFP. Arrows point to an example of eYFP and CRF in the same neuron. AC, anterior commissure; AMY, amygdala; BLA, basolateral amygdala; CeC, capsular central amygdala; CeM, medial central amygdala; cst, commissural stria terminalis; Hip, Hippocampus; IC, internal capsule; LV, lateral ventricle; Str, Striatum; Th, Thalamus.

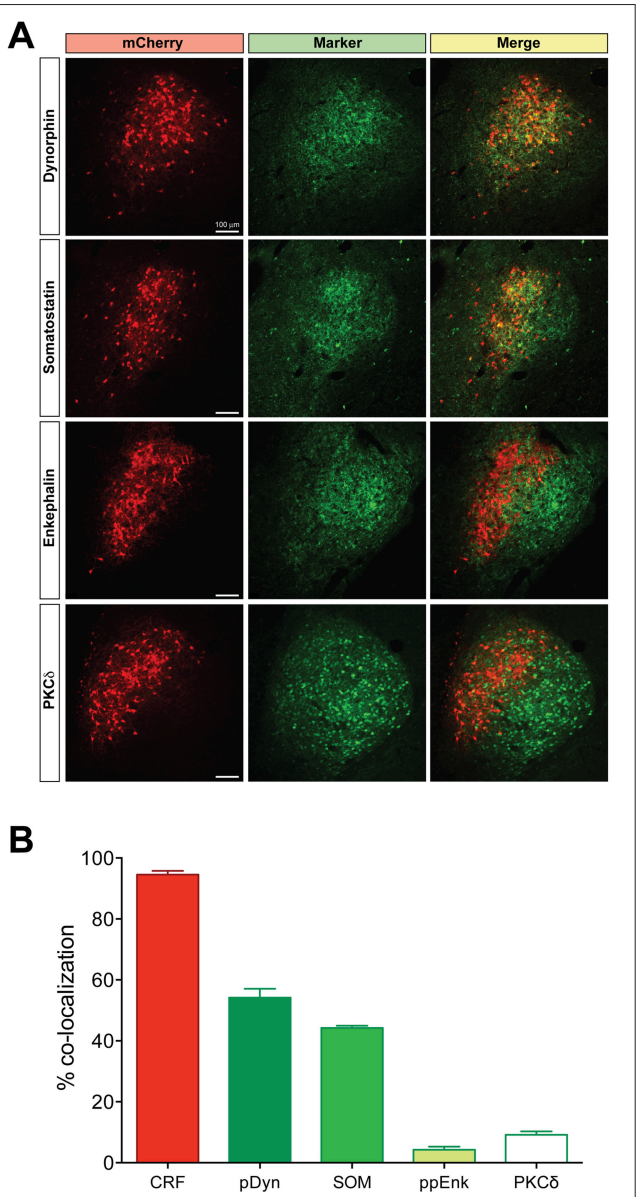

FIGURE 2 | Coexpression of other neuropeptides in CeL CRF neurons. (A) A large percentage of CRF neurons identified by expression of Cre-dependent mCherry are immunoreactive for dynorphin and somatostatin, while few express enkephalin or PKCδ. Scale bars, 100 μm. Medial is to the left. **(B)** Quantification of mCherry expression with neuropeptide immunoreactivity; CRF $n = 3$ rats, 12 amygdala sections/rat; dynorphin $n = 4$ rats, 6 amygdala sections/rat; somatostatin $n = 4$ rats, 6 amygdala sections/rat; enkephalin $n = 4$ rats, 6 amygdala sections/rat; PKCδ, $n = 6$ rats, 10–12 amygdala sections/rat.

and recent studies demonstrate an active role for CeL SOM+ neurons in conditioned fear in mice (Li et al., 2013; Penzo et al., 2015). We determined that approximately 44.2 ± 0.7% of CeL CRF neurons co-localize with SOM+ neurons. In addition to SOM+ neurons, there is a distinct GABA-ergic subpopulation of CeL neurons in mice that expresses protein kinase C delta (PKCδ), but not CRF, and suppresses fear conditioning (Ciocchi et al., 2010; Haubensak et al., 2010). We similarly found that CRF and PKCδ are present in distinct populations in the rat amygdala with only approximately 9.2 ± 1.1% of CRF neurons co-expressing PKCδ. Also, CRF-expressing cells were consistently more medial than PKCδ-expressing cells in the CeL (**Figure 2A**).

Projections from CeL CRF Neurons Outside the CeA

We examined neuronal projections from CeL CRF neurons using mCherry or ChR2-eYFP as a histological marker (**Figures 3–6**). We detected projections to several regions identified previously in nonselective tract tracing studies of the CeL (Petrovich and Swanson, 1997; Veinante and Freund-Mercier, 1998; Zahm et al., 1999; Bourgeais et al., 2001; Dong et al., 2001). The largest and densest were to the PBN and the LC (**Figure 3**). Fibers were present in both the lateral and medial PBN and in the mesencephalic trigeminal nucleus, and extended caudally within the medial PBN to the LC. CRF fibers there appeared to be interdigitated and orthogonal to the dorsolateral LC dendritic field (**Figure 3D**).

We also observed a substantial projection from the CeL to the dorsolateral and especially the ventral BNST (**Figures 4A–C**). Dorsolateral CRF fibers appeared to cluster around the oval nucleus and also extend into the adjacent dorsal striatum. In addition, a small projection was detected slightly ventrolateral to the ventral BNST in the substantia innominata and ventral pallidum (**Figure 4C**). Caudal to the BNST, CRF projections were present in the most lateral portion of the lateral hypothalamus (LH) along its entire anterior-posterior axis traveling through the nigrostriatal bundle (**Figure 4D**).

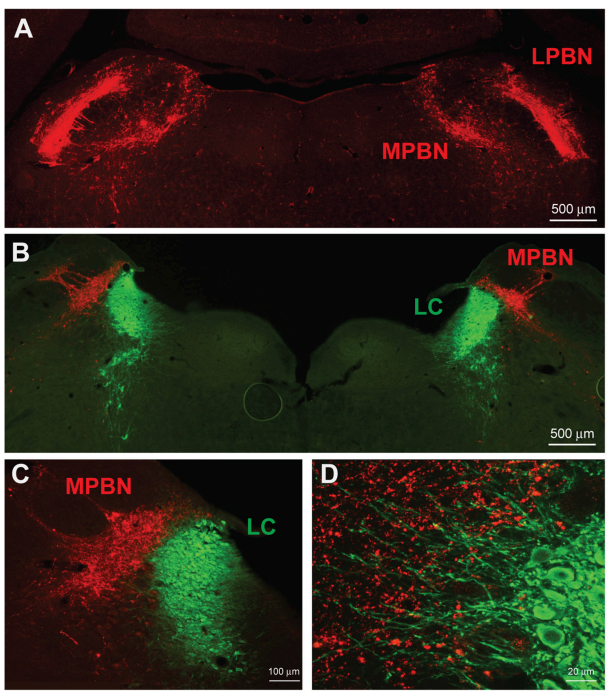

FIGURE 3 | CeL CRF neurons project strongly to brainstem nuclei. (A) After injection of AAV-hSyn-DIO-mCherry into the CeL, mCherry expressing fibers were detected in the lateral and medial parabrachial nuclei (Bregma −9.0). Scale bar, 500 µm. **(B)** mCherry expressing fibers were also detected in the medial parabrachial nucleus just lateral to the locus coeruleus (Bregma -9.6). Red, mCherry; Green, Tyrosine Hydroxylase. Scale bar, 500 µm. **(C,D)** High-magnification examples of mCherry fibers from the CeL and noradrenergic LC neurons. CeL fibers appear to run orthogonally to noradrenergic dendrites extending laterally from the LC core into the medial parabrachial nucleus. Scale bars, 100 µm in panel **(C)**; 20 µm in panel **(D)**. MPBN, medial parabrachial nucleus; LPBN, lateral parabrachial nucleus; LC, locus coeruleus.

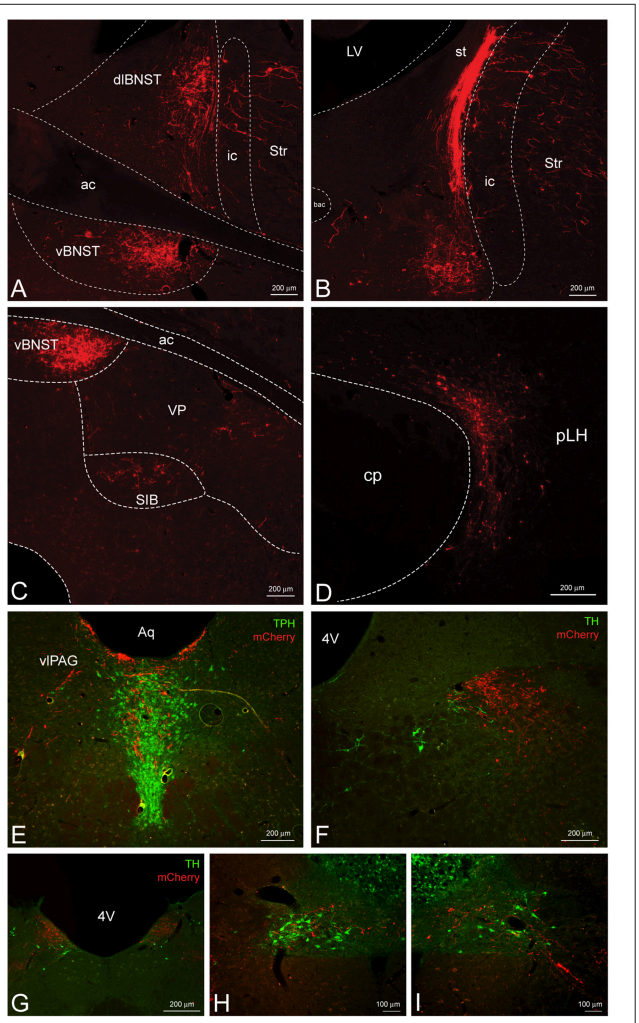

FIGURE 4 | CeL CRF neurons provide inputs to other limbic brain structures. (A,B) A dense bundle of mCherry expressing fibers from the CeL were observed in the dorsolateral and ventral BNST. **(A)** Rostrally, fibers clustered mainly in the oval nucleus of the dorsal bed nucleus and in the subcommisural zone of the ventral bed nucleus (Bregma −0.12). **(B)** Caudally, dense fibers of the stria terminalis were present in the dorsal region (Bregma −0.6). Scale bars, 200 µm. **(C)** Less dense projections were detected ventral and lateral to the ventral BNST in the substantia innominata and the ventral pallidum (Bregma −0.12). Scale bar, 200 µm. **(D)** Fibers were detected throughout the lateral hypothalamus (Bregma -4.20) within the nigrostriatal bundle. Scale bar, 200 µm. **(E)** Fibers also projected dorsomedially into the caudal dorsal raphe nucleus and ventrolateral periaqueductal gray (Bregma −7.7). TPH, tryptophan hydroxylase. Scale bar, 200 µm. **(F–I)** Some fibers projected as far as the nucleus tractus solitarius where they came in close contact to noradrenergic processes and cell bodies in the most caudal regions. Bregma (−12.9) – (−14.0). TH, tyrosine hydroxylase. Scale bars, 200 µm in panel **(F)**; 200 µm in panel **(G)**; 100 µm in panel **(H)**; 100 µm in panel **(I)**. ac, anterior commissure; ic, internal capsule; Str, striatum; st, stria terminalis; VP, ventral pallidum; SIB, substantia innominata; cp, cerebral peduncle; pLH, posterior lateral hypothalamus; Aq, central aqueduct; vlPAG, ventrolateral periaqueductal gray; 4V, fourth ventricle.

Most of these appeared to be fibers of passage with small projections extending medially into the LH. Caudal to the hypothalamus, we observed CRF fibers coursing into the ventrolateral periaqueductal gray, and eventually into the caudal aspect of the serotonergic dorsal raphe nucleus (**Figure 4E**). Deep in the brainstem caudal to the LC, we detected a small projection to the nucleus tractus solitarius (NTS) throughout much of its anterior-posterior axis (**Figures 4F–I**). At the most anterior aspect, CRF fibers were localized to the lateral NTS and overlapped with tyrosine hydroxylase positive processes but not somata (**Figure 4F**). Further posterior, CRF fibers clustered within the medial NTS as it coursed toward the 4th ventricle (**Figure 4G**), and fibers eventually terminated in the caudal ventrolateral NTS around noradrenergic cell bodies (**Figures 4H,I**).

CRF signaling in the dopaminergic ventral tegmental area (VTA) has garnered much attention due to its significant role in relapse to drug seeking (Shalev et al., 2010). However, the source of CRF in the VTA has remained controversial (Grieder et al., 2014; Zhao-Shea et al., 2015). CRF fibers from the CeL were present traveling through the dorsolateral substantia nigra pars compacta, most likely as fibers of passage on their way to the brainstem (**Figures 5A–D**). However, upon closer examination we detected minor collateral projections within the rostral VTA (**Figure 5E**).

Projections from CeL CRF Neurons within the CeA

Since the CeL sends dense projections to the CeM (Petrovich and Swanson, 1997), we investigated whether CRF expressing CeL neurons contribute to these projections. Surprisingly, following microinjection of AAV-hSyn-DIO-mCherry into the CeA, we detected very few mCherry-expressing projections from the CeL to the adjacent CeM (**Figures 1C,6A**). We next used AAV-Ef1α-DIO-ChR2-eYFP to express ChR2-eYFP in CeL CRF neurons (**Figure 6B**) and to detect light-evoked inhibitory postsynaptic currents (IPSCs) in CeA neurons that did not express ChR2-eYFP. To compare data across animals and brain slices, we mapped the spatial position of each recorded neuron to a common reference frame. First, we established a scale using the intermediate capsule between the BLA and CeA as a guide. We defined the distance between where the intermediate capsule meets the external capsule and the ventral border of the BLA as equal to 100 relative units (**Figure 6A**). We then mapped the position of each cell onto a common Cartesian coordinate system with the Y axis parallel to the intermediate capsule and the origin at the most ventral part of the ovoid cluster of CeL CRF cell bodies (**Figures 6A,E**). The position of each neuron was expressed as relative units along both axes. To assess the accuracy of this method, we mapped the position and size of the CRF cell body cluster in slices from 12 rats. We found that the coordinates of points defined by the intersection of the oval border of the CeL CRF cell body cluster with its maximal dorsal-ventral and medial-lateral diameters were consistent across slices (**Figure 6E**). The borders of the CeL CRF cell body cluster were also consistent when expressed in relative units using the bottom of the intermediate capsule as the origin for the coordinate system (bottom X: 22.9 ± 2.2, Y: 19.7 ± 1.8; top X: 27.0 ± 2.0, Y: 55.0 ± 1.8; medial edge X: 35.5 ± 2.1, Y: 44.0 ± 1.5; lateral edge X: 13.7 ± 1.4, Y: 41.3 ± 1.3).

Stimulation of ChR2 evoked IPSCs in 8 of 18 (44.4%) non-CRF CeL neurons. In contrast only 8 of 71 (11.3%) of CeM neurons demonstrated an IPSC, concurring with our histological findings of sparse mCherry fluorescence and eYFP immunoreactivity of fibers within the CeM (**Figures 1C,6A**). Picrotoxin (100 μM) blocked light-evoked IPSCs more than 97.7 ± 0.9% (n = 4; **Figure 6C**), as well as blocking spontaneous

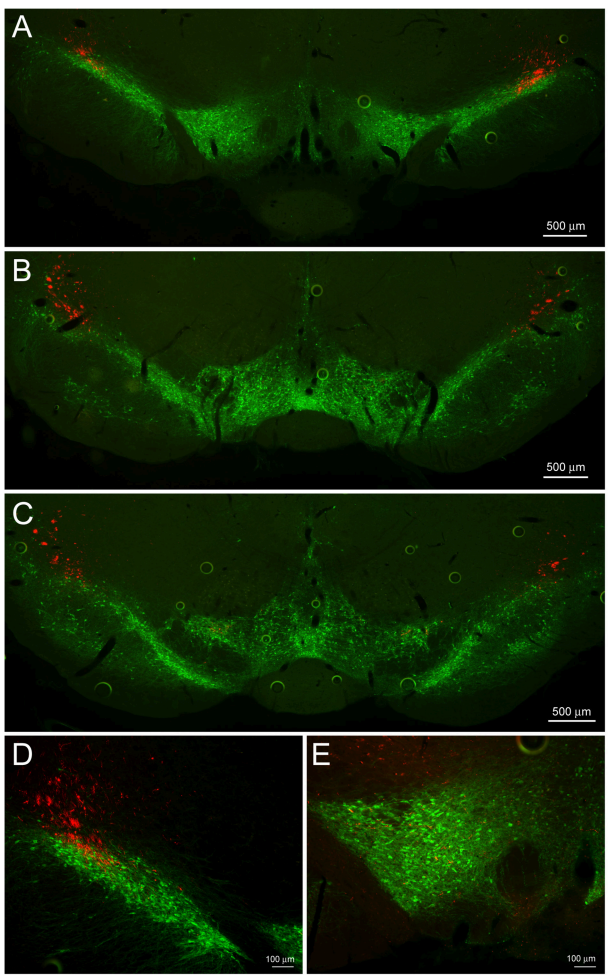

FIGURE 5 | CeL CRF projections to the substantia nigra and VTA. (A) Representative example of CeL CRF fibers in the rostral VTA and substantia nigra pars compacta (SNc; Bregma −5.0). Scale bar, 500 μm. **(B)** CeL CRF fibers were observed projecting through the SNc, but not contacting the VTA slightly more caudally (Bregma -5.5). Scale bar, 500 μm. **(C)** CeL CRF fibers were present at the most caudal aspects of the VTA and SNc (Bregma −6.1). Scale bar, 500 μm. **(D)** CeL CRF fibers course through the most dorsolateral region of the SNc. Scale bar, 100 μm. **(E)** Low density collaterals were present in the rostral VTA surrounding dopamine neurons. Scale bar, 100 μm. Green, tyrosine hydroxylase.

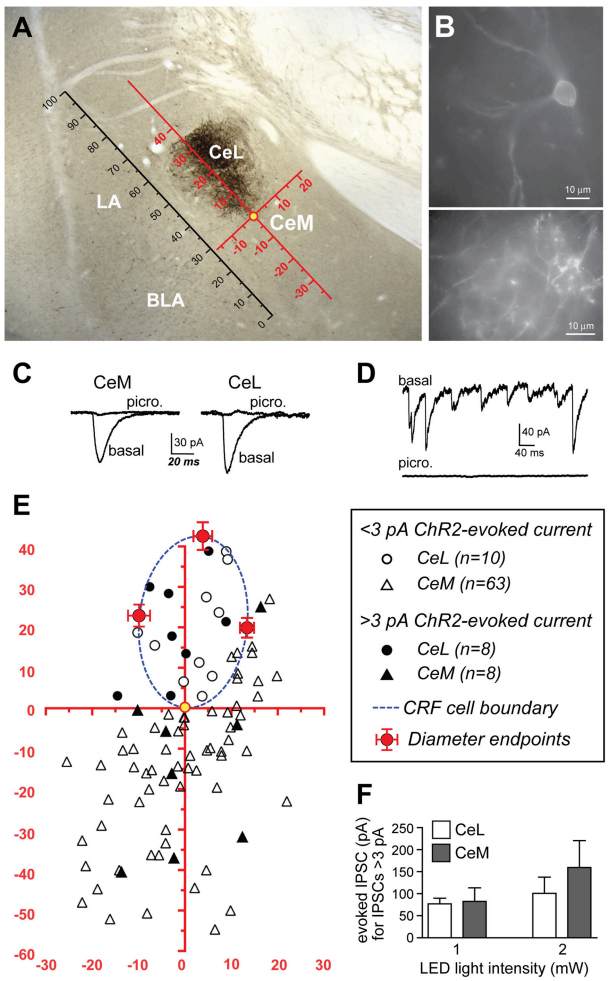

FIGURE 6 | ChR2 stimulation of CRF CeL terminals evokes IPSCs in a subset of CeA neurons. (A) Coronal section through the CeA with superimposed reference frame to identify the location of recorded neurons across slices. **(B)** Example of live CeL neurons (Scale bar, 10 μm) and fibers (Scale bar, 50 μM) expressing ChR2-eYFP. **(C)** Examples of IPSCs evoked after stimulation of ChR2 in CRF CeL inputs, which were blocked by picrotoxin. **(D)** Picrotoxin also blocked spontaneous IPSCs. **(E)** Diagram showing distribution of recorded cells relative to the cluster of CRF cell bodies and dendrites in the CeL (dotted blue circle). Filled symbols represent neurons with IPSC responses; open symbols are neurons without evoked IPSC responses. One CeL neuron with an IPSC response was found outside the cluster of CRF cell bodies and dendrites but within the confines of the CeL. **(F)** Similar magnitude of evoked IPSCs in CeL and CeM neurons and at two different LED intensities. BLA, basolateral amygdala; CeL, lateral central amygdala; CeM, medial central amygdala; LA, lateral amygdala.

IPSCs (**Figure 6D**), which is consistent with previous studies demonstrating that CeA CRF neurons are GABA-ergic (Veinante and Freund-Mercier, 1998; Cassell et al., 1999; Day et al., 1999). The CeM neurons exhibiting light-evoked IPSCs were scattered rather than clustered in a subregion of the CeM (**Figure 6E**). The IPSC amplitudes were not different between CeM and CeL neurons at 2 vs. 1 mW of LED illumination (**Figure 6F**), indicative of a weak input-output relationship for ChR2 as

TABLE 1 | IPSC kinetics for spontaneous and evoked IPSCs in CeM neurons.

	ChR2-evoked IPSC	Spontaneous IPSC	Electrically-evoked IPSC
Rise tau (ms)	0.79 ± 0.06	0.89 ± 0.06	0.97 ± 0.13
Decay tau (ms)	8.39 ± 1.25	10.01 ± 0.98	10.79 ± 1.34
Half-width (ms)	9.22 ± 0.60	9.77 ± 0.84	11.31 ± 1.16
Peak amplitude (pA)	159.6 ± 65.0	78.3 ± 11.3	196.1 ± 39.0
Area under the curve	1787 ± 425	885 ± 136	2433 ± 514

The kinetic values for each IPSC event in a given cell were determined and then all events for that cell were averaged. No significant differences were observed between the three classes of IPSCs for any measure (One-way ANOVA). Data shown are from 8 CeM cells with ChR2-evoked currents and 10 additional cells with electrically evoked IPSCs < 200 pA (peak amplitude approximately matching those of ChR2-evoked currents, since larger IPSCs exhibit longer half-widths), with spontaneous IPSCs determined from IPSC events in the same traces except at the time of evoked IPSCs (at 111 ms into the 1 s sweep).

described (Stuber et al., 2011). Together, these results suggest that CRF inputs target a small subset of CeM neurons. Nearly all CeM neurons showed spontaneous ISPCs that were greatly inhibited by picrotoxin (**Figure 6D**), demonstrating that most CeM neurons can respond to synaptically released GABA. Many cells also exhibited electrically evoked IPSCs, with kinetics similar to those seen for spontaneous IPSCs and ChR2-evoked IPSCs (**Table 1**) and previously reported for CeA IPSCs (Delaney and Sah, 2001; Naylor et al., 2010). Thus, our optogenetic results indicate that CeL CRF neurons send GABA-ergic projections to almost half of their neighboring non-CRF neurons in the CeL, but only to a small number of neurons in the CeM.

Chemogenetic Activation of CeI CRF Neurons Induces CRF1 Receptor-Dependent c-Fos Expression in the CeA

Since the *Fos* promoter is rapidly induced in strongly activated neurons, *Fos* mRNA and Fos protein are commonly used as surrogate markers of recent neuronal activation (Kaczmarek and Chaudhuri, 1997). To identify patterns of activation downstream of CeL CRF neurons, we examined Fos immunoreactivity following activation of Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) expressed in CeL CRF neurons. Systemic administration of clozapine-N-oxide (CNO, 2 mg/kg) induced Fos expression (**Figures 7A,B**) in CRF CeL neurons expressing hM3Dq-mCherry, but not in CRF neurons expressing hM4Di-mCherry or mCherry alone [$F_{(2,\ 10)} = 351$, $p < 0.0001$]. These results suggest that CeL CRF neurons are relatively inactive at baseline. We also found substantial induction of Fos in non-CRF neurons (**Figures 7C,D**) throughout the CeL [$F_{(2,10)} = 27$, $p < 0.0001$] and in some cells of the CeM [$F_{(2,10)} = 43$, $p < 0.0001$]. In the CeL, the proportion that expressed Fos was less than the proportion that exhibited IPSCs after ChR2 stimulation, while in the CeM these proportions were similar (compare **Figure 7F** and **Figure 6E**).

Since CeL CRF neurons are GABA-ergic, we were surprised to find that DREADD stimulation excited a subpopulation of CeA

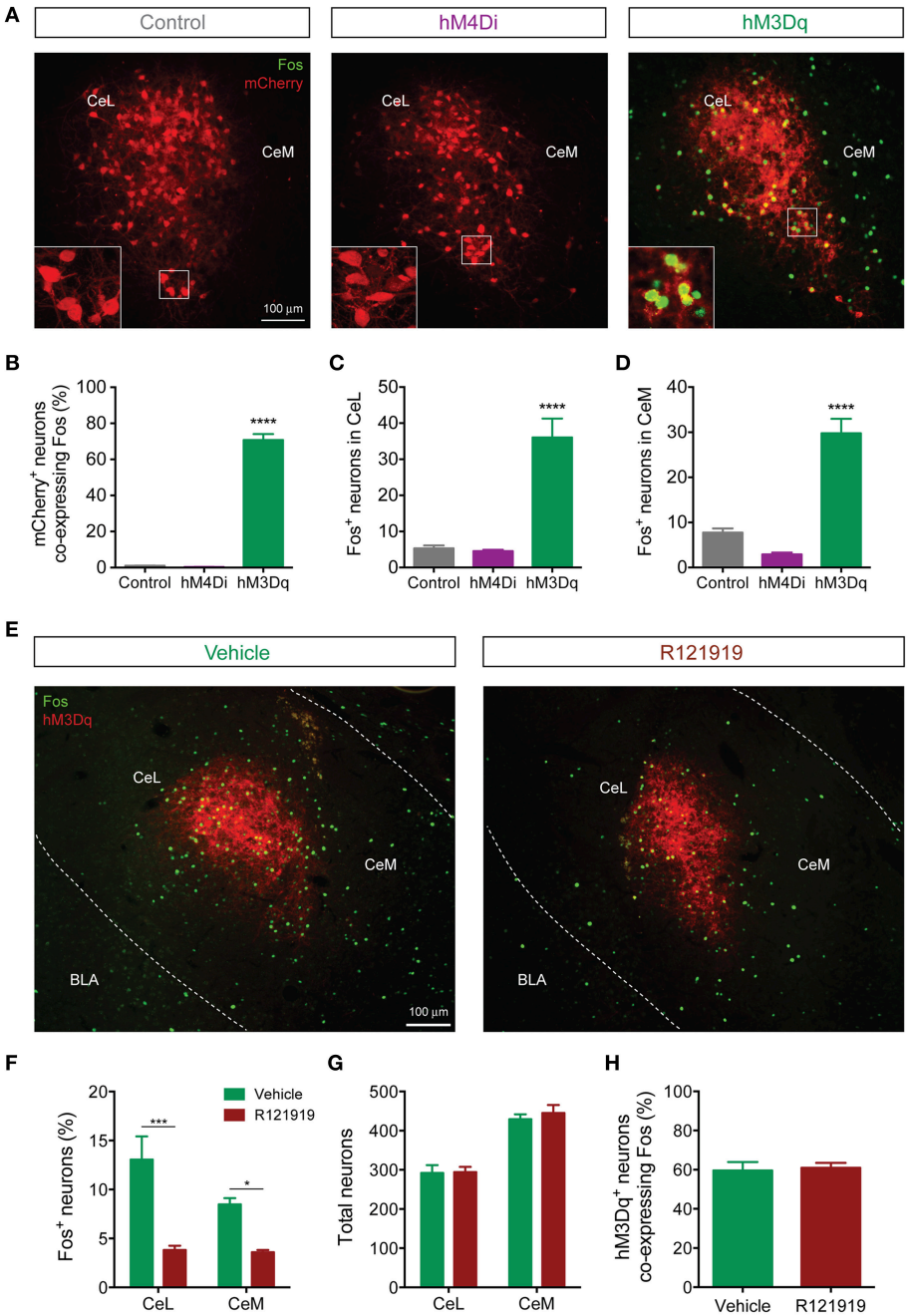

FIGURE 7 | CeL CRF neurons activate non-CRF neurons in the CeL and CeM. (A) Representative overlay images of Fos immunoreactivity and mCherry fluorescence in control, hM4Di, and hM3Dq-expressing CeL neurons. Scale bar, 100 µm. Insets show high-magnification examples of mCherry expressing neurons immunostained for Fos. **(B)** Percentage of mCherry+ neurons co-expressing Fos following administration of CNO. **(C,D)** Fos induction in non-CRF neurons of the CeL **(C)** and the CeM **(D)** after administration of CNO. $^{****}p < 0.001$ compared with Control or hM4Di, $n = 4$–5 rats, 10 sections/rat, Tukey's multiple comparisons test. **(E)** Representative overlay images of Fos immunoreactivity and native mCherry fluorescence in hM3Dq-expressing cells from vehicle- or R121919-treated rats. Scale bar, 100 µm. **(F)** Percentage of Fos+ neurons in the CeL and CeM after administration of R121919. **(G)** Total neuron counts per amygdala section are equivalent between groups. **(H)** Percentage of hM3Dq neurons expressing Fos after administration of CNO is equivalent between groups. $^{***}p < 0.001$, $^{*}p < 0.05$, $n = 4$–5 rats, 10–12 sections/rat, Tukey's multiple comparisons test.

neurons. We hypothesized that CRF released onto local CRF1 receptors was responsible, since CRF1 receptors are expressed in the CeA (Van Pett et al., 2000), and in the mouse CeL CRF1 receptor activation enhances spontaneous glutamate release (Silberman and Winder, 2013). To test this hypothesis, we treated rats with 10 mg/kg of the selective CRF1 receptor antagonist

R121919 (Chen et al., 2004) prior to activation of hM3Dq. This treatment reduced Fos expression in non-CRF neurons in both CeL and CeM [$F_{drug(1,14)} = 40.13$, $p < 0.0001$] (**Figures 7E,F**). Importantly, the total number of neurons (**Figure 7G**) and the proportion of hM3Dq-mCherry positive CRF neurons expressing Fos were similar between R121919 and vehicle-treated groups, indicating that the hM3Dq-driven activity of CeL CRF neurons was not impaired by CRF1 receptor blockade [$p = 0.786$, $t_{(7)} = 0.282$; **Figure 7H**]. These results demonstrate that stimulation of CeL CRF neurons with hM3Dq excites a subpopulation of non-CRF neurons in the CeL and CeM in a CRF1 receptor-dependent manner, presumably through local release of CRF.

DISCUSSION

Until recently our knowledge about the anatomy of CRF systems has rested on traditional neuroanatomical methods and inferences about CRF function through administration of drugs that act at CRF receptors. To gain more direct access to CRF neurons to study their functional neuroanatomy we generated a novel BAC transgenic *Crh*-Cre rat. Using Cre-dependent reporters we found Cre recombinase expression in neurons of the CeL and the dorsolateral BNST. There was strong concordance of Cre-dependent transgene expression and CRF immunoreactivity in the CeL, indicating lack of ectopic expression of Cre recombinase in this area. CRF CeL projections were similar to targets of CRF CeL cells identified in previous neuroanatomical studies. However, little is known about their local projections within the CeA, and we found that CRF CeL neurons projected to other non-CRF CeL cells, and also to a smaller number of CeM neurons. These intra-CeA CRF projections exhibited both inhibitory effects, indicated by evoked GABA currents, and excitatory effects, indicated by increased Fos expression, which were prevented by blocking CRF1 receptors. These findings indicate that CRF CeL neurons are a mixed population of interneurons and projection neurons that encode both inhibitory and excitatory information.

Although we found CRF neurons in the CeL and dorsal BNST, CRF cells were absent from the ventral BNST, PVN, and other brainstem and forebrain regions where CRF neurons have been reported (Merchenthaler, 1984; Wang et al., 2011). Thus, despite the size of our BAC vector (~224 kb), Cre expression was limited to two major CRF cell populations, possibly due to incomplete capture of all regulatory elements in the integrated BAC transgene. Although we do not know the precise reason for restricted CRF expression in our animals, it is notable that CRF neurons of the dorsolateral BNST and the CeL share several common features, including medium spiny neuron morphology (Cassell and Gray, 1989; Phelix and Paull, 1990; Sun and Cassell, 1993), expression of the phosphatase STEP (Dabrowska et al., 2013b), and production of GABA (Cassell et al., 1999; Day et al., 1999; Dabrowska et al., 2013b). In contrast, PVN CRF neurons produce glutamate (Dabrowska et al., 2013a) and ventral BNST CRF neurons may also be glutamatergic (Dabrowska et al., 2013a). The *Crh* gene also is regulated differently in these populations of neurons. For example, while corticosterone suppresses CRF expression in the PVN, it up-regulates expression in the CeA and dorsolateral BNST (Swanson and Simmons, 1989; Makino et al., 1994). This differential regulation could involve PKC signaling since we previously found that production of pro-CRF mRNA and protein in the CeA, but not in the PVN, is impaired in PKC epsilon knockout mice (Lesscher et al., 2008). Additionally a recent study identified novel CRF expressing neurons in the VTA, but this expression was only detectable in animals undergoing nicotine withdrawal (Grieder et al., 2014). The detailed mechanisms responsible for heterogeneity in phenotypic characteristics and control of CRF expression among subpopulations of CRF neurons remain to be explored, but the present findings suggest our *Crh*-cre rats may prove useful for selective study of one major subtype of CRF neurons.

Using viral delivery of Cre-dependent reporters to identify CeL CRF neurons, we found robust CRF projections from CeL to the brainstem, terminating in the medial and lateral PBN and the LC (**Figure 8**). There were also extensive projections to the diencephalon, terminating in the dorsal and ventral BNST and the LH. This pattern of connectivity concurs with previously reported CeA CRF projections in the rat (Moga and Gray, 1985; Sakanaka et al., 1986; Van Bockstaele et al., 1998; Reyes et al., 2011). We did not, however, observe projections to the pontine reticular nuclei, as reported in earlier neuroanatomical tracing studies (Gray and Magnuson, 1992; Fendt et al., 1997). A previous study of neuropeptide afferents from the CeA found CRF neurons in the CeL that contained retrograde tracer after injections into the dorsal vagal complex (Gray and Magnuson, 1987). Our results refine this finding by demonstrating that CeL CRF fibers specifically innervate the NTS. Since the NTS provides noradrenergic input to the extended amygdala that plays a role in drug withdrawal and anxiety (Smith and Aston-Jones, 2008), it will be interesting to determine whether a reciprocal connection between CeL CRF neurons and the NTS exists, and whether this circuit is recruited during withdrawal states.

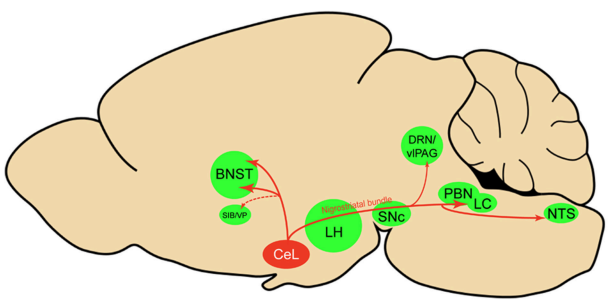

FIGURE 8 | Sagittal rat brain schematic of CeL CRF neuron projections. BNST, bed nucleus of the stria terminalis; CeL, lateral central amygdala; DRN, dorsal raphe nuclei; LC, locus coeruleus; LH, lateral hypothalamus; NTS, nucleus tractus solitarius; PBN, parabrachial nucleus; SIB, Substantia innominata; SNc, substantia nigra pars compacta; vlPAG, ventrolateral periaqueductal gray; VP, ventral pallidum.

Within the CeA, despite extensive CeL innervation of the CeM (Petrovich and Swanson, 1997), we surprisingly found few CeL CRF projections to the CeM. Instead, we found that CeL CRF neurons preferentially innervate other CeL neurons. Given the sparseness of projections to CeM, we speculate that CeL CRF projection neurons act in parallel with CeM projection neurons to regulate behavior, and that the small number of direct CeL CRF projections to the CeM, and potentially more extensive indirect projections *via* non-CRF neurons in the CeL, coordinate the actions of CeL and CeM systems on behavior. Interesting questions for the future are whether individual CeL CRF neurons project to many or a restricted set of targets and whether the same or different CeL CRF neurons serve as interneurons and projection neurons. Future studies using *Crh*-Cre rats and Cre-dependent tracing tools and actuators should allow us to unravel this circuitry in greater detail.

Despite CeL CRF neurons being GABA-ergic, activation of the excitatory DREADD hM3Dq in these neurons induced expression of Fos in several non-CRF neurons of the CeL and CeM. Thus, CRF CeL neurons can generate both excitatory (Fos) and inhibitory (GABA IPSCs) responses in CeL and CeM neurons. Fos induction following activation of CRF CeL neurons involved CRF release since it was substantially reduced by administration of a CRF1 receptor antagonist. Depending on the synapse, activation of CRF1 receptors can activate neurons by enhancing glutamatergic transmission. For example, in the rat lateral capsular CeA, CRF acting at CRF1 receptors enhances glutamatergic transmission from parabrachial efferents (Ji et al., 2013), and CRF increases the frequency of spontaneous EPSCs in the mouse CeL through actions at CRF1 and CRF2 receptors (Silberman and Winder, 2013). The actions of CRF on excitatory neurotransmission in the rat CeL have yet to be determined.

Given our limited knowledge of intra-CeA circuitry, we can envision several mechanisms by which activating CRF CeL neurons could generate both inhibitory and excitatory responses. First, GABA and CRF may affect different target neurons, with GABA released at synapses and CRF released non-synaptically to signal via local volume transmission that results in excitation of CeA neurons through convergent disinhibition and CRF signaling. A somewhat similar situation has been recently described for innervation of the cerebral cortex and striatum by histaminergic neurons of the hypothalamus that also release GABA (Yu et al., 2015). Alternatively, excitatory effects of CRF may be partly suppressed by concurrent GABA release, for example where CRF acting at CRF1 receptors enhances GABA release, as has been demonstrated in the rat CeM (Herman et al., 2013). On the other hand, in cells having a depolarized Cl^- reversal potential, activation of $GABA_A$ receptors could synergize with CRF to directly activate post-synaptic neurons (Staley and Proctor, 1999), although this may be more speculative for adult neurons. Finally, stimulus intensity and duration may affect GABA and CRF release differently, leading to a range of inhibitory and excitatory responses on the same target neuron population. Future optogenetic and chemogenetic studies using *Crh*-Cre rats could help to determine if the actions of GABA and CRF occur at the same or at different neurons and to elucidate mechanisms by which these transmitters act.

The generation of several *Crh*-Cre mouse lines has facilitated our understanding of CRF circuits and their roles in several behavioral states. At least three *Crh*-Cre mouse lines have been reported, and have been used to demonstrate roles for CRF neurons in fear conditioning (Gafford et al., 2014), fear extinction (Gafford et al., 2012), anxiety and avoidance behaviors (Gafford et al., 2012; McCall et al., 2015), and binge-like alcohol consumption (Pleil et al., 2015). However, a recent review (Chen et al., 2015) indicates that two of these lines, the *Crh*-BAC transgenic (Alon et al., 2009) and CRFp3.0Cre (Martin et al., 2010) exhibit ectopic Cre transgene expression, whereas *Crh*-IRES-Cre mice (Taniguchi et al., 2011) express Cre with high fidelity to endogenous CRF across the brain. Since our current rat line is a BAC transgenic, our determination of Cre and CRF fidelity in the amygdala (**Figure 1**) was critically important. Although our rat shows limited expression of Cre, it provides a tool to study the role of GABAergic CRF neurons of the amygdala and dorsolateral BNST to not only complement work done with *Crh*-Cre mice, but to permit investigation of more complex behaviors such as operant conditioning and sophisticated learning tasks that cannot easily be studied using mice.

The anatomy of projections from CeL to CeM has been examined recently in mouse models of fear conditioning (Ciocchi et al., 2010; Haubensak et al., 2010; Li et al., 2013), although without a focus on amygdala CRF neurons or employing *Crh*-Cre mice. An anatomical framework has emerged in which fear-related cues excite BLA neurons, which in turn enhance firing in a CeL cell subpopulation termed On-cells that inhibit a separate CeL subpopulation termed Off-cells, the net result of which is to disinhibit CeM neurons. The subsequent increase in CeM activity mediates conditioned fear *via* projections downstream to somatic and autonomic brainstem nuclei. In mice the PKCδ neuron subpopulation in the CeL represents Off-cells (Haubensak et al., 2010), which tonically suppress CeM neurons to inhibit fear responses. On-cells in mice are at least partially SOM+ (Li et al., 2013). In contrast, the role of CeL CRF neurons in this fear control circuit has been largely overlooked. Here we provide evidence consistent with rat CRF neurons being mostly a subpopulation of On-cells based on co-expression of SOM in about 40% of CRF neurons and sparse projections to the CeM. Future challenges will be to dissect the relative contribution of CRF neurons (SOM+ and SOM-) to fear-related circuitry and behavior, and to determine whether CRF neurons with unique neuropeptide co-expression profiles provide distinct inputs to local and distant projection targets.

In summary, we present a novel transgenic Cre driver rat line that permits selective targeting of CRF-expressing GABAergic neurons of the extended amygdala. The *Crh*-Cre rat will be an important tool for dissecting extended amygdala CRF systems in the control of fear and anxiety, as well as stress-sensitive behaviors, such as feeding and drug seeking. Furthermore, species-specific phenotypic differences can be evaluated by comparing *Crh*-Cre rats with *Crh*-Cre mice, which should help unify conclusions about CRF circuits across rodent species.

AUTHOR CONTRIBUTIONS

Study concept and design: MP, EM, FH, PJ, RM. Acquisition of data: MP, EM, FH, RK, RM, AB, VK, GD, EC. Analysis and interpretation of data: MP, EM, FH, GD, OG, RM. Drafting of the manuscript: EM, FH. Critical revision of the manuscript for important intellectual content: MP, RM. Statistical analysis: MP, FH. Obtained funding: FH, OG, PJ, RM. Administrative, technical, and material support: EC, KR. Study supervision: FH, PJ, RM.

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Paraventricular Hypothalamic Mechanisms of Chronic Stress Adaptation

James P. Herman[1] and Jeffrey G. Tasker[2]*

[1] Department of Psychiatry and Behavioral Neuroscience, University of Cincinnati, Cincinnati, OH, USA, [2] Department of Cell and Molecular Biology, Tulane Brain Institute, Tulane University, New Orleans, LA, USA

The hypothalamic paraventricular nucleus (PVN) is the primary driver of hypothalamo–pituitary–adrenocortical (HPA) responses. At least part of the role of the PVN is managing the demands of chronic stress exposure. With repeated exposure to stress, hypophysiotrophic corticotropin-releasing hormone (CRH) neurons of the PVN display a remarkable cellular, synaptic, and connectional plasticity that serves to maximize the ability of the HPA axis to maintain response vigor and flexibility. At the cellular level, chronic stress enhances the production of CRH and its co-secretagogue arginine vasopressin and rearranges neurotransmitter receptor expression so as to maximize cellular excitability. There is also evidence to suggest that efficacy of local glucocorticoid feedback is reduced following chronic stress. At the level of the synapse, chronic stress enhances cellular excitability and reduces inhibitory tone. Finally, chronic stress causes a structural enhancement of excitatory innervation, increasing the density of glutamate and noradrenergic/adrenergic terminals on CRH neuronal cell somata and dendrites. Together, these neuroplastic changes favor the ability of the HPA axis to retain responsiveness even under conditions of considerable adversity. Thus, chronic stress appears able to drive PVN neurons *via* a number of convergent mechanisms, processes that may play a major role in HPA axis dysfunction seen in variety of stress-linked disease states.

Keywords: corticotropin-releasing hormone, vasopressin, hypothalamo–pituitary–adrenal axis, glucocorticoids, neuroendocrine system

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****Correspondence:***
James P. Herman
jpherman87@gmail.com

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INTRODUCTION

The hypothalamo–pituitary–adrenocortical (HPA) axis is required for appropriate adaptation to external or internal challenge. The so-called HPA axis "stress response" culminates in the release of glucocorticoids by the adrenal gland, which acts at multiple sites throughout the body (and brain) to mobilize energy resources. This redistribution of energy provides essential fuels for meeting real or anticipated challenges to homeostasis or well being (i.e., "stressors") (1). While important for immediate adaptation, prolonged exposure to glucocorticoids can create a metabolic challenge of its own. Consequently, activation of the HPA axis is kept in control by negative feedback, wherein glucocorticoids inhibit their own release (2).

The HPA axis is controlled by peptidergic neuroendocrine neurons located in the medial parvocellular division of the hypothalamic paraventricular nucleus (PVN) (**Figure 1**). These neurons are responsible for the release of corticotropin-releasing hormone (CRH), which acts as the

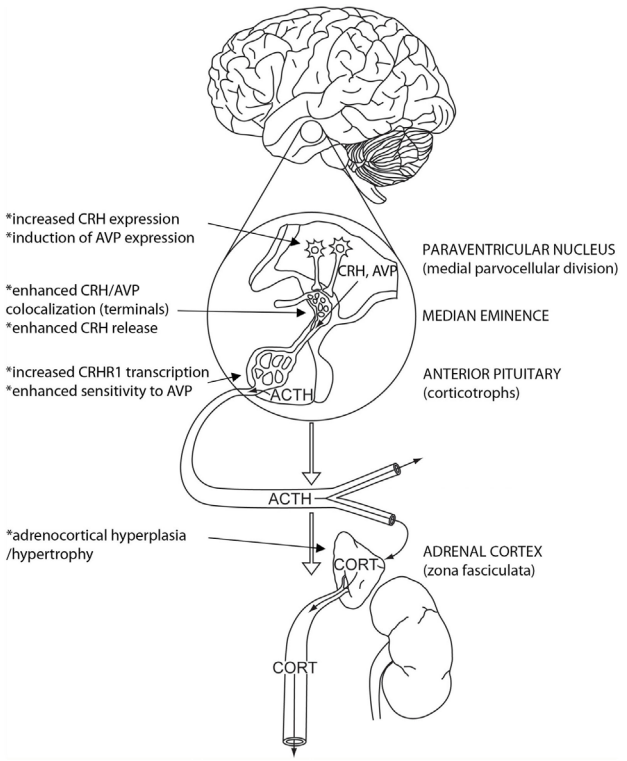

FIGURE 1 | **Plasticity across the hypothalamo–pituitary–adrenocortical (HPA) axis following chronic stress**. HPA axis activation is initiated by CRH (and AVP)-containing neurons in the medial parvocellular paraventricular nucleus (PVN), which releases ACTH secretagogues into hypophysial portal vessels in the median eminence. ACTH is released from the pituitary and travels *via* the systemic circulation to the adrenal cortex, where it causes the synthesis and secretion of glucocorticoids [e.g., cortisol or corticosterone (CORT)]. Under conditions of chronic stress, there are marked changes in cellular reactivity across the HPA axis, including (1) increased CRH and AVP expression in, and enhanced excitability of CRH neurons in the PVN, (2) increased CRH/AVP colocalization and CRH release in the median eminence, (3) increased CRHR1 and V1b gene expression in and sensitivity to CRH and AVP of pituitary corticotropes, and (4) adrenal hypertrophy and enhanced adrenal sensitivity to ACTH. All changes are consistent with increased potential for HPA axis activation. Modified from Myers et al. (4), with permission.

obligate factor responsible for adrenocorticotrophic hormone (ACTH) release in most organisms (3). Upon stimulation, CRH is released into hypophysial portal vessels in the external lamina of the median eminence, where it is transported to the anterior pituitary gland to promote ACTH release. ACTH then travels *via* the systemic circulation to the adrenal cortex, where it triggers the synthesis and release of glucocorticoids.

Importantly, PVN CRH neurons also synthesize arginine vasopressin (AVP), which is co-stored and co-released with CRH in the median eminence (5, 6). While having minimal effects on its own, AVP can synergize with CRH to greatly amplify ACTH release (7). Under unstimulated conditions, AVP expression in CRH neurons is very low and likely plays a minimal role in ACTH secretion. However, following prolonged activation (e.g., adrenalectomy or as we will see below, chronic stress) (8, 9), parvocellular AVP production is markedly increased, suggesting a role in chronic drive of the HPA axis. Electron microscopy studies indicate depletion of AVP from CRH terminals in the median eminence following acute stress (10), consistent with co-release in response to drive of PVN neurons.

Both CRH and AVP act at the level of the pituitary corticotrope to modulate the release of ACTH. CRH binds to corticotropin-releasing hormone R1 receptors (CRHR1), causing activation of adenylate cyclase and subsequent release of ACTH (11). Deletion of the CRH gene blocks both basal and stress-induced ACTH release, indicative of the obligatory nature of CRH for HPA axis activation (12). In contrast, AVP does not drive ACTH release on its own but complements the actions of CRH (*via* binding to AVP1B receptors) (13). Together, CRH and AVP provide for a broad range of corticotrope response following PVN stimulation.

It is important to note that a subset of PVN CRH neuron project centrally and may be of functional importance in behavioral regulation. Lesions of the parvocellular PVN reduce anxiety-like behaviors in a novel environment, suggesting a role in emotional regulation (14). A recent study indicates that optogenetic inhibition of PVN CRH neurons reduces stress-induced grooming and enhances locomotion and rearing following stress, whereas stimulation induces grooming and reduces exploratory behaviors (15). Collectively, these data suggest that PVN CRH (and possibly AVP) neurons may be involved in coordinating behavioral as well as neuroendocrine responses to stress.

Parvocellular CRH neurons also express numerous other neuropeptides, including angiotensin II, cholecystokinin, and neurotensin (16). The role of these other peptides in HPA axis function has yet to be clarified. In addition, parvocellular PVN neurons have the capacity to release the excitatory neurotransmitter glutamate (17). Given the presence of presynaptic glutamate receptors in the median eminence, it is possible that glutamate may also influence local release of peptide at the level of the neurovascular junction (18). Indeed, blockade of GluR5-containing kainate receptors in the median eminence inhibits stress-induced ACTH release (19), suggesting a role for local glutamatergic signaling in HPA axis control.

The PVN is one of the primary sites of glucocorticoid negative feedback regulation of the HPA axis. Negative feedback is largely mediated by glucocorticoid receptors (GRs), which are activated mainly when glucocorticoid levels are elevated (e.g., during stress responses) (20). The GR is richly expressed in the medial parvocellular PVN and is co-localized with CRH (21, 22), placing it in prime position to control output of the very neurons that activate the HPA axis. Within the PVN, GR-mediated negative feedback of CRH neuronal activation ("fast feedback") is likely mediated by non-genomic glucocorticoid signaling at or near the cell membrane (23, 24). Rapid inhibition of CRH neurons is key to limiting the duration of glucocorticoid secretion following acute stress, as genomic feedback would not be sufficiently fast to terminate HPA axis activation in a timely fashion. Fast feedback is mediated by glucocorticoid-dependent mobilization of endocannabinoid production in putative CRH neurons, which cause inhibition of presynaptic glutamate release *via* retrograde signaling at type 1 cannabinoid (CB1) receptors (23). While there

is evidence to suggest that membrane effects involve the classical GR (25, 26), the exact mechanism of glucocorticoid action remains to be delineated (27).

Genomic feedback effects on CRH neurons likely occur at longer poststimulation latencies and may be mediated by ligand-dependent nuclear translocation and subsequent interactions of the GR with cognate DNA binding elements or other transcription factor complexes. Genomic actions of GR may be involved in glucocorticoid-mediated inhibition of CRH and AVP gene expression in hypophysiotrophic neurons following adrenalectomy (28, 29). However, there are data to suggest that rapid effects of stress on CRH gene transcription may be glucocorticoid-independent. Rapid inhibition of CRH heteronuclear RNA (hnRNA) expression appears to be mediated by rapid stress-induced increases in expression of the inducible cyclic AMP early repressor (ICER) isoform of the cyclic AMP response element modulator (CREM), which binds to the CRH promoter and blocks transcription (30). Transcriptional repression of CRH by ICER/CREM is not dependent on a glucocorticoid surge, suggesting an alternative mechanism.

Glucocorticoids also signal through the mineralocorticoid receptor (MR). The MR is occupied at low circulating levels of glucocorticoids and is not thought to mediate glucocorticoid feedback effects (20). However, there are data suggesting that occupation of the MR is required for appropriate regulation of stress responses in some contexts (31), and certain rapid glucocorticoid actions in the hippocampus and basolateral amygdala are mediated by MR activation (32, 33). While there is evidence for MR expression in the medial parvocellular PVN (34), its role in the local regulation of HPA axis function is unexplored.

CHRONIC STRESS-INDUCED NEUROPEPTIDERGIC PLASTICITY IN THE PVN

Chronic exposure to stress produces regimen-dependent alterations in PVN production of CRH mRNA. Most stressors that involve non-social interventions (e.g., repeated immobilization, repeated footshock, chronic unpredictable/variable stress, repeated predator exposure) produce upregulation of PVN CRH mRNA expression (9, 35–37). For example, in our hands, we have observed increased PVN CRH mRNA expression in all studies using chronic variable stress (1- to 4-week exposure to a random assort of stressors twice/day, at unpredictable times) (38–41). Indeed, the magnitude of CRH mRNA upregulation is remarkably similar across studies (generally falling between 40 and 60%), despite studies being conducted at two different institutions and over several years (38–41). Thus, it is evident that increased drive to the PVN effectively enhances CRH gene expression in these regimens. The exact mechanism driving elevations in CRH gene expression is not completely understood but probably involves increases in transcription mediated by repeated activation of cAMP (42, 43). Transcription may be partially (but not completely) counter-balanced by enhanced glucocorticoid-mediated degradation of CRH mRNA (44), likely as a feedback homeostatic control mechanism.

The impact of chronic stress on CRH gene transcription is accompanied by enhanced CRH peptide production in PVN cell bodies and decreased storage in the median eminence (45). The latter observation suggests an increase in turnover of CRH, which is consistent with chronic activation. In the cell soma, increased peptide synthesis is accompanied by an increase in the physical size of the CRH neurons (46), similar to that seen in magnocellular AVP and oxytocin neurons following dehydration and lactation (47, 48). Increased cell size may reflect a general drive on transcription and protein synthesis, perhaps as a way of adapting to increased energetic demand. These data are consistent with increase in protein synthesis as well as in capacity for release, which may partially underlie baseline glucocorticoid hypersecretion and stress facilitation seen following chronic stress (below).

Chronic stress also increases AVP gene transcription in parvocellular PVN neurons (49). The dynamics of chronic stress-induced AVP and CRH transcriptional responses differ considerably. Exposure to habituating, homotypic stressors (e.g., restraint) results in the loss of PVN CRH transcriptional responses over time, in terms of both hnRNA and mRNA expression (50). Moreover, CRH neurons lose the ability to mount transcriptional responses to a novel acute (heterotypic) stress (e.g., hypertonic saline) following repeated stress exposure. In contrast, while AVP hnRNA and mRNA responses also habituate with repeated restraint, robust transcriptional responses are observed when challenged with a heterotypic stressor, stronger than that seen in stress-naïve controls (49). Together, the data suggest that AVP may play an important role in maintaining or potentiating PVN (and by extension, HPA axis) drive after homotypic stress exposure.

Chronic variable stress reduces expression of the GR in the medial parvocellular PVN. Since both CRH and AVP gene/protein expression are negatively regulated by glucocorticoids, it is possible that reduced GR may play a role in the observed upregulation of both genes during chronic stress. Indeed, expression of GR is negatively correlated with CRH mRNA in the PVN (9, 51), suggestive of a mechanism for stress-induced upregulation. However, it is important to note that downregulation of GR mRNA is not observed in all stress regimens, despite elevated CRH, suggesting that other mechanisms may also contribute to driving PVN gene expression. In this regard, it is important to note that the efficacy of GR signaling may be negatively modulated by posttranscriptional mechanisms. For example, nuclear GR signaling can be affected at the level of receptor translocation (as seen in aging) (52) or binding to nuclear factors that modulate transcriptional activity (53). Regarding the latter possibility, GR binding to different isoforms of steroid receptor coactivator protein 1 can dramatically affect the transcription of CRH and CRH gene methylation (54).

CELLULAR IMPACT OF CHRONIC STRESS ON MEDIAL PARVOCELLULAR PVN NEURONS

Chronic stress produces multiple cellular adaptations in CRH neurons that are consistent with enhanced excitability

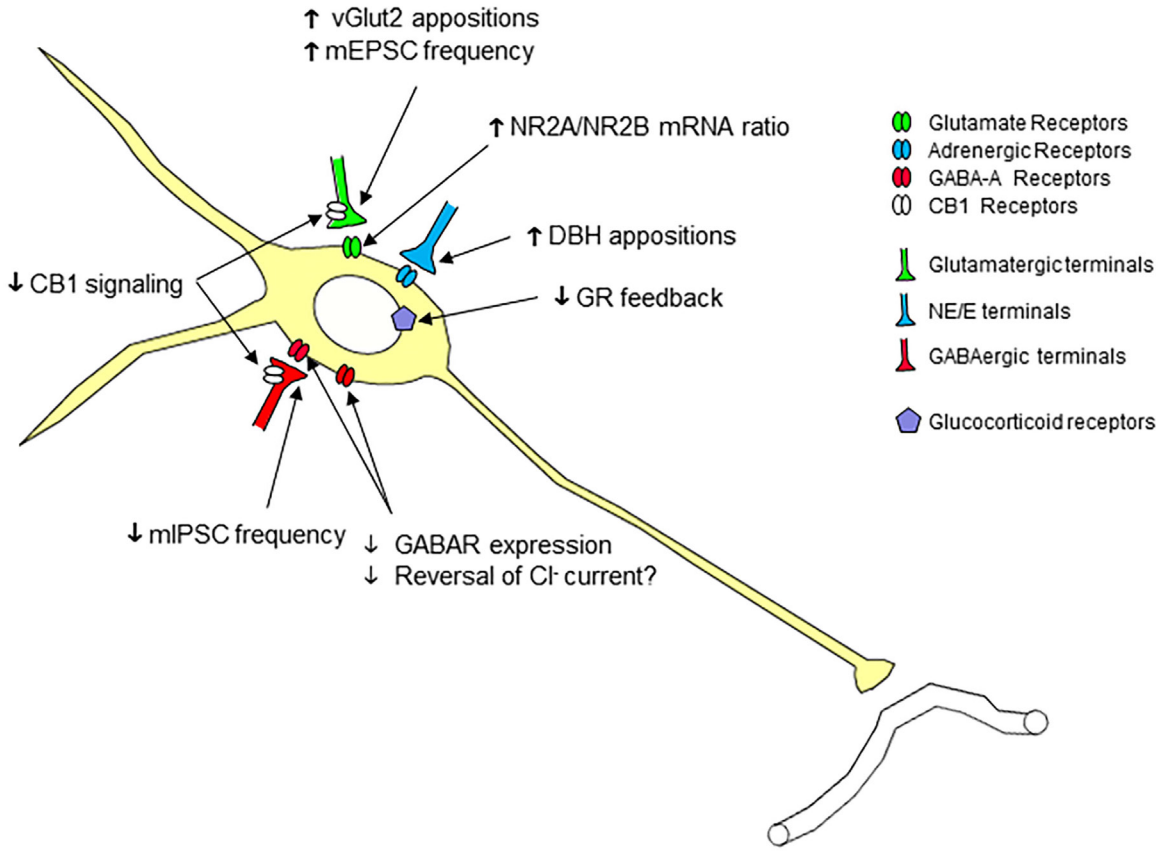

FIGURE 2 | **Cellular plasticity in parvocellular paraventricular nucleus neurons following chronic stress**. Chronic stress causes a number of neuroplastic changes in parvocellular neurons consistent with increased excitability, including (1) increased number of (excitatory) glutamergic (VGluT2) terminal appositions, (2) enhanced excitatory synaptic inputs (increase in mEPSC frequency), (3) enhanced NR2A/NR2B ratio, predictive of increased NMDA receptor calcium permeability, and (4) increased number of (putative excitatory) norepinephrine/epinephrine terminal appositions. In addition, there is evidence for reduced inhibition of the PVN neurons, in terms of (1) reduced GABAergic synaptic inputs (decrease in mIPSC frequency), (2) decreased expression of synaptic and extrasynaptic $GABA_A$ receptor subunits, (3) decreased cannabinoid type 1 receptor signaling, and (4) reduced GR expression/signaling, in some, but not all, studies.

(**Figure 2**). Chronic variable stress reduces mIPSC frequency (but not amplitude) in putative CRH neurons, indicative of loss of inhibitory inputs (55, 56). Chronic variable stress exposure also decreases the expression of $GABA_A$ receptor α5, β1, β2, and δ subunits (55, 57), consistent with reduced GABAergic inhibition. Importantly, receptors expressing δ subunits are largely extrasynaptic and are thought to mediate tonic inhibition (58). The loss of extrasynaptic receptors, therefore, would be expected to reduce tonic GABAergic inhibition of PVN neurons. In addition, there is evidence supporting a reversal of chloride current in CRH neurons after stress exposure, essentially rendering GABA less effective at the $GABA_A$ receptor and resulting in reduced synaptic inhibition (59). Loss of inhibitory GABAergic transmission is linked to marked glucocorticoid-dependent downregulation of the potassium-chloride co-transporter, KCC2, resulting in enhanced intracellular chloride ion concentration and a weakened membrane chloride gradient (59). Notably, chronic variable stress also causes downregulation of KCC2 in CRH neurons, consistent with lasting impairments in inhibitory GABA signaling (60).

There is evidence to suggest enhanced excitability of PVN neurons following chronic stress. Baseline excitatory drive of parvocellular PVN neurons is enhanced by chronic stress exposure, manifest as increased mEPSC frequency (56). In addition, ionotrophic *N*-methyl-D-aspartate receptor subunits are modulated by chronic variable stress in a manner consistent with enhanced postsynaptic glutamate receptivity. Chronic stress causes a selective decrease in expression of NR2B in the medial parvocellular PVN, whereas NR1 and NR2A expression are unaffected. As calcium permeability of NR2A containing channels is greater than that of NR2b channels, these data predict an enhanced excitability of the NMDA receptor complex following chronic stress (61).

Enhanced PVN excitability may also be affected by chronic stress-induced loss of endocannabinoid signaling in PVN afferents. Animals exposed to chronic restraint stress lose depolarization-induced suppression of both glutamate and GABA release at parvocellular PVN neurons (62). Given the reversal of the chloride gradient following chronic stress, both effects are consistent with reduced inhibition of PVN

neurons. Loss of inhibitory control appears to be mediated by a GR-dependent inhibition of CB1 receptor signaling, likely mediated by the loss of CB1 terminals on PVN neurons. Thus, chronic restraint stress likely produces a loss of presynaptic cannabinoid inhibition, effectively blocking endocannabinoid-mediated glucocorticoid-dependent fast feedback inhibition of the HPA axis (62). However, it is important to note that glucocorticoid-induced endocannabinoid signaling is not affected following chronic variable stress (56), indicating that different mechanisms may regulate PVN responses under conditions of predictable vs. unpredictable stress exposure.

Chronic stress exposure affects markers of cellular activation in the PVN. Neuronal activation causes rapid induction of the immediate early gene, *cfos*, largely as the result of depolarization (63, 64). The resulting protein, Fos, binds with other cofactors of the *jun* family to form a potent transcriptional activation complex acting at activator protein 1 (AP1) response elements on DNA (65). Not surprisingly, acute stress produces a marked increase in PVN *cfos* mRNA/Fos expression, consistent with cellular activation (63, 64). However, the acute Fos response to homotypic stressor exposure (e.g., restraint, noise) wanes over repeated exposure (66–68), suggesting habituation of cellular activation. In contrast, PVN *cfos* gene expression/Fos protein induction by a heterotypic stressor is preserved or enhanced in some paradigms [e.g., chronic cold plus acute restraint (69)] but not others [e.g., chronic variable stress plus novelty, chronic social stress plus open arm (elevated plus maze) exposure] (70, 71). The fact that the acute Fos activation is reduced in the latter paradigms indicates some degree of cross-habituation to stressor exposure, at least as far as Fos induction is concerned. The lower *cfos* mRNA/Fos response may also be associated with the relative (low) intensity of the evocative stimuli with respect to other stimuli occuring in the unpredictable stress or social stress paradigms. Of course, it is important to consider that the magnitude of the Fos response may not be an accurate reporter of cellular activation in the context of repeated drive, given that other intracellular changes [e.g., increased CRH expression (68), enhanced excitability (56)] may compensate for habituation in the AP1 pathway.

Chronic stress also causes enduring changes in immediate early gene family members, consistent with chronic activation. For example, expression of FosB/delta FosB, a Fos family member that aggregates in the cell over repeated activations, is upregulated in the PVN by morphine withdrawal (72).

CHRONIC STRESS NEUROPLASTICITY OF PVN INPUTS

Chronic stress-induced enhancement of PVN response capacity may be driven in large part by neuroplasticity in afferent connections (**Figure 2**). Work from our group suggests that chronic variable stress increases glutamatergic inputs to CRH neurons, as determined by elevations in the number of vesicular glutamate transporter 2 (VGluT2) appositions onto both dendrites and somata (46) and increases in excitatory synaptic inputs to PVN neurons (56). Both observations suggest that the enhanced drive is mediated by additional synaptic contacts. Moreover, early-life maternal enrichment, which is linked to reduced stress reactivity, decreases VGluT2 synaptic contacts and excitatory synaptic inputs in CRH neurons (73), further suggesting that the glutamate innervation is an important determinant of PVN excitability.

Chronic stress also increases the number of dopamine beta hydroxylase-positive appositions with CRH dendrites and somata, consistent with enhanced noradrenergic (and adrenergic) innervation (46). A sizable body of evidence suggests that PVN norepinephrine (NE) drives HPA axis responses in a stressor-dependent fashion (74, 75), suggesting the capacity for stress-induced enhancement of NE innervation to drive CRH neurons. Lesion of PVN-projecting NE/E neurons using DBH-conjugated saporin (DSAP) reduces ACTH responses to an acute stress in rats previously exposed to chronic variable stress, indicating that adrenergic input is required for the excitatory drive of CRH neurons under these conditions. However, corticosterone responses are not affected by PVN NE/E depletion, consistent with compensation at the level of the adrenal gland (see below) (76). Notably, DSAP also blocks chronic stress-induced increases in PVN synaptophysin and VGluT2 expression, suggesting that lesions may interfere with glutamatergic neuroplasticity (76).

Subsequent studies have compared the impact of chronic stress on PVN innervation in males and females. Overall, medial parvocellular PVN innervation, determined from the density of synaptophysin staining, is greater in females than in males. However, in response to chronic stress, synaptic density markedly increases in males but decreases in females (77). Enhanced PVN innervation in males is accompanied by increased VGluT2 innervation of CRH neurons (unpublished data). Despite clear evidence for chronic stress-induced decrements in inhibitory synaptic inputs (55, 56), we did not observe a loss of GABAergic appositions onto CRH neurons in either males or females (unpublished data). These results suggest that the loss of GABAergic input to PVN neurons may not be simply due to reduced synaptic afferents but rather may reflect differences in release probability. Chronic stress effects on GABAergic inputs may also be connected to modifications in GABAergic signaling efficacy, mediated by sex differences in postsynaptic reversal of chloride gradients or reduced efficacy of presynaptic CB1 signaling, noted above. These data imply a marked difference in PVN synaptic physiology in males and females, the significance of which remains to be evaluated.

Not all presynaptic changes are linked to enhanced excitability. Work from our group has demonstrated that glucagon-like peptide 1 (GLP-1) inputs to the PVN (from the caudal nucleus of the solitary tract) are powerful drivers of HPA axis stress responses (78, 79). Moreover, exogenous GLP-1 enhances HPA axis facilitation following chronic variable stress, suggesting a role in driving CRH neuronal responsiveness (79). However, chronic stress results in profound inhibition of the expression of the GLP-1 precursor preproglucagon (PPG) in NTS neurons, accompanied by loss of GLP-1 innervation to the medial parvocellular PVN (80). Reduction of PPG gene expression is glucocorticoid-dependent, suggesting that chronic stress-induced glucocorticoid secretion removes an important excitatory drive to the PVN (80). Removal of GLP-1 PVN innervation may represent a feedback mechanism that limits excessive drive of the HPA axis following chronic stress.

CHRONIC STRESS AND PVN AFFERENTS

The PVN gates output of the HPA axis, acting essentially as a "final common pathway." Consequently, PVN output is subject to upstream changes in stress-sensitive projections (81). Lesion studies suggest that damage to key limbic structures following chronic stress causes marked changes in PVN activation. Damage to the posterior subregion of the bed nucleus of the stria terminals potentiates HPA axis stress responses and increases PVN *cfos* gene expression following chronic variable stress. The posterior BST provides strong GABAergic input to the PVN, suggesting that chronic stress reduces BST inhibition of the HPA axis (82). However, lesions of the anterior subregion of the BST reduce HPA axis responses to acute stress but enhance ACTH release and PVN *cfos* mRNA expression following chronic stress, consistent with a change in the functional connectivity of this region with the PVN as a result of repeated stress (83). The anterior BST sends both excitatory (CRH-containing) and inhibitory (GABAergic) projections to the PVN (84, 85), raising the possibility that the relative weighting of these inputs is modified by repeated stress exposure.

Lesions of the paraventricular thalamic nucleus (PVT) have profound effects on both habituation and sensitization of the HPA axis and PVN responses to stress (86). This region of the limbic thalamus does not connect directly with the PVN and thus requires intermediary relays to access CRH neurons. Damage to the PVT has no effect on acute stress responses but blocks chronic stress-induced facilitation of HPA axis responses and PVN Fos induction to a novel stress (86). Moreover, damage to this region blocks habituation of HPA axis responses to repeated stressor exposure (86). These data indicate a role for this structure in relaying information on stress chronicity to the PVN.

It is of interest to note that regions controlling acute stress reactivity do not necessarily modulate chronic stress responses. Lesions of the central nucleus of the amygdala, locus coeruleus, or ventral subiculum have no effect on somatic end points, HPA axis activity, or PVN neuropeptide plasticity following chronic stress, despite profound actions on acute stress reactivity (38, 39). Thus, circuits mediating PVN plasticity following chronic stress may differ from those controlling acute reactivity.

We recently used FosB immunostaining to map brain regions selectively activated by chronic variable stress, as compared to a chronic heterotypic (habituation) stress regimen (restraint). Our data identified a small set of interconnected structures that expressed FosB after chronic variable stress: the infralimbic cortex (IL), posterior hypothalamus, and NTS (87). Both the posterior hypothalamus and NTS are connected with the IL and send excitatory projections to the PVN (88–90), consistent with a possible role in potentiation of the excitatory drive to the HPA axis following chronic stress.

Selective inhibition of GR signaling further implicates two of these brain regions, the IL and NTS, in alterations to PVN drive by chronic stress. Lentiviral knockdown of GR in the IL (but not prelimbic cortex) produces a marked potentiation of HPA axis and PVN Fos induction to a novel stress in animals exposed to CVS (91), a phenomenon linked to interneuron-mediated decreases in IL output (92). Implants of the GR antagonist mifepristone in the NTS inhibit PVN excitability following chronic stress, suggesting that the glucocorticoids may play a role in inhibiting the NTS drive to the PVN (93). These data implicate both regions as sites of glucocorticoid feedback (perhaps at the genomic level) and suggest that they function to set HPA reactivity consequent to chronic stress.

IMPACT OF CHRONIC STRESS ON PVN OUTPUT

Chronic stress leaves a cumulative record of HPA activation, including adrenal hypertrophy (due to elevated ACTH exposure), thymic atrophy (as a result of glucocorticoid-mediated cell death), and increased PVN FosB/delta FosB expression. All effects are believed to be a direct consequence of central drive of CRH neurons by stressors. However, it is important to consider the dynamics that underlie the progression of chronic stress endpoints. In general, the largest somatic and HPA axis effects are observed in the first few days of a chronic stress regimen. For example, exposure to chronic social stress causes a profound initial weight loss that is then maintained across the duration of the stress regimen (94). Similarly, increases in resting glucocorticoid secretion are greatest across the first few days of chronic stress and can diminish significantly over time. Given evidence for adaptation within chronic stress regimens, it is likely that the enduring effects of chronic stress on PVN activation are mediated by a combination of marginal increases in baseline drive and episodic activation of CRH neurons as a result of each stress exposure.

It is important to note that chronic stress activation of the HPA axis may be adjusted downstream of the CRH neuron (**Figure 1**). Chronic stress results in marked changes in pituitary corticotropes, including an upregulation of proopiomelanocortin (ACTH precursor protein) synthesis and increases in both CRHR1 and vasopressin 1b (V1b) receptor mRNA expression (13, 95), suggesting mechanisms that would enhance capacity for ACTH release. Interestingly, chronic stress triggers increases in pituitary V1b, but not CRHR1 binding, again predicting a role for AVP in potentiation of stress responses at the level of the pituitary (11). We recently reported an increase in the sensitivity of pituitary ACTH secretion to CRH stimulation *in vivo* and *in vitro* following chronic variable stress (56).

Chronic stress also causes changes at the level of the adrenal cortex. Exposure to 2 weeks of chronic variable stress results in marked hyperplasia and hypertrophy of the adrenal gland, as well as a marked enhancement of adrenal sensitivity to ACTH (96). Prior studies indicated that adrenal sensitivity to ACTH is modified by splanchnic nerve cuts (which remove innervation of the adrenal cortex) (97), suggesting that chronic stress-related changes in adrenal size and responsiveness are mediated by increased sympathetic activation.

There are marked differences in HPA axis regulatory mechanisms in males and females. Basal total corticosterone levels are higher in females than in males [e.g., Figueiredo et al. (98)]. However, circulating corticosteroid-binding globulin is higher in females than males, with the net effect of negating the sex differences in resting corticosterone levels (however, stress-induced

free corticosterone remains higher in females than males) (99). In addition, estrogens play a major role in driving HPA axis activation in females and are responsible for elevated glucocorticoids during proestrus and estrus in rodents (98, 100, 101). Interestingly, the effect of estrogens on the HPA axis is particularly pronounced at the level of the adrenal gland: estradiol inhibits restraint-induced ACTH release but strongly enhances the sensitivity of the adrenal to ACTH, which results in a net increase in stress-induced glucocorticoid release (102).

ALTERNATIVE PVN RESPONSES TO CHRONIC STRESS: SOCIAL SUBORDINATION

While upregulation of PVN function is commonly observed after most chronic stress regimens, it is important to note that stress regimens with strong social or metabolic components produce more variable results. For example, in rats, chronic social stress appears to decrease PVN CRH mRNA expression in subpopulations of subordinate individuals (103). Decreased CRH mRNA is observed in individuals that exhibit short defeat latencies to repeated social defeat and in individuals showing a stress-non-responsive phenotype in a colony-based subordination paradigm (104), suggesting an enhanced downregulation of response capacity in the most severely affected animals. In mice, results have been more variable. Some studies report upregulation of CRH mRNA, particularly in subsets of animals that do not engage the resident in a repeated intruder stress paradigm (designated as "stress-susceptible" individuals) (105). Chronic social stress (housing with aggressive conspecific) results in transient CRH mRNA increases that normalize over time, despite subsequent development of severe adrenal insufficiency (106). Other studies do not report changes in CRH following repeated defeat stress (although samples were not stratified with respect to submissive behaviors) (107). However, social stress-induced increases in AVP mRNA expression have been reported by multiple groups (107–109), once again suggesting that prolonged activation of the HPA axis during social stress may be driven by this important ACTH co-secretagogue.

Unfortunately, the data on PVN function following social stress are largely limited to gene expression, limiting appreciation of cellular and neuroplastic adaptations that may be important to understanding mechanisms underlying social stress and its related pathologies. Moreover, these studies do not explore possible dynamic changes in PVN glucocorticoid signaling that are specific to social stress. These interesting discrepancies between PVN responses to environmentally imposed and social stress require additional attention, as they may inform essential differences between diseases linked to HPA axis hyper- vs. hypo-responsiveness (e.g., depression vs. PTSD/chronic fatigue).

SUMMARY

The weight of evidence overwhelmingly supports a dynamic neuroplasticity in the PVN as a result of chronic stress. Chronic stress has a demonstrable impact on cellular biosynthetic activity (cell size, neuropeptide expression); cell physiology (reduced inhibition and enhanced excitation); neurotransmission (receptor expression, innervation patterns); glucocorticoid feedback (rapid and genomic, local and upstream); and storage and release of ACTH secretagogues in the median eminence. Nearly all of these changes are predictive of enhanced potential for CRH and co-secretagogue release, which would be instrumental in driving HPA axis activity. Many of the most dramatic effects are related to upregulation of AVP signaling capacity in CRH neurons and at the pituitary, and underscore the potential importance of this co-localized and co-released peptide as an amplifier of the CRH signal normally initiated by these neurons. The mechanisms in place for promoting CRH neuronal activity occur in the context of a heightened glucocorticoid signal, indicating that neural drive is sufficient to largely overcome feedback inhibition under conditions of chronic stress. All of these findings highlight the importance of maintaining HPA axis stress reactivity even in the context of chronic stress, thereby defending the organism from real or perceived adversity.

Enhancement of the "potential energy" of the HPA axis is likely adaptive within the context of a period of prolonged, but manageable adversity. Indeed, upregulation of the PVN may be thought of as a component of "allostatic load," an "adaptation through change" that keeps the system on line in the event of need (110). However, conditions of prolonged drive may have the capacity to push the system beyond the point of "adaptative" value and contribute to psychological and physiological pathologies associated with chronic stress. Likewise, prolonged engagement of PVN drive in the absence of an appropriate context would be sufficient to promote pathological features of HPA axis activation under inappropriate (e.g., non-stressed) conditions, as observed in diseases linked to stress (e.g., enhanced CRH and AVP mRNA expression and impaired dexamethasone suppression in depression).

Overall, as the "final common pathway" for stress integration by the brain, the PVN is ultimately responsible for both normal and pathological features of HPA axis stress responses. Despite progress to date, mechanisms underlying chronic stress-related PVN drive, modification of PVN secretagogue signaling, and synaptic plasticity are ill-defined. The significance of sex differences in PVN function is virtually unexplored. Failure to address these key issues in what is arguably the simplest component of stress reactivity imperils our ability to develop strategies to mitigate stress and diseases of stress adaptation.

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Dr. JH wrote the article, along with intellectual and editorial contributions from Dr. JT.

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REVIEW
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Ancient interaction between the teneurin C-terminal associated peptides (TCAP) and latrophilin ligand-receptor coupling: a role in behavior

Rebecca Woelfle[1], *Andrea L. D'Aquila*[1], *Téa Pavlović*[1], *Mia Husić*[1] and *David A. Lovejoy*[1,2]*

[1] Department of Cell and Systems Biology, University of Toronto, Toronto, ON, Canada, [2] Protagenic Therapeutics Inc., New York, NY, USA

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***Correspondence:**
David A. Lovejoy,
Department of Cell and Systems Biology, University of Toronto, 25 Harbord Street, Toronto, ON M5S 3G5, Canada
david.lovejoy@utoronto.ca

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Teneurins are multifunctional transmembrane proteins that are found in all multicellular animals and exist as four paralogous forms in vertebrates. They are highly expressed in the central nervous system, where they exert their effects, in part, by high-affinity binding to latrophilin (LPHN), a G-protein coupled receptor (GPCR) related to the adhesion and secretin GPCR families. The teneurin C-terminal associated peptides (TCAPs) are encoded by the terminal exon of all four teneurins, where TCAPs 1 and 3 are independently transcribed as soluble peptides, and TCAPs 2 and 4 remain tethered to their teneurin proprotein. Synthetic TCAP-1 interacts with LPHN, with an association with β-dystroglycan, to induce a tissue-dependent signal cascade to modulate cytoskeletal dynamics. TCAP-1 reduces stress-induced behaviors associated with anxiety, addiction and depression in a variety of models, in part, by regulating synaptic plasticity. Therefore, the TCAP-1-teneurin-LPHN interaction represents a novel receptor-ligand model and may represent a key mechanism underlying the association of behavior and neurological conditions.

Keywords: stress, synapse, molecular evolution, neuroendocrine interactions, adhesion GPCR

Introduction

The molecular association of adhesion proteins and G protein-coupled receptors (GPCRs) became established in the evolution of eukaryote and later multicellular organisms, and their relationship with energy metabolism would be essential to the survival of these systems. Ultimately, such fundamental molecular transducing systems would have been evolutionarily retained and incorporated into newly evolving signaling and biochemical pathways. The teneurin-latrophilin (LPHN) intermolecular trans-synaptic pairing formed at the earliest stages of metazoan evolution, and is currently the only known example of a trans-synaptic molecular adhesion coupling that is common between invertebrates and vertebrates. However, although the teneurins are well established to play a role in adhesion, these multifunctional transmembrane proteins are also associated, in part with paracrine signaling via a peptide termed "teneurin C-terminal-associated peptide (TCAP)," found in the terminal exon of each of the four teneurin genes. Its primary structure is highly conserved throughout animal evolution and is associated with modulating numerous stress-associated

behaviors. In this review, we posit that the teneurin/TCAP-LPHN unit represents one of the most fundamental signaling systems to regulate neuronal metabolism and associated behaviors.

Discovery and Structure of Teneurins, TCAP, and Latrophilins

The teneurin and LPHN ligand receptor system represents an early evolving system that has become associated with the coordination of cellular development, sensory regulation and control of energy systems to promote the survival of cells in multicellular organisms. On the distal extracellular tip of each teneurin is a short amino acid sequence termed "TCAP" that has actions independent of the full-length teneurin protein. Also, TCAP possesses a compelling structural homology with a number of bioactive peptide ligands associated with the secretin family of GPCRs. The teneurin TCAP protein binds and activates the LPHNs, a family of adhesion- associated GPCRs, to regulate numerous neurological and physiological activities.

Teneurins

The teneurins (**Figure 1**) comprise a family of glycosylated type II transmembrane proteins that were originally discovered in *Drosophila* as tenascin-like molecule accessory (ten-a) (Baumgartner and Chiquet-Ehrismann, 1993), tenascin-like molecule major (ten-m) (Baumgartner et al., 1994) and odd oz (odz) (Levine et al., 1994), by two independent groups in a search intended to identify orthologs of the vertebrate tenascins (Baumgartner et al., 1994) and tyrosine phosphorylated proteins (Levine et al., 1994). However, they were eventually established to be structurally and functionally distinct from the tenascins, despite the high degree of conservation of their epidermal growth factor (EGF)-like repeats (Tucker et al., 2006). The name "teneurins" reflects the protein's high level of expression in the developing and adult nervous system, as well as its association with ten-m (Oohashi et al., 1999; Lovejoy et al., 2006). Teneurin genes encode large proteins that are composed of approximately 2800 amino acids and contain an N-terminal intracellular domain, a single span transmembrane domain and a large highly conserved C-terminal extracellular domain (Rubin et al., 1999; Tucker and Chiquet-Ehrismann, 2006), consistent with the architecture of prokaryote polymorphic proteinaceous toxins (see below). The intracellular region contains two EF-hand-like domains, typical of calcium-binding proteins, as well as two polyproline regions which serve as c-Cbl-associated protein/ponsin binding sites, facilitating interaction between teneurin-1 and the cytoskeleton (Nunes et al., 2005). On the highly conserved extracellular side, there are eight tenascin-type EGF-like repeats, a region of conserved cysteine residues, and a unique stretch of 26 tyrosine-aspartate (YD)-repeats (Minet and Chiquet-Ehrismann, 2000; Young and Leamey, 2009). Among eukaryotic proteins, the 26 YD repeats occur only in teneurins.

Subsequently, the vertebrate orthologs of ten-a and ten-m were discovered. Mouse *Doc4*, the first vertebrate member of the teneurin family, was identified in a screen for novel genes that were expressed in response to perturbation of protein folding in the endoplasmic reticulum (Wang et al., 1998) and since then, several laboratories independently described the *ten-a* and *ten-m/odz* homologs in zebrafish (Mieda et al., 1999), chicken (Mieda et al., 1999; Rubin et al., 1999), mouse (Oohashi et al., 1999), rat (Otaki and Firestein, 1999), human (Minet et al., 1999; Minet and Chiquet-Ehrismann, 2000), and *Caenorhabditis elegans* (Drabikowski et al., 2005). In most invertebrates, only one teneurin copy has been identified, with the exception of insects, where two paralogs have been discovered (Tucker et al., 2012). However, unlike invertebrates, four teneurin paralogs have been reported in most vertebrates.

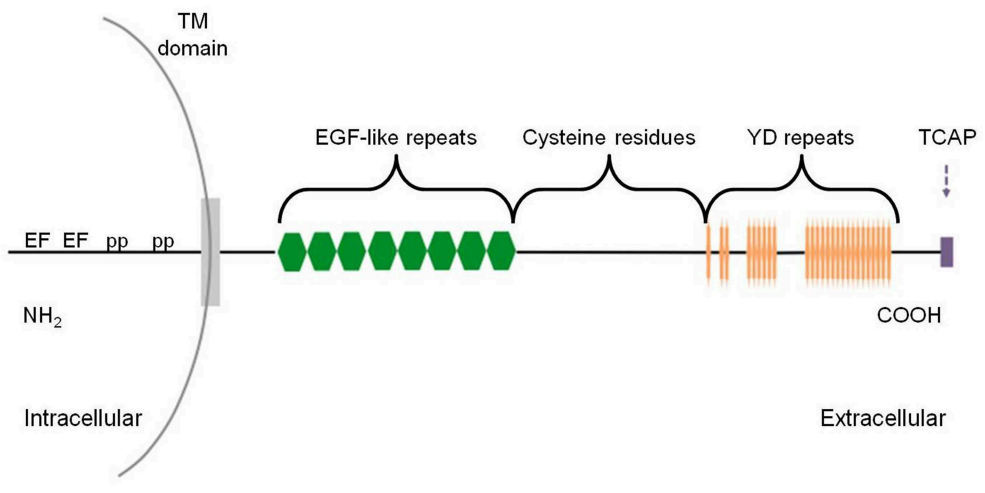

FIGURE 1 | Schematic of teneurin-TCAP protein structure. The intracellular amino terminus contains polyproline (pp) sites and EF-hand-like Ca^{2+} binding motifs (EF). The extracellular domain is composed of eight epidermal growth factor (EGF)-like repeats, a cysteine-rich region, followed by 26 tyrosine-aspartic acid (YD) repeats. Finally, the carboxy terminus contains the TCAP structure with 40–41 residues. Drawing is not to scale.

In metazoans, it has been postulated that the teneurins arose from a single ancestral gene. Comparison of the gene organization among human *Ten-1*, *Drosophila ten-a* and *ten-m* and the *C. elegans ten-1* reveals the presence of both conserved intron locations and exon sequences (Minet and Chiquet-Ehrismann, 2000). Sequence comparisons of teneurins show that it is not possible to classify any of the vertebrate teneurins specifically with either *Drosophila ten-a or ten-m*. However, phylogenetic analysis suggests that insects and vertebrates separated before the teneurin gene started to duplicate in each indicating that the two insect *ten-m* (teneurin) homolog genes are the result of a lineage-specific duplication. Therefore, the insect teneurin ancestor gene duplicated once to allow for two teneurin paralogs, *ten-a and ten-m*, whereas the duplication of the teneurins in the vertebrates occurred before the vertebrates radiated into their respective classes.

Recent evidence indicates that the original transfer of teneurin-like homologs originated from a prokaryotic transmembrane polymorphic proteinaceous toxin gene into the genome of a choanoflagellate. Subsequently, it became associated with another gene possessing a set of EGF- like domain repeats, which are a hallmark of metazoan genomes (Tucker et al., 2012; Tucker, 2013). Before the advent of single-celled eukaryotes (protists), prokaryotes were the initial form of life on the planet. Prokaryotes were likely the major food source of the first eukaryotes. As the first heterotrophic (non-phototrophic) multicellular organisms evolved, prokaryotes and protists would have been the fundamental nutrient sources. Choanoflagellates are thought to be the most closely related protist lineage to the Metazoa (Ni et al., 2012; Ramulu et al., 2012; Tucker et al., 2012; Tucker, 2013).

Teneurins and their invertebrate homolog, ten-m, have been found in most metazoans studied to date. In vertebrates, teneurins have been functionally implicated in neurite outgrowth (Rubin et al., 1999), cell adhesion (Rubin et al., 2002), and neuronal pathfinding. In neuroblastoma cells, overexpression of teneurin-2 led to enhanced neurite elongation, enlarged growth cones, increased filopodia formation and co-localization with actin-containing filopodia (Rubin et al., 1999, 2002). Similar effects were also observed in chicken dorsal root ganglia explants plated on recombinant teneurin-1 YD-repeats (Mieda et al., 1999). In mice, a missense mutation at the C-terminus of teneurin-4 results in delayed gastrulation and neural tube defects prior to embryonic lethality (Lossie et al., 2005). Knockout mice lacking teneurin-3 show abnormalities in mapping of ipsilateral projections, indicating that teneurin-3 has a role in axonal guidance within the visual cortex. These mice also exhibit deficits when performing visually mediated behavioral tasks, however the deficits were described as mild, suggesting functional redundancy with other teneurins. Similar actions are observed within the invertebrate teneurins. The *Drosophila* ten-a and ten-m are required for correct matching of olfactory projection neurons and receptor neurons, leading to proper olfactory mapping (Hong et al., 2012). Thus, the conservation of the teneurin gene, its function, and duplication in multicellular organisms argues for its early essential role in species survival and evolutionary success.

Teneurin C-terminal Associated Peptide (TCAP)

The discovery of TCAP in rainbow trout occurred after the reports of teneurin and *ten-m* by an independent study, searching for homologs of corticotropin-releasing factor (CRF) (Qian et al., 2004). Alignment with other genomic sequences uncovered a 40-residue carboxy-terminal sequence located in the final 3′ exon of teneurin, now known as TCAP (Qian et al., 2004). Flanked by a cleavage motif on the amino terminus and an amidation motif on the carboxy terminus, TCAP contains features characteristic of an endogenous bioactive peptide (Qian et al., 2004). In comparing teneurin-3 orthologs, a high degree of conservation was observed across the zebrafish, mouse and human, and the TCAP portion embedded within the teneurin carboxy terminal was found to be the most highly conserved sequence of the final exon; this resistance to change is highly suggestive of functional importance. The TCAP sequence alone bore the closest resemblance to the CRF peptide family, in terms of amino acid sequence, than any other known peptide sequences. Since then, it has been shown to possess sequence similarity to all of the secretin GPCR family peptide ligands (Lovejoy et al., 2006; Lovejoy and de Lannoy, 2013). It has since been hypothesized that the original TCAP-like peptide introduced into the Metazoa by lateral gene transfer spawned the evolution of the peptide ligands of the secretin family of GPCRs. This hypothesis is further supported by the close relationship among the cognate receptors (see further discussion below).

Recent studies on the evolution of prokaryote polymorphic proteinaceous toxins indicate that the C-terminal region of the teneurins is derived from the toxin "payload" resulting from the original gene transfer in choanoflagellates (Zhang et al., 2012). This confirmed previous studies on the biological efficacy of the TCAPs. The TCAP family of peptides was first discovered in a search for novel CRF homologs (Qian et al., 2004). Since then, four TCAPs have been identified in vertebrates, annotated as TCAP-1,-2, -3, and -4, based on their location in the four teneurin genes. All TCAPs are the same size as CRF, but possess less than 20% sequence similarity to the CRF family of peptides (Lovejoy et al., 2006) and differ markedly in structure (Tan et al., 2012). However, the TCAP family is highly conserved across the metazoans.

In the genome, TCAPs are annotated as being part of the extracellular domain of teneurin but recent studies indicate that some members of the TCAP family may possess functions that are independent of the larger teneurins. Rodent brain teneurin-1 (Zhou et al., 2003) and TCAP-1 (Wang et al., 2005) mRNA expression is distinct in some regions such as in the limbic areas, but overlap in others areas such as the olfactory bulb and cerebellum suggesting that the teneurin gene could be differentially regulated. Northern blot studies in the adult brain and embryonic hypothalamic cell culture demonstrated that only TCAP-1 and -3 can be independently synthesized from the larger teneurins, (Chand et al., 2012a).

Latrophilins

A receptor for the teneurins and TCAP has recently been characterized through an independent identification of the black widow spider toxin component, α-latrotoxin. This led to the discovery of

the LPHNs, a family of GPCRs (Lelianova et al., 1997). Like the teneurins, recent studies have indicated that the LPHNs, evolved, in part, by lateral gene transmission from prokaryotes to an early metazoan ancestor (Zhang et al., 2012). Based on sequence comparisons of other GPCRs known at the time, LPHN was a novel member of the secretin family of GPCRs. Since then, recent analyses have placed the receptors within the adhesion family of GPCRs (Silva et al., 2011). There are three types of LPHNs (-1, -2, and -3), and both LPHN-1 and -3 are expressed in the brain, whereas LPHN-2 shows expression in non-neural tissues (Silva and Ushkaryov, 2010). The authors suggest that LPHN may play a role in the regulation of the presynaptic release of neurotransmitters in a system that regulates the role of calcium.

LPHNs possess a ligand-binding domain that is characterized as a "hormone binding-domain" as identified by Perrin et al. (1998) based on the characterization of the ligand binding domain of the CRF receptors. This region is found in a number of the adhesion GPCRs (Matsushita et al., 1999; Vakonakis et al., 2008). The toxin, α-latrotoxin, acts a potent agent via its interaction with the hormone-binding domain of LPHN-1 to regulate presynaptic neurotransmitter function by influencing, in part, the voltage-gated calcium channels (Davydov et al., 2009; O'Sullivan et al., 2014). Specifically, α-latrotoxin has been shown to release both γ-aminobutyric acid (Storchak et al., 2002) and acetylcholine (Lelyanova et al., 2009). However, because it produces neurotransmitter release in both calcium containing and calcium free media, it was thought to act on different receptor sites (Storchak et al., 2002). The stimulation of neurons by α-latrotoxin leads to a fusion of synaptic vesicles even in the absence of action potentials, calcium ionophores or hypertonic sucrose media (Boudier et al., 1999; Storchak et al., 2002).

At the time of the LPHN protein discovery, the endogenous ligands were not known, but were later shown to interact with synaptic scaffolding proteins and were generally implicated in presynaptic function leading to neurotransmitter release (Lelianova et al., 1997; Vakonakis et al., 2008; O'Sullivan et al., 2014). The LPHNs, which possess three paralogous genes and proteins and a number of splice variants, were eventually established to be part of the adhesion family of G-protein coupled receptors and were subsequently termed Lec 1, 2, and 3 by virtue of their lectin-like-binding regions (Lelianova et al., 1997; Matsushita et al., 1999). The LPHNs consist of multidomain regions possessing a rhamnose-binding lectin-like domain, an olfactomedin-like domain, and a hormone-binding domain similar to that previously established for the CRF family of receptors (Perrin et al., 1998; Vakonakis et al., 2008). As mentioned previously, it was thought that LPHNs were associated with the Secretin family (Family B) group of GPCRs, as both LPHN and the secretin family both contain a unique hormone-binding domain (Fredricksson et al., 2003; Nordstrom et al., 2009; Schiöth et al., 2010). However, more recently, LPHNs have been found to belong to the Adhesion family of GPCRs (Silva et al., 2011).

Several studies support a significant functional and physical interaction between teneurins and LPHNs, which is integral for the maintenance of the stability of the synaptic cleft. Silva et al. (2011) show that teneurin-2 binds and activates LPHN-1 with a Kd of less than 2 nM. Moreover, a C-terminal fragment of teneurin-2, comprising the region that encompasses TCAP-2 likewise binds LPHN-2 inducing release of intracellular calcium stores. The authors suggest, in the case of teneurin-2, the TCAP-2 region likely remains tethered to the teneurin-2 protein to produce its physiological and behavioral effects. In this study, there was little evidence to suggest that teneurins-1, -3 or -4 bound to the LPHN-1 receptor, and suggest that teneurin-2 was one of the cognate ligands for LPHN-1. However, a later study, confirmed that teneurin-2 did, indeed, bind to LPHN-1 with similar affinity, but teneurin-4 also bound the receptor with a physiologically similar, albeit somewhat lower affinity than teneurin-2 (Boucard et al., 2014). In this study, the lectin domain region of LPHN-1 was essential for full affinity of the teneurin ligand, which is consistent with the lower affinity and activity established for the C-terminal TCAP-2 containing fragment previously reported (Silva et al., 2011). As previously mentioned, the lectin domain is the most highly conserved region of LPHNs, which further supports the ancient ligand receptor interaction and coevolution of teneurin and LPHN.

Dystroglycans and Additional Proteins in the Teneurin-LPHN Complex

Given the complexity of the teneurin and LPHN proteins, it is unsurprising that the association of these two protein systems results from complex stoichiometry (**Figure 2**). Additional proteins including dystroglycans, fibronectin leucine-rich transmembrane (FLRT) proteins and neurexins have also been associated with the organization and signaling of this system (Sugita et al., 2001; O'Sullivan et al., 2014). Research shows that the FLRT proteins bind to the lectin domain of LPHNs and are involved in excitatory synapse development, axon guidance, and cell migration in the hippocampus and cortex (O'Sullivan et al., 2012). Moreover, a number of studies indicate a functional and physical association of the teneurin-TCAP protein and dystroglycans. Dystroglycans were initially established as intercellular adhesion proteins in a number of tissues. They arise as single precursor proteins that are then cleaved into α and β forms where they remain associated in the membrane. The α subunit is extracellularly exposed and binds a number of extracellular matrix proteins in non-neuronal cells, including laminin, perlecan, and agrin. Neurexins have been implicated as extracellular binding sites for neuronal α-dystroglycan. Similar to laminin, perlecan, and agrin, the extracellular sequences of neurexins contain a laminin-neurexin-sex hormone-binding/laminin G domain (Sugita et al., 2001). The β-dystroglycan form, on the other hand, consists of a smaller transmembrane region and a cytoplasmic tail. There is little exposure of this subunit to the extracellular space. The intracellular region of the β subunit binds to dystrophin and utrophin, both of which interact with the actin cytoskeleton (Hemler, 1999; Henry and Campbell, 1999). Thus, the dystroglycan complex serves, in part, to couple extracellular matrix proteins with the intracellular matrix. The significance of this arrangement is underscored by deletion studies indicating that loss of the dystroglycan gene results in embryonic lethality (Henry and Campbell, 1998).

Dystroglycan is a main component of the transmembrane protein complex, dystrophin-glycoprotein complex, originally

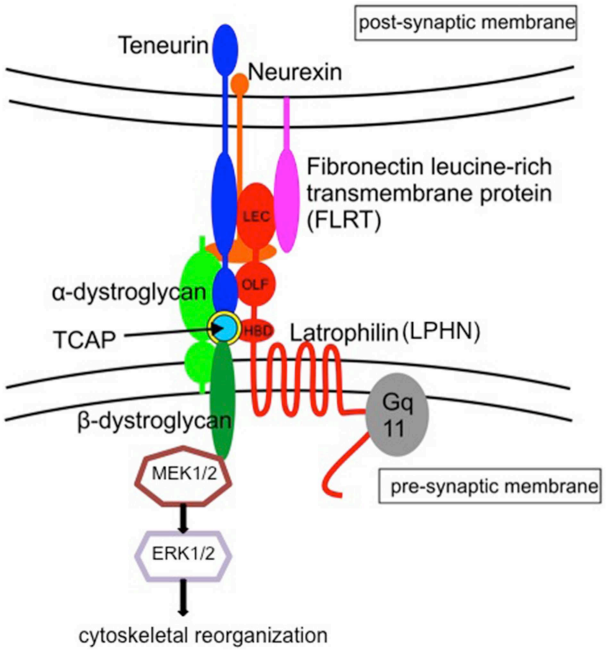

FIGURE 2 | Schematic interpretation of the teneurin/TCAP-latrophilin complex in the trans-synaptic region. Current evidence suggests that teneurins interact with both the lectin-like (LEC) and olfactomedin-like (OLF) binding domains. We further postulate that TCAP interacts with the hormone-binding domain (HBD) of latrophilin, and associates with β-dystroglycan to activate the MEK-ERK1/2 pathway leading to cytoskeletal reorganization and synaptic plasticity (Adapted from Chand et al., 2012b). Teneurin/TCAP and latrophilins, together with α–dystroglycan, neurexin and fibronectin leucine-rich transmembrane protein (FLRT) play a role with the stabilization of the trans-synaptic region.

discovered in skeletal muscle (Masaki and Matsumura, 2010). In muscle, dystroglycan acts as a scaffold providing a crucial link between extracellular matrix and the cytoskeleton (Henry and Campbell, 1996; Masaki and Matsumura, 2010), and is also present at the neuromuscular junction where it links rapsyn and acetylcholine receptor, thereby aiding in acetylcholine receptor clustering (Banks et al., 2003). Mice that are homozygous null mutants for dystroglycan result in embryonic lethality, which indicates it is critical for development (Williamson et al., 1997). However, dystroglycan is expressed in non-muscular tissue as well and has functional properties that are not muscle-specific (Henry and Campbell, 1996). Dystroglycan plays an important role in kidney epithelial morphogenesis by its interaction with laminin (Henry and Campbell, 1996), as well; neuronal dystroglycan is responsible for the maintenance of Schwann cell myelination and axonal guidance (Masaki and Matsumura, 2010).

In Trzebiatowska et al. (2008) initially reported an association of the teneurins with dystroglycans. It was concluded that the teneurins have partly related and redundant functions in *C. elegans* development, particularly with respect to the organization and maintenance of the basement membrane in tissues associated with the gonad, pharynx and hypodermis. Both teneurins and dystroglycans bind laminins (Rubin et al., 1999; Henry et al., 2001), a key component of the basement membrane and extracellular matrix in non-neural tissues. Although these studies pointed to a functional relationship between teneurins and dystroglycans, a physical association was not established. In an independent study, Chand et al. (2012b) identified α-dystroglycan as a candidate target for further investigation, when it was found to have the highest level of mRNA up-regulation as ascertained by gene array studies following the treatment of synthetic mouse TCAP-1 on cultures of immortalized mouse hypothalamic cells. It was subsequently determined that fluoresceinisothiocyanate (FITC)-labeled mouse TCAP-1 with lysine substitutions for arginine residues 8 or 37 (FITC-K8/37-mTCAP-1) showed significant co-localization with immunoreactive β-dystroglycan although an association with α-dystroglycan was equivocal. However, although FITC-K37-mTCAP-1 was internalized into the cell via clathrin-independent but caveolin-associated vesicle transport, no co-labeling of this TCAP-1 analog with either of the dystroglycan subunits could be discerned (Chand et al., 2013). These findings were partially resolved when TCAP-1 and teneurin-1 was investigated in the mouse testes. Here, the studies indicated a clear relationship of teneurin-1 with α-dystroglycan, and TCAP-1 with β-dystroglycan (Chand et al., 2014). However, in these studies, teneurin immunoreactivity was found at the cell membrane, whereas TCAP-1 immunoreactivity was typically found within the cytosol. Thus, it was not clear if the intact teneurin-TCAP protein was binding to both dystroglycan subunits. Although these studies show a different interaction profile of the teneurins and TCAPs with respect to dystroglycans, it is not clear whether a similar arrangement occurs in the brain. Thus, based on these studies, the authors suggested that while the dystroglycans were associated with the teneurins and TCAPs, they were not the cognate receptors.

In the brain, the arrangement of the dystroglycan complex with components of the extracellular matrix differs as laminin, perlecan, and agrin are not typically found (Sugita et al., 2001). Indeed, as basement membrane is found in non-neural tissues, it is similarly lacking in the brain. However, because of the high expression of dystroglycans in the brain, additional ligands were sought. There is a strong association of both dystroglycan subunits with the neurexins (Sugita et al., 2001; Reissner et al., 2014). Neurexins are neuron-specific cell surface proteins, and although only three vertebrate neurexin genes have been identified, there are thought to be over a thousand alternative splice-generated forms (Ullrich et al., 1995). The neurexin structure can be complex in that it includes an extracellular domain consisting of a number of laminin-neurexin, sex steroid-binding globulin domains that are interspersed with EGF-like domains, although this region is variable depending upon the splice variant. Dystroglycans specifically bind to this region under the appropriate glycosylation and appears to compete with α-latrotoxin, as neurexin has been identified as an alternate viable receptor for α-latrotoxin (Sudhof, 2001; Sugita et al., 2001).

Evidence for Latrophilins as Cognate Receptors of Teneurins and TCAPs

Several lines of evidence indicate that TCAP is an endogenous ligand of the LPHNs, and these arguments can be divided into

evolution and phylogenetic, cellular expression, molecular and physiological aspects. At the evolutionary and phylogenetic level, TCAP appears to have evolved about the same time as the LPHNs at an early stage of metazoan evolution (Zhang et al., 2012; Chand et al., 2013; Lovejoy and de Lannoy, 2013). TCAP-like sequences are found in most metazoans and possess sequence similarity to a diverse range of hormones associated with the secretin GPCR family of ligands in vertebrates, and toxins found in both invertebrates and vertebrates. All TCAPs are associated, in part, with the expression of the teneurins. Our studies indicate that while mRNA expression of all TCAPs show a relationship with the intact teneurin, TCAP-1 is transcribed independently and TCAP-3 may also do so (Chand et al., 2013). The teneurin expression shows a strong overlap with the LPHNs, and TCAP and teneurins are found in all tissues known to express the LPHNs, including the amygdala, hippocampus, and prefrontal cortex (Arcos-Burgos et al., 2010; Silva et al., 2011). At the molecular level, teneurins have been established to bind to LPHNs at physiologically compatible affinity at the lectin-like domain (Silva et al., 2011; Boucard et al., 2012). This domain is the most highly conserved region of LPHNs, further indicating the ancestry and importance of teneurin-latrophilin binding.

Neurological Actions of the Teneurin/TCAP-LPHN Complex

There are a number of synaptic adhesion proteins, although only the teneurins, LPHNs and FLRTs have been conserved between invertebrates and vertebrates (Tucker et al., 2006; Boucard et al., 2014). Therefore, the teneurin-latrophilin pairing is a likely candidate to understand the phylogenetically earliest mechanism that lead to neuronal transmission. Interneuronal transmission is essential for neuron viabilities. Tan et al. (2011) showed that intracerebroventricular (ICV) administration of synthetic mTCAP-1 increased spine density in hippocampal neurons. Interestingly, LPHN-1 has been localized on nerve terminals near synaptic clefts but not on dendritic spines whereas teneurin-2 has been found on dendritic spines, suggesting that the two types of receptors may have different structure-function attributes (Silva et al., 2011). Also, as mentioned previously, post-synaptic FLRTs regulate glutamatergic synapse density in hippocampal cells; thus, providing support for their role in the trans-synaptic cell adhesion complex (O'Sullivan et al., 2014).

This interaction suggests a plausible mechanism for TCAP action: the LPHN-teneurin trans-synaptic complex stabilizes the synapse (along with the accessory proteins, dystroglycan, and neurexins) and the TCAP region of the C-terminal region of teneurin is required for high affinity binding to LPHN. Then, the presence of free TCAP, either in the synthetic form or in the form of a natural transcribed ligand, may bind to the hormone binding domain of LPHN and compete, in part, for the teneurin binding. Once bound, TCAP may regulate the actions/coupling of the receptor depending on the LPHN.

TCAP affects pathways related to anxiety and emotionality. Wang and associates screened rats by their response to the acoustic startle test, and then divided the rats into either low or high reactive groups based on their results (Wang et al., 2005). When treated with ICV TCAP-1, high reactive rats had a reduced acoustic startle response, whereas low reactive rats had an increased response, indicating that TCAP has a normalizing effect on behavior. It is proposed that TCAP has a general action across all synapses: synaptic circuits that promote a stress response would be disrupted leading to a decrease in stress behavior because of the inability to perceive and carry out the stress. In animals with a reduced sympathetic response and a higher parasympathetic response would be, likewise, inhibited and show a reduced inhibition of the stress response and so both would show a normalization of behavior. In the case of findings from Tan et al. (2011), the increase in spine density may reflect a normal homeostatic mechanism to increase the connections after synthetic TCAP-1 acted to break existing synapses. In the short term, the post-synaptic regions would be expected to increase dendrite spine number to maintain the signal. Then, in the long term and under repeated TCAP-1 administration (and assuming TCAP-1 reduces neurotransmitter release) the spine density would be reduced due to continued reduction of the neurotransmitter signal.

As part of the LPHN trans-synaptic complex, teneurins function to maintain neural networks by promoting synapse connectivity and increasing neurite outgrowth (Rubin et al., 1999; Young and Leamey, 2009). High levels of teneurins in the central nervous system are found on axon surfaces (Wang et al., 2005), and there is significant teneurin expression in the interconnected neurons found in chicken and mouse (Kenzelmann et al., 2008). Since the length of teneurin and LPHNs together can span the synaptic cleft (Silva et al., 2011), this allows teneurins to interact with adjacent neurons thus increasing efficiency of the synapse.

Additionally, teneurins are integral to the normal development of visual, auditory, and olfactory pathways (Young and Leamey, 2009; Hong et al., 2012). For example, teneurin-3 is required for binocular vision, as it regulates mapping of ipsilateral axons connecting the ventral retina to the dorsal lateral geniculate nucleus (Young and Leamey, 2009). Moreover, teneurin-1 is expressed prominently in the L5 and L6 regions of the neocortex (O'Sullivan et al., 2014) and in the piriform cortex, a region associated with processing of odor and pheromone signals (Wang et al., 2005). Ultimately, teneurins are important for basic brain maturation and function, as they regulate synaptic plasticity and modulate sensory processes.

A number of biological functions both *in vitro* and *in vivo* have been attributed to the TCAP family of peptides. The first studies found that in immortalized mouse neurons, TCAP-1 treatment had a dose-dependent effect on cAMP levels, teneurin-1 gene expression and cell proliferation (Qian et al., 2004; Wang et al., 2005; Al Chawaf et al., 2007b). Further, TCAP-1 is neuroprotective in hypothalamic neurons by increasing superoxide dismutase-1, superoxide dismutase-1 copper chaperone and catalase enzyme levels (Trubiani et al., 2007). In unstressed hypothalamic cells and primary hippocampal neurons, TCAP-1 treatment increases expression of α-actinin-4, β-actin and β-tubulin; and also induced neurite outgrowth, dendritic arborization, and axon fasciculation (Al Chawaf et al., 2007a). TCAP-1 also inhibits brain-derived neurotrophic factor expression and translation in hypothalamic neurons (Anantharaman and Aravind, 2003).

The receptor complex and signal transduction system for TCAP-1 has been recently established. Gene array studies initially established that the β-dystroglycan gene was significantly upregulated in TCAP-1 treated immortalized hypothalamic neurons. Immunohistochemical studies established that FITC-tagged synthetic TCAP-1 strongly co-localized with β-dystroglycan at the plasma membrane. In addition, the MEK-ERK1/2 signal transduction system associated with β-dystroglycan was, likewise, stimulated by TCAP-1 and could be blocked by a MEK inhibitor (Chand et al., 2012b) Subsequently, TCAP-1 stimulated ERK1/2-dependent phosphorylation of the cytoskeleton regulatory proteins, filamin, and stathmin. These studies indicate that TCAP-1 signals through a novel pathway associated with the dystroglycan complex to regulate cytoskeletal dynamics. Moreover, it provides an understanding of how TCAP and the teneurins can regulate neurite and neuronal process formation. Recently, studies on the carboxy-terminal region of teneurins on cytoskeletal elements in *Drosophila* showed a similar mechanism (Hong et al., 2012; Mosca et al., 2012). The two *Drosophila* teneurin proteins, ten-m and ten-a, have been implicated in neuromuscular synapse organization. Following teneurin perturbation, proper microtubule organization was impaired at presynaptic terminals, and post-synaptic spectrin cytoskeleton, α- and β-spectrin, an essential component of the membraneous subsynaptic reticulum, was significantly reduced. The most severe effects seen were cytoskeletal, thus, teneurins and TCAP play a vital role in pre-synaptic and post-synaptic cytoskeleton organization in *Drosophila*. (Hong et al., 2012; Mosca et al., 2012).

Cognitive and Behavioral Effects of the Teneurin/TCAP-LPHN System

Studies of the LPHNs have indicated a number of neurological effects specifically in the limbic region, and recent work has aimed at elucidating the neuronal expression of LPHNs and their role in psychiatric disorders. For instance, high *Lphn1* expression was found in the rat hippocampus and high *LPHN3* mRNA expression was found in human mesolimbic brain regions such as the amygdala and prefrontal cortex (Arcos-Burgos et al., 2010; Silva et al., 2011). *Lphn1* knockout studies have revealed some of its neurological actions. Female mice who were homozygous null mutants for *Lphn1* were not able to attend to their litters compared to wild-type mice, resulting in the death of their pups regardless of the genotype of the pups themselves (Martinez et al., 2010). As well, Silva and Ushkaryov (2010) found that *Lphn1* knockout mice showed increased aggression and schizophrenia-like behaviors compared to wild-type mice.

High *lphn3* expression was also examined in zebrafish dopamine neurons in the posterior tuberculum, a brain area with similar functions as the mammalian ventral tegmental area (Lange et al., 2012). Furthermore, loss of the *Lphn3* gene has been implicated in impaired dopamine system development and the pathogenesis of cocaine addiction and attention-deficient hyperactive disorder (ADHD) (Lange et al., 2012; Wallis et al., 2012). A hyperactive motor phenotype was seen in *lphn3*-mutant zebrafish, as indicated by their fast swimming behavior (Lange et al., 2012). Moreover, mutated *LPHN3* gene may be implicated in developmental synaptic abnormalities underlying ADHD (O'Sullivan et al., 2014). Similar to FLRTs, LPHN-3 controls glutamatergic synapse development and abundance of synaptic inputs in mouse pyramidal neurons. It is suggested that a mutation in the lectin domain of LPHN3 impairs binding of teneurin-1 to LPHN in the mouse neocortex (O'Sullivan et al., 2014). Interestingly, rats that repeatedly self-administered cocaine show upregulated *Lphn1* levels in the striatum (Lynch et al., 2008). Thus, LPHNs may be important components in dopamine-mediated mechanisms and emotional regulation. Interestingly, GPCRs have been of major pharmaceutical interest, with about one-third of research being directed at them (Martinez et al., 2010). This further supports the importance of this research of the Teneurin/TCAP-LPHN ligand-receptor interaction and its medicinal applications in respect to neurological conditions. Further studies indicate that the teneurin/TCAP-latrophilin system may have a number of effects on cell and organismal metabolism.

TCAP-1 has emerged as a novel candidate in the integration and modulation of a number of psychiatric disorders including stress, anxiety, and addiction (Tan et al., 2008). Acute administration of TCAP-1 into the basolateral nucleus of the amygdala in rats modulated acoustic startle behavior by increasing the startle response in the low-startle group and decreasing startle response in the high-startle group (Wang et al., 2005). Other anxiety studies, using an elevated plus maze and open field tests, showed that TCAP-1 modulates CRF-regulated behaviors. Repeated intravenous (IV) administration of TCAP-1 had an anxiolytic effect on CRF-induced responses in both the elevated plus maze and open field tests (Al Chawaf et al., 2007b). However, ICV administration of TCAP-1 yielded differential behavioral responses based on the presence or absence of a stress challenge (Tan et al., 2008). In the absence of a CRF-mediated stressor, TCAP-1 had mild anxiolytic effects in the elevated plus maze and open field tests of behavior however, in the presence of a stressor TCAP-1 had anxiogenic effects in the elevated plus maze and open field tests (Tan et al., 2008). Interestingly, the TCAP-1 induced effects on behavior are long lasting, with effects persisting 21 days after TCAP-1 injection (Wang et al., 2005), suggesting that TCAP-1 may regulate neuronal plasticity in the brain. Further *in vivo* studies strengthened the notion of a functional link between the TCAP-1 and CRF-mediated systems. TCAP-1 blocked CRF-mediated c-fos synthesis in the hippocampus and amygdala of the adult rat (Tan et al., 2009) and increased spine density in the CA1 and CA3 neurons of the rodent hippocampus but not in the amygdala (Tan et al., 2011). Moreover, repeated ICV and IV TCAP- 1 administration ablates CRF-induced cocaine-seeking reinstatement in rats (Kupferschmidt et al., 2011; Erb et al., 2014).

Summary

As indicated by evolutionary and phylogenetic, molecular and physiological aspects, we posit that TCAP is the endogenous

ligand for LPHN. This cognate ligand-receptor coupling has been retained since their ancient interaction approximately one billion years ago. Endogenous TCAP-teneurin is bound to a trans-synaptic LPHN cell adhesion complex and is associated with FLRTs, neurexins, and dystroglycans. Ultimately, this complex is integral to maintenance of synaptic plasticity and regulating pathways associated with behavior. While little is known about the mechanisms behind the expression of TCAP-1, evidence strongly suggests that it can be transcribed independently of teneurin-1 and is translated and processed outside of the classical ER/Golgi pathway. This, as well as its distinct functions and cellular expression compared to teneurin-1, indicate that the two have independent roles within the cell.

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REVIEW
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An Update on CRF Mechanisms Underlying Alcohol Use Disorders and Dependence

Isabel Marian Hartmann Quadros, Giovana Camila Macedo, Liz Paola Domingues and Cristiane Aparecida Favoretto*

Department of Psychobiology, Escola Paulista de Medicina, Universidade Federal de São Paulo, São Paulo, São Paulo, Brazil

Alcohol is the most commonly used and abused substance worldwide. The emergence of alcohol use disorders, and alcohol dependence in particular, is accompanied by functional changes in brain reward and stress systems, which contribute to escalated alcohol drinking and seeking. Corticotropin-releasing factor (CRF) systems have been critically implied in the transition toward problematic alcohol drinking and alcohol dependence. This review will discuss how dysregulation of CRF function contributes to the vulnerability for escalated alcohol drinking and other consequences of alcohol consumption, based on preclinical evidence. CRF signaling, mostly *via* CRF1 receptors, seems to be particularly important in conditions of excessive alcohol taking and seeking, including during early and protracted withdrawal, relapse, as well as during withdrawal-induced anxiety and escalated aggression promoted by alcohol. Modulation of CRF1 function seems to exert a less prominent role over low to moderate alcohol intake, or to species-typical behaviors. While CRF mechanisms in the hypothalamic–pituitary–adrenal axis have some contribution to the neurobiology of alcohol abuse and dependence, a pivotal role for extra-hypothalamic CRF pathways, particularly in the extended amygdala, is well characterized. More recent studies further suggest a direct modulation of brain reward function by CRF signaling in the ventral tegmental area, nucleus accumbens, and the prefrontal cortex, among other structures. This review will further discuss a putative role for other components of the CRF system that contribute for the overall balance of CRF function in reward and stress pathways, including CRF2 receptors, CRF-binding protein, and urocortins, a family of CRF-related peptides.

Keywords: alcohol, addiction, alcohol self-administration, neuropeptides, animal models, mesocorticolimbic system, sensitization, alcohol-related aggression

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****Correspondence:***
Isabel Marian Hartmann Quadros
isabel.quadros@unifesp.br

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OVERVIEW

For a few decades now, a critical role for stress in the induction, maintenance, and relapse to drug and alcohol dependence has been increasingly investigated. Indeed, drug dependence has been hypothesized as a "stress surfeit disorder" (1). Dysregulation of brain stress systems would contribute to the transition from drug-taking, which is primarily motivated by reward-seeking, toward escalated drug-taking that becomes mainly instigated by dysphoria and negative reinforcement. As the very primary player mediating brain stress responses, the corticotropin-releasing factor [CRF; also known as corticotropin-releasing hormone (CRH)] has received great attention in drug dependence.

This review will discuss evidence provided by preclinical studies focusing on the critical participation of CRF and its closely related peptides urocortins, in escalated alcohol drinking, and other important alcohol-related behaviors. Also, apart from its role in more traditional brain stress circuits, this review will discuss increasing evidence for the CRF system as a direct modulator of brain reward pathways, suggesting that CRF/urocortin signaling may also modulate alcohol's effects at earlier stages of alcohol dependence. The impact of alcohol exposure on CRF/urocortin signaling and plasticity will be discussed, as potential neuroadaptive mechanisms recruited during the establishment of alcohol dependence. Pharmacological manipulations targeting CRF receptors are presented as promising tools for reducing excessive alcohol drinking, withdrawal-related anxiety as well as alcohol-induced aggression and behavioral sensitization induced by alcohol. The participation of the hypothalamic–pituitary–adrenal (HPA) stress axis is addressed at specific points, but specifically as a downstream target engaged by brain CRF [for reviews on HPA axis and alcohol, refer to Stephens and Wand (2) and Edwards et al. (3)]. Finally, considerations are made on a few human alcohol studies evaluating polymorphisms in CRF-related genes. This review does not attempt to discuss alcohol studies using genetically modified animals for CRF-related genes, a subject vastly covered by a recent review on CRF systems and alcohol (4).

We refer to several review articles published on CRF and drug/alcohol dependence [e.g., Ref. (5–7)] as well as a recent review specifically focusing on CRF mechanisms involved in alcohol use disorders (AUDs) and alcohol-induced neuroplasticity (4).

ALCOHOL AND ALCOHOL USE DISORDERS

Alcohol is the most widely consumed psychotropic substance worldwide. Likewise, the harmful use of alcohol ranks within the five main risk factors for disease, disability, and death in the globe (8). AUDs are responsible for ~6% of all deaths throughout the world, even considering potential beneficial effects of low-risk alcohol drinking (8). Consequences of alcohol misuse can be both acute (e.g., acute intoxication, heavy episodic drinking, driving under the influence of alcohol, alcohol-related violence) and chronic (e.g., AUDs, gastrointestinal diseases including liver cirrhosis, cancers), and cause harm to the drinker, to other individuals, and to society in general. Thus, alcohol consumption is responsible for a large burden in public health, with serious economic and social impact.

Alcohol-related health harm is proportional to the amount of alcohol consumed, with a dose–response relationship. About 13% of the alcohol-drinking population will show a pattern of heavy episodic drinking, or "binge" drinking, which is more commonly associated with alcohol-related injury, including driving accidents and violent behavior (9). AUDs are the main neuropsychiatric condition promoted by alcohol consumption and encompass both the harmful use of alcohol and alcohol dependence. Alcohol dependence affects ~5% of the adult (15 years and older) population worldwide (8) and consists of a variety of behavioral, cognitive, and physiological symptoms that follow repeated alcohol use. According to the International Classification of Diseases, main features of alcohol dependence include a strong desire to consume alcohol; difficulties in controlling the frequency and amount of alcohol consumption; persistent use despite perceived harmful consequences; a higher priority given to obtaining and using alcohol relative to other activities and obligations; increased tolerance; and sometimes a physiological withdrawal state (10).

Despite the complexity of biological, environmental, and social factors that influence drug and AUDs, animal models have been instrumental in providing evidence for behavioral, pharmacological, and neurobiological mechanisms underlying acute and repeated alcohol exposure [for a review, see Ref. (11)]. This review will focus primarily on findings stemming from preclinical studies and how they contribute to our understanding of CRF/urocortin mechanisms in alcohol dependence. Of particular interest are models of voluntary alcohol consumption, including home cage drinking (e.g., two-bottle choice, with intermittent, limited, or continuous access conditions; the "drinking in the dark" model, etc.) and operant self-administration (animals respond by pressing a lever or nose poking in order to obtain an alcohol reward). Using these experimental models, it is also possible to promote escalated, excessive alcohol intake by using protocols of repeated and prolonged voluntary access to the substance, usually followed by one or multiple cycles of withdrawal. Examples are procedures such as the alcohol-deprivation effect (12) and dependent-like alcohol consumption in subsets of chronically drinking rats and mice [e.g., Ref. (13, 14)]. Other widely used procedures for escalated drinking rely on forced alcohol exposure in order to obtain reliable and consistent exposure to high alcohol concentrations, such as the alcohol vapor chamber (15, 16), as well as alcohol-containing liquid diet [see also Ref. (17) for a review]. Such excessive alcohol exposure is usually associated with withdrawal symptoms upon short-term withdrawal, and dysphoria and anxiety during protracted abstinence. These negative features trigger and motivate increased levels of voluntary alcohol self-administration [e.g., Ref. (18); see review by Heilig and Koob (5)].

Different from other drugs of abuse, such as cocaine, amphetamines, and morphine, pharmacological mechanisms for alcohol include a variety of targets in the central nervous system. Synaptic actions of alcohol have been described with several neurotransmitter-gated ionotropic receptors (e.g., GABAA, glutamate NMDA and AMPA receptors, serotonin 5-HT3 receptors, etc.), ion channels (e.g., different subtypes of calcium and potassium channels), as well as intracellular downstream signaling proteins also involved in metabotropic receptors cascades (e.g., diacylglycerol, protein kinase A, protein kinase C, etc.), as extensively reviewed by Lovinger and Roberto (19). Additionally, presynaptic effects of alcohol may be observed, with a facilitation or potentiation of presynaptic GABA release, but not glutamate, as revealed by electrophysiological studies using slice preparations or isolated neurons [reviewed by Lovinger and Roberto (19) and Siggins et al. (20)]. Chronic alcohol exposure produces consistent neuroadaptive changes in the function of both ionotropic and metabotropic glutamate receptors, for example, with the upregulation of NMDA receptors [e.g., Ref. (21–25)]. Likewise, GABAA receptors are markedly affected in their subunit composition, sensitivity, and function by chronic

alcohol treatment [see reviews by Grobin et al. (26) and Kumar et al. (27)]. These and many other changes in synaptic function, which vary in different brain regions, are considered critical for the development of tolerance and dependence to alcohol (19).

The effects of alcohol are importantly modulated by neuropeptides, including opioid peptides, neuropeptide Y, orphanin/nociceptin, and orexin. This review will focus on the family of neuropeptides comprised of CRF and its closely related peptides, the urocortins. The next section will briefly describe the physiology of CRF/urocortin signaling in the brain, and subsequently we will discuss the critical role of CRF signaling in AUDs.

A GLANCE AT CRF/UROCORTIN SYSTEMS IN THE BRAIN

The 41-amino acid neuropeptide CRF was identified in 1981 by Vale and colleagues and has long been associated with neural, endocrine, autonomic, and immune responses to stress (28). Acting as a neuromodulator, the availability of CRF is critically determined by the CRF-binding protein (CRF-BP), a glycoprotein that regulates the extracellular availability of CRF to bind to its receptors (29, 30). CRF exerts its effects *via* interaction with two G-protein coupled receptors, namely CRFR1 and CRFR2 (CRF receptor 1 and 2, respectively), which are found in several variants (31). However, CRF has a 10-fold higher affinity for CRFR1 relative to CRFR2 (32). Urocortins, another set of peptides in the CRF family, are the main endogenous ligands for CRFR2, showing similar affinity for both receptor subtypes (urocortin 1), or binding almost exclusively to CRFR2 [urocortins 2 and 3; (33)]. Activation of both CRFR1 and CRFR2 preferentially lead to the activation of cyclic-AMP second messenger pathways (34). The two receptor subtypes are differentially distributed in the brain, with overlapping regions (35). CRFR1 are more ubiquitously found. In rodents, high densities of CRFR1 are found in the anterior hypophysis, cerebral cortex, cerebellum, amygdala, hippocampus, and striatum (36), whereas CRFR2 are more limited to the mesencephalon, raphé nuclei, lateral septum, amygdala, and hypothalamus (37), as recently reviewed by Phillips et al. (4). Although not the focus of the current review, CRF and urocortin peptides, as well as receptors and binding protein, are widely distributed in peripheric organs and tissues, including the gastrointestinal tract, cardiovascular, and immune systems, where they integrate systemic stress responses and participate in other functions [see reviews by Fekete and Zorrilla (33), Pan and Kastin (38), Stengel and Taché (39)].

As a major modulator of systemic neuroendocrine stress responses, hypothalamic CRF drives the HPA axis, with secondary modulation by the neuropeptide arginine vasopressin [e.g., Ref. (28, 40, 41)]. Produced by parvocellular and magnocellular neurons of the paraventricular nucleus of the hypothalamus (PVN), AVP has a limited capacity to activate the HPA axis by itself (4, 42). However, the neuropeptide AVP seems to increase the effects of CRF on HPA axis by synergistic activation of its V1b receptor in the anterior pituitary (4, 42, 43). Produced by parvocellular neurons of the PVN, CRF is released in the median eminence to reach the anterior hypophysis (or pituitary), where it binds to densely expressed receptors, CRFR1 (44). Adrenocorticotropic hormone (ACTH) is then secreted into the blood stream by the adenohypophysis (45). Once secreted, ACTH stimulates the production and release of glucocorticoids from the cortex of adrenal glands (cortisol in humans, corticosterone in rodents). Glucocorticoids will then act upon high- and low-affinity receptors (mineralocorticoid and glucocorticoid receptors, respectively), widely distributed in the periphery and the brain. Cortisol and corticosterone promote adaptive responses to environmental challenges and stressors, including changes in energy metabolism, physiological, and behavioral responses. HPA axis function is importantly regulated *via* inhibitory feedback by glucocorticoids, ultimately reducing activity of PVN neurons and CRF release. For a more complete discussion on HPA axis signaling and functions, we refer to reviews by Herman et al. (45), McEwen (46), Myers et al. (47), Sapolsky et al. (48), and Ulrich-Lai and Herman (49).

Wide distribution of cell bodies and fibers with immunoreactivity for CRF is found in stress-related pathways involving amygdalar nuclei [especially the central nucleus of the amygdala (CeA)], the bed nucleus of the stria terminalis (BNST), and the PVN, with ascending projections to forebrain structures as well as descending innervations to the brain stem (31). High levels of CRF are also detected in the hippocampus, thalamus, locus coeruleus, raphé nuclei, and other mesencephalic structures (50, 51). Detection of neurons and fibers containing urocortin peptides are limited to fewer brain structures and projection sites in rodents, although a wider distribution of brain urocortin can be found in human and non-human primates (38, 52, 53). As extensively reviewed by Fekete and Zorrilla (33), urocortin 1 (Ucn1) is primarily synthesized in the centrally projecting Edinger–Westphal nucleus, a midbrain structure, and other secondary sites. Descending fibers of Ucn1 are found in many regions, including the substantia nigra, the dorsal raphe nucleus, and periaqueductal gray, while ascending fibers of Ucn1 are found in the lateral septum, BNST, hypothalamus, and other structures. Urocortin 2 (Ucn2) is synthesized in the PVN and other hypothalamic nuclei, as well as in the locus coeruleus, but its projection targets are unknown (33). Urocortin 3 (Ucn3) is produced in hypothalamic and amygdala regions. Ucn3 fibers project from the amygdala to the hypothalamic ventral premammillary nucleus, while fibers from undertermined origins are also found in other hypothalamic regions, in lateral septum, BNST, medial amygdala, and ventral hippocampus [for reviews, see Ref. (33, 54)].

Briefly, biological functions mediated by CRF and urocortin peptides include not only stress responses but also regulation of appetitive responses, such as feeding and exploratory behaviors, and a variety of social behaviors (38, 55, 56). In several of these functions, CRF receptor subtypes often play different roles (57, 58). For example, stress and anxiety-like responses are thought to be primarily initiated by CRF/urocortin 1 activation of CRFR1 signaling, while CRFR2 activation has been associated with anxiolytic effects and/or termination of stress responses (38, 59). As another example, CRF and urocortins inhibit feeding behavior likely *via* CRFR2 signaling, although both CRFR1 and CRFR2 are involved in stress-induced anorexigenic effects (54). Thus, biological responses involving CRF and urocortins are wide and

complex, and recruitment of specific CRF receptors will vary according to each particular behavior and/or pathology, in a brain region-dependent manner.

Through actions on CRFR1 and/or CRFR2, CRF and urocortin peptides set the pace for brain monoaminergic function in regions, such as the locus coeruleus (primary site for noradrenergic neurons in the brain), the dorsal and median raphé (serotonin neurons), and the ventral tegmental area (VTA), where reward-related dopamine neurons are located [e.g., Ref. (57, 60–62)]. Of particular interest to this review, dopamine neurons in the VTA receive CRF inputs from fibers that originate in the BNST, the CeA and, to a lesser extent, the hypothalamic PVN (63). In the VTA, CRF stimulates firing of dopamine neurons likely *via* CRFR1 signaling, modulating dopamine output to the nucleus accumbens (64). On the other hand, when CRF is directly applied in the nucleus accumbens, it can increase dopamine release and promote appetitive behavior (65). Exposure to acute stress is well known to induce activation of this dopamine pathway arising from the VTA and projecting to the nucleus accumbens and the prefrontal cortex, typically promoting increased drug-taking and/or drug-seeking behaviors [see review by Holly and Miczek (66)]. Interestingly, the ability of stress to trigger activation of dopamine brain reward pathways may be a downstream event from stress-induced increased CRF signaling within the VTA. For example, acute exposure to footshock stress promotes marked increases in CRF levels in the VTA and is associated with dopamine increases in the VTA and with reinstatement of cocaine-seeking behavior in cocaine-experienced rats (67). Remarkably, these CRF effects seem to be primarily mediated by CRFR2 in the VTA (68). More recently, acute or repeated social defeat stress were also shown to phasically increase CRF levels in the VTA, but with regional heterogeneity (anterior vs. posterior subregions of VTA), while repeated stress promotes long-term increases in CRF tonus in both subregions (69). In this case, both CRFR1 and CRFR2 modulated cocaine-seeking behaviors in rats with a history of social stress, according to the subregion of the VTA (69). Evidence for the involvement of CRF/urocortin signaling in alcohol drinking and other alcohol-related behaviors will be specifically addressed in the following sections.

MODULATION OF ALCOHOL CONSUMPTION BY PHARMACOLOGICAL MANIPULATIONS OF CRF/UROCORTIN SIGNALING

Extensive evidence points to a critical role for CRF and CRFR1 receptors in escalated alcohol consumption [(70–74); see review by Phillips et al. (4)]. As summarized in **Table 1**, antalarmin – a non-peptidergic CRFR1 antagonist – reduces free-choice alcohol drinking in rats given intermittent access to alcohol (72). Mice chronically exposed to alcohol vapor showed reduced escalated alcohol self-administration when treated with antalarmin prior to the withdrawal drinking session (75). In this study, control mice showed no changes in alcohol self-administration when treated with antalarmin, suggesting that only excessive drinking, in this case withdrawal-induced drinking, was sensitive to CRFR1 treatment (75). Binge drinking in C57BL6 mice was attenuated by another CRFR1 antagonist, CP-154,526, using the drinking-in-the-dark protocol (76). However, alcohol consumption was only reduced in high drinking conditions (blood alcohol ~80 mg/dl), not in moderate drinking conditions (blood alcohol ~40 mg/dl or lower). Other systemically administered CRFR1 antagonists were also effective in attenuating drinking-in-the-dark consumption, such as CP 376,395 and NBI-27914, despite non-selective effects reducing water and calory intake as well (77). The possibility that reduced alcohol drinking due to CRFR1 blockade could be secondary to broader effects on fluid and calory intake needs to be further investigated (77). A couple of studies failed to observe an attenuation of alcohol intake by CRFR1 antagonist when animals had continuous access to the drug [e.g., Ref. (78, 79)].

Overall, these studies suggest that systemic CRFR1 antagonists present selective actions in reducing escalated alcohol drinking, but not moderate drinking, as summarized in **Table 1** [e.g., Ref. (75, 76, 88)]. One particular study suggests that these effects are likely extra-hypothalamic and independent from HPA axis activation, since adrenalectomized mice show similar binge alcohol intake as controls, and CRFR1 blockade remains effective in attenuating drinking in adrenalectomized animals (86). Non-selective peptidergic CRF receptor antagonists, such as alpha-helical CRF or D-Phe-CRF, usually present a similar profile of effects as those of selective CRFR1 antagonists. In particular, icv infusion of D-Phe-CRF has been shown to reduce escalated alcohol drinking (106) and prevent reinstatement of stress-induced alcohol seeking (82, 107), just like more selective CRFR1 antagonists (see **Table 1**). When a selective CRFR1 antagonist was given chronically *via* icv minipumps, however, reduced drinking was only observed on the first drinking day and not on the following test sessions, suggesting that the chronic, continuous blockade of CRFR1 may not be as effective (84).

Important brain regions for the anti-drinking effects of selective CRFR1 and non-selective CRFR1/2 antagonists include brain reward and stress-related regions. For example, infusion of CP-154,526 into the VTA attenuated binge drinking in C57BL/6J mice (89). A different study also showed a reduction in two-bottle choice drinking in both rats and mice after intra-VTA infusion of CP-154,526 (74). CRFR1 signaling in the dorsal raphé nucleus (DRN) is also recruited for escalated alcohol drinking in rats and mice, likely due to a CRFR1 modulation of serotonergic output to the prefrontal cortex (74, 84). On the other hand, while CRFR1 in the median raphé nucleus may not modulate drinking (84), blockade of CRFR1/2 in this region prevents stress-induced reinstatement of alcohol seeking (103, 111). Infusion of either the non-selective antagonist D-Phe-CRF or antalarmin into the CeA also attenuates binge drinking (73) and withdrawal-induced alcohol self-administration (108–110). Notably, the preferential, but non-selective, activation of CRFR1 by CRF itself, whether icv or in different brain regions, produces inconsistent outcomes in alcohol drinking [e.g., Ref. (100, 101, 104, 105)]. Nonetheless, infusion of CRF into the ventricles or into the median raphé promotes reinstatement of alcohol-seeking behavior (103).

Using a different procedure, high alcohol-preferring P rats were exposed to three cycles of voluntary alcohol drinking

TABLE 1 | Summarized effects of pharmacological manipulations of CRF/urocortin targets on alcohol-drinking studies.

Receptor	Mechanism	Drug	Drug administration	Results	Reference
CRFR1	Antagonist	Antalarmin	ip	Reduced escalated[a] drinking	Lodge and Lawrence (71), Cippitelli et al. (72), Hansson et al. (70), Chu et al. (75), Marinelli et al. (80), Lowery-Gionta et al. (73)
				Reduced ethanol seeking	Marinelli et al. (80), Funk et al. (81)
				No effect on escalated[a] drinking	Yang et al. (79), Molander et al. (78)
			Into CeA	Reduced escalated[a] drinking	Lowery-Gionta et al. (73)
CRFR1	Antagonist	CP-154,526	ip	Reduced stress-induced reinstatement of ethanol seeking	Le et al. (82)
				Reduced escalated[a] drinking	Correia et al. (83), Hwa et al. (84), Lowery et al. (85, 86), Overstreet et al. (87), Sparta et al. (76, 88)
			icv (*chronic minipump*)	No effect on escalated[a] drinking	Hwa et al. (84)
			Into VTA	Reduced escalated[a] drinking	Hwa et al. (74), Sparta et al. (89)
			Into DRN	Reduced escalated[a] drinking	Hwa et al. (74, 84)
			Into MRN	No effect on escalated[a] drinking	Hwa et al. (84)
CRFR1	Antagonist	CP 376,395	ip	Reduced escalated[a] drinking	Giardino and Ryabinin (77), Hwa et al. (84), Simms et al. (90)
			icv (*chronic minipump*)	Reduced escalated[a] drinking only on day 1; no effect in remaining days	Hwa et al. (84)
CRFR1	Antagonist	CRA-1000	ip	Reduced escalated[a] drinking	Overstreet et al. (87, 91), Lowery-Gionta et al. (73)
CRFR1	Antagonist	LWH-63	ip	Reduced escalated[a] drinking	Lowery-Gionta et al. (73)
			sc	Reduced escalated[a] drinking	Sabino et al. (92)
				Modestly increased limited access alcohol and water drinking	Sabino et al. (92)
CRFR1	Antagonist	MJL-1-109-2	ip	Reduced escalated[a] drinking	Funk et al. (81)
				No effect on drinking	Sabino et al. (92)
CRFR1	Antagonist	MPZP	sc	Reduced escalated[a] drinking	Gilpin et al. (93), Richardson et al. (94)
				No effect on escalated[a] drinking	Ji et al. (95)
CRFR1	Antagonist	MTIP	ip	Reduced stress-induced reinstatement of ethanol seeking	Gehlert et al. (96)
				Reduced escalated[a] drinking	Gehlert et al. (96)
CRFR1	Antagonist	NBI-27914	ip	Reduced escalated[a] drinking	Lowery-Gionta et al. (73), Giardino and Ryabinin (77)
CRFR1	Antagonist	NBI-27914	ip	No effect on escalated[a] drinking	Molander et al. (78)
CRFR1	Antagonist	R121919	ip	No effect on escalated[a] drinking	Yang et al. (79)
			sc	Increased escalated[a] drinking	Sabino et al. (92)
				Prevented stress-induced suppression of drinking	Sabino et al. (92)
				No effect on escalated[a] drinking	Sabino et al. (97)
				Reduced escalated[a] drinking	Funk et al. (81), Roberto et al. (24), Roltsch et al. (98)
CRFR1	Antagonist	SSR125543	Into NAcc	Reduced escalated[a] drinking	Knapp et al. (99)
			Into AMY, DRN	No effect on escalated[a] drinking	Knapp et al. (99)
CRFR1/2	Agonist	CRF	icv	No effect on escalated[a] drinking	O'Callaghan et al. (100)
				Reduced escalated[a] drinking	Bell et al. (101), Thorsell et al. (102)
				Reinstated CRF-induced ethanol seeking	Le et al. (103)
			Into DRN	No effect on drinking	Weitemier and Ryabinin (104)
			Into LS	Reduced escalated[a] drinking and water intake	Ryabinin et al. (105)
			Into MRN	Reinstated CRF-induced ethanol seeking	Le et al. (103)
			Into NAcc	Further augmented escalated[a] drinking	Knapp et al. (99)
CRFR1/2	Agonist	CRF	Into CeA, DRN, VTA, PVN	No effect on escalated[a] drinking	Knapp et al. (99)
CRFR1/2	Agonist	Ucn 1	Into DRN	No effect on drinking and reduced water intake	Weitemier and Ryabinin (104)
			Into LS	Reduced escalated[a] drinking	Ryabinin et al. (105)
CRFR1/2	Antagonist	D-Phe-CRF	icv	Reduced escalated[a] drinking	Valdez et al. (106)

(Continued)

TABLE 1 | **Continued**

Receptor	Mechanism	Drug	Drug administration	Results	Reference
				Reduced reinstatement of ethanol seeking induced by the combination of stress and ethanol-cues	Liu and Weiss (107)
				Reduced stress-induced reinstatement of ethanol seeking	Le et al. (82), Liu and Weiss (107)
				No effect on cue-induced reinstatement of ethanol seeking	Liu and Weiss (107)
			Into CeA	Reduced escalated[a] drinking	Finn et al. (108), Funk et al. (109, 110)
			Into MRN	Reduced yohimbine-induced reinstatement	Le et al. (111)
			Into MRN	Reduced stress-induced reinstatement	Le et al. (103)
CRFR1/2	Antagonist	Alpha-helical CRF	icv	Increased drinking in low preference animals	O'Callaghan et al. (100)
				Reduced escalated[a] drinking	Lowery et al. (86)
				No effect on drinking in high preference animals	O'Callaghan et al. (100)
CRFR2	Agonist	Ucn 3	icv	Reduced escalated[a] drinking	Sharpe and Phillips (112), Valdez et al. (113), Lowery et al. (86)
			Into CeA	Reduced escalated[a] drinking	Funk and Koob (114)
				Increased drinking in control animals	Funk and Koob (114)
CRFR2	Antagonist	Astressin-2B	Into VTA	Reduced escalated[a] drinking	Albrechet-Souza et al. (115)
			Into CeA	No effect on escalated[a] drinking	Albrechet-Souza et al. (115)
CRFR2	Antagonist	Antisauvagine-30	Into DRN	No effect on drinking	Weitemier and Ryabinin (104)
CRF-BP	Antagonist	$CRF_{(6-33)}$	Into VTA	Reduced escalated[a] drinking	Albrechet-Souza et al. (115)
			Into CeA	No effect on escalated[a] drinking	Albrechet-Souza et al. (115)

[a]*Escalated drinking refers to ethanol intake that is above baseline or control levels, as observed in protocols of alcohol deprivation, "binge" drinking (as in drinking-in-the-dark protocol), intermittent/limited access conditions, withdrawal from ethanol vapor or liquid diet exposure, prolonged ethanol access conditions, etc.*
ip, intraperitoneal; sc, subcutaneous; icv, intracerebroventricular; AMY, amygdala; CeA, central nucleus of the amygdala; BLA, basolateral amygdala; DRN, dorsal raphé nucleus; MRN, median raphé nucleus; PVN, paraventricular nucleus of the hypothalamus; VTA, ventral tegmental area; NAcc, nucleus accumbens; LS, lateral septum.

(5 days), with 2-day withdrawal periods in between cycles (99). During withdrawal periods, rats received the infusion of a CRFR1 antagonist, SSR 125543, into the nucleus accumbens prior to exposure to restraint stress. Escalated drinking was promoted by the multiple cycles of withdrawal and stress exposure, and was attenuated by the blockade of CRFR1 in the accumbens during withdrawal periods (99). Using the same protocol, CRFR1 blockade in the DRN or the amygdala failed to affect escalated alcohol drinking (99). Interestingly, intra-accumbens infusion of CRF itself during the withdrawal periods (as a substitute for restraint stress) augmented alcohol consumption but had no effects on drinking when microinjected into the VTA, amygdala, DRN, or the paraventricular nucleus of the hypothalamus (99). Thus, plastic changes in accumbal CRFR1 seem to be recruited during cycles of withdrawal and stress, which underlie escalated drinking.

A few studies also suggest the involvement of urocortin peptides and CRFR2 in the modulation of escalated alcohol drinking. In mice, infusion of Ucn 1 (a non-selective agonist at CRFR1/2) into the lateral septum, but not into the dorsal raphé, significantly blunts binge alcohol drinking, likely due to a preferential action on CRFR2 (105). The selective activation of CRFR2 with icv administration of Ucn 3 reduced alcohol intake in mice (86, 112) and rats (113). Interestingly, this attenuation of escalated alcohol consumption can also be observed after infusion of Ucn 3 into the CeA of rats withdrawn from chronic alcohol vapor (114). On the other hand, blockade of CRFR2 in the CeA with astressin-2B failed to affect binge alcohol drinking in mice (115). Thus, these studies suggest that it is the activation, not the blockade of CRFR2, which prevents or attenuates excessive alcohol drinking, icv, or in regions such as the lateral septum or the CeA. However, one recent study reported decreased alcohol intake after infusion of a selective CRFR2 antagonist into the VTA (115), suggesting that different roles for CRFR2 may emerge from different brain pathways.

In summary, both subtypes of CRF receptors seem to be important during escalated alcohol drinking. While consistent and extensive evidence weighs toward CRFR1 modulation of excessive alcohol intake, increasing studies are currently suggesting that a balance between CRFR1 and CRFR2 activation/blockade may be critical in determining the final outcome. In particular brain regions such as the CeA, it seems that the inhibition of CRFR1 signaling and/or facilitation of CRFR2 function will blunt excessive alcohol drinking. On the other hand, blockade of either CRFR1 or CRFR2 in the VTA is effective in attenuating drinking (74, 89, 115). Thus, both CRFR1 and CRFR2 are important pharmacological targets that can contribute to treatment of AUDs. Furthermore, targets, such as the CRF-BP, have just started to emerge as interesting modulators of alcohol drinking, as recently suggested by Albrechet-Souza et al. (115).

CRF/UROCORTIN MODULATION OF OTHER ALCOHOL-RELATED BEHAVIORS

Conditioned Place Preference

Conditioned place preference is a common model for indirectly assessing drug reward and drug seeking, by associating drug-induced reinforcing effects to a particular environment [for

reviews, see Ref. (116–118)]. In this model, alcohol has reliably produced conditioned reward as evidenced by increased time spent in the alcohol-paired environment, relative to a different saline-paired environment, when the animal is tested in the absence of the drug (i.e., conditioned preference).

Genetic manipulations of the CRF system seem to affect the conditioned reinforcing effects of alcohol. Olive et al. (119) showed that transgenic CRF knockout mice fail to present preference to the alcohol-paired environment after conditioning sessions with a moderate dose of alcohol (2 g/kg), in a protocol that promotes significant preference in wild-type controls. Nonetheless, with a higher conditioning dose of alcohol (3 g/kg), both wild-type and knockout mice presented conditioned preference for the alcohol-associated context (119). These results suggest that CRF may have a facilitatory role in alcohol-conditioned reward, but other mechanisms contribute as well.

Urocortin1 knockout mice failed to present preference to the alcohol-paired environment (2 g/kg conditioning dose), and this strain also presents decreased alcohol intake and preference in a two-bottle choice protocol (120). Nonetheless, alcohol-conditioned aversive effects were preserved in this strain, suggesting that different mechanisms underlie conditioned reward and conditioned aversion (120). Furthermore, CRFR2 knockout mice do not display alcohol-induced conditioned place preference (120). Since Ucn1 acts on both CRFR1 and CRFR2, authors suggest that Ucn1-mediated activation of CRFR2 would be critical for the conditioned effects of alcohol. These were the only two studies assessing conditioned place preference for alcohol and CRF/urocortin systems. Determination of specific mechanisms requires assessment of the role of CRFR1 in this model, and further pharmacological studies.

Behavioral Sensitization

Similar to other drugs of abuse, repeated injections of alcohol promotes neuroadaptation in brain reward pathways, rendering the brain more vulnerable and sensitive to drug-induced reward and stimulation [see reviews by Robinson and Berridge (121, 122), Steketee and Kalivas (123), Vanderschuren and Pierce (124)]. Behaviorally, this neurochemical sensitization may be accompanied by progressively augmented motor stimulation responses or "behavioral sensitization" [e.g., Ref. (125–129)]. Different from other drugs, however, alcohol-induced locomotor sensitization is not easily demonstrated in rats[1] (130, 131) but is well described in some inbred and outbred strains of mice [e.g., Ref. (132–135); see also Ref. (136) for a report on adolescent macaques]. Also, a putative role for sensitized behavioral responses to alcohol has been suggested as a vulnerability factor for alcohol dependence in humans (137, 138).

Components of the CRF system play a critical role in alcohol-induced behavioral sensitization, as also reviewed by Phillips et al. (4). Studies with transgenic mice show that CRF and CRFR1 are required for alcohol sensitization (139, 140). Mice homozygous for CRF gene depletion do not show a sensitized locomotor response to alcohol after repeated injections, while heterozygous animals develop normal levels of sensitization (140). CRFR1 knockout mice failed to develop alcohol sensitization and also show a reduction in the acute response to alcohol (119, 139). On the other hand, knockout mouse lines for CRFR2 or urocortin 1 both develop normal levels of sensitization (139), suggesting no particular role for CRFR2 in alcohol sensitization.

As shown in **Table 2**, pharmacological blockade of CRFR1 receptors with CP-154,526 (in doses of 10 mg/kg and higher; ip) consistently abolishes the expression of a sensitized locomotor stimulation after a 10-day alcohol treatment regimen, without affecting the acute response to alcohol in DBA/2J mice (139, 141). During the induction phase, the effects of the CRFR1 antagonist are less clear, with significant attenuation of alcohol sensitization only at a high 30 mg/kg dose of CP-154,526 [no effects at lower doses; (139, 141)]. No studies were found assessing the pharmacological manipulation of CRFR1 in different inbred or outbred lines of mice other than DBA/2J, nor were these CRFR1 pharmacological manipulations tested in particular brain sites, as shown in **Table 2**. Future studies should broaden our understanding of the critical CRF/CRFR1 mechanisms underlying alcohol sensitization. Furthermore, while glucocorticoids seem to play a role in alcohol sensitization (139, 140, 142–144), it remains to be determined whether glucocorticoid involvement in this process is directly mediated by upstream CRF signaling.

Alcohol-Escalated Aggression

Moderate doses of alcohol may promote escalated levels of aggressive behaviors in mice, rats, monkeys, and humans [see reviews from Miczek et al. (156, 157)]. In mice, alcohol-heightened aggression seems to involve serotonin transmission arising from the DRN and involving different serotonin receptors and terminal regions [e.g., Ref. (158–161)]. Interestingly, CRF receptors are well established critical modulators of serotonin function in the dorsal raphé [e.g., Ref. (57, 58, 162), for reviews], and we have observed a critical role for raphé CRFR1 in the modulation of alcohol-related aggression in male mice (145). Systemic administration of two different CRFR1 antagonists (CP-154,526 and MTIP) was shown to dose-dependently reduce aggression not only in mice that were consuming a low dose of alcohol (1.0 g/kg) but also in mice under control conditions (after water drinking). However, when the CRFR1 antagonists were administered intra-dorsal raphé, a selective reduction in alcohol-escalated aggression was reported, with no other effects on species-typical aggressive behavior (145). Furthermore, the anti-aggressive effects of CP-154,526 seem to rely upon serotonin transmission, with increased serotonin output to the prefrontal cortex as a result of CRFR1 antagonism in alcohol-drinking animals (145).

Alcohol Withdrawal-Induced Effects on Anxiety-Like Behaviors

In models with extensive and prolonged exposure to alcohol (e.g., alcohol vapor chamber or feeding from an alcohol-containing liquid diet), animals may present alcohol withdrawal

[1] This affirmation refers exclusively to locomotor sensitization induced by alcohol. Neurochemical sensitization of the dopamine reward system and sensitization to other behaviors and drugs (including cross-sensitization between alcohol and other drugs; withdrawal-induced sensitization of anxiety-like behaviors, etc.) have been extensively demonstrated in rats.

TABLE 2 | Pharmacological manipulations of CRF/urocortin targets on alcohol-related behaviors.

Target	Drug (action, route adm.)	Experimental design/treatment	Results	Reference
Behavioral sensitization				
CRFR1	CP-154,526 (antagonist, ip)	DBA/2J mice. Ethanol injections (2.5 g/kg, ip) for 10 days, followed by ethanol challenge (1.5 g/kg, ip). Pretreatment with antagonist during acquisition or expression of behavioral sensitization	CRFR1 antagonist blocked the expression of ethanol-induced locomotor sensitization when administered prior to the challenge, but not during repeated ethanol treatment (*induction*)	Fee et al. (141)
		DBA/2J mice. Ethanol injections (2.5 g/kg, ip) for 10 days, followed by ethanol challenge (1.5 g/kg, ip). Pretreatment with antagonist during acquisition or expression of behavioral sensitization	CRFR1 antagonist blocked ethanol-induced locomotor sensitization when administered during the induction phase or prior to the challenge (*expression*)	Pastor et al. (139)
Aggression				
CRFR1	CP-154,526 or MTIP (antagonist, ip)	CFW Swiss-derived mice. Operant ethanol self-adm (1.0 g/kg, oral, ~twice/week for 6 weeks); antagonist injection immediately after drinking, 10 min before aggressive confrontation	Reduced alcohol-escalated aggression and species-typical aggression	Quadros et al. (145)
	CP-154,526 or MTIP (antagonist, into DRN)	CFW Swiss-derived mice. Operant ethanol self-adm (1.0 g/kg, oral, ~twice/week for 6 weeks); antagonist infusion immediately after drinking, 10 min before aggressive confrontation	Selective reduction of alcohol-escalated aggression, with no effect on species-typical aggressive behaviors	Quadros et al. (145)
Elevated plus maze				
CRFR1	MTIP (antagonist, ip)	Wistar and msP rats. Ethanol injection (3 g/kg, ip) administered 12 h before elevated plus maze test. Antagonist administered 30 min prior test	Reduction of anxiogenic effects elicited by acute ethanol withdrawal	Gehlert et al. (96)
CRFR1/2	Alfa-helical CRF (9–41) (antagonist, icv)	Wistar rats. Ethanol liquid diet (2–3 weeks); antagonist administered 8 h into ethanol withdrawal. Anxiety-like behavior tested 30 min after drug adm	Reduction of anxiogenic effects elicited by ethanol withdrawal	Baldwin et al. (146)
		Wistar rats. Ethanol liquid diet (16 days); antagonist administered 8 h into ethanol withdrawal. Anxiety-like behavior tested 30 min after drug adm	No change in the anxiogenic effects induced by ethanol withdrawal	Rassnick et al. (147)
	Alfa-helical CRF (antagonist, into CeA)	Wistar rats. Ethanol liquid diet (16 days); antagonist administered 8 h after ethanol withdrawal. Behavioral test conducted 30 min after drug adm	Reduction of anxiogenic effects elicited by ethanol withdrawal	Rassnick et al. (147)
	D-Phe-CRF (antagonist, icv)	Wistar rats. Ethanol liquid diet (21 days) + 6 weeks withdrawal; antagonist administered prior to restraint stress (15 min of stress). Elevated plus maze test was conducted after exposure to the stressor	Conditions of protracted abstinence and restraint stress, *per se*, failed to affect anxiety-like behavior. When combined, restraint stress-induced anxiogenic effects in animals preexposed to ethanol, which was attenuated by the antagonist	Valdez et al. (148)
CRFR2	Ucn 3 (agonist, icv)	Wistar rats. Operant self-adm (daily 30-min session, 22 days) followed by ethanol liquid diet (21 days). Agonist administered 2 h into ethanol withdrawal. Elevated plus maze was conducted 10 min after drug adm	Reduction of anxiogenic effects elicited by ethanol withdrawal	Valdez et al. (113)
Social interaction				
CRFR1	CP-154,526 (antagonist, ip)	Sprague-Dawley rats. Ethanol liquid diet (3 weeks, 5 days/week); antagonist administered 4 h after ethanol removal, during first two withdrawal periods. Restraint stress (45 min) was applied 3 days after the final ethanol withdrawal. Social interaction test 30 min after stress	The antagonist reduced social avoidance promoted by ethanol withdrawal in combination with an acute stressor	Breese et al. (149)
		Sprague-Dawley rats. Ethanol liquid diet (3 weeks, 5 days/week); antagonist administered 4 h after ethanol removal, during first two withdrawal periods. Social interaction test 5–48 h after final ethanol withdrawal	5-h into ethanol withdrawal, social avoidance was reduced by the antagonist. Neither 24 or 48 h of withdrawal produced social deficits	Wills et al. (150)
		Sprague-Dawley rats. Ethanol liquid diet (3 weeks, 5 days/week); antagonist administered 4 h after ethanol removal, during first two withdrawal periods. Social interaction test conducted 5 h after the final ethanol withdrawal	The antagonist blocked social avoidance induced by ethanol withdrawal	Overstreet et al. (91)
CRFR1	CP-154,526 (antagonist, ip)	P rats. Ethanol liquid diet (3 weeks, 5 days/week); antagonist administered 4 h after ethanol removal, during first two withdrawal periods. Social interaction test conducted 5–6 h after the final ethanol withdrawal	Antagonist blocked ethanol-withdrawal-induced social anxiety in P rats	Overstreet et al. (151)

(Continued)

TABLE 2 | **Continued**

Target	Drug (action, route adm.)	Experimental design/treatment	Results	Reference
CRFR1	CRA-(1000) (antagonist, ip)	Sprague-Dawley rats. Control liquid diet (11 days) with two non-consecutive days of restraint stress (60 min; days 6 and 11). Antagonist administered 30 min before restraint stress. Starting on day 12, animals received ethanol liquid diet (5 days). Social interaction tested 5 h into ethanol withdrawal	The antagonist prevented the facilitatory effect of stress on ethanol-withdrawal anxiety	Breese et al. (152)
		Sprague-Dawley rats. Ethanol liquid diet (17 days); drug administered 30 min before social interaction test (5–6 h into ethanol withdrawal)	The antagonist blocked ethanol-withdrawal-induced social anxiety	Knapp et al. (153)
		Sprague-Dawley rats. Ethanol liquid diet (3 weeks, 5 days/week); antagonist administered 4 h into ethanol withdrawal, during the first two withdrawal periods, or 30 min before social interaction test (5 h into final ethanol withdrawal)	The antagonist reduced ethanol-withdrawal-induced social anxiety, when administered during the first withdrawal periods (preventing withdrawal sensitization), or 30 min prior to the social interaction test	Overstreet et al. (91)
CRFR1	SSR 125543 (antagonist, into CeA, DRN, or dorsal BNST)	Sprague-Dawley rats. Control liquid diet (12 days) with two non-consecutive days of restraint stress (60 min; days 6 and 12). Antagonist infused 15 min before restraint stress. Then, exposure to ethanol liquid diet (5 days). Social interaction test 5–6 h into ethanol withdrawal	Infusion of a CRFR1 antagonist into CeA, DRN, and dorsal BNST prevented the stress-potentiation of ethanol-withdrawal anxiety	Huang et al. (154)
	SSR 125543 (antagonist, into DRN, amygdala, or NAcc)	Inbred alcohol-preferring (iP) rats. Single-bottle continuous access (3 days), then two-bottle choice continuous access (3 weeks; 5 days/week). Antagonist administered 15 min before restraint stress (60 min), which occurred 4 h into ethanol withdrawal, during the first two withdrawal periods. Social interaction 5–6 h after the final ethanol withdrawal	Infusion of the antagonist into DRN and amygdala prevented social avoidance induced by the combination of ethanol withdrawal and restraint stress. No effects of the antagonist when infused into the NAcc	Knapp et al. (99)
	SSR 125543 (antagonist, ip)	Sprague-Dawley rats. Control liquid diet (13 days) with two non-consecutive days of cytokine or chemokine treatment (days 7 and 13). Antagonist administered 15 min before treatment. Then, animals received ethanol liquid diet (5 days). Social interaction tested 24 h into ethanol withdrawal	Pretreatment with cytokine or chemokine elicited social anxiety after withdrawal from short-term exposure to ethanol. Social avoidance was prevented by the CRFR1 antagonist given during pretreatment phase	Knapp et al. (155)
CRFR1/2	CRF (agonist, icv)	Sprague-Dawley rats. Control liquid diet with two non-consecutive days of CRF infusion (days 1 and 6). Then, animals received ethanol liquid diet (5 days). Social interaction test was conducted 5 h into ethanol withdrawal	Pretreatment with CRF increased the social anxiety induced by withdrawal from one cycle of ethanol exposure	Overstreet et al. (91)
CRFR1/2	CRF (agonist, into DRN, amygdala, NAcc, VTA, or PVN)	Inbred alcohol-preferring (iP) rats. Single-bottle continuous access (3 days), then two-bottle choice continuous access (3 weeks; 5 days/week). CRF given 4 h into ethanol withdrawal during the first two withdrawal periods. Social interaction test 5–6 h into final ethanol withdrawal	Infusion of CRF into DRN or amygdala increased ethanol withdrawal-induced social anxiety. No effects after CRF infusion into NAcc, VTA, or PVN	Knapp et al. (99)
	CRF (agonist, into CeA, BLA, DRN, dBNST, vBNST, hippocampus CA1, or PVN) and SSR 125543 (CRFR1 antagonist, ip)	Sprague-Dawley rats. Control liquid diet (12 days) with two non-consecutive days of CRF injection on different brain sites (days 6 and 12). In other experiments, CRFR1 antagonist was administered (ip) 15 min before CRF infusion. Then, animals received ethanol liquid diet (5 days). Social interaction test 5–6 h into ethanol withdrawal	Pretreatment with CRF into CeA, DRN, BLA, or dorsal BNST increased ethanol withdrawal-induced social anxiety. No effects when CRF was infused into ventral BNST, CA1, or PVN. The administration of the CRFR1 antagonist prevented the enhanced withdrawal anxiety induced by CRF infusions into CeA, DRN, and dorsal BNST	Huang et al. (154)
CRFR2	Antisauvagine-30 (antagonist, icv)	Sprague-Dawley rats. Ethanol liquid diet (3 weeks, 5 days/week); antagonist administered 4 h into ethanol withdrawal, during the first two withdrawal periods. Social interaction test 5 h into final ethanol withdrawal	No effects in ethanol withdrawal-induced social anxiety	Overstreet et al. (91)
	Ucn 3 (agonist, icv)	Sprague-Dawley rats. Control liquid diet (12 days) with two non-consecutive days of CRF injection (days 6 and 12). Then, animals received ethanol liquid diet (5 days). Social interaction test 5–6 h into ethanol withdrawal	No effects on ethanol withdrawal-induced social anxiety	Huang et al. (154)

ip, intraperitoneal; icv, intracerebroventricular; CeA, central nucleus of the amygdala; BLA, basolateral amygdala; DNR, dorsal raphe nucleus; PVN, paraventricular nucleus of the hypothalamus; BNST, bed nucleus of the stria terminalis (d, dorsal; v, ventral); VTA, ventral tegmental area; NAcc, nucleus accumbens.

effects within a few hours or days post termination of alcohol treatment, as well as protracted abstinence signs. Increases in anxiety-like behavior are a well characterized consequence of alcohol withdrawal, particularly within the first hours of withdrawal. Using the elevated plus maze, withdrawal-induced anxiety-like behavior was attenuated by icv administration of the non-selective CRFR1/R2 antagonist, alpha-helical CRF (146). However, a subsequent study only observed anti-anxiety effects when alpha-helical CRF was injected into the CeA, but not icv (147). In protracted abstinence from a 3-week alcohol exposure, anxiogenic effects in the plus maze emerged upon exposure to a stressor, selectively in alcohol "dependent" subjects. This effect was prevented by centrally blocking CRF receptors prior to the stressor (148). Specific roles for CRFR1 or CRFR2 mechanisms in this particular model are still inconclusive. On the one hand, the activation of CRFR2 with urocortin 3 reduced anxiety behavior after withdrawal from chronic alcohol (113). On the other hand, selectively blocking CRFR1 receptors reduced anxiety-like behavior after withdrawal from a single alcohol injection (96).

A greater number of studies addressed social anxiety as an index of alcohol withdrawal, as assessed in social interaction tests, as shown in **Table 2**. Most commonly, these studies exposed rats to an alcohol liquid diet, with a single or repeated cycles of withdrawal (e.g., one cycle consists of 5 days of alcohol diet, followed by 2 days of alcohol withdrawal). This protocol promotes reduced social interaction when animals are tested a few hours into withdrawal, usually after the third cycle of alcohol exposure (see experimental designs on **Table 2**). Pharmacological antagonism of CRFR1 consistently attenuates repeated alcohol-withdrawal-induced social anxiety. Different CRFR1 antagonists are effective in reducing social avoidance when administered shortly prior to the social interaction test [e.g., Ref. (91, 149, 153)]. Interestingly, blocking CRFR1 signaling during the initial withdrawal days of the cycles of alcohol exposure/withdrawal also prevents the typical social avoidance response, even though animals are tested in the absence of the antagonist [e.g., Ref. (91, 149–151)]. This suggests that cumulative neuroadaptations involving CRF occur over the repeated cycles of exposure and withdrawal, and by blocking CRFR1 receptors attenuates the deleterious effects of withdrawal on social anxiety. A few brain regions have been shown to participate in the CRFR1 modulation of withdrawal-related anxiety, including the DRN and the CeA, but not the nucleus accumbens (99).

Furthermore, exposure to stressors or to an infusion of CRF may additionally aggravate alcohol withdrawal-related social anxiety with a single cycle of alcohol diet exposure (91, 154), or with a two-bottle choice protocol (99). Infusion of CRF directly into the DRN, CeA, BNST, and the basolateral amygdala (BLA), either prior to alcohol exposure or during withdrawal periods of drinking cycles, also potentiates social avoidance associated with alcohol withdrawal (99). These effects of CRF are likely mediated by CRFR1, since urocortin 3 infusion does not replicate the CRF potentiation of alcohol withdrawal (154), neither does a CRFR2 antagonist block withdrawal-induced social anxiety (91).

CONSEQUENCES OF ALCOHOL EXPOSURE ON THE CRF/UROCORTIN SYSTEM

Extensive and consistent evidence supports alcohol's actions on the CRF systems, modulating both HPA axis function as well as extra-hypothalamic CRF signaling in different brain regions. However, as shown in **Table 3**, consequences of alcohol exposure on CRF/urocortin system and the HPA axis may vary according to several variables, such as the route of alcohol administration, duration of alcohol exposure, alcohol withdrawal period, age, sex, animal strain, and CRF-related targets. Because the primary interest of this review is focused on alcohol and CRF-related peptides and signaling, data on ACTH and corticosterone were only included from studies that also directly assessed CRF-related targets. For reviews analyzing alcohol's effects on HPA axis, with focus on glucocorticoid systems, please refer to Rose et al. (163), Stephens and Wand (2), and Edwards et al. (3).

As shown in **Table 3**, acute alcohol exposure, whether administered ip or by gavage, promotes activation of the HPA axis, as evidenced by dose-dependent increases on plasma ACTH and corticosterone with doses of 1 g/kg and higher [e.g., Ref. (41, 164, 169, 172)]. Increases in ACTH can also be elicited with icv administration of alcohol (174) and can be shown *in vitro* in a hypothalamus–pituitary preparation (166). Importantly, alcohol's actions on ACTH and corticosterone release seem to be primarily mediated by alcohol-induced CRF activation in the hypothalamus [see review by Rivier (192)]. Pretreatment with antagonists of CRF receptors as well as with anti-CRF antibody, both prevent or attenuate alcohol-induced increases in ACTH and corticosterone [e.g., Ref. (164, 165, 174)]. Consistent with this hypothesis, heteronuclear RNA (precursor of mRNA) for CRF and CRF peptide levels are reliably increased in the hypothalamus after acute alcohol administration (164, 169, 174, 176). Hypothalamic CRF mRNA levels are not as reliably affected by acute alcohol, perhaps due to the kinetics of transcription processes (172, 176). In hypothalamic cell culture, alcohol incubation produced increases in CRF mRNA, CRF promoter activity, as well as CRF peptide, in a process that requires adenylate cyclase-PKA signaling (186). Extra-hypothalamic sites, such as the central amygdala, also show augmented release of CRF peptide as a result of acute alcohol administration (184). Concerning CRF receptors, mRNA levels may be affected by acute alcohol exposure, but results are scarce and inconsistent (172, 176), as shown in **Table 3**.

Chronic or repeated alcohol exposure promote less consistent changes on CRF/urocortin function, when components of the CRF system are analyzed under alcohol influence or *immediately* (within 60 min) after removal from alcohol exposure. Some reports suggest the development of tolerance in HPA axis responses after repeated alcohol treatment, with lower ACTH and corticosterone responses to alcohol [e.g., Ref. (172, 175); but see Ref. (41, 167, 168)]. Concerning CRF contents in the hypothalamus and/or pituitary, there are reports of no changes (41, 172), decreases (41, 166, 177, 178), or increases (170) in CRF peptide, mRNA, and/or hnRNA after chronic alcohol. For example, in the PVN, alcohol-induced upregulation of CRF hnRNA and alcohol-induced CRF

TABLE 3 | Consequences of ethanol exposure on CRF/urocortin systems.

Target	Tissue/brain region	Ethanol administration	Withdrawal period	Other manipulations	Animal/age	Results	Reference
ACTH	Blood	*ip injection*: acute (3 g/kg)	0–3 h	Astressin (non-selective CRF receptor antagonist; 3 mg/kg iv)	Rats: Sprague-Dawley (male) – adult	Acute ethanol increases ACTH levels (peak at 30 min; baseline restored after 3 h). ACTH response to ethanol is blunted by pretreatment with astressin	Rivier and Lee (164)
ACTH	Blood	*ip injection*: acute 1.5–3 g/kg	0–30 min	Non-selective CRF receptor antagonists: α-helical CRF_{9-41} (25 μg, icv) and astressin (0.3–3 mg/kg, iv)	Rats: Sprague-Dawley (male) – adult	Acute ethanol increased ACTH levels, with peak 15 min after ethanol injection. Pretreatment with astressin attenuated the increased ACTH response to ethanol. Prior injection of α-helical CRF failed to alter ethanol-induced ACTH response	Rivier et al. (165)
ACTH	Pituitary	*Acute ethanol exposure* in hypothalamic–pituitary superfusion or pituitary superfusion (various concentrations)	During acute ethanol incubation		Rats: Sprague-Dawley (male) – adult	Incubation of hypothalamus–pituitary superfusion with acute ethanol produced dose-dependent increases on ACTH release (peak at 20 mg % ethanol). Acute ethanol on pituitary superfusion also produced increased ACTH release (peak at 40 mg % ethanol)	Redei et al. (166)
ACTH, CORT	(A) and (B) blood (C) pituitary-derived cells	(A) *ip injection*: acute 0.3–3 g/kg (B) *Vapor chamber*: 7 days (various concentrations) (C) *Cell culture incubation*: exposure to acute ethanol (during incubation with CRF – 4 h) or prolonged ethanol (24 h prior to CRF incubation)	(A) 15-min postinjection (B) Immediately after removal from chamber (C) Immediately after incubation	Anti-CRF serum, CRF (0.004–2.5 nM)	Rats: Sprague-Dawley (male) – adult	(A) Lowest dose of ethanol (0.3 g/kg) increased CORT but not ACTH levels, while 1 and 3 g/kg induced high levels of both hormones. Previous administration of anti-CRF serum abolished ethanol-induced ACTH release. (B) Higher concentrations of ethanol increased CORT secretion upon immediate withdrawal. (C) Acute exposure to 0.2% ethanol failed to affect CRF-induced ACTH release, but prolonged exposure (24 h) declined CRF-induced ACTH response	Rivier et al. (41)
ACTH, CORT	Blood	*ip injection*: acute 1–3 g/kg *Intragastric injection*: acute 1–3 g/kg *Liquid diet*: 4 or 6 nights (12 h/day)	30 min–4 h		Rats: Sprague-Dawley – adult	*Acute ip and ig injection* of ethanol (2 and 3 g/kg) increased plasma ACTH levels, with peak release 30 min after injection; returning to basal levels in 180 min. *Liquid diet*: ethanol-fed rats presented lower levels of ACTH than pair-fed animals, but both groups presented similar plasma CORT response	Ogilvie et al. (167, 168)
ACTH, CORT	Blood	*Oral gavage* (1 ml, 75% ethanol)[a]	0–1 h		Rats: Wistar (male) – adult	Ethanol increased plasma ACTH (peak at 16 min) and CORT levels (peak at 5 min and elevation remains for 60 min)	Laszlo et al. (169)
ACTH, CORT	Blood	*Vapor chamber*: 7 days	3 h	iv CRF (0.3–10 μg) or footshock (1.0 mA, 0.5 s, 2 shocks/min; during 10 min)	Rats: Sprague-Dawley (male) – adult	At 3-h withdrawal, ethanol-exposed rats showed higher levels of ACTH and CORT. After CRF stimulation, controls show dose-dependent increases in ACTH levels, while ethanol-exposed rats showed increases in ACTH with no dose dependency. At the highest CRF dose, ethanol-exposed rats showed lower ACTH response than controls. Footshock induced similar ACTH response in both groups	Rivier et al. (170)

(Continued)

TABLE 3 | Continued

Target	Tissue/brain region	Ethanol administration	Withdrawal period	Other manipulations	Animal/age	Results	Reference
ACTH, CORT	Blood, pituitary	*Vapor chamber*: 7 days during the second gestational week. Pups were fed by foster mothers and euthanized at 21 days old. Pituitary maintained in short-term culture	28 days	Exposure to inescapable shocks (1.5 mA, 1 s, 25 times over 10 min) when pups were 21 days old, prior to euthanasia; CRF (0.01–100 nM)	Rats: Sprague-Dawley – prenatal/pup	Ethanol exposure during the second week of gestation potentiated plasmatic ACTH but not CORT response to shock stress. The ACTH released from the pituitary of ethanol-exposed pups was decreased after 3 h incubation with CRF and increased after incubation with higher CRF dose, when compared with control pups	Lee et al. (171)
ACTH, CORT, POMC (mRNA)	Blood, pituitary, and hypothalamus	*Oral gavage*: (4.5 g/kg/day) – 1 or 14 days	30 min after last adm		Rats: Fischer (male) – adult	Ethanol-induced acute increases in ACTH and CORT levels. After 14 days, ethanol's effects on ACTH were abolished and CORT responses were reduced. Neither protocol changed POMC mRNA levels in pituitary. Hypothalamic POMC mRNA levels were reduced after acute, but not chronic ethanol	Zhou et al. (172)
ACTH, CORT; POMC (mRNA)	Blood, pituitary	*Liquid diet* (gestational): ethanol (~13 g/kg/day) during all pregnancy (withdrawal upon birth)	~4 months (exposure during prenatal period; tissue collected as adults)		Rats: Sprague-Dawley (male and female) – prenatal/adult	Prenatal ethanol exposure failed to affect plasmatic CORT and ACTH levels, or pituitary POMC mRNA	Glavas et al. (173)
ACTH, POMC (hnRNA and mRNA)	Blood, pituitary	*ip injection*: acute (3 g/kg) *icv injection*: (5 μl)	0–60 min after ethanol adm	CRF antibody (0.4 ml/kg)	Rats: Sprague-Dawley (male) – adult	*ACTH*: both ip and icv ethanol injection induced increased plasma ACTH levels, a response that was prevented by anti-CRF antibody pretreatment. *POMC*: pituitary POMC primary transcript is increased 15, 30, and 60 min after ethanol ip and 15 min after ethanol icv. Anti-CRF antibody attenuated ethanol-induced POMC transcription	Lee et al. (174)
CORT	Blood	*Vapor chamber*: 6 h daily, during 2 or 8 days (in adolescence)	Immediately after removal from chamber, ranging from 1.5 to 5.5 h of vapor exposure		Rats: Sprague-Dawley (male) – adolescent	On the 2nd day of ethanol vapor exposure, adolescent rats presented increased levels of CORT after 3.5 and 5.5 h of ethanol vapor. On the 8th day, CORT levels were increased after exposure of 4.5 and 5.5 h to ethanol vapor	Logrip et al. (175)
CRF (hnRNA and mRNA)	Parvicellular PVN	*Ip injection*: acute (3 g/kg)	3 h	Astressin (non-selective CRF receptor antagonist; 15 μg, icv)	Rats: Sprague-Dawley (male) – adult	CRF heteronuclear RNA was increased after ethanol, as well as the combination of ethanol + astressin. CRF mRNA levels were unaffected	Lee and Rivier (176)
CRF (hnRNA and mRNA)	Parvicellular PVN	*Ip injection*: acute (3 g/kg)	20 min–3 h		Rats: Sprague-Dawley (male) – adult	CRF heteronuclear RNA was increased at 20 and 40 min after ethanol administration. CRF mRNA levels were unaffected	Rivier and Lee (164)
CRF (hnRNA and peptide)	PVN, median eminence (ME)	*Intragastric injection*: daily (4.5 g/kg), 3 days of treatment, ethanol challenge 7 days later	0–1 h		Rats: Sprague-Dawley (male) – adult	In PVN: ethanol pretreatment did not affect basal CRF hnRNA levels. Preexposure to ethanol reduced ethanol-induced upregulation of CRF hnRNA. In medial eminence (external zone): reduced CRF peptide levels in rats with prior ethanol history (after 7 days withdrawal)	Lee et al. (177, 178)
CRF (hnRNA)	Parvicellular PVN	*Icv injection*: acute (5 μl)	0–60 min after ethanol adm		Rats: Sprague-Dawley (male) – adult	PVN CRF hnRNA is increased 30 min after ethanol treatment	Lee et al. (174)

(Continued)

TABLE 3 | Continued

Target	Tissue/brain region	Ethanol administration	Withdrawal period	Other manipulations	Animal/age	Results	Reference
CRF (mRNA)	BLA	*Intermittent ethanol drinking*: daily access to one-bottle ethanol for 1 h, during 18 days, under water restriction condition. Intake ~2.5 g/kg/day for adolescents; ~2.3 g/kg/day for adults	60 days		Rats: Long-Evans (male) – adolescent – adult	BLA pre-pro-CRF mRNA levels were decreased in rats exposed to ethanol during adulthood, but not in those exposed during adolescence	Falco et al. (179)
CRF (mRNA)	CeA	*Liquid diet*: for 10–12 days (intake ~11.5 g/kg/day)	Immediately after ethanol removal		Rats: Sprague-Dawley (male) – 120 g (~5 weeks old)	Ethanol liquid diet increased pre-pro-CRF mRNA levels in CeA	Lack et al. (23)
CRF (mRNA)	CeA	*Vapor chamber*: intermittent exposure (8 h ethanol vapor/8 h air), for 8 on/off cycles. Ethanol (1.6 g/kg, ip) injected prior to every ethanol vapor session. Stress-induced ethanol self-adm (60-min session for 4 days; 120 min in the last session)	Immediately after removal from ethanol exposure; 2 weeks withdrawal; 2 weeks withdrawal + 4 h after the final self-adm session	4-h food restriction prior to ethanol self-adm sessions	Mice: C57BL/6N (male) – adult	Immediately after ethanol vapor exposure, there were no changes in CeA CRF mRNA levels. After 2 weeks of withdrawal, CeA CRF mRNA levels were increased, with further increases 4 h after the last drinking session	Eisenhardt et al. (180)
CRF (mRNA)	CeA, BNST	*Vapor chamber*: 7 weeks of ethanol exposure (17 h/day)	3 weeks		Rats: Wistar (male) –adult	Chronic ethanol exposure increased CRF mRNA levels in CeA, but not in BNST	Sommer et al. (18)
CRF (mRNA)	Hypothalamus	*Oral gavage* (4.5 g/kg/day) – 1 or 14 days	30 min after final adm		Rats: Fischer (male) – adult	No changes in CRF mRNA levels after acute or chronic ethanol	Zhou et al. (172)
CRF (mRNA)	Hypothalamus	*Vapor chamber*: 7 days during the second gestational week. Pups were fed by foster mothers and euthanized at 21 days old	28 days		Rats: Sprague-Dawley – prenatal/ pup	Ethanol exposure during the second week of gestation increased hypothalamic CRF mRNA levels in the offspring	Lee et al. (171)
CRF (mRNA)	Parvicellular PVN	*Liquid diet* (gestational): ethanol (~13 g/kg/day) during all pregnancy (withdrawal upon birth)	~4 months (exposure during prenatal period; tissue collected as adults)		Rats: Sprague-Dawley (male and female) – prenatal/ adult	Prenatal ethanol exposure failed to affect CRF mRNA levels in parvicellular PVN in males and females, when adults	Glavas et al. (173)
CRF (mRNA)	Parvicellular PVN	*Vapor chamber*: 7 days	Immediately after removal from chamber		Rats: Sprague-Dawley (male) – adult	Increased CRF mRNA levels in PVN after 3 or 7 days of ethanol vapor exposure	Rivier et al. (170)
CRF (mRNA)	PVN	*Vapor chamber*: 6 h daily, during 15 days (in adolescence). Also, 20 days later, rats received intragastric ethanol (4.5 g/kg) challenge	2 h after ethanol challenge (adulthood)		Rats: Sprague-Dawley (male) – adolescent/ adult	Rats exposed to ethanol vapor during adolescence presented decreased levels of CRF mRNA in PVN after ethanol challenge in adulthood	Allen et al. (181)
CRF (mRNA)	PVN	*Vapor chamber*: 6 h daily, during 8 or 15 days (in adolescence). Also, 20 days later, rats received intragastric ethanol (4.5 g/kg) challenge	6 h after 8 or 15 days of vapor exposure (adolescence); or 2 h after ethanol challenge (adulthood)		Rats: Sprague-Dawley (male and female) – adolescent/ adolescent-adult	During adolescence, there were no differences in PVN CRF mRNA levels after ethanol vapor exposure. Adolescent ethanol vapor exposure also failed to affect CRF mRNA levels in adult rats exposed to an ethanol challenge after 20 days of withdrawal	Logrip et al. (175)
CRF (peptide)	Amygdala	*Liquid diet*: 2–3 weeks	2–12 h		Rats: Wistar (male) –adult	10–12 h of withdrawal increases CRF immunoreactivity in amygdala dialyzates	Merlo-Pich et al. (182)

(Continued)

TABLE 3 | Continued

Target	Tissue/brain region	Ethanol administration	Withdrawal period	Other manipulations	Animal/age	Results	Reference
CRF (peptide)	BNST	*Liquid diet*: ethanol for 2 weeks, ~10 g/kg/day. Diet removal for 7.5 h + reexposure to ethanol diet	0–7.5 h		Rats: Long-Evans (male) – adult	Chronic ethanol had no effect on BNST CRF baseline levels (while still exposed to alcohol). During withdrawal, only ethanol-fed rats showed elevated levels of extracellular CRF in BNST, from 4.5 to 7.5 h. Upon reexposure to ethanol, CRF levels returned to baseline. Ethanol-fed animals exposed to control diet after abstinence, presented even further increases in CRF levels	Olive et al. (183)
CRF (peptide)	CeA	*Ip injection*: acute (2–2.8 g/kg)	Immediately – 180 min		Rats: Sprague-Dawley (male) – adult	Doses of 2.4 and 2.8 g/kg induced increased release of CRF in the CeA 120 min after administration, the effect was sustained until 180-min postinjection	Lam and Gianoulakis (184)
CRF (peptide)	CeA, VTA	*Drinking in the dark*: 1 cycle = 2-h ethanol access for 3 days, 4-h access in the 4th day. 3 days of withdrawal between cycles	0–24 h		Mice: C57BL/6J (male) – adult	CRF immunoreactivity was increased in CeA immediately after 1 or 6 cycles of drinking; in VTA only after 1 cycle. CRF immunoreactivity was also increased 18–24 h after 3 cycles of drinking	Lowery-Gionta et al. (73)
CRF (peptide)	Hypothalamus	*Drinking in the dark* (prenatal exposure): two-bottle choice (ethanol in saccharin or saccharin alone), 4 h/day during all gestational period. Upon birth, dams were gradually weaned out of ethanol over 6 days	40–50 days (prenatal exposure; euthania in late adolescence)		Mice: C57BL/6J (male) – prenatal/ adolescent	Increased levels of CRF immunoreactivity in the hypothalamus of mice with prenatal ethanol exposure	Caldwell et al. (185)
CRF (peptide)	Hypothalamus	*Liquid diet*: 2 weeks. Acute ethanol exposure in hypothalamic superfusion (various concentrations)	Immediately after ethanol removal		Rats: Sprague-Dawley (male) – adult	Chronic ethanol exposure increased CRF release pulse frequency in a hypothalamic *in vitro* preparation. CRF content in hypothalamus was lower in ethanol-exposed rats than in controls. Incubation with acute ethanol produced dose-dependent increases on CRF release from hypothalamus, effects that were blunted in ethanol diet-exposed rats. Potassium-stimulated CRF release was also reduced in ethanol preexposed rats	Redei et al. (166)
CRF (peptide)	Hypothalamus	*Oral gavage* (1 ml, 75% ethanol)[a]	0–1 h	Cycloheximide (protein synthesis inhibitor; 10 or 30 mg/kg, ip)	Rats: Wistar (male) – adult	Ethanol increased hypothalamic CRF contents after 30 and 60 min. Cycloheximide reduced ethanol-induced CRF levels	Laszlo et al. (169)
CRF (peptide)	Median eminence, hypothalamus	*Vapor chamber*: 7 days (various concentrations of ethanol)	Immediately after removal from vapor chamber		Rats: Sprague-Dawley (male) – adult	Higher doses of ethanol reduced CRF immunoreactivity in median eminence, but not in hypothalamus	Rivier et al. (41)
CRF (promoter, mRNA and peptide)	Hypothalamic cell culture	*Ethanol incubation* (25 mM, during 1–4 h)	Immediately after ethanol incubation	Forskolin (adenylyl cyclase activator; 25 μM); PKA inhibitors H89 (10 μM), Rp-cAMP (250 μM)	Hypothalamic cells derived from Sprague-Dawley rats (6–7 days old)	Acute ethanol incubation increased CRF peptide levels (after 1 and 4 h), increased CRF mRNA (peak at 1 h incubation), and increased CRF promoter activity (after 2 h). These effects were potentiated with the combined incubation of ethanol and forskolin, except for ethanol-induced CRF mRNA, which was slightly blunted by forskolin. PKA inhibitors abolished the effects of ethanol incubation	Li et al. (186)

(Continued)

TABLE 3 | Continued

Target	Tissue/brain region	Ethanol administration	Withdrawal period	Other manipulations	Animal/age	Results	Reference
CRF-BP (mRNA)	CeA	*Liquid diet*: for 10–12 days (intake ~11.5 g/kg/day)	Immediately after ethanol removal		Rats: Sprague-Dawley (male) – 120 g (~5 weeks old)	No alterations in CRF-BP in the CeA after chronic ethanol exposure	Lack et al. (23)
CRFR1 (mRNA)	Amygdala	*Extended ethanol drinking*: three-bottle choice (water, ethanol 5%, and ethanol 10%) during 70 days, followed by 2 weeks of withdrawal and reexposure to three-bottle choice for 2 weeks. Then, quinine-adulterated ethanol for 2 weeks, followed by ethanol reexposure for 1 week	Immediately after the final ethanol exposure		Mice: Swiss (male) – adult	Mice with moderate ethanol intake profile (± 9.4 g/kg/day) presented increased CRFR1 mRNA levels in amygdala, while animals with high (± 11.5 g/kg/day) and low (± 5.2 g/kg/day) ethanol intake profile showed no alterations in CRFR1 mRNA	Correia et al. (83)
CRFR1 (mRNA)	Amygdala	*Operant self-administration*: after training lever-pressing for saccharin, 3 days of access to ethanol + saccharin solution (30-min session/day; ~1 g/kg ethanol)	20 days		Rats: Wistar (male) – adult	No correlations between alcohol consumption and CRFR1 mRNA expression in the amygdala	Pickering et al. (187)
CRFR1 (mRNA)	CeA, MeA, BLA	*Vapor chamber*: intermittent exposure (8 h ethanol vapor/8 h air), for 8 on/off cycles. Ethanol (1.6 g/kg, ip) injected prior to every ethanol vapor session. Stress-induced ethanol self-adm (60-min session for 4 days; 120 min in the last session)	Immediately after removal from ethanol exposure; 2 weeks withdrawal; 2 weeks withdrawal + 4 h after the final self-adm session	4-h food restriction prior to ethanol self-adm sessions	Mice: C57BL/6N (male) – adult	Immediately after ethanol vapor exposure, CRFR1 mRNA levels were decreased in CeA but increased after 2 weeks of withdrawal and 4 h after the last self-administration session. In MeA and BLA, CRFR1 levels were also increased after withdrawal and after the drinking session	Eisenhardt et al. (180)
CRFR1 (mRNA)	CeA, MeA, BLA, BNST	*Vapor chamber*: 7 weeks of ethanol exposure (17 h/day)	3 weeks		Rats: Wistar (male) – adult	Chronic ethanol exposure increased CRFR1 mRNA in BLA and MeA, but not CeA and BNST	Sommer et al. (18)
CRFR1 (mRNA)	CeA, MeA, BLA, Nacc, Cg	*Two-bottle choice*: continuous access to ethanol and water, 24 h/day for 15 days	Immediately after ethanol removal		Rats: alcohol-preferring (msP) (male) – adult	Chronic ethanol consumption decreased CRFR1 mRNA levels in CeA, MeA, and NAcc, but not in BLA or Cg	Hansson et al. (188)
CRFR1 (mRNA)	DRN	*Drinking in the dark*: three daily 1-h sessions (2-h interval between sessions), 5 days/week, for 3 weeks (intake ~9 g/kg/day)	3 h		Rats: alcohol-preferring (P) (male) – adolescent	Increased CRFR1 mRNA in the DRN of ethanol binge-drinking rats	McClintick et al. (189)
CRFR1 (mRNA)	Hypothalamus	*Operant self-administration*: after training lever-pressing for saccharin, 3 days of access to ethanol + saccharin solution (30-min session/day; ~1 g/kg ethanol)	20 days		Rats: Wistar (male) – adult	Strong positive correlation of alcohol consumption and hypothalamic CRFR1 mRNA expression	Pickering et al. (190)
CRFR1 (mRNA)	Parvicellular PVN	*Intragastric injection*: daily (4.5 g/kg), 3 days of treatment, ethanol challenge 7 days later	3 h		Rats: Sprague-Dawley (male) – adult/adult	Ethanol pretreatment did not affect basal CRFR1 expression. Preexposure to ethanol reduced ethanol-induced upregulation of CRFR1 mRNA	Lee et al. (177, 178)

(Continued)

TABLE 3 | Continued

Target	Tissue/brain region	Ethanol administration	Withdrawal period	Other manipulations	Animal/age	Results	Reference
CRFR1 (mRNA)	Pituitary	*Liquid diet* (gestational): ethanol (~13 g/kg/day) during all pregnancy (withdrawal upon birth)	~4 months (exposure during prenatal period; tissue collected as adults)		Rats: Sprague-Dawley (male and female) – prenatal/adult	Prenatal ethanol exposure reduced CRFR1 mRNA levels in the anterior pituitary of male but not female rats	Glavas et al. (173)
CRFR1 (mRNA)	Pituitary	*Oral gavage* (4.5 g/kg/day) – 1 or 14 days	30 min after final adm		Rats: Fischer (male) – adult	Acute, but not chronic, ethanol reduced CRFR1 mRNA in the anterior pituitary	Zhou et al. (172)
CRFR1 (mRNA)	PVN, amygdala	*Ip injection*: acute (3 g/kg)	3 h	Astressin (non-selective CRF receptor antagonist; 15 µg, icv)	Rats: Sprague-Dawley (male) – adult	Ethanol increased CRFR1 mRNA in parvicellular PVN, an effect not prevented by astressin. In magnocellular PVN, CRFR1 mRNA is increased after combined ethanol plus astressin administration. No ethanol-induced changes in amygdala CRFR1 mRNA levels	Lee and Rivier (176)
CRFR2 (mRNA)	Amygdala	*Operant self-administration*: after training lever-pressing for saccharin, 3 days of access to ethanol + saccharin solution (30-min session/day; ~1 g/kg ethanol)	20 days		Rats: Wistar (male) – adult	No correlations between alcohol consumption and CRFR2 mRNA expression in the amygdala	Pickering et al. (187)
CRFR2 (mRNA)	CeA, MeA, BLA	*Vapor chamber*: intermittent exposure (8 h ethanol vapor/8 h air), for 8 on/off cycles. Ethanol (1.6 g/kg, ip) injected prior to every ethanol vapor session. Stress-induced ethanol self-adm (60-min session for 4 days; 120 min in the last session)	Immediately after removal from ethanol exposure; 2 weeks withdrawal; 2 weeks withdrawal + 4 h after the final self-adm session	4-h food restriction prior to ethanol self-adm sessions	Mice: C57BL/6N (male) – adult	Immediately after ethanol vapor exposure, there were no effects on CRFR2 mRNA levels in CeA. After 2-week withdrawal, no changes were observed in CRFR2 mRNA levels in CeA, MeA, and BLA. Ethanol self-administration increased CRFR2 mRNA in CeA and BLA, but not in MeA in ethanol-exposed mice, relative to air-exposed controls	Eisenhardt et al. (180)
CRFR2 (mRNA)	CeA, MeA, BLA, BNST	*Vapor chamber*: 7 weeks of ethanol exposure (17 h/day)	3 weeks		Rats: Wistar (male) – adult	Chronic ethanol exposure decreased CRFR2 mRNA levels only in BLA	Sommer et al. (18)
CRFR2 (mRNA)	DRN	*Drinking in the dark*: three daily 1-h sessions (2 h interval between sessions), 5 days/week, for 3 weeks (intake ~9 g/kg/day)	3 h		Rats: alcohol-preferring (P) (male) – adolescent	Decreased CRFR2 mRNA in the DRN of ethanol binge-drinking rats	McClintick et al. (189)
CRFR2 (mRNA)	Hypothalamus	*Operant self-administration*: after training lever-pressing for saccharin, 3 days of access to ethanol + saccharin solution (30-min session/day; ~1 g/kg ethanol)	20 days		Rats: Wistar (male) – adult	Strong positive correlation of alcohol consumption and hypothalamic CRFR2 mRNA expression	Pickering et al. (190)
CRFR2 (mRNA)	VMH, SON	*Ip injection*: acute (3 g/kg)	3 h		Rats: Sprague-Dawley (male) – adult	CRFR2 mRNA in VHM or SON is not affected by ethanol	Lee and Rivier (176)
CRFR2 (protein)	LS, DRN	*Ip injection*: chronic (2 g/kg, daily), 0, 1, or 7 days	24 h		Mice: C57BL/6J (male) – adult	Higher CRFR2 binding in dorsomedial DR after 7 days of ethanol exposure. No changes in LS	Weitemier and Ryabinin (191)

(Continued)

TABLE 3 | Continued

Target	Tissue/brain region	Ethanol administration	Withdrawal period	Other manipulations	Animal/age	Results	Reference
POMC (mRNA)	Hypothalamus	*Operant self-administration:* after training lever-pressing for saccharin, 3 days of access to ethanol + saccharin solution (30-min session/day; ~1 g/kg ethanol)	20 days		Rats: Wistar (male) – adult	No correlations between alcohol consumption and hypothalamic POMC mRNA expression	Pickering et al. (190)
Ucn1	npEW, LS, DRN	*Ip injection:* chronic (2 g/kg, daily), 0, 8, or 15 days	20 min		Mice: C57BL/6J and DBA/2J (male) – adult	Repeated ethanol exposure (8 or 15 days) had no effect on Ucn1 cell count in npEW but reduced Ucn1 fibers in the LS and DR (DR only at 8 days). No strain differences	Weitemier and Ryabinin (191)

[a]*Estimated dose in the range of 3.0–3.5 g/kg ethanol.*

ip, intraperitoneal; icv, intracerebroventricular; iv, intravenous; CeA, central nucleus of the amygdala; MeA, medial nucleus of the amygdala; BLA, basolateral amygdala; DRN, dorsal raphe nucleus; PVN, paraventricular nucleus of the hypothalamus; Cg, cingulate gyrus; BNST, bed nucleus of the stria terminalis; LS, lateral septum; npEW, Edinger–Westphal nucleus; VMH, ventromedial hypothalamus; SON, supraoptic nucleus; VTA, ventral tegmental area; NAcc, nucleus accumbens.

release were reduced in rats with a history of alcohol exposure (166, 177, 178). In the CeA, repeated alcohol was reported to increase detection of CRF mRNA or immunoreactivity [(73, 193); but see Ref. (188)], although a lack of alteration was also found (180). In amygdalar nuclei, a reduction in CRFR1 gene expression, but not CRFR2, was detected after alcohol vapor exposure (180) or after 15 days of voluntary of alcohol drinking in an alcohol-preferring strain (188). After extended access to alcohol for over 90 days, only mice with a profile of moderate alcohol intake showed increased CRFR1 mRNA in the amygdala, which was not the case for mice with high or low intake profiles (83). In the BNST, no changes in CRF immunoreactivity were seen while animals still had access to alcohol in a liquid diet, but increases in CRF emerged after 4 h into withdrawal from alcohol (183). Interestingly, repeated alcohol failed to change the number of urocortin1 cells in the Edinger–Westphal nucleus, but it decreased urocortin1 fibers to the lateral septum and the DRN (191).

Short-term withdrawal (here defined as an arbitrary window of 1–24 h after the final alcohol exposure) from repeated/chronic alcohol administration is usually associated with increased anxiety-like behavior and escalated alcohol self-administration, as discussed in previous sections. Acute withdrawal (i.e., 3 h) from a 7-day alcohol vapor exposure was characterized by increased plasma ACTH and corticosterone levels, and also associated with increased CRF mRNA levels in the PVN (170). However, maximal CRF-mediated ACTH response was reduced in alcohol-exposed rats (170). A different study showed that after 1-day withdrawal from chronic alcohol liquid diet, CRF immunoreactivity was not changed in the hypothalamus but was reduced in the amygdala, hippocampus, and frontal cortex (194). These changes were also accompanied by lower plasma corticosterone concentrations at acute withdrawal. In a classic study on acute alcohol withdrawal, dialyzate CRF levels in the amygdala are shown to increase in great proportions throughout the first 12 h of withdrawal from chronic alcohol vapor exposure (182). Increases in CRF immunoreactivity in CeA are also observed after repeated cycles of binge alcohol drinking in mice, persisting for 18–24 h into alcohol withdrawal (73). In another stress-related region, the BNST, CRF peptide levels increase after 4.5 h into withdrawal from an alcohol liquid diet. Remarkably, CRF levels in the BNST return back to baseline levels when rats regain access to the alcohol-containing liquid diet (183). However, these effects of acute alcohol withdrawal may be age-dependent. Exposure to alcohol vapor or binge drinking for 8–15 days in adolescent rats produces no changes in CRF transcripts or peptides in the PVN (175, 195), although a reduced CRF immunoreactivity in the CeA is observed (195). As shown in **Table 3**, a few studies also reported changes in CRF receptors' transcripts after short-term alcohol withdrawal in the PVN (177, 178) and the DRN (189), as well as in CRFR2 binding levels in the DRN (191).

Finally, critical adaptations in the CRF/urocortin are recruited during protracted abstinence from chronic alcohol exposure (i.e., at least 7 days of withdrawal, but usually longer than 2 weeks). For example, rat's exposure to repeated alcohol administrations followed by a 7-day withdrawal, show reduced CRF peptide levels in the median eminence of the hypothalamus, despite no changes

in CRF or CRFR1 transcripts in the PVN (177, 178). However, animals preexposed to the drug show a blunted upregulation of CRF and CRFR1 transcripts in the PVN in response to an alcohol challenge (177, 178). These data, and others, support a dampened function of HPA axis function after chronic alcohol followed by prolonged withdrawal, which is also supported by evidence of low baseline corticosterone levels after 3-weeks withdrawal from chronic alcohol [e.g., Ref. (194)]. As shown in **Table 3**, prenatal exposure to alcohol also dysregulates elements of the HPA axis in adolescent and adults animals, although differences in neuroadaptations arise according to the duration and methodology of *in utero* alcohol exposure [e.g., Ref. (171, 173, 185)]. Chronic exposure to alcohol during adolescence may lead to reduced HPA axis response to alcohol in early adulthood, as shown by reduced CRF mRNA in the PVN (181). However, such outcome was not observed when adolescents were exposed to alcohol as binge drinking (195). Thus, recruitment of neuroadaptive changes in CRF/urocortin elements in the HPA axis depend on age of alcohol exposure, the duration and protocol for alcohol exposure (vapor, liquid diet, oral intake), as well as age at time of assessment of CRF/urocortin targets.

In the amygdala, and particularly within the CeA, there is consistent evidence of upregulated CRF signaling after protracted withdrawal from chronic alcohol exposure. For example, there are increased levels of CRF transcript in the CeA (18, 180) or immunoreactivity for CRF in the amygdala (194) after 3 or more weeks of withdrawal from alcohol. These changes are accompanied by increased CRFR1 gene expression within the CeA and/or in other amygdalar nuclei (BLA, MeA). Changes in CRFR2 transcripts in the amygdala were less consistent within different studies (18, 180). One study also reported a decrease in CRF mRNA in the BLA in adult, but not adolescent rats after 18 days of restricted access to alcohol drinking followed by a 60-day withdrawal (179). Although BNST levels of CRF seem to be upregulated during acute withdrawal (183), BNST may not be particularly engaged in protracted abstinence, as no changes in transcripts for CRF or CRFR1 were seen in this region (18).

In summary, alcohol exposure importantly impacts CRF/urocortin components and function, both within the HPA axis and in extra-hypothalamic sites. In general, acute alcohol reliably upregulates CRF transcripts and/or peptide within the hypothalamus, promoting activation of the HPA axis with increases in ACTH and corticosterone. When CRF/urocortin signaling is assessed shortly after chronic alcohol exposure, relevant but quite variable and inconsistent effects may be detected, depending on brain region, alcohol exposure protocol, and the age of animals. After protracted abstinence from alcohol, a more reliable profile emerges, characterized by hyperactivity of brain CRF signaling, particularly within the amygdala. Although the studies are limited, existing research suggests the involvement of urocortins as targets for alcohol's effects, as well as changes in CRFR2 mRNA/protein levels, and therefore these molecules should be further investigated. Also, target brain regions were highly limited to the hypothalamus, amygdala, and BNST, with a few exceptions. A wider characterization of alcohol modulation of CRF/urocortin signaling should include the VTA, accumbens, DRN, prefrontal cortex, and other reward-related structures, which may contribute to our understanding of alcohol-induced neuroadaptations that influence alcohol reward and withdrawal.

INVOLVEMENT OF CRF SYSTEMS IN HUMAN ALCOHOLISM

This review will discuss a few human studies regarding HPA axis function in AUDs. For a vast discussion on this literature, we also recommend reviews by Lovallo (196); Rose et al. (163); Stephens and Wand (2). As described in animal studies, human studies confirm an activation of HPA axis by alcohol as demonstrated by increases in plasma cortisol after an acute alcohol administration (197). This cortisol response seems to require ACTH release from the hypophysis, as alcohol failed to increase cortisol levels in patients with lesions in the anterior hypophysis (197). In heavy drinkers and dependent subjects, there is an attenuation of alcohol-induced cortisol response (198), as well as reduced cortisol and ACTH levels upon CRF stimulation (199–201). Dependent individuals also show an attenuated cortisol response to moderate mental and physical stressors (202–205). Upon alcohol withdrawal, plasma cortisol is elevated during the first week, but with protracted abstinence, cortisol levels drop below normal range (206–208). Together, these studies suggest a blunted HPA function in response to drugs and stressors in alcoholics, supporting a contribution of the HPA axis in the transition to dependence (209).

Studies targeting the CRF system and alcohol in humans mostly rely on genetic approaches, with evidence of associations between AUDs and polymorphisms in genes encoding for CRFR1 and CRF-BP. Chen et al. (210) showed that variations in the CRFR1 gene are associated with alcohol dependence. In another study, patterns of alcohol consumption were assessed in a teenage sample, as well as in a sample of dependent adults. In teenagers with little to moderate alcohol exposure, polymorphisms in CRFR1 gene were associated with the amount of alcohol consumed, but not with the frequency of drinking episodes (211). In alcohol-dependent adults, an association was also detected between CRFR1 polymorphism and the amount of alcohol consumed (211). Different studies show gene–environment interactions, in which variants of the CRFR1 gene interact with a history of exposure to stressful events and predict heavy alcohol drinking (212, 213) and age of drinking initiation (213). On the other hand, protective effects for alcohol dependence were shown for a gene–environment interaction involving a CRFR1 haplotype and childhood sexual abuse in an Australian population (214).

Electroencephalogram phenotypes have been identified for anxiety-prone alcoholics (215, 216). A "linkage scan" indicated CRF-BP as a candidate gene for electroencephalogram profiles in two distinct populations. Further, genetic variations in CRF-BP were associated with anxiety disorders and AUDs (217). There is also evidence that polymorphisms in CRF-BP modulate stress-induced craving in heavy drinkers (218) and are involved in the relationship between negative reactivity to stress and negative consequences to alcohol consumption (219). Thus, CRF-BP gene variations may play particular a role in stress-related alcohol dependence. Furthermore, an interaction of polymorphisms in CRFR1 and CRF-BP genes predicts increased risk for AUDs in

schizophrenic patients (220). Thus, genetic studies, so far, point to some polymorphisms in CRFR1 and CRF-BP genes, which can contribute to the susceptibility for AUDs.

SUMMARY AND PERSPECTIVES

Increasing evidence suggests that a recruitment of CRF/urocortin mechanisms is more prominent in individuals with increased sensitivity to and/or vulnerability for alcohol-induced effects (5). After repeated and chronic exposure to alcohol, rats present hyperactive extra-hypothalamic CRF activity, as indicated by increases in CRF immunoreactivity and/or increases in mRNA for CRF and its receptors in amygdala nuclei and the BNST (18, 182, 183). Consistently, increased alcohol seeking and intake can be attenuated after the administration of antagonists to CRF type 1 receptors (systemically, icv or intra-amygdala), particularly in animals with previous alcohol history (5, 18, 81, 106, 109, 110) or in high alcohol-consuming mice (76). While blocking CRFR1 attenuates alcohol drinking, this effect can also be achieved with the activation of CRFR2 signaling, suggesting opposite roles for CRFR1 and CRFR2 in the modulation of excessive alcohol intake [e.g., Ref. (86, 105, 113)]. However, manipulation of CRF receptor signaling in different brain regions may reveal differential effects and interactions between CRFR1 and CRFR2 [e.g., Ref. (73, 99, 105, 115)]. Pharmacological studies start to unveil a role for both CRF receptors within brain reward pathways, as well as CRF-BP, as critical modulators of escalated alcohol drinking [e.g., Ref. (74, 84, 89, 115)]. Furthermore, the finding that different CRF mechanisms and pathways may be engaged during escalated drinking or alcohol withdrawal-related anxiety (99) gives rise to an intriguing path for further investigation. Moreover, CRF/urocortin signaling is also recruited during other alcohol-related effects, including alcohol-induced behavioral sensitization, alcohol-escalated aggression, and alcohol withdrawal-related anxiety. Determination of specific mechanisms for the engagement of CRF, urocortins, CRF-BP, and CRF receptors, after acute and chronic alcohol exposure still remain to be clarified. While effects of alcohol on the HPA axis, the amygdala, and other stress-related structures have been more widely characterized, promising contributions of CRF/urocortin signaling in brain reward pathways and other structures are starting to emerge. Finally, there is hope that new molecules targeting components of the CRF/urocortin system will be made available for testing in preclinical experimental settings and advancing to clinical trials, providing helpful therapeutic tools for the treatment of AUDs.

AUTHOR CONTRIBUTIONS

IQ and GM proposed and outlined the contents of the review. IQ, GM, LD, and CF wrote different sections of the text, prepared the corresponding tables, and organized the bibliography. IQ revised the manuscript text and tables. All authors carefully read, revised, and approved the entire text.

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Naltrexone Prevents in Males and Attenuates in Females the Expression of Behavioral Sensitization to Ethanol Regardless of Maternal Separation

Suzi E. Kawakami, Isabel M. H. Quadros and Deborah Suchecki*

Department of Psychobiology, Escola Paulista de Medicina – Universidade Federal de São Paulo (UNIFESP), Sao Paulo, Sao Paulo, Brazil

Maternal separation alters the activity of the opioid system, which modulates ethanol-induced stimulation and behavioral sensitization. This study examined the effects of an opioid antagonist, naltrexone (NTX), on the expression of behavioral sensitization to ethanol in adult male and female mice submitted to maternal separation from postnatal days (PNDs) 2 to 14. Whole litters of Swiss mice were either not separated [animal facility rearing (AFR)] or separated from their mothers for 3 h [long maternal separation (LMS)]. Starting on PND 90, male and female AFR and LMS mice received daily i.p. injections of saline (SAL) or ethanol (EtOH, 2.2 g/kg) for 21 days. Locomotor activity was assessed in cages containing photoelectric beams, once a week, to examine the development of behavioral sensitization. Five days after the end of the chronic treatment, animals were submitted to four locomotor activity tests spaced by 48 h, to assess the expression of behavioral sensitization. In all tests, animals received two i.p. injections with a 30-min interval and were then assessed for locomotor response to different treatment challenges, which were: SAL/SAL, SAL/EtOH (2.2 g/kg), NTX 2.0 mg/kg (NTX2)/EtOH, and NTX 4.0 mg/kg (NTX4)/EtOH. Regardless of maternal separation, EtOH-treated male and female mice displayed increased locomotor responses to EtOH during the 21-day treatment, indicating the development of behavioral sensitization. In the SAL/EtOH challenge, EtOH-treated LMS and AFR male and female mice exhibited higher locomotor activity than their SAL-treated counterparts, indicating the expression of sensitization. The coadministration of either dose of NTX blocked the expression of locomotor sensitization in both AFR and LMS male mice with a history of EtOH sensitization. In females, a significant attenuation of EtOH sensitization was promoted by both NTX doses, while still maintaining an augmented stimulant response to EtOH. Importantly, maternal separation did not interfere in this phenomenon. These results indicate that expression of behavioral sensitization was importantly modulated by opioidergic mechanisms both in male and female mice and that maternal separation did not play a major role in either development or expression of this EtOH sensitization.

Keywords: neonatal stress, locomotor sensitization, opioid system, alcohol

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****Correspondence:***
Isabel M. H. Quadros
isabel.quadros@unifesp.br

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INTRODUCTION

Maternal care is essential for the proper development of altricial mammals, whose central nervous system maturation takes place postnatally. The ontogenesis of the hypothalamic–pituitary–adrenal (HPA) axis stress response also occurs during the first weeks of life in mice and rats and is regulated by maternal presence, which tonically inhibits its activation by most stressors. Maternal care is responsible for maintaining the pups' HPA axis quiescent; specifically stroking of the anogenital area inhibits the ACTH stress response, whereas lactation reduces corticosterone secretion (1–5). This inhibition is demonstrated by separating the offspring from its mother for periods of 8–24 h, resulting in elevated stress-induced ACTH and corticosterone stress responses (2, 4, 6–8).

Disruption of the mother–infant relationship produces long-term alterations in numerous behaviors and brain systems (9), including vulnerability to drug abuse (10) and changes in the activity of the opioid system (11). A considerable number of studies have shown that separation of pups from their mothers for long periods of time during the first 2 weeks of life [3–6 h/day; hereby referred to as long maternal separation (LMS)] affects brain opioid levels (12–15). Increases in immunoreactivity for Met-enkephalin peptides are detected in regions associated with reward and emotional behaviors, including the medial prefrontal cortex (12, 13), while less consistent changes are found in dynorphin-B levels, depending on the brain region and separation protocol (11). Additionally, LMS animals show greater sensitivity to morphine, an opioid agonist (16), compared to control animals. Changes in the opioid system are particularly relevant within the context of drug abuse, since these neuropeptides are involved in motivation and reward, regulating the activity of the dopaminergic mesolimbic system by means of μ-, δ-, and κ-opioid receptors (17–19). However, the effects of LMS on drug addiction-related behaviors appear to be sexually dimorphic for it increases self-administration of psychostimulants (10), morphine (20), and ethanol in males (21–24), but not in females (13, 25). LMS also modifies behavioral sensitization to cocaine (26) and ethanol in females, but not in males (27), indicating a strong influence of sex and paradigm used to evaluate the neurobiological aspects involved in drug addiction.

Locomotor sensitization is defined as an augmented behavioral response, e.g., locomotor activity, to the stimulant effects of drugs upon repeated administration. This paradigm has been used to study neuroadaptive changes induced by chronic EtOH administration, which may contribute to EtOH addiction. Interestingly, opioids seem to play a key role in the motivational aspects of drug and alcohol abuse in several animal models, including behavioral sensitization [for reviews, see Ref. (28, 29)]. Non-selective opioid receptors antagonists, such as naltrexone (NTX) or naloxone, decrease EtOH-induced stimulant effect (30, 31) and inhibit the development of behavioral sensitization to ethanol (32, 33). However, the expression of behavioral sensitization to EtOH is not affected by these opioid receptor antagonists (32, 34). In the present study, we evaluated the effect of NTX, a non-selective opioid antagonist of important clinical value in the treatment of alcohol dependence [for reviews, see Ref. (18, 35)], on the expression of behavioral sensitization to ethanol in maternally separated adult mice. For this, we employed higher NTX doses than previous studies (32), and also tested both male and female mice to investigate possible sex differences [as opposed to only testing males, as in Ref. (32–34)]. Additionally, we tested the effect of an early life stress manipulation, which could further modulate the expression of ethanol sensitization (26, 27) and the sensitivity to NTX effects (11–16).

MATERIALS AND METHODS

Animals

Swiss mice were mated in the animal facility of the Department of Psychobiology and daily inspected for the presence of pups. The day of the birth was designated postnatal day (PND) 0. On PND 1, litters were culled to 5 males and 5 females. Animals were maintained in a controlled 12-h light–dark cycle (lights on at 7:00 a.m. and off at 7:00 p.m.) and temperature (23 ± 2°C). Food and water were provided *ad libitum* throughout the entire study. Animal manipulations and protocols were approved by the Ethics Committee in Research (CEP# 521/07), and the experiments were carried out in accordance with Brazilian regulations on the use and care of animals.

Neonatal Manipulations

From PND 2 to 14, pups were subjected to daily maternal separation for 180 min (LMS) or not separated until weaning on PND 22 [animal facility rearing (AFR)]. In the LMS group, whole litters were removed from the nest, at ~12:00 hours, and placed in separate cages on a heating pad set at 33°C, whereas the mothers remained in the home cage and, at the end of the allotted period, litter and mother were reunited in the home cage. Once a week during the separation, half of the old bedding material was mixed with clean material, in order to prevent excessive ammonia accumulation and still keeping olfactory familiarity from the old bedding material. AFR litters were handled during cage cleaning (three times a week). Weaning took place on PND 22 and two to three litters from the same group were housed in plastic cages (10–15 animals).

Drugs

Ethanol (Synth) was prepared fresh every day, in a concentration of 15% w/v, in 0.9% saline (SAL) and administered at a dose of 2.2 g/kg (i.p.). The opioid antagonist, NTX (Sigma-Aldrich) was also prepared before use, in 0.9% SAL, and the doses were chosen based on a previous study (33).

Behavioral Sensitization

Habituation (Hab)

At PND 90, AFR and LMS male and female mice (8–9 litters/manipulation) were individually tested in Opto-Varimex activity cages (Columbus Instruments, Columbus, OH, USA), which detect locomotion by interruptions of horizontal photoeletric beams, for 15 min.

Development Phase

Fourty-eight hours after the habituation test, the animals were allocated into two treatment groups, and received daily injections of SAL or 2.2 g/kg EtOH, i.p, for 21 days (n = 10–13 animals/sex/group), since studies from our lab have shown that this treatment induces consistent levels of behavioral sensitization (36, 37) (**Figure 1**). Locomotor activity was measured once a week (days 1, 7, 14, and 21 of treatment), immediately after the treatment, for 15 min.

Expression Phase

Five days after the last administration of the development/induction phase, animals were submitted to 4 challenges spaced by 48 h. In all challenges, animals received two i.p. injections spaced by 30 min (**Figure 1**). All mice were submitted to drug challenges in the following order: SAL/SAL, SAL/EtOH (2.2 g/kg), NTX (2.0 mg/kg)/EtOH (2.2 g/kg), and (NTX, 4.0 mg/kg)/EtOH (2.2 g/kg). Immediately after the second administration, the animals were placed in the activity cages, and locomotor activity was measured for 15 min. All procedures were carried out in the afternoon (between 12:00 and 17:00 hours).

Statistical Analysis

Locomotor response to habituation was compared between groups (AFR, LMS) by Student's t-test. The locomotor response during the development phase was analyzed by three-way analysis of variance (ANOVA) with group (AFR, LMS), treatment (SAL, EtOH), and day (repeated measure) as main factors. During the expression phase, the locomotor response was analyzed by a three-way ANOVA for repeated measures, with group (AFR, LMS), pretreatment (SAL, EtOH), and challenge as the repeated measure (Sal/Sal, Sal/EtOH, NTX2/EtOH, and NTX4/EtOH). Males and females were analyzed separately. When appropriate, *post hoc* analysis was carried out using Newman–Keuls test, with the level of significance set as $p \leq 0.05$.

RESULTS

Habituation

Pairwise comparison showed no differences in locomotion between LMS and AFR in either male [$t_{(46)} = 0.31$; $p > 0.05$] or female mice [$t_{(47)} = 1.51$; $p > 0.05$] (**Table 1**).

Development of Behavioral Sensitization

Male Mice

ANOVA revealed main effects of treatment [$F_{(1,44)} = 25.41$, $p < 0.01$] and day [$F_{(3,132)} = 4.36$, $p < 0.01$] and an interaction between treatment and day [$F_{(3,132)} = 10.32$, $p < 0.01$], with no differences between AFR and LMS manipulations (**Figure 2**). Newman–Keuls tests for the treatment × day interaction showed that EtOH-treated mice presented increases in locomotor activity on test days 7, 14, and 21, relative to SAL-treated animals ($p < 0.05$). EtOH-induced hyperactivity was also higher on days 14 and 21, when compared to responses to EtOH on days 1 and 7 ($p < 0.01$).

Female Mice

ANOVA revealed main effects of treatment [$F_{(1,45)} = 45.80$, $p < 0.01$] and day [$F_{(3,135)} = 28.01$, $p < 0.01$] and interaction between these factors [$F_{(3,135)} = 22.85$, $p < 0.01$], with no group

TABLE 1 | Locomotor activity (counts), during habituation, of male and female mice kept with their mothers for the entire developmental period [animal facility rearing (AFR)] or submitted to long maternal separation (LMS), from postnatal days 2 to 14.

	Males	Females
AFR	1209.57 ± 369.82 (23)	1573.75 ± 523.22 (24)
LMS	1183.04 ± 212.20 (25)	1375.68 ± 387.89 (25)

The values are presented as mean ± SD. Number of animals/group is shown in parenthesis.

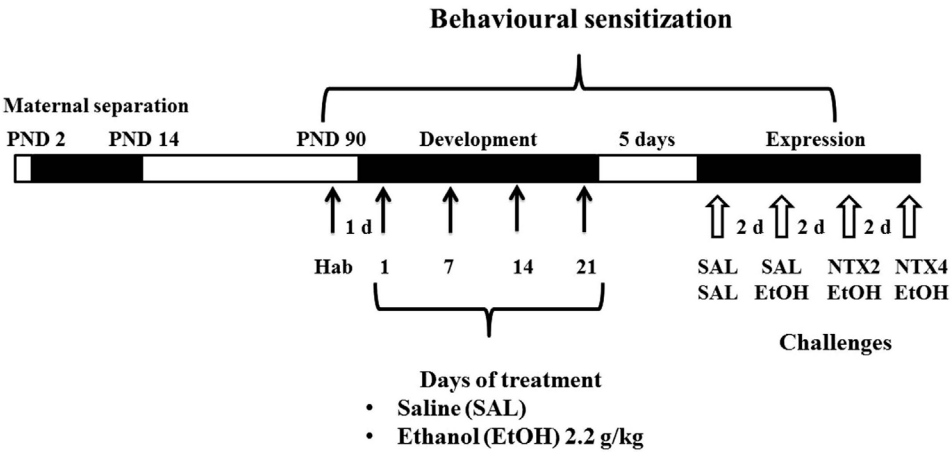

FIGURE 1 | Schematic representation of the experimental design. Black horizontal bars represent daily saline (SAL) or ethanol (EtOH) i.p. administration. The numbers represent the locomotor activity tests. White arrows represent two i.p. administrations, first with SAL or NTX (2.0 or 4.0 mg/kg) and second with SAL or EtOH.

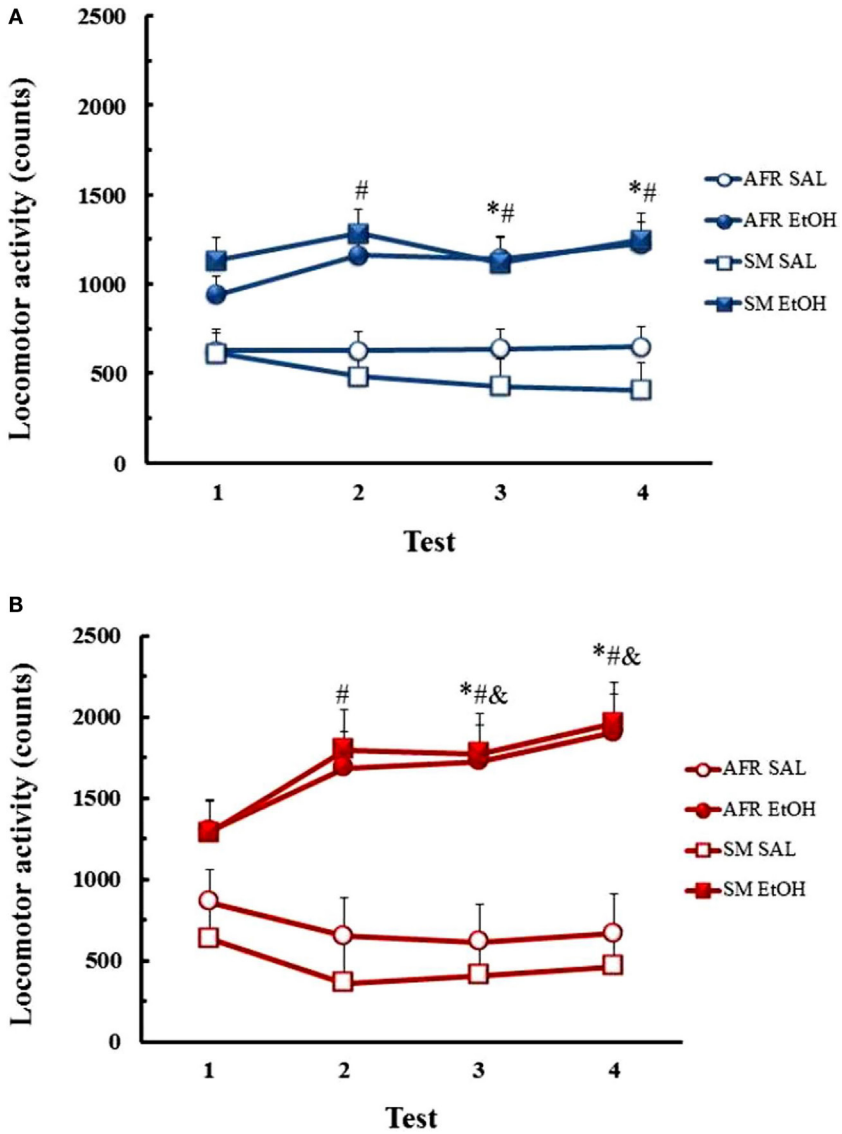

FIGURE 2 | Locomotor activity counts (mean ± SEM) throughout the course of saline (SAL) or ethanol (EtOH) chronic treatment in AFR and LMS male (A) and female mice (B). Number of animals/group for each condition (sex, group, treatment) was 10–13. * – Different from saline-treated groups; # – different from day 1; & – different from day 7.

effects (AFR vs. LMS). Analysis of the interaction showed that the locomotor activity of EtOH-treated mice was higher on days 7, 14, and 21 than on day 1 of treatment ($p < 0.01$) and on days 14 and 21 compared to day 7 ($p < 0.01$). EtOH-treated mice presented higher locomotor activity than SAL-treated mice on days 7, 14, and 21 of treatment ($p < 0.01$).

Expression of Behavioral Sensitization

Males

Three-way ANOVA revealed main effects of pretreatment [$F_{(1,44)} = 5.6872$, $p < 0.03$] and challenge [$F_{(3,132)} = 19.841$, $p < 0.00001$] and an interaction between these factors [$F_{(3,132)} = 12.395$, $p < 0.00001$], with no group effect (**Figure 3**). *Post hoc* analysis of the interaction showed that mice with a history of EtOH treatment displayed higher locomotor activity than SAL-pretreated counterparts in the SAL/EtOH challenge ($p < 0.0005$). Such differences were no longer observed during the NTX2/EtOH and NTX4/EtOH challenges, suggesting that EtOH-induced expression of sensitization was prevented by NTX. Moreover, in mice with a previous history of EtOH treatment, locomotor response to EtOH was the highest during the SAL/EtOH challenge, with significant reductions when NTX was administered with EtOH (NTX2/EtOH and NTX4/EtOH challenges, p's < 0.005). In SAL-pretreated male mice, no changes in locomotor response were observed with any of the drug challenges, relative to the SAL/SAL challenge.

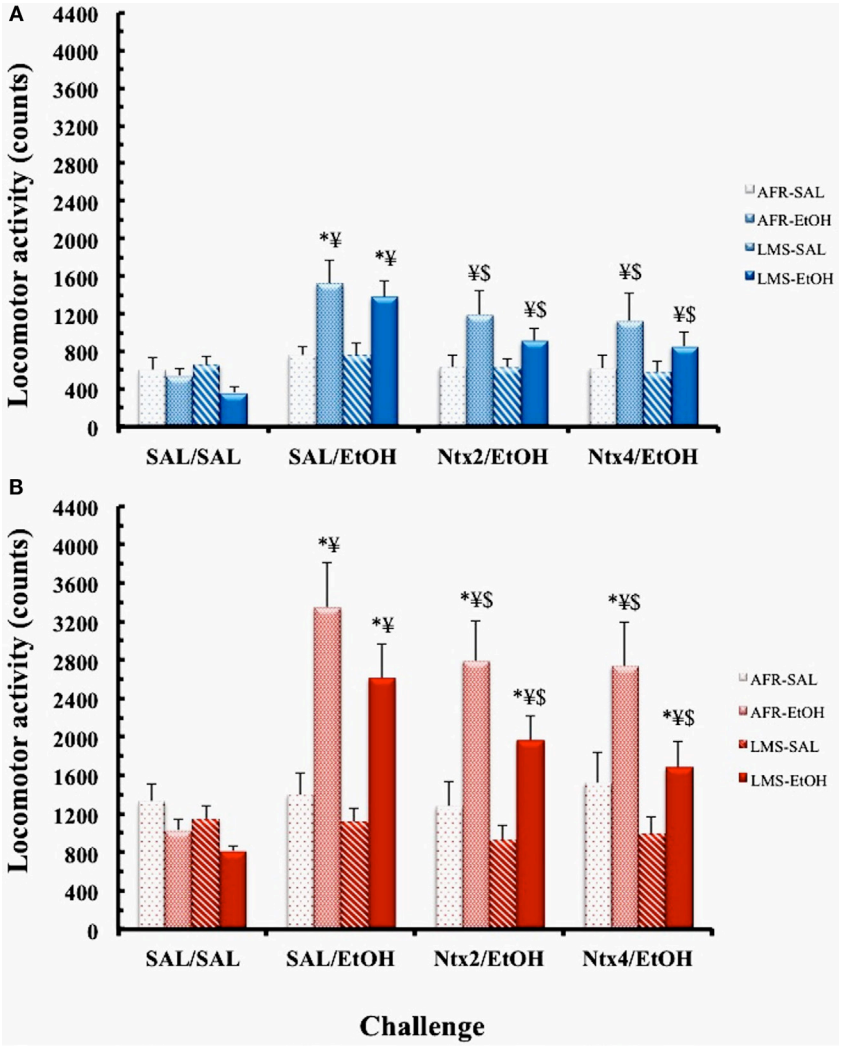

FIGURE 3 | Locomotor activity counts (mean ± SEM) of saline (SAL)- or ethanol (EtOH)-pretreated AFR and LMS males (A) or females (B) in the challenges SAL/SAL, SAL/EtOH, naltrexone 2.0 mg/kg – NTX2/EtOH, and naltrexone 4.0 mg/kg – NTX4/EtOH. EtOH dose was 2.2 g/kg (i.p.). Number of animals/group for each condition (sex, group, treatment) was 10–13. * – different from SAL-treated groups; ¥ – different from SAL/SAL challenge, $ – different from SAL/EtOH challenge.

Females

A three-way ANOVA showed main effects of group [$F_{(1,45)} = 4.303$; $p < 0.05$], pretreatment [$F_{(1,45)} = 12.983$; $p < 0.001$], and challenge [$F_{(3,135)} = 22.48$; $p < 0.00001$] and an interaction between these two latter factors [$F_{(3,135)} = 23.14$; $p < 0.00001$]. Newman–Keuls analysis of the overall group effect showed that LMS animals exhibited lower locomotor activity than AFR mice ($p < 0.03$). Analysis of the pretreatment × challenge interaction revealed that mice with a history of EtOH preexposure displayed higher locomotor activity than SAL-pretreated ones in the SAL/ETOH ($p < 0.0005$), NTX2/EtOH ($p < 0.001$), and NTX4/EtOH ($p < 0.005$) challenges. However, the sensitized response to EtOH was significantly attenuated by co-treatment with both doses of NTX (NTX2 or NTX4; p's < 0.005). In mice pretreated with SAL, no changes in locomotor behavior were induced by challenges with EtOH or NTX/EtOH coadministration.

DISCUSSION

The results of the present study showed that the non-selective opioid antagonist, NTX, blocked the expression of behavioral sensitization to EtOH in male mice, and attenuated this phenomenon in females, regardless of the neonatal manipulation. Moreover, maternal separation had no impact on either the development or the expression of EtOH sensitization. Both males and females showed significant augmentation of locomotor responses to EtOH during the 21-day treatment (development of sensitization) and maintained a sensitized stimulant response when challenged with EtOH during the expression tests.

In previous studies from our group, repeated EtOH administration induced behavioral sensitization in mice (36–39). Although an initial study reported that LMS could accelerate the development of EtOH sensitization in female, but not male mice (27), this was not confirmed using a more robust, 21-day treatment protocol (39), as replicated in our current findings. Thus, the facilitatory effects of LMS on EtOH sensitization seem to only emerge with weaker sensitizing regimens (e.g., 5 EtOH injections), and in females. However, EtOH-induced corticosterone responses were higher in chronically EtOH-treated LMS males than in EtOH-treated controls, with no changes observed in females (39). Such changes in corticosterone response to chronic EtOH were not observed when mice received fewer EtOH treatments (27). Thus, LMS may modulate different behavioral and physiological/hormonal responses to EtOH in a sex-dependent and exposure-dependent manner.

A role for opioid receptor modulation of acute EtOH locomotor stimulation was reported by studies showing that opioid antagonists reduced EtOH-induced hyperactivity in mice (30–32), despite controversial findings (40). Coadministration of non-specific opioid receptor antagonists, such as NTX and naloxone, blocked the development of EtOH locomotor sensitization (32, 33). However, in mice previously sensitized to EtOH, NTX and naloxone failed to prevent the expression of EtOH sensitization (32, 34). In contrast, the present study showed important effects of NTX preventing and/or attenuating the expression of EtOH sensitization in males and females, while not inducing locomotor effects in animals with no previous EtOH history. This finding contrasts with Abrahao and colleagues, who reported no effect of NTX on the expression of EtOH sensitization (34). However, the NTX dose was considerably lower in that study, 0.1 mg/kg (34). Indeed, in a pilot study using male mice with no neonatal manipulation, we observed significant reduction of EtOH sensitization using NTX doses of 1 mg/kg and higher, but not with a 0.5 mg/kg dose (data not shown). The dose range necessary to block the expression of sensitization in male mice in our study (2 mg/kg) was similar to that required for preventing the development of EtOH sensitization as reported by Pastor and Aragon [1 or 2 mg/kg (33)].

Despite acting as non-selective antagonists at opioid receptors, both NTX and naloxone have preferential effects on μ-receptors, rather than on δ-receptors (30, 33, 41). Thus, our findings support an additional role for μ-receptors in the modulation of the expression of EtOH sensitization, besides mediating the development of EtOH sensitization (32, 33). Indeed, Pastor and Aragon showed that both NTX and a selective antagonist at μ-receptors, CTOP, blocked the development of EtOH sensitization, which was not affected by a delta-receptor antagonist, naltrindole. Moreover, the facilitation of EtOH sensitization after a period of EtOH consumption was absent in a recombinant line of mice with reduced expression and function of μ-receptors, CXBK mice (42). Altogether, these studies point to μ-receptors as the critical target for NTX's effects on EtOH sensitization. A putative mechanism for NTX effects relies on the modulation of dopamine neurons projecting from the ventral tegmental area (VTA) to the nucleus accumbens (NAcc), a pathway involved in drug-stimulation, sensitization, and reward [e.g., Ref. (18, 19, 43)]. Opioid receptors, including μ-receptors, are located in both regions, where they modulate dopamine output directly (in the NAcc) or indirectly (via GABA interneurons in VTA) (18, 19). Indeed, local administration of NTX into the VTA or the NAcc inhibits acute EtOH-induced stimulation in mice (29). Thus, it would be expected that μ-receptor blockade in either or both brain regions could participate in the prevention/attenuation of the expression of EtOH sensitization reported in this study.

Remarkably, while NTX treatment blocked the expression of EtOH sensitization in males, in females there was only an attenuation of EtOH-sensitized response. In males, NTX administration, at both doses, blocked the expression of behavioral sensitization, since there were no longer differences in locomotor behavior between SAL- and EtOH-pretreated mice in the challenges. In females, both doses of NTX reduced, but failed to completely prevent a sensitized response to EtOH, suggesting a sexual dimorphism in the behavioral response to this opioid antagonist. In agreement with these findings, several studies report on sexual differences in response to opioidergic drugs. For example, NTX treatment is less effective in women than in men (44, 45), men are more vulnerable to opioid addiction than women (46), and males are more responsive to analgesic drugs than females, in several species (47). In a recent study carried out with rats bred for increased preference for ethanol, a low acute dose of NTX was effective to block spontaneous ethanol intake in male, but not in female, rats (48). NTX is also capable to reduce the intake of a highly palatable sucrose solution only in LMS males, but not in females (49). Sex steroid hormones appear to regulate the density of opioid receptors in the hypothalamus, with increased density of μ-opioid receptors during proestrus, and changes in μ receptor density in other limbic regions induced by hormone replacement in ovariectomized rats (50). Interestingly, full agonists of μ receptors in males act as partial agonists in female rats and primates (51), suggesting smaller affinity for these receptors in females. Thus, the reduced efficiency of NTX in blocking the expression of EtOH sensitization in female mice may be due to a smaller affinity/efficacy in μ-opioid receptor signaling in females.

In the present study, the only effect of maternal separation was seen in female mice, which displayed lower locomotor activity than their AFR counterparts during the expression, but not during the development of behavioral sensitization. Few studies had compared the induction and/or expression of behavioral sensitization between maternally separated male and female animals, with contradictory results. LMS has been reported to increase induction of behavioral sensitization to cocaine in male and female mice, but only males exhibited increased expression to a cocaine challenge (26). As mentioned in the Introduction, development of EtOH sensitization was facilitated in LMS female, but not in male mice, using a weaker sensitizing protocol (27), but not with a stronger one, with more prolonged EtOH treatment (39). Amphetamine sensitization was also not modified by maternal separation in rats (52, 53). However, LMS reduces the response rate for intracranial self-stimulation in female, but not in male rats (54).

In conclusion, NTX blocked the expression of EtOH-induced behavioral sensitization in male mice, while significantly attenuating EtOH sensitization in females, with no effects of neonatal

manipulation. The only detectable effect of maternal separation was an overall reduced locomotor behavior of female mice during the expression tests for EtOH sensitization.

AUTHOR CONTRIBUTIONS

SK planned and performed the experiments described, analyzed the results, and wrote the original version of the manuscript. SK, IQ, and DS participated in defining experimental design, data analysis, and interpretation of results, as well as revising and writing the final version of the manuscript.

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REVIEW
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Neuropeptide Y in Alcohol Addiction and Affective Disorders

Annika Thorsell[1] and Aleksander A. Mathé[2]*

[1] Center for Social and Affective Neuroscience, Department of Clinical and Experimental Medicine, Linköping University, Linköping, Sweden, [2] Department of Clinical Neuroscience, Karolinska Institutet, Stockholm, Sweden

Neuropeptide Y (NPY), a neuropeptide highly conserved throughout evolution, is present at high levels in the central nervous system (CNS), as well as in peripheral tissues such as the gut and cardiovascular system. The peptide exerts its effects *via* multiple receptor subtypes, all belonging to the G-protein-coupled receptor superfamily. Of these subtypes, the Y1 and the Y2 are the most thoroughly characterized, followed by the Y5 subtype. NPY and its receptors have been shown to be of importance in central regulation of events underlying, for example, affective disorders, drug/alcohol use disorders, and energy homeostasis. Furthermore, within the CNS, NPY also affects sleep regulation and circadian rhythm, memory function, tissue growth, and plasticity. The potential roles of NPY in the etiology and pathophysiology of mood and anxiety disorders, as well as alcohol use disorders, have been extensively studied. This focus was prompted by early indications for an involvement of NPY in acute responses to stress, and, later, also data pointing to a role in alterations within the CNS during chronic, or repeated, exposure to adverse events. These functions of NPY, in addition to the peptide's regulation of disease states, suggest that modulation of the activity of the NPY system *via* receptor agonists/antagonists may be a putative treatment mechanism in affective disorders as well as alcohol use disorders. In this review, we present an overview of findings with regard to the NPY system in relation to anxiety and stress, acute as well as chronic; furthermore we discuss post-traumatic stress disorder and, in part depression. In addition, we summarize findings on alcohol use disorders and related behaviors. Finally, we briefly touch upon genetic as well as epigenetic mechanisms that may be of importance for NPY function and regulation. In conclusion, we suggest that modulation of NPY-ergic activity within the CNS, *via* ligands aimed at different receptor subtypes, may be attractive targets for treatment development for affective disorders, as well as for alcohol use disorders.

Keywords: neuropeptide Y, receptor subtypes, anxiety, stress, depression, post-traumatic stress disorder

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Robert Warren Gould, Vanderbilt University, United States

****Correspondence:***
Annika Thorsell
annika.thorsell@liu.se

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INTRODUCTION

Neuropeptide Y (NPY), a 36 amino acid neuropeptide, was originally isolated from porcine brain using a method detecting the C-terminal amide. NPY belongs to the pancreatic polypeptide (PP) family of biologically active peptides, together with two other members, PP and peptide YY (1). The amino acid sequence for porcine NPY was determined in 1985 (2), and it was subsequently

determined that the amino acid sequence is identical for species such as human, rat, porcine, and guinea pig (3). Indeed, both mRNA and peptide sequence display a high degree of conservation throughout evolution (4–6), possibly indicating preserved functional relevance.

Expression and synthesis of the PP-family of peptides is a multiple step process, also well conserved between species. The PP-family of peptides are synthesized as large protein precursors; for NPY, the 98 amino acid precursor peptide is proteolytically processed into three separate peptide products: an N-terminal signal peptide, NPY, and a 30 amino acid C-terminal flanking peptide (C-PON). The strong evolutionary conservation can be seen for both the NPY peptide and the C-PON, with the rat and human sequences showing 100 and 93% homology, respectively (7).

Expression of NPY within the Central Nervous System (CNS)

Neuropeptide Y is predominantly expressed in cells originating from the neural crest, and it is one of the most highly expressed neuropeptides within the CNS; an expression that has been shown to be present in, but not limited to, neurons (7–9). NPY is expressed at high levels in brain regions involved in regulation of affective behavior, energy homeostasis, and memory function and plasticity. These include among others, the hypothalamus, in particular the arcuate and the paraventricular nuclei, the hippocampal formation, the amygdala, periaqueductal gray, locus coeruleus, and septum (7, 10, 11).

The amygdala is a central neurobiological substrate for mediation of stress- and anxiety-related behaviors and has strong NPY-ergic innervation. Within the amygdala, the central amygdala constitutes an output relay for the functional consequences of amygdala activation by fearful stimuli and, together with the the lateral/basolateral complex mediate anti-stress effects of NPY (12, 13).

The dorsolateral portion of the periaqueductal gray matter (PAG) has been suggested to tonically inhibit the amygdala. The PAG is involved in the behavioral output of fear responses, with subcompartments differentially involved in defensive behaviors (14). The septum is a key component in a behavioral inhibition system partaking in regulation of anxiety states. However, while important, lesions of the septum that affect anxiety-related behaviors most likely reflect effects on fibers passing through this structure, most likely belonging to hippocampal output. The dorsal hippocampus is an important component of neuronal circuitry controlling anxiety-related behaviors and stress responses and septo-hippocampal circuits are likely to be important for fear-related behaviors. Expression of NPY is high in hippocampal regions (15).

The numerous functions of NPY within the CNS, as well as its extensive expression, contribute to making the NPY system one of the most well-studied neuro-hormonal systems.

NPY Receptor Subtypes and Function

Neuropeptide Y exerts its actions *via* four functionally relevant receptor subtypes, the Y1, Y2, Y4, and Y5 (16–19). All NPY receptors cloned belong to the superfamily of G-protein-coupled receptors but differ in their ligand affinity profiles (20–23). The Y1 receptor subtype requires the full peptide to be activated, while the Y2-subtype also can be bind C-terminal fragments of NPY. The Y4 receptor preferentially binds PP and may be referred to the pp1 receptor (17). The Y5 subtype binds similar ligands as the Y1 (24). NPY receptors couple *via* Gi/o proteins to several downstream signaling pathways, including inhibition of adenylyl cyclase, activation of mitogen-activated protein kinase, regulation of intracellular calcium (Ca2+) concentrations, and activation of G-protein-coupled, inwardly rectifying potassium (K+) channels (25, 26).

The predominantly postsynaptic Y1 receptor requires the intact NPY sequence for recognition and activation and is the subtype mediating antianxiety and antidepressant actions of NPY (13). Activation of the Y1 receptor decreases levels of experimental anxiety, alleviates post-traumatic stress disorder (PTSD) and depression-like behavior, predominantly *via* actions in the amygdala and hippocampus (13, 27–29). The presynaptic Y2 receptor is, in addition to intact NPY, also activated by C-terminal fragments of NPY, such as NPY 13–36 and NPY 3–36 (20). The Y2 subtype functions as a heteroreceptor, affecting presynaptic release of NPY and classical neurotransmitters, including GABA and glutamate, as well as norepinephrine (20, 30, 31). The Y4 receptor has low affinity for NPY and is primarily the target for PP, which, as mentioned also, is a member of the PP-family of peptides (32). The Y5 receptor was initially thought to be the exclusive receptor regulating NPYs effect on feeding behavior (33); however, the orexigenic effects of NPY have since been determined to also involve the Y1 and the Y2 receptor subtypes (34–36).

Within the mammalian CNS, NPY receptor subtypes are expressed in regions overlapping with NPY expression and involved in regulation of anxiety and stress, depression, energy homeostasis, and memory function. These regions include the previously mentioned amygdala, hypothalamus, and hippocampus, and also the periaqueductal gray (37), septum (38), and the locus coeruleus (39).

STRESS AND ANXIETY

Early Findings

An early finding for CNS action of NPY was a long-lasting synchronization of the EEG pattern (40). This is similar to the effects of sedative/anxiolytic compounds such as bensodiazepines or barbiturates. Furthermore, i.c.v. administration of central NPY suppressed baseline as well as novelty-induced locomotor activity (41). Another early finding, the prevention of formation of gastric erosions, also indicated a role of NPY in regulation of stress-related events and, possibly, anxiety-related behavior (42). Early on, anxiolytic-like effects of NPY were demonstrated using the elevated plus-maze (EMP), the social interaction test, as well as "conflict tests" such as the Geller–Seifter and the Vogel punished drinking conflict test (43, 44). Here, spatial or social exploration is suppressed by fear of open spaces, and unfamiliar conspecifics, respectively, and restored by bensodiazepines, and

also NPY (29, 43, 45). In the light–dark compartment test, a model conceptually related to the elevated plus-maze similar findings was reported (46). Finally, in the fear-potentiated startle model, which is based on fear potentiation rather than inhibition of behavior, NPY effectively reverses the potentiation of the acoustic startle response, which occurs upon presentation of a conditioned fear stimulus, but does not affect basal, unconditioned startle (45).

Stress and Homeostasis

Responses that may be beneficial in an acute situation may become adverse under repeated circumstances. This is in particular true for stress responses, which when chronically activated may cause damage and become the basis for or accelerate disease conditions (47). Stress as a term refers to evolutionary highly conserved processes involving perception, appraisal, and response to threatening, challenging, and/or possibly harmful stimuli. Homeostasis refers to consistency of internal parameters within a normal range, while allostasis is the process of reestablishing homeostasis beyond the normal range that entails changing the homeostasis to match external demands in response to a challenge. Allostasis links the brain with the endocrine and immune systems to coordinate appropriate responses to a stressor (48, 49).

In addition to being intrinsically harmful, (chronic) stress and accompanying alterations in homeostatic balance have also been shown to increase vulnerability to addiction (50–52). Drug intake and withdrawal also in themselves act as stressors leading to a disruption of the homeostatic state and constitute a mechanism underlying progression from drug/alcohol use to abuse (53, 54). Furthermore, repeated exposure to and withdrawal from drug use leads to increased sensitivity to stress and an increased behavioral stress response (55). A hallmark of addiction is the risk for relapse following a period of abstinence. During progression from drug use to abuse, alterations in hypothalamic as well as extra-hypothalamic structures, such as the amygdala, lead to an increased stress sensitivity (51, 55–59). Stress-induced relapse is a model frequently used in preclinical settings and involves exposure to a stressor (for example, yohimbine or foot shock (60–63), for example) which then leads to the reinstatement of a previously extinguished behavior, i.e., drug taking.

Affective disorders including anxiety and depression affect as many as 1 in 4 individuals during their lifetime, and are, together with alcohol use disorders, major causes of "Years of life lived with disability" in all ages and "Years of life lost because of premature death" as a consequence of illness itself and due to depression comorbidity with, e.g., cardiovascular disease and a high suicide rate (64–69). Depression is more prevalent in women, while alcohol use disorders are more prevalent in men (65, 70). The frequency and prevalence of these disorders are increasing due to demographic changes (longer life expectancy) and, possibly, due to improved diagnostic procedures.

Currently available pharmacological treatments have limited efficacy, about one-third of patients do not respond or are only partial responders. Thus, there is a major unmet medical need, and neuropeptide systems may offer opportunities to develop novel treatments to alleviate it.

NPY in Stress and Anxiety-Related Behavior

As previously mentioned, central administration of NPY was early on shown to mimic anxiolytic and sedative effects of compounds such as barbiturates and benzodiazepines. Later studies, using both rats and mice, have confirmed and extended these initial findings to an extensive range of experimental models, including conflict tests, fear-potentiated startle, and different mazes (43–45, 71). Consistent with effects of NPY administration, overexpression of NPY was shown to exert anxiolytic effects. Using an NPY transgenic rat model with hippocampal NPY overexpression (72), it was demonstrated that increased hippocampal NPY activity led to a behavioral insensitivity to restraint stress on the elevated plus maze, absent fear suppression of behavior in a punished drinking test, and impaired spatial learning in the Morris water maze. Additionally, localized overexpression of NPY within the amygdala led to decreased anxiety, as well as alcohol intake, in rats, further confirming a role for endogenous NPY in regulation of anxiety-related behavior (73). This was also confirmed by the finding that rats with an innate higher number of NPY-positive cells in the central amygdala displayed less anxiety-like behavior in the light–dark box model (74). However, while the amygdala has long been known to regulate fear and anxiety-related behavior, as well as being considered a site of storage of fear memories, newer findings suggest that the prefrontal cortex (PFC) is essential in the regulation of amygdala-dependent memories and fear expression (75). Dysregulation of fear related memories are of especial importance in patients with PTSD. Within the PFC, activation of the prelimbic cortex (PrL) enhances the expression of fear, while an elevated activity in the infralimbic cortex (IL) enhances fear extinction. It was recently shown that the pyramidal neurons in the PrL receive a direct inhibitory input, which is mediated by bipolar NPY(+)-GABAergic projection neurons in the IL (76). Additionally, infusion of NPY into the IL impairs retrieval of fear extinction without affecting depression-like behavior or working memory (77). Further, NPY is markedly reduced in several brain regions in a well-defined rat model of PTSD, exposure to predator scent (78–80) and, crucially, direct NPY administration into the CNS or intranasally administered NPY counteracts PTSD symptoms (81–85). Amygdala–PFC connections have indeed been demonstrated to be of great clinical relevance in PTSD (86). Interestingly, changes in the Npy gene (rs16147 T>C polymorphism) represent a risk factor for expression of negative affect in individuals exposed to adversity in early childhood (87).

The Y1 receptor subtype has been shown to mediate the anxiolytic effect of NPY within the amygdala (13, 88), with the presence of the receptor being required for this effect (27). Additionally, administration of Y1 receptor antagonists into the lateral ventricles or the basolateral nucleus of the amygdala induced anxiogenic effects in rats (89, 90). In contrast, activation of the Y2 receptor subtypes is anxiogenic, following ventricular administration or local injection into the basolateral amygdala (91, 92). This is consistent with the proposed localization of Y2 receptors presynaptically (93) and has been suggested to be due to a self-regulatory mechanism where activation of the Y2 receptor leads to decreased release of NPY (and regulation of

GABA/glutamate dependent on neurobiological substrate). In line with this, blockade of Y2 receptors could be expected to be anxiolytic. Indeed, using different antagonists aimed at the Y2 receptor anxiolytic-like effects have been demonstrated in animal models, including the elevated plus-maze and conflict tests (94–96). Additionally, Y2 receptor knockout showed a low-anxiety phenotype in the elevated plus maze and open field tests, suggesting that in addition to limiting NPY-release, the Y2 receptor may counterbalance anxiolytic effects of NPY (95, 96). Deletion of Y2 receptors also lead to reduced neuronal activation in brain areas of interest following stress exposure (97). Furthermore, brain region-specific deletions of the Y2 receptor gene within the basolateral and central amygdala generated an anxiolytic phenotype (98). On the contrary, administration of NPY (13–36), a Y2 receptor specific agonist, into the vicinity of the locus coeruleus produced anxiolytic effects (39), indicating regional differences in effects of Y2 receptor activation.

Although Y4-knockout mice display reduced anxiety-related and antidepressant activity, as well as enhanced locomotor activity in behavioral tests (99, 100), direct activation of the Y4 receptor did not show any effect on anxiety-related behavior (101). A putative interaction between Y2 and Y4 receptor subtypes was suggested by an amplified anxiolytic-like effect of a double Y2/Y4 knockout (102). However, further elucidation of the involvement of the Y4 receptor subtype is hampered by the lack of specific ligands aimed at the receptor. The Y5 subtype has also been implicated in regulation of affective behavior, but it is difficult to determine the contribution of this receptor subtype due to its close relationship with the Y1 subtype. Conflicting results exist as to whether Y5 antagonists may reduce food intake and have anxiolytic or antidepressant-like effects (103, 104). In an animal model, the Y1 and Y5 receptors were shown to have overlapping functions as well as expression in regions regulating anxiety. Conditional removal of the Y1 receptor in Y5 receptor-expressing neurons in juvenile mice leads to higher anxiety but no changes in hypothalamus–pituitary–adrenocortical axis activity, under basal conditions or after acute restraint stress (105).

NPY AS AN ANTIDEPRESSANT

The relationship between anxiety and depression is that of overlapping conditions. Symptoms of anxiety and depression commonly co-exist, and both disorders are thought to reflect maladaptive changes in stress-responsive systems (106).

In depression, there have been reports on reductions in gray matter volume and glial density in regions mediating the cognitive aspects of depression, i.e., the PFC and the hippocampus (107). In addition, functional studies show that activity within the amygdala and subgenual cingulate cortex is chronically increased in depressed individuals while reverting back to normal levels with successful treatment (108, 109). In rodents, exposure to chronic mild stress, a model used to induce a depressive-like state, increased activity, measured as c-Fos response, within the amygdala, medial habenula, and IL in rats susceptible to the stress effects (110). Within the hippocampus, NPY modulates synaptic activity and inhibits hippocampal excitability, having distinct effects on memory function (30, 111).

Indeed, central administration of NPY was shown to exert antidepressant-like effects in the forced swim test (FST), indicated by a dose-dependent increase in swimming and a decreased immobility (112). In another study, intracerebroventricular administration of NPY in olfactory bulbectomized rats, a rodent model of depressive-like symptoms, resulted in attenuation of increased behavioral irritability (113), indicating a possible therapeutic role of NPY in reducing depression-like behaviors. NPY has also been shown to reverse tricyclic antidepressant treatment-resistant depression induced by central administration of adrenocorticotropic hormone (114). Furthermore, NPY has been shown to modulate effects of antidepressant treatments such as imipramine and for exploratory treatments such as agmantine and other neuropeptides (115–117).

With regard to receptor subtypes mediating the antidepressant effects of NPY, activation of the Y1 receptor subtype has been shown to have direct antidepressant-like effects, as well as to modulate effects of antidepressant treatment (28, 115). Recently, intranasal administration of both NPY as well as a peptidergic Y1-agonist to rats was shown to have antidepressant-like effects (82, 83, 118) Furthermore, it was recently shown that chronic treatment with a Y5 receptor antagonist produced antidepressant-like effects in the rat chronic mild stress model and reversed depressive-like behavioral changes in the FST and prevented degeneration of astrocytes in the mPFC (104, 119).

Reduced NPY expression, both mRNA and protein, may constitute a risk for depression and anxiety-related behaviors. In a study of Fawn Hooded rats, an animal model of depression, decreased NPY concentrations were found in hippocampus compared to control animals (120). In another genetic animal model of depression, the Flinders Sensitive Line (FSL) rats, decreased NPY protein was found in the hippocampal CA region, while Y1 binding sites were increased. On the other hand, NPY was increased in the arcuate nucleus of the hypothalamus, compared to the non-depressed control Flinders Resistant Line (FRL) rats (121–123). For gene expression, Y1 receptor mRNA was decreased in several cortical and limbic regions in FSL rats compared with FRL rats (122). Considering the increased prevalence of depression with age, the observation that cell loss of the NPY-positive cells in the dentate gyrus is enhanced in the depressed FSL animals as they age compared to the FRL line confirm their use as a depression model (124). Consistent with these findings, decreased NPY in selected brain regions has been found in several models of dysregulated emotionality and stress, such as learned helplessness (125), maternal separation/deprivation (126, 127), chronic mild stress (128), social isolation (129), PTSD (79), and acute as well as early-life stress (130, 131), as well as in animal models of alcohol use such as an alcohol-preferring rat strain (132).

MODULATION OF NPY EXPRESSION AND ACTIVITY BY STRESS OR TREATMENT

Exposure to stress or fearful stimuli, or treatment with anxiolytic or antidepressant drugs, affects CNS expression and function of NPY, the relationship being bidirectional. Acute stress significantly decreased NPY expression within the amygdala, an effect

accompanied by anxiogenic behavior as measured on the elevated plus-maze (130). The reverse relationship was found when the stressor was applied repeatedly, indicating an innate mechanism for adaptations in amygdala NPY dependent on context or exposure frequency (133).

With regard to treatments, antidepressants administered orally to rats increased NPY in frontal cortical regions and the hypothalamus, as did electroconvulsive (ECS) treatment (134, 135). Lithium treatment was shown to increase NPY protein and mRNA levels in several brain regions in rats, such as the striatum, hippocampus, frontal and occipital cortices, and the entorhinal cortex (136–139). Early studies of ECS demonstrated that hippocampal and cortical NPY levels increased after repeated ECS in rats (140–142). Treatment of experimental animals with SSRIs has yielded differential effects on NPY levels in different brain regions. Interestingly, antidepressant effects of exercise may be related to alterations in hippocampal NPY levels (143, 144).

NPY IN ALCOHOL USE DISORDERS

Substance use disorders correlate significantly with prevalence of mood and anxiety disorders that develop independent of intoxication and withdrawal (145–147). Specifically, overconsumption of alcohol is commonly associated with anxiety and depression (148). Animal models have shown that acute ethanol administration produces dose-dependent anxiolytic effects short term (149), while acute high doses produce withdrawal-induced anxiety (150). Additionally, greater alcohol intake has been associated with states of anxiety (151). The highly prevalent comorbidity has generated interest in anxiolytic and antidepressant drugs as putative treatment targets in alcohol use disorders (152).

Accumulating evidence points to a key role of NPY in the modulation of the development of alcohol dependence. Alcohol consumption is increased in mice with a null mutation of the NPY gene, but decreased in transgenic NPY overexpressing subjects (153). Furthermore, differences either in electrophysiological responses to exogenous NPY or in peptide concentrations in specific brain regions have been found in rat strains selected for high and low alcohol preference (132, 154, 155). Further support for an involvement of NPY and its receptors in the behavioral consequences of alcohol dependence come from animals with a history of alcohol dependence induced *via* alcohol vapor exposure. Here, changes in NPY-like immunoreactivity (156), stress-responsivity (55), and brain activation patterns (157) were seen. These findings indicate that alterations in NPY-related systems may underlie some of the behavioral changes induced by a history of alcohol vapor exposure, and suggesting that NPY, or analogs thereof, may modulate alcohol-induced behavioral modifications. Alterations in NPY system expression and function are seen for many drugs of abuse (158).

Administration of NPY into the ventricles reduced alcohol intake in alcohol-preferring P rats (159), as well as in vapor-exposed animals (160), while this effect was absent in animals without a history of dependence or the appropriate genetic background (161). Reduction of alcohol intake following NPY infusion in predisposed animals might relate to its anxiolytic effect, since alcohol dependence is accompanied by an increased sensitivity to stress (55). This relates to the clinical context, in that, clinical studies have shown a correlation between anxiety levels and subsequent alcohol abuse (152).

The amygdala is a central neurobiological substrate in stress- and anxiety-related behavior, as well as in modulation of alcohol intake (162, 163). Amygdala lesions disrupt anxiety-related behavior and reduce alcohol consumption (164, 165). Some lines of alcohol-preferring rats also exhibit higher anxiety-like behaviors and lower amygdala NPY levels (166). However, as an illustration of the complexity of alcohol use disorders, increased alcohol intake due to selective breeding can also be accompanied by reduced anxiety-like behavior in rodents (166). An infusion of NPY into the central nucleus of the amygdala in alcohol-preferring rats normalizes both anxiety behaviors (assessed using the light/dark box exploration test) and alcohol intake (167). Conversely, direct injection of NPY into the paraventricular nucleus of the hypothalamus actually increases alcohol consumption (168), an effect that may illustrate the caloric content of ethanol. Additionally, elevated NPY signaling in the nucleus accumbens and/or striatum may contribute to the increased sensitivity to ethanol-induced behavioral sensitization. Reduced expression of ethanol-induced behavioral sensitization was seen following activation of Y2 receptors in the nucleus accumbens (169).

Alcohol use disorders are characterized by, among other things, escalated consumption over time, an inability to stop intake despite adverse consequences, and relapse to alcohol taking following periods of abstinence. Periods of consumption are interspersed with periods of alcohol withdrawal and abstinence. Alcohol withdrawal induces acute anxiety (170), which can be alleviated by known anxiolytics. Alcohol withdrawal affects NPY expression, and withdrawal-induced decreases of NPY within the central amygdala likely contribute to increased GABAergic tone in alcohol-dependent animals. It has been shown that application of exogenous NPY normalizes dependence-induced increases in GABA release in CeA (171).

NPY Receptor Function in Alcohol Use Disorder

Neuropeptide Y infusion into the CNS reduces alcohol intake in animal models of escalated intake, and an overexpression of NPY within the amygdala reduces alcohol intake in a choice model (72, 73). The direct effect of NPY in reducing alcohol intake is most likely due to an increased activation of the Y1 receptor subtype. It was recently demonstrated that Y1 receptor activation in the bed nucleus of the stria terminalis suppressed binge alcohol drinking and that the underlying mechanism was an enhanced inhibitory synaptic transmission specifically in CRF neurons *via* a Gi-mediated, PKA-dependent postsynaptic mechanism (163). Furthermore, central infusion of NPY, a Y1 receptor agonist, and a Y2 receptor antagonist significantly blunted binge-like alcohol drinking in C57BL/6J. In that study, binge-like alcohol drinking reduced NPY and Y1 immunoreactivity in the central nucleus of the amygdala, while 24 h of alcohol abstinence after a history of binge-like drinking promoted increases of Y1 and Y2R expression. The binge-like alcohol drinking augmented the ability of NPY to inhibit GABA (172).

Some data indicate that Y2 receptor antagonism proposedly leads to increased NPY in the synaptic cleft, thereby functioning as an indirect Y1 receptor agonist. Indeed, results from Y2 knockout mice in models of alcohol intake (173), as well as in anxiety and depression models (95), indicate that antagonism of the Y2 receptor may modify these behaviors. Furthermore, central administration of the Y2 receptor antagonist BIIE0246 suppressed self-administration of a sweetened alcohol solution in rats, and post-dependent animals showed increased sensitivity to this effect (174, 175). However, using a different small molecule, non-peptidergic antagonist, JNJ-31020028 (176), in high alcohol-preferring rats as well as outbred Wistar rats, no effect on alcohol intake-related behaviors or relapse to alcohol seeking could be detected (177). The differential effects may be due to structural differences in the used ligands.

With regard to the Y5 receptor subtype, NPY activity at this receptor subtype can modulate ethanol reinforcement in mice (178). Furthermore, in a high alcohol-preferring rat line, antagonism at the Y5 receptor can reduce alcohol intake (179).

In addition to increased alcohol intake, a hallmark of addiction is the risk to relapse following periods of prolonged abstinence (52–54). The potential role of NPY as well as NPY receptor ligands in preventing relapse of alcohol intake in dependent animals has been explored in several experiments. Thus, NPY-administered ICV blocked reinstatement of alcohol seeking induced by the pharmacological stressor yohimbine, an alpha-2 adrenoreceptor antagonist (161).

CLINICAL STUDIES

Decreased levels of CSF NPY have been found in patients with affective disorders, patients who had a history of suicide attempt, PTSD, and dementia (180–185). PTSD patients have lower plasma and CSF NPY levels than healthy controls (186, 187). Challenge studies have also demonstrated differences between in PTSD patients and controls. In healthy subjects, intravenous administration of yohimbine has been reported to induce anxiety as well as relapse to alcohol seeking and craving for alcohol, increased plasma NPY, highlighting the role of NPY in regulating anxiety (187, 188); this effect was attenuated in PTSD. NPY levels were also positively correlated with cortisol levels and behavioral performance under stress (189–191). The reduced levels of NPY in CSF have been shown to be accompanied with reductions in NPY immunoreactivity and mRNA in postmortem brain tissue (192). In a study with patients with depression and anxiety, serum NPY levels were lower in the patients than in the controls. Serum NPY levels were increased by treatment with escitalopram and venlafaxine in the patients with depression, but not in the patients with anxiety (193). Regulation of NPY levels in circulation is regulated in part by the enzyme dipeptidylpeptidase 4 (DPP4). DPP4 has been shown to have lower activity in depressed patients, an effect reversed by antidepressive treatment (194, 195).

GENETICS AND EPIGENETICS OF NPY

Affective disorders as well as alcohol use disorders have strong genetic contributions (196, 197). In an early study of the genetics of alcohol dependence, linkage analysis on the F2 intercross progenies of P and non-preferring rats revealed a chromosomal region containing a NPY precursor gene (198, 199). Within the Npy gene, a number of functional single-nucleotide polymorphisms (SNPs) exist within the Npy gene. NPY haplotypes were found to predict levels of NPY mRNA in postmortem brain and levels of plasma NPY, as well as emotion-induced activation of the amygdala (200). A SNP (rs16147) located in the promoter region alters NPY expression *in vitro* and seems to account for more than half of the variation in expression *in vivo* (87). In depression, reductions in NPY levels are associated with a preproNPY SNP (201, 202). The rs16147 SNP was associated with impaired antidepressant treatment response in patients with anxious depression (203, 204), and low-expression NPY genotypes were also found to be overrepresented in subjects with major depression (205).

Within the Npy gene, a thymidine(1128)-to-cytosine(1128) polymorphism (T1128C; rs16139), which results in a substitution of Leu(7) by Pro(7) in the signal peptide part of pre-pro-NPY, was identified in relation to serum cholesterol (206). The minor allele of the Leu7Pro polymorphism in the NPY gene has been associated with higher processing into mature NPY and higher CSF NPY levels (201). The cytosine 1128 (Pro7) allele was shown to be rare in a depression population and has been suggested to play a protective role against depression (202, 207). Based on animal literature, the Pro7 allele was suggested to be associated with elevated alcohol consumption in humans. Indeed, the frequency of Pro7 allele was higher in European Americans subjects with alcohol dependence compared with healthy controls. These findings suggest that in humans, the Pro7 allele of leu7pro may be a genetic vulnerability for pathological alcohol consumption and dependence. Conflicting data exist with regard to the contribution of the Pro7 allele in alcohol dependence (208–210). Other NPY gene polymorphisms have been associated with alcohol dependence including a polymorphism at the 602 position in the 5′ region and a C to T substitution at the 5671 position (211). Additionally, in rhesus macaques, it was suggested that a polymorphism within the Npy gene promoter may be associated with susceptibility to alcohol use disorders (212). Furthermore, the increased drinking of P rats may be related to NPY-ergic activity in this selectively bred rat line (213–215).

Neuropeptide Y Y5 receptor variants have also been found to contribute to the etiology of panic disorder in a population of German patients, supporting the evidence for a risk locus on chromosome 4q31–q34 in anxiety disorders (216). With regard to the Y1 and Y2 receptor subtypes, limited data on SNPs are available. For the Y2, haplotypes containing a SNP within the first intron (rs17376826 SNP) have strong associations with body mass index, but relations to stress, anxiety, or alcohol addiction have not been examined (217). Additional Y2 polymorphisms, rs4425326 and rs6857715, have been associated with severe alcohol dependence, comorbid alcohol and cocaine, and cocaine dependence in European American population (218). Furthermore, the prevalence of current smokers was greater among Japanese men having the rs4425326 C-allele compared to ex-smokers (219).

Epigenetic mechanisms have so far only to a limited extent been shown to be involved in stress- and nutritional-regulation

of NPY expression and function. Perinatal malnutrition has stress-like effects on offspring in animal models (220–224) and has been shown to alter DNA methylation of CpG dinucleotides in the proximal promoter region of the NPY gene within the hypothalamus at 16 and 100 days of age, compared to control rats (225). Additionally, rearing of newborn rats on a high-carbohydrate diet, shown to induce hyperinsulinemia, increases acetylation of lysine 9 in histone 3 (H3K9) for the NPY gene, without changes in histone methylation (H3K9). These findings were consistent with the changes in the expression levels of NPY, suggesting that epigenetic mechanisms regulate NPY levels in response to nutritional stress, at least within the hypothalamus. Within the amygdala, NPY protein levels were shown to be decreased in the CeA and MeA of rats with access to intermittent alcohol during adolescence (226). The authors additionally showed that histone H3K9/14 acetylation was decreased in the Npy promoter in the amygdala of alcohol-exposed adult rats compared to controls.

In the context of stress and affective disorders, the epigenetic contribution to regulation of NPY expression and function needs to be further elucidated.

The SNP in the rat NPY gene promoter (C/T; rs105431668) affects *in vitro* transcription and DNA-protein interactions. In a rat model of depression, the FSL-line, and its counterpart, the FRL line, the presence of the C-allele enables binding of a transcription factor (CREB2) and a histone acetyltransferase (Ep300). It was determined that the C-allele is only present in the FRL rat line and that its presence correlates with increased hippocampal levels of NPY mRNA and H3K18 acetylation, a gene-activating histone modification maintained by Ep300 (227, 228). This finding illustrates a direct epigenetic mechanism for regulation of NPY expression and function. At the very least, the finding opens up an interesting avenue of exploration for genetic/epigenetic interactions in affective disorders. Furthermore, this suggests that different populations due to their genetics may be differentially susceptible to exposure to stressful, adverse events both during development and in adulthood.

As far as other epigenetic mechanisms, histone acetylation has been shown to affect anxiety-related behavior as well as NPY expression within the amygdala. Specifically, more pronounced deficits in histone acetylation were suggested to be involved in lower NPY expression in the amygdala of P rats, and, thereby, operative in controlling anxiety-like and alcohol-drinking behavior (229, 230). With regard to small RNAs, here microRNA (miRNA) and NPY, a study indicated that deletion of Dicer, an enzyme cleaving pre-miRNAs into miRNAs, in mice leads to decreased expression of NPY mRNA within the hypothalamus (231). However, the authors indicated that this may be a compensatory mechanism due to the genetic modification, and not a direct cause–effect relationship. Thus, miRNA involvement in NPY gene expression remains to be elucidated.

CONCLUDING REMARKS

Affective disorders, including anxiety and PTSD, and alcohol use disorders are major causes of "Years of life lived with disability" in all ages and "years of life lost because of premature death."

Pathophysiologies are insufficiently understood, and currently available drugs in the clinic are only partially effective. While dysregulation of the monoaminergic systems may be a sufficient cause, there is ample evidence that dysregulation of the glutamatergic signaling and changes in neuropeptides, in particular NPY may result in same phenotypes. Consequently, there is an urgent unmet medical need to develop novel treatments that would focus on those targets. Above review of NPY illustrates its important role in physiology as well as pathophysiology of several brain disorders with dysregulated emotionality and points to its potential as a therapeutic agent that can be administered intranasally.

AUTHOR CONTRIBUTIONS

Both AT and AM particpated in drafting, writing, and editing the manuscript.

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Neuroendocrine and Peptidergic Regulation of Stress-Induced REM Sleep Rebound

Ricardo Borges Machado[1,2,3] and Deborah Suchecki[3]*

[1] Department of Psychology, Psychosomatic Research Group, Universidade Ibirapuera, São Paulo, Brazil, [2] Department of Pharmacy, Psychosomatic Research Group, Universidade Ibirapuera, São Paulo, Brazil, [3] Department of Psychobiology, Escola Paulista de Medicina, Universidade Federal de São Paulo, São Paulo, Brazil

Sleep homeostasis depends on the length and quality (occurrence of stressful events, for instance) of the preceding waking time. Forced wakefulness (sleep deprivation or sleep restriction) is one of the main tools used for the understanding of mechanisms that play a role in homeostatic processes involved in sleep regulation and their interrelations. Interestingly, forced wakefulness for periods longer than 24 h activates stress response systems, whereas stressful events impact on sleep pattern. Hypothalamic peptides (corticotropin-releasing hormone, prolactin, and the CLIP/ACTH$_{18–39}$) play an important role in the expression of stress-induced sleep effects, essentially by modulating rapid eye movement sleep, which has been claimed to affect the organism resilience to the deleterious effects of stress. Some of the mechanisms involved in the generation and regulation of sleep and the main peptides/hypothalamic hormones involved in these responses will be discussed in this review.

Keywords: sleep, stress, prolactin, CLIP, serotonin, CRH, homeostasis, REM sleep

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****Correspondence:***
Ricardo Borges Machado
machado.ricardoborges@gmail.com

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INTRODUCTION

The purpose of the present review is not to present a detailed description of the neural basis of sleep generation and maintenance. For that, we refer to a number of previous review papers that cover this subject (1–5).

Sleep is a fundamental behavior for the individual's survival and is redundantly regulated by the interaction of several neurotransmitter and neuropeptide systems acting on several brain structures, mainly located in the hypothalamus and brain stem [for review, see Ref. (6)]. In humans and rodents, sleep is classified into two main stages: non-rapid eye movement sleep (NREMS) and rapid eye movement sleep (REMS), which electroencephalographic features, in rats, are represented in **Figure 1**. In humans, NREMS encompasses three stages, N1, N2, and N3, characterized by synchronized, high amplitude, and low frequency cortical waves, whereas in rats, NREMS or slow wave sleep can be distinguished into two substages, differing in the amplitude of these slow waves (low- and high-amplitude waves). REMS or paradoxical sleep (used for rats, since they show very little eye movements) is characterized by desynchronized, high frequency, low-amplitude cortical waves, very similar to wakefulness, and hippocampal theta waves. In addition, muscle atonia is a main tonic feature of this sleep stage (7, 8).

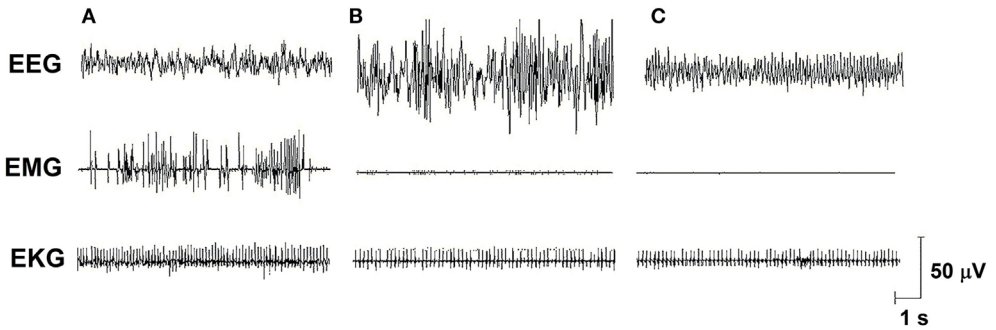

FIGURE 1 | **Electro-oscillographic signs of wakefulness and sleep in the *Wistar* rat**. **(A)** Active wake (with low voltage and fast EEG frequency, concomitant a high EMG activity and EKG is fast), **(B)** non-rapid eye movement sleep (NREMS—high amplitude and slow wave EEG, activity in EMG is low and EKG is low), **(C)** rapid eye movement sleep (REMS—theta 6–8 Hz activity is present in this medial EEG, EMG is almost quiet, and EKG shows an intermediate activity). EEG, electroencephalogram (obtained from a medial frontoparietal bipolar deviation); EMG, electromyogram (from the trapezius muscle); EKG, electrocardiogram (from intercostal electrodes). Signs were calibrated with 50-μV pulse. Data from our group.

SLEEP REGULATION AND HOMEOSTASIS

Circadian and Homeostatic Mechanisms

Sleep is regulated by a combination of homeostatic and circadian mechanisms. The homeostatic process refers to sleep needs or pressure, and the circadian one, entrainment to the light/dark cycle. Besides the homeostatic factor, the circadian aspect is important and is related to the animal's expression of daily preference for sleep/rest. Also, the duration of the sleep episodes appears to be greater in animals that are at the top of the food chain, since preys need to monitor the environment constantly to ensure their integrity, thus sleeping very short bouts (9, 10). The interaction between homeostatic (called "S process") and circadian factors (called "C process") in sleep regulation led some authors to propose a model in which the two processes would act together. Sleep begins wherever there is a conjunction of larger homeostatic pressure (need for sleep) and greater circadian predisposition (proximity to the phase of the cycle that sleep normally occurs), whereas it ends when this interaction decreases (11, 12).

Sleep homeostasis depends, among many factors, on the length and quality of the preceding waking period. Therefore, longer periods of waking lead to greater compensatory sleep, also known as rebound sleep. Interestingly, brief periods of sleep deprivation (SD) (3–6 h) result only in increased NREMS without affecting REMS (13). A total of 12–24 h of total sleep deprivation increases both NREMS and REMS (14–16), whereas total SD for 96 h induces a very pronounced increase in REMS (17). In 1960, few years after the discovery of REMS, William Dement (18) reported, for the first time in humans, that selective REMS deprivation induces a compensatory increase of this specific phase, e.g., REMS rebound. These effects have been replicated in rats, using the platform method that produces a complete suppression of REMS and some loss of NREMS (19, 20).

Effects of Stress

It is interesting to note that REMS deprivation induces the activation of the hypothalamic–pituitary–adrenal (HPA) axis, with increased production (21, 22) and release (23) of corticotropin-releasing hormone (CRH), and increased ACTH (24, 25) and corticosterone plasma levels (22, 26–28). This constellation of neuroendocrine changes indicates the stressful nature of this manipulation, thereby leading some authors to question whether sleep rebound would be an outcome of stress exposure. It soon became clear that stressful events can alter sleep pattern in a stimulus- and length-related fashion, inasmuch as immobilization stress increases REMS (29), social conflict (30) and exposure to cold (31) induce NREMS, whereas unpredictable footshock stress decreases REM sleep (32, 33). One to two hours of immobilization stress in the beginning of the dark phase, increase REMS, but longer periods of stress blunt the expression of sleep rebound (34), similar to what is seen with repeated immobilization (29). These opposite effects between acute and chronic exposure to the stress seem to be due to an adaptation phenomenon, which is also observed with footshock stress (32, 35). Either increased or reduced stress-induced REMS appear to be related to corticosterone secretion, in an inverted U shape fashion (36, 37).

HORMONAL REGULATION OF STRESS-INDUCED SLEEP REBOUND

HPA Axis

Abnormal functioning of the HPA axis substantially changes the sleep pattern, as observed in Addison's disease patients who exhibit adrenal insufficiency and display more NREMS and less REMS (34); this sleep abnormality can be corrected by corticoid replacement therapy (38). Conversely, patients with Cushing's syndrome, who exhibit exaggerated cortisol secretion, display less NREMS and more awakenings during the night, which can also be corrected by interventions that decrease production of glucocorticoids (GCs) (39, 40). These findings strongly suggest that optimal GC concentrations are essential for normal sleep patterns in humans (41, 42). In rats, a similar phenomenon is also observed, with high levels of corticosterone being especially detrimental to NREMS (43).

The 41-aminoacid peptide CRH, its mRNA, and receptors are increased in stress situations, such as electric footshock (44), immobilization (45), restriction of food (46), and sleep deprivation (21, 23). CRH stimulates the release of other proopiomelonocortin (POMC)-derived peptides, including β-endorphin and alpha-melanocyte-stimulating hormone (α-MSH) (47–49). It differentially stimulates the activity of the prohormone convertases (PC1 and PC2, mainly), which cleave POMC in its bioactive peptides (50, 51).

CRH reduces REM and NREMS and increases awakenings when injected intravenously (52) or after i.c.v. administration (53), whereas CRH type 1 receptor (CRH-R1) antagonists promote sleep (54, 55). CRH effects on sleep homeostatic regulation are also evident, for its administration immediately after sleep deprivation increases REMS rebound (56). Conversely, α-helical-CRH_{9-41}, a CRH-R1 antagonist inhibits REMS rebound induced by immobilization (57). Recently, we have demonstrated that both i.c.v. CRH or α-helical-CRH_{9-41} administration during REMS deprivation impairs sleep homeostasis, thereby decreasing the length of REMS episodes in the theta band energy (6.0–9.0 Hz), and decreases the time of REM and NREMS in the recovery period (58).

Corticotropin-Like Intermediate Peptide (CLIP or $ACTH_{18-39}$)

CLIP is a POMC derivative, well known for inducing long REMS episodes (59, 60). ACTH cleavage to CLIP and α-MSH is mediated by prohormone convertase 2 (PC2) and is stimulated by serotonin, *via* $5\text{-}HT_{2C}$ receptors (61–63). This process occurs in the melanocytes of the pituitary *pars intermedia* and in two other distinct locations in the brain: the arcuate nucleus—Arc (and peri-arcuate) in the basomedial hypothalamus and in a cell group of the nucleus of the solitary tract (NST) (64, 65). There are also CLIP-containing fibers, originating in the Arc and projecting to the lateral, paraventricular, basal and preoptic hypothalamic regions, dorsal and medial raphe nucleus (DRN and MRN, respectively), and septal area (66–70) (**Figure 2**).

Initial studies on the effects of stress-induced REMS rebound show that 1 h of immobilization stress increases REMS 3 h after the end of the stressor, a time point that corresponds to the increased content of phosphorylated CLIP in the Arc (71, 72). Conversely, micro-infusion of CLIP into the DRN increases REMS, similar to that observed after acute immobilization stress (73–75). It has been hypothesized that stress induces 5-HT release from the raphe nuclei, which projections to the Arc lead to CLIP processing from POMC. When the stress finishes, 5-HT is reuptaken, whereas CLIP synthesis and release from the Arc still go on. Interestingly, immunoreactive CLIP fibers are found in the DRN, which are postulated to trigger the dendritic release of serotonin (76) by a process of extracellular diffusion (67). The dendritic release of 5-HT within the DRN inhibits further 5-HT synthesis and release, *via* $5HT_{1A}$ autoreceptors (77, 78), a process commonly observed during REMS (79, 80). Confirmation of this mechanism was obtained by electrical stimulation of the DRN leading to release of 5-HT and indol compounds in the Arc, followed, 3 h later, by increased REMS (81) (**Figure 2**). It is worth mentioning that REMS induced by CLIP, more precisely the amino-terminal fragment $ACTH_{20-24}$ (Val-Lys-Tyr-Pro), is characterized by extremely long episodes, usually above 7 min. This effect on REMS is not obtained with ACTH ($ACTH_{1-39}$) or other active fragments, such as $ACTH_{18-24}$ (60).

Prolactin and Prolactin-Releasing Peptide

Prolactin (PRL) is a polypeptide hormone consisting of 199 amino acid residues (in rats and mice, by 197 amino acid residues), which is synthesized and secreted by the anterior pituitary lactotrophs, under tonic dopaminergic inhibitory control (82, 83); the main function of pituitary PRL is to stimulate lactation in mammals (84). In humans, PRL is secreted mostly in the second half of the night (85), with a very clear circadian rhythm (86).

This hormone exhibits anxiolytic effects (87), inhibits the development of stress-induced gastric ulcers (88), and mitigates hormonal and neuronal responses to various stressors (89, 90). PRL also has an important neurotrophic effect and is involved with neurogenesis in various brain structures and cell cultures (91–96), preventing stress-induced decrease in neurogenesis in an animal model of chronic stress (96). PRL is closely associated with physiological stress response (97–99), and immunoreactive neurons are found in the lateral hypothalamus, Arc, and surrounding areas, thereby innervating other hypothalamic nuclei, amygdala, nucleus accumbens, olfactory cortex, septum, the reticular formation region, parabrachial region, locus coeruleus, periaqueductal gray, and DRN (100), and presenting practically the same distribution of POMC and its derivatives, as evidenced by double labeling studies (101). Interestingly, such structures are particularly involved in the regulation of sleep and stress response (**Figure 2**).

Numerous evidence indicates that PRL may be another mediator of stress-induced REMS rebound phenomenon. On the one hand, movement restriction (102) and ether vapor exposure (103) increase PRL plasma levels and time in REMS in rodents. On the other hand, PRL infusion in the dorsolateral hypothalamus or in the lateral ventricle increases REMS during the light phase (104, 105). Recently, we also showed that footshock stress applied concomitantly to REMS deprivation produces substantial REMS rebound, by virtue of very long REM episodes. Plasma PRL and hypothalamic serotonin (5-HT) were the likely mediators of the major increase in REMS episode length (37). Serotonin stimulates PRL release (106, 107) and PRL also increases serotonin synthesis (108–111) (**Figure 2**). In addition, both PRL micro-infusion into the DRN (112) and systemic administration of PRL increases REMS (113), whereas anti-PRL antibody suppresses REMS in rats (114), and this phase of sleep is naturally reduced in mice genetically deficient for PRL (115).

Prolactin-releasing peptide (PRL-RP) acts on a seven-transmembrane G protein-coupled receptor promoting PRL release *in vitro* and *in vivo* (116, 117). Central PRL-RP administration increases REMS in rats in parallel to PRL circulating levels (118, 119). Intriguingly, high PRL-RP concentrations also increase NREMS and have no effect on REMS (118, 119). In a somewhat contradictory way, PRL-RP has the ability to reduce the oscillatory activity in sections of the reticular thalamic tissue (120), which plays a fundamental role in the generation of NREMS (121).

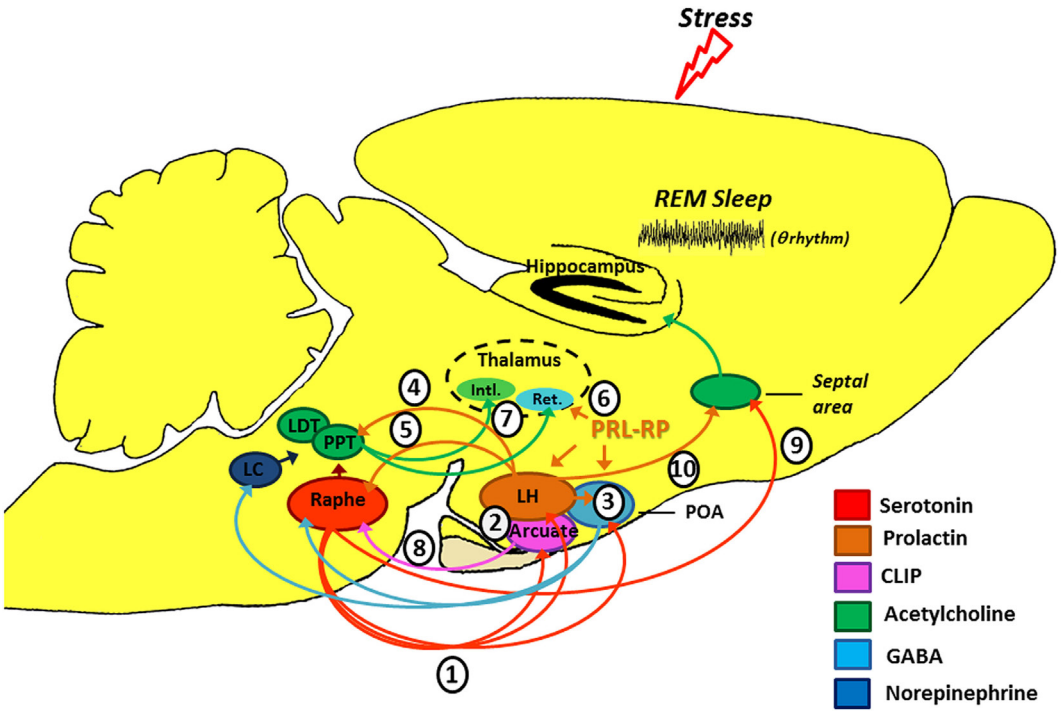

FIGURE 2 | Schematic model of the possible modulation of stress-induced rapid eye movement sleep rebound by PRL, CLIP, and PRL-RP. LC, *locus coeruleus*; LDT, laterodorsal tegmental nucleus; PPT, pedunculopontine tegmental nucleus; LH, lateral hypothalamic area; POA, preoptic area; Arcuate, arcuate nucleus of hypothalamus; Intl., thalamic intralaminar nuclei; Ret., reticular thalamic nucleus. For more details, please refer to the main text.

CONCLUDING REMARKS

Several experimental stressors (1) (122, 123) and SD protocols (124, 125) promote serotonin release from the dorsal raphe (see **Figure 2**). Ascending serotonergic projections stimulate CLIP and PRL production and release by the Arc and lateral hypothalamic area (LH), respectively (2), which depend on gene transcription and protein synthesis (hence, the rebound happens only a few hours after exposure to the stressful stimulus). Serotonin- and PRL-dependent self-stimulating neural circuits (and probably PrRP of the anterior preoptic area) (3), producing GABAergic inactivation of the DRN and LC on pontine nuclei (pedunculopontine tegmental/laterodorsal tegmental) (126). Furthermore, PRL can also activate the pontine cholinergic neurons directly (4) (127). Prolactinergic projections to DRN (5) (100, 101) induce serotonin release (112) that, at first, can feedback on the system leading to additional production and release of CLIP and PRL. However, 5-HT excess can stimulate self-receptors (5-HT_{1A}) in the DRN, thereby inhibiting its activity (128, 129). All these phenomena contribute positively to REM sleep expression. Additionally, PrRP reduces the oscillatory activity of the reticular thalamic neurons (Ret.) (6) (120), an important structure for the generation of synchronized sleep (121), which also has positive effect on REM sleep. Without the inhibitory influence of the DRN and LC (130, 131), pontine cholinergic nuclei (7) stimulate the thalamic intralaminar nuclei (Intl.), which induces cortical activation during desynchronized sleep and inhibits GABAergic neurons of thalamic reticular nucleus (132–134). We should emphasize that CLIP has inhibitory action (8) on serotonin release by the DRN (71), thereby contributing also to halt the suppressive activity that it has on pontine nuclei. It is noteworthy that there is a serotonergic ascending pathway, from the DRN to the septal area (9) that is responsible for the inhibitory activity in the cholinergic basal forebrain area, essential for hippocampal theta rhythm during REM sleep (135). This region also has a high density of PRL receptors (136) and can also be a potential site of prolactin action. Thus, PRL (10) also contributes to REM sleep expression.

REMS in mammals is involved with various functions, such as brain maturation of neonates (10), maintenance of minimal brain activity during sleep [which would allow a quick wake up and avoid a possible coma during sleep (137)], memory consolidation (138, 139), and maintenance of brain monoaminergic neurotransmitter systems (140–143), among others. Recently, however, studies have brought the attention to the importance of REMS in emotion regulation and the main adaptive function of REMS rebound after stress. This is well exemplified by studies showing that patients with posttraumatic stress disorder (PTSD) display longer latency to REMS, and short REM episodes and sleep fragmentation (144, 145). In a recent work, Mellman and colleagues assessed the sleep of individuals after a traumatic event and found a negative correlation between the duration of REMS episodes and development of PTSD. Furthermore, PTSD patients display reduced EEG high frequency activity during REMS, indicating low cognitive activation during this

sleep phase (146), thereby pointing to the need for long and consolidated REMS events to elaborate and integrate traumatic memories in conscious level (147). Therefore, we do not hesitate to propose, for reasons of evolutionary logic, that in rodents, increased REMS observed after some kinds of stress has a similar role (148).

AUTHOR CONTRIBUTIONS

RM and DS contributed equally to this work.

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Effects of corticotropin releasing factor (CRF) on sleep and temperature following predictable controllable and uncontrollable stress in mice

Laurie L. Wellman[1], *Linghui Yang*[2] *and Larry D. Sanford*[1]*

[1] Sleep Research Laboratory, Department of Pathology and Anatomy, Eastern Virginia Medical School, Norfolk, VA, USA,
[2] West China Hospital of Sichuan University, Sichuan, China

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***Correspondence:**
Larry D. Sanford,
Sleep Research Laboratory, Department of Pathology and Anatomy, Eastern Virginia Medical School, 700 Olney Road, PO Box 1980, Norfolk, VA 23501, USA
sanfordl@evms.edu

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Corticotropin releasing factor (CRF) is a major mediator of central nervous system responses to stressors, including alterations in wakefulness and sleep. However, its role in mediating stress-induced alterations in sleep has not been fully delineated. In this study, we assessed the role of CRF and the non-specific CRF antagonist, astressin (AST), in regulating changes in sleep produced by signaled, escapable shock (SES) and signaled inescapable shock (SIS), two stressors that can increase or decrease sleep, respectively. Male BALB/cJ mice were surgically implanted with transmitters (DataSciences ETA10-F20) for recording EEG, activity and core body temperature by telemetry and a cannula for intracerebroventricular (ICV) microinjections. After baseline (Base) sleep recording, mice were presented tones (90 dB, 2 kHz) that started 5.0 s prior to and co-terminated with footshock (0.5 mA; 5.0 s maximum duration). SES mice ($n = 9$) always received shock but could terminate it by moving to the non-occupied chamber in a shuttlebox. Yoked SIS mice ($n = 9$) were treated identically, but could not alter shock duration. Training with SES or SIS was conducted over 2 days to stabilize responses. Afterwards, the mice received saline, CRF [0.4 μg (0.42 mM) or AST (1.0 μg (1.4 mM)] prior to SES or SIS. Sleep was analyzed over 20 h post-stress recordings. After administration of saline, REM was significantly greater in SES mice than in SIS mice whereas after CRF or AST, REM was similar in both groups. Total 20 h NREM did not vary across condition or group. However, after administration of saline and CRF, NREM episode duration was significantly decreased, and NREM episode number significantly increased, in SIS mice compared to SES animals. SES and SIS mice showed similar stress induced hyperthermia (SIH) across all conditions. These data demonstrate that CRF can mediate stress-induced changes in sleep independently of SIH, an index of hypothalamic-pituitary-adrenal axis activation.

Keywords: corticotropin releasing factor, stress, predictability, controllability, escape learning, stress-induced hyperthermia

Introduction

Corticotropin releasing factor (CRF) is a major mediator of central nervous system responses to stressors (Koob and Bloom, 1985; Heinrichs et al., 1995; Koob, 1999; Koob and Heinrichs, 1999; Bakshi and Kalin, 2000; Deussing and Wurst, 2005) including alterations in wakefulness and sleep (González and Valatx, 1998; Chang and Opp, 2002). CRF antagonists attenuate behavioral responses to stress (e.g., Aloisi et al., 1999; Basso et al., 1999; Deak et al., 1999) whereas intracerebroventricular (ICV) administration of CRF can produce many of the signs associated with anxiety in humans, including increased wakefulness (Ehlers et al., 1986; Marrosu et al., 1990; Chang and Opp, 1998), increased activity, and an exaggerated startle response (Swerdlow et al., 1986; Heilig et al., 1994). In the absence of stressors, CRF contributes to the regulation of spontaneous waking (Opp, 1995, 1997; Chang and Opp, 1998, 2001).

CRF has been implicated in the control of rapid eye movement sleep (REM) (González and Valatx, 1997) and some authors have argued that CRF promotes REM. However, there actually have been few studies examining the role of CRF in regulating stress-induced alterations in sleep, and these have yielded conflicting data. This is exemplified with the work on restraint stress and sleep. For example, González and Valatx (1997) reported that the ICV administration of the broad CRF antagonist, αHelCRF (α-helical CRF9-41), prior to restraint stress at the beginning of the dark period prevented the subsequent increase in REM that can occur after restraint (e.g., Rampin et al., 1991; Bonnet et al., 1997; Meerlo et al., 2001), but did not alter spontaneous REM, NREM, or wakefulness in non-stressed animals. In contrast, Chang and Opp found no effect of restraint stress applied at the beginning of the dark period on subsequent sleep, and also found no effect of the non-specific CRF antagonist, astressin (AST), on sleep after restraint (Chang and Opp, 2002). There also are conflicting data on the effects of CRF on REM recovery after sleep deprivation. CRF antagonists administered during sleep deprivation reduce recovery REM in rats (González and Valatx, 1998) and mice (Kimura et al., 2010) whereas the repeated administration of CRF during the actual recovery period blocked the increase in REM in sleep-deprived humans (Schüssler et al., 2006).

Fear conditioning with inescapable shock (IS), an uncontrollable stressor, and the presentation of fearful contexts and cues associated with IS are followed by significant reductions in REM that occur in the first few hours after exposure (Sanford et al., 2003a,b,c; Pawlyk et al., 2005). In mice trained with IS, ICV administration of CRF enhances the reduction in REM following fearful contexts whereas ICV administration of AST attenuates fear-induced reductions in REM (Yang et al., 2009). By comparison, training with escapable shock (ES), and reminders of ES, can produce significant enhancements in post-stress REM (Sanford et al., 2010). Microinjections of either saline or AST prior to ES training produce similar, significant enhancements in post-stress REM relative to a non-shocked handling control condition whereas the increases in REM are blocked by pre-training treatment with CRF (Yang et al., 2011b). Direct comparisons of the effects of manipulating CRF on alterations in sleep associated with controllable and uncontrollable stress have not been made; however, the results to date are consistent with a suppressing effect of CRF on REM after exposure to stress.

In summary, studies administering antagonists prior to or during the presentation of stressors or sleep deprivation have led to conclusions that CRF can promote REM whereas studies administering CRF in stress paradigms suggest that that it can suppress REM. In the current study, we compared the effects of CRF and AST on stress-induced alterations in sleep in mice trained with auditory-signaled variants of ES and IS (SES and SIS, respectively), which also produce equivalent physical stress but directionally different alterations in REM (Yang et al., 2011a). We concurrently examined stress-induced hyperthermia (SIH), an increase in core body temperature induced by both physiological and psychological stress (Vinckers et al., 2009), as a measure of the acute stress response. This study design enabled us to determine the effects of CRF and AST on stressors that produce predictable increases and decreases in REM.

Methods

Subjects

Male BALB/cJ mice ($n = 18$) were obtained from Jackson Laboratory, Bar Harbor, Maine. The mice weighed 20–25 g at arrival. Animals were individually housed with food and water available *ad libitum*. The mouse colony room was kept on a 12:12 light-dark cycle and ambient temperature was maintained at $24 \pm 1.5^{\circ}$C. Throughout the experimental procedures, measures were taken to minimize unnecessary pain and discomfort of the animals.

Surgery

All mice were implanted intraperitoneally with telemetry transmitters (DataSciences ETA 10-F20) for recording EEG, body temperature and activity as previously described (Tang and Sanford, 2002). EEG leads from the transmitter body were led subcutaneously to the head, and the free ends were placed into holes drilled in the dorsal skull to allow recording cortical EEG. In the same surgery, the mice were stereotaxically implanted with a cannula to allow ICV microinjections. A hole was drilled in the skull 1.00 mm lateral and 0.5 mm posterior to Bregma and the tip of a 26-gauge stainless steel infusion cannula was placed 2.00 mm below the skull surface into the right ventricle. The cannula was secured to the skull with dental cement and a stylus was inserted to maintain patency. All surgeries were performed under aseptic conditions and with the mice under isoflurane (as inhalant: 5% induction; 1–2% maintenance) anesthesia. Ibuprofen (30 mg/kg, orally) was continuously available in each animal's drinking water for 24–48 h preoperatively and for a minimum of 72 h postoperatively to alleviate potential postoperative pain. Antibiotics (gentamicin 5–8 mg/kg and Procaine penicillin 100,000 IU/kg) were given subcutaneously preoperatively to prevent infection. Dexamethazone [0.4 mg (0.2 ml total dosage)] was administered subcutaneously preoperatively to reduce brain swelling. All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Experimental Animals and were approved by Eastern Virginia Medical School's Animal Care and Use Committee.

Baseline and Control Recordings

The mice were housed and studied in the same room. Cages and bedding were changed 2 days prior to recording onset for each phase of the experiment and then not disturbed until that phase was complete.

The mice were allowed a post-surgery recovery period of 19–20 days prior to beginning the experiment. Undisturbed baseline (Base) recordings were then obtained for 2 days. Afterwards, the mice were habituated to two daily sessions of the handling procedures needed for administering microinjections. Sleep also was recorded following a 30 min exposure to a novel chamber [an enclosure of approximately the same dimensions as the shock chambers (20 × 40 × 30 cm)] with an open top and walls and floor constructed of clear Plexiglas™. This recording session controlled for handling and exposure to a non-cage environment and was used as a handling control (HC) for comparisons across treatment conditions.

Signaled Escapable and Inescapable Shock (SES/SIS) Training Procedures

The mice were randomly assigned to either SES ($n = 9$) or SIS ($n = 9$) conditions. Training was conducted in a shuttlebox (Model E10-15SC, Coulbourn Instruments, Whitehall, PA) consisting of two chambers divided by a guillotine door. Opening and closing of the guillotine door, as well as the tone (90 dB, 2 kHz, 10 s maximum) and footshock (0.5 mA; 5.0 s maximum) administration were controlled by Coulbourn Graphic State (GS) software (version 2.1). Electric footshock was produced via Coulbourn Precision Regulated Animal Shockers and administered via grid floors of a shuttlebox. Training started between the third and fourth hour after lights on. The entire training procedure was of approximately 30 min duration. For the first 5 min (pre-shock period), the mice were allowed to freely explore the shuttlebox followed by presentation of 20 shocks. The tone was presented 5 s prior to the footshock to signal its onset. When footshock was administered, the SES mice were then able to move through the door to the unoccupied chamber. This movement caused interruption of photo-beam sensors which was detected by GS software and terminated shock presentation and tone. If the animal did not move to the unoccupied chamber, the shock and tone co-terminated after 5 s. Footshock for SES mice was *escapable,* but not avoidable, for movement prior to footshock onset did not prevent shock presentation. SES and SIS mice were simultaneously trained as yoked pairs such that SIS mice received identical amounts of shock as a mouse in the SES group, but could not alter shock duration by their actions. After the final shock, the animals remained undisturbed in the shuttlebox for 5 additional min (post-shock period) and then were returned to their home cages.

The mice received 2 days of 20 (1.0 min intervals) tone-shock pairings without drug administration to stabilize responses. On four subsequent days, the mice were administered saline (SAL), CRF or AST prior to SES or SIS. The experiment was pseudo-counterbalanced in that the series of CRF microinjections were completed before starting the series of AST microinjections. SAL was administered such that approximately half the mice received SAL on each of the four training days. Each shock presentation day with injection was separated by 1 week.

Drugs and Microinjections

After recovery from surgery, ICV location of the cannula was verified with administration of angiotensin (200 ng in 0.2 μl ICV) and observation for drinking. A positive angiotensin-induced drinking response was shown by all mice included in the study.

CRF and AST [cyclo(33)[D-Phe12,N1e21,38,Glu30,Lys33] h/rCRF(12-41)] were obtained in powder form from Sigma-Aldrich (St. Louis, MO) and were diluted to the desired concentrations in pyrogen-free SAL. Concentrations [CRF 0.4 μg (0.42 mM); AST: 1.0 μg (1.4 mM)] used in this experiment were based on dosages used in previous studies (Sanford et al., 2008; Yang et al., 2009).

For microinjections, an injection cannula (33 ga.), which projected 1.0 mm beyond the tip of the implanted guide cannula, was inserted and secured in place. The injection cannula was connected to lengths of polyethylene tubing that in turn were connected to a 5.0 μl Hamilton syringe. The injection cannula and tubing had been pre-filled with the solution to be injected. The solutions in a volume of 0.2 μl were slowly infused over 1 min and administered 15 min prior to beginning shock training.

Sleep Recording and Determination of Sleep State

Sleep and wakefulness of the animals were monitored in the colony room. Recording started at the fifth hour after lights on. The transmitters for the mice were activated with a magnetic switch, and their home cages were placed on a telemetry receiver (Model RPC-1, Data Sciences International). When the animals were not on study, the transmitters were inactivated. Signals from the transmitter were detected by the receiver, and were processed and saved by Data Sciences International software for subsequent visual scoring. Sleep and wakefulness were determined by a trained observer in 10-s epochs using SleepWave software (Biosoft Studio). Each epoch was scored either as NREM, REM, active wakefulness (AW), or quiet wakefulness (QW), based on EEG and gross whole body activity as previously described (Tang and Sanford, 2002). Twenty hours uninterrupted sleep recordings were collected for Base and after each experimental session.

Data Analyses

Data were analyzed using Sigmaplot 12 software (Systat Software, Inc. San Jose, CA). The data were primarily analyzed with two-way mixed factorial ANOVA procedures (Group X Treatment Day with repeated measures on Treatment Day. When appropriate, *post-hoc* comparisons among means were conducted using Holm-Sidak tests. Differences were considered significant at $p < 0.05$.

Results

Amounts of REM, NREM and total sleep did not differ across the two SAL recording days. We therefore collapsed data for these 2 days and made comparisons across Base, HC, SAL, CRF, or AST prior to shock training. Analyses were conducted on the total 20 h recordings and on the 8 h light and 12 h dark periods.

Effects of CRF and AST on Stress-induced Alterations in REM

Total REM and REM Percentage

The ANOVA for total 20 h REM amounts revealed a significant Treatment Day effect [$F_{(4, 64)} = 2.85$, $p < 0.03$] and a Group X Treatment Day interaction [$F_{(4, 64)} = 3.55$, $p < 0.02$]. *Post-hoc* comparisons across Treatment Days revealed a significant difference in REM between the SES and SIS groups only on the SAL day (**Figure 1A**). Amounts of REM during Base, and on the HC, CRF and AST recording days did not significantly differ between groups. Thus, administration of either CRF or AST reduced or eliminated the differences normally seen between mice trained with SES and SIS, i.e., CRF reduced REM in the SES mice to levels seen in the SIS mice and AST attenuated the reduction in REM in the SIS mice to levels that were not significantly different from those in the SES mice. The difference between groups on the SAL day was found primarily during the dark period which was also characterized by a significant Group X Treatment Day interaction [$F_{(4, 64)} = 2.98$, $p < 0.03$] and similar findings across Treatment Days (**Table 1**). The analysis for light period total REM found a significant Treatment Day effect [$F_{(4, 64)} = 5.67$, $p < 0.01$]. REM was reduced on the CRF Treatment Day compared to Base, HC, and AST (**Table 1**), but there were no significant differences between groups.

A similar Main Effect for Treatment Day [$F_{(4, 64)} = 4.92$, $p < 0.01$] and a Group X Treatment Day interaction [$F_{(4, 64)} = 3.92$, $p < 0.01$] were found for the analyses of total 20 h REM percentage (of total sleep time) and a significant interaction for dark period REM percentage [$F_{(4, 64)} = 3.45$, $p < 0.02$]. Differences between SES and SIS mice also were found only on the SAL Treatment Days (**Figure 1B**, **Table 1**). As with total REM amounts, the analysis for the light period did reveal any Group differences, though there was a significant Treatment Day effect [$F_{(4, 64)} = 10.22$, $p < 0.001$]. REM percentage was reduced on the CRF Treatment Day compared to Base, HC, SAL, and AST (**Table 1**).

Number of REM Episodes and REM Episode Duration

There were no significant differences in number of REM episodes (**Figure 1C**) or REM episode duration (**Figure 1D**) in the analyses of the 20 h recording period. However, there was a significant Treatment Day effect for number of REM episodes during the light period [$F_{(4, 64)} = 4.41$, $p < 0.01$] and the *post-hoc* analysis found significantly reduced REM episodes on the CRF day compared to the Base day (**Table 1**). There also was a significant Treatment Day effect for REM episode duration during the light period [$F_{(4, 64)} = 3.71$, $p < 0.01$] and the *post-hoc* analysis found significantly reduced REM episode duration on the CRF day compared to HC (**Table 1**). No other comparisons were significant.

Effects of CRF and AST on Stress-induced Alterations in NREM, Total Sleep Time, and Wakefulness

NREM and Total Sleep Time

Total 20 h TST (**Figure 2A**) and NREM amounts (**Figure 2B**) did not vary across condition or group. However, there was a difference across Treatment Days for light period NREM [$F_{(4, 64)} = 3.44$, $p < 0.02$] and TST [$F_{(4, 64)} = 4.15$, $p < 0.01$]. Light period NREM was reduced on the CRF day compared to the HC day and light period TST was reduced on the CRF day compared to the HC and AST Treatment Days (**Table 2**). No other comparisons were significant.

The analysis of NREM episodes for the 20 h recording period (**Figure 2C**) revealed significant effects for Group [$F_{(1, 16)} = 10.305$, $p < 0.01$] and Treatment Day [$F_{(4, 64)} = 8.483$, $p < 0.001$]. This effect was primarily due to the increased number of NREM episodes in the SIS mice compared to the SES mice on the SAL and CRF treatment days compared to Base and HC. Virtually identical results were found for the analyses of the light [Group ($F_{(1, 16)} = 7.949$, $p < 0.02$) and Treatment Day [$F_{(4, 64)} = 5.504$, $p < 0.001$)] and dark [Group ($F_{(1, 16)} = 4.973$, $p < 0.02$] and Treatment Day [$F_{(4, 64)} = 6.567$, $p < 0.001$)] periods (**Table 2**).

There was a main effect for Treatment Day in the analysis of the NREM episode duration (**Figure 2D**) for the total 20 h recording period [$F_{(4, 64)} = 7.669$, $p < 0.001$). NREM episode duration was reduced on the SAL and CRF treatment days compared to Base and to the HC Treatment Day. There also was a significant Treatment Day effect during the light period [$F_{(4, 64)} = 5.588$, $p < 0.001$]; however, the reductions in NREM episode duration for the SAL and CRF treatment days were significantly reduced only compared to the HC treatment day (**Table 2**).

The analysis of NREM episode duration for the dark period revealed a significant effect for Treatment Day [$F_{(4, 64)} = 6.610$, $p < 0.011$] and a significant Group X Treatment Day [$F_{(4, 64)} = 2.569$, $p < 0.05$]. The duration of dark period NREM episodes were significantly reduced in the SIS mice than in the SES mice on the SAL and CRF treatment days (**Table 2**).

Active Waking and Quiet Waking

Total 20 h amounts of active waking did not significantly differ across groups or conditions (**Figure 3A**). By comparison, the analysis of quiet waking revealed a significant Treatment Day effect [$F_{(4, 64)} = 5.13$, $p < 0.01$]. Quiet waking was greater on the CRF day than on the HC or AST treatment days (**Figure 3B**). There also was significant effect for Quiet waking in the light period [$F_{(4, 64)} = 9.274$, $p < 0.001$]. Light period quiet waking was significantly greater during Base and on the SAL and CRF treatment days than on the HC or AST treatment days (**Table 2**).

Effects of CRF and AST on Stress-induced Alterations in Core Body Temperature

We examined changes in core body temperature hourly across the first 4 h of recording to assess potential differences in SIH produced by SES (**Figure 4A**) and SIS (**Figure 4B**) and the effects of CRF and AST. The ANOVAs revealed a main effect for Treatment Day for h 1 [$F_{(4, 64)} = 9.083$, $p < 0.001$]; h 2 [$F_{(4, 64)} = 11.721$, $p < 0.001$] and h 3 [$F_{(4, 64)} = 3.013$, $p < 0.03$]. In h 1, compared to Base, core body temperature was significantly increased for HC, SAL, CRF, and AST. In h 2, temperature in the HC treatment did not differ significantly from that in Base, but remained elevated in SAL, CRF, and AST. There also was a Group effect in h 2 [$F_{(1, 16)} = 4.505$, $p < 0.05$] and

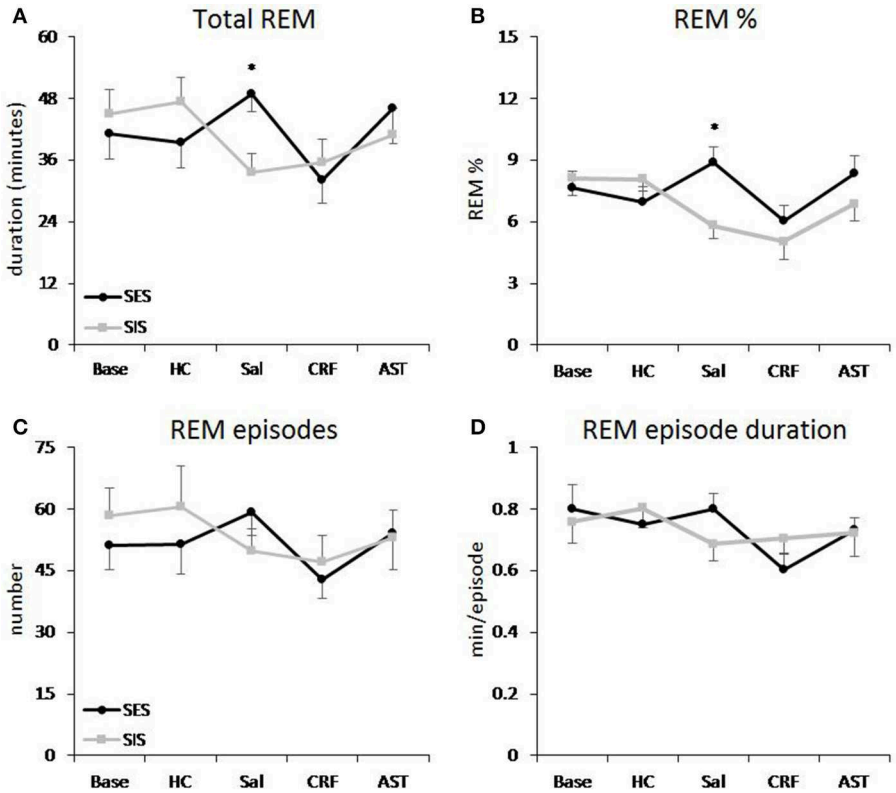

FIGURE 1 | REM sleep plotted as 20 h totals for baseline (Base), handling control (HC) and for days on which the mice received ICV injections of saline (SAL), corticotropin releasing factor (CRF) or astressin (AST) prior to signaled, escapable shock (SES) or to signaled, inescapable shock (SIS) training. (A) Total REM; **(B)** REM % (total REM/total sleep time × 100%); **(C)** REM episodes; **(D)** average REM episode duration. Comparisons of SES ($n = 9$) and SIS ($n = 9$): $^*p < 0.05$. Values are means ± SEM.

temperature was greater in the SES mice (36.83°C) than in the SIS mice (36.33°C). In h 3, temperature remained significantly higher in the CRF and AST treatment condition compared to Base whereas the difference for SAL did not reach significance ($p = 0.07$). There were no significant differences between SES and SIS, and by h 4, temperature had returned to Base levels in all conditions.

Discussion

The present results support previous studies (Sanford et al., 2010; Yang et al., 2011a,b) demonstrating that signaled and non-signaled controllable and uncontrollable stress are followed by directionally different alterations in REM in the post-stress period even though the acute stress response (as indicated by SIH) is similar. The results also indicate that centrally acting CRF is a significant factor in the reduction in REM that follows uncontrollable stress. This conclusion is supported by findings that differences in REM between SES and SIS trained mice are reduced by ICV administration of CRF and AST, with CRF resulting in reduced REM in both groups and with AST attenuating the reduction in REM in the SIS group. SIS, but not SES, also produced significant changes in NREM architecture, but not total amount, which were ameliorated by antagonizing CRF. The increases in the number of NREM episodes and decreases in NREM episode duration after SIS suggests more fragmented sleep even though the amount of sleep did not change. Thus, our results indicate a strong role for CRF in mediating stress-induced reductions in REM and a likely role for CRF in disrupting sleep continuity after certain types of stressors. Despite significant differences in REM, SIH was similar for SIS and SES treatments with and without co-administration of CRF or AST. These data suggest that central CRF may play a minimal role in modulating stress-induced alterations in temperature in this model or that sleep may be more sensitive to perturbations of the central CRF system than are peripheral indices of the stress response.

Stressor Controllability and Predictability and Sleep

Whether an animal perceives stressors as controllable and/or predictable appears to be an important factor in the effects of stress (Abbott et al., 1984; Adell et al., 1988). Both controllability and predictability can significantly influence post-stress sleep, and there appears to be the potential for interactions between the type of stress-related information available to the animal, its processing of that information, and the subsequent effects on sleep. In previous work, we demonstrated that auditory cues

TABLE 1 | Selected REM sleep parameters for the 8 h light period and 12 h dark period during baseline (Base), handling control (HC), and for days on which the mice received ICV injections of saline (Sal), corticotropin releasing factor (CRF) or astressin (AST) prior to training with signaled, escapable shock (SES) or signaled, inescapable shock (SIS).

		Base	HC	Sal	CRF	AST
LIGHT PERIOD						
REM total	SES	13.4 ± 2.4	15.5 ± 2.5	12.6 ± 2.0	5.7 ± 1.6$^{+\#\wedge}$	15.2 ± 4.9
	SIS	16.4 ± 2.6	16.2 ± 2.1	9.1 ± 1.7	9.0 ± 2.6$^{+\#\wedge}$	11.7 ± 2.4
REM %	SES	6.1 ± 0.8	5.7 ± 0.7	5.6 ± 0.8	2.9 ± 0.7$^{+\#¤\wedge}$	5.7 ± 1.4
	SIS	6.6 ± 0.9	6.11 ± 0.6	3.5 ± 0.6	2.6 ± 0.5$^{+\#¤\wedge}$	4.3 ± 0.8
REM episodes	SES	11.5 ± 2.9	10.5 ± 1.4	9.8 ± 1.5	4.9 ± 1.1$^{\#}$	10.6 ± 2.8
	SIS	12.5 ± 2.1	11.9 ± 2.1	7.8 ± 1.3	6.8 ± 1.5$^{\#}$	9.8 ± 2.2
REM duration	SES	0.7 ± 0.07	0.72 ± 0.07	0.65 ± 0.07	0.46 ± 0.10$^{+\#}$	0.59 ± 0.06
	SIS	0.67 ± 0.05	0.77 ± 0.07	0.58 ± 0.05	0.58 ± 0.06$^{+\#}$	0.64 ± 0.13
DARK PERIOD						
REM total	SES	27.8 ± 4.8	24.0 ± 3.6	36.4 ± 3.1*	26.4 ± 4.0	30.9 ± 3.9
	SIS	28.6 ± 3.4	31.2 ± 3.3	24.6 ± 2.0	26.5 ± 2.9	29.3 ± 4.0
REM %	SES	8.3 ± 1.1	8.0 ± 1.0	11.1 ± 0.9*	7.6 ± 0.9	10.1 ± 0.9
	SIS	9.5 ± 0.9	9.6 ± 0.8	7.7 ± 0.8	6.6 ± 1.4	8.9 ± 1.2
REM episodes	SES	15.3 ± 3.2	10.1 ± 1.8	13.2 ± 1.2	11.0 ± 1.6	11.0 ± 1.6
	SIS	11.1 ± 1.1	12.3 ± 2.0	11.4 ± 1.2	11.1 ± 1.5	11.2 ± 1.2
REM duration	SES	0.87 ± 0.1	0.78 ± 0.06	0.90 ± 0.06	0.70 ± 0.04	0.83 ± 0.06
	SIS	0.82 ± 0.08	0.83 ± 0.07	0.76 ± 0.07	0.79 ± 0.05	0.78 ± 0.06

*Comparisons between SES (n = 9) and SIS (n = 9): *p < 0.05; Comparisons to Base: $^{+}p < 0.05$; Comparisons to HC: $^{\#}p < 0.05$; Comparisons to Sal: ¤p < 0.05; Comparisons to AST: ^p < 0.05. Values are means ± SEM. Total REM and REM Duration are reported in minutes; REM% was calculated as total REM/total sleep time × 100%, and REM Episodes are reported as number of episodes.*

that predict shock can modify its effects on sleep and, contrary to contexts associated with non-signaled escapable footshock, do not produce conditioned increases in REM (Yang et al., 2011a). Learning the appropriate escape response (control) is also necessary for increases in REM (Machida et al., 2013).

The state of the animal at the time of stress also appears to be important. The brief manual restraint necessary for conducting microinjections is itself a stressor that can impact the animals and significantly alter subsequent sleep (Tang et al., 2007). It can also influence the effects of other stressors on sleep (Tang et al., 2007). For example, in rats, 5 min of manual restraint administered prior to 20 inescapable footshocks prevented the decrease in light period REM normally observed when inescapable footshock is presented without prior restraint. In a previous study reporting on the effects of SIS and SES without prior microinjections, we found an increase in NREM after shock training in SIS mice compared to SES mice (Yang et al., 2011a) that we did not see in the present study. This difference may have been due to the handling needed to complete the microinjections, as all other conditions were virtually the same across studies. Thus, our results should be interpreted in the context of the effects of CRF and AST on two interacting stressors, brief restraint and SES or SIS, as well as the complex emotions and memories and potential learning associated with the two conditions.

CRF and the Regulation of Sleep and Arousal

Several lines of existing data indicate that CRF is a significant regulator of stress-induced alterations in sleep and arousal; however, its actual role remains to be clarified. Studies administering antagonists prior to or during the presentation of stressors (González and Valatx, 1997) or sleep deprivation (González and Valatx, 1998; Kimura et al., 2010) have led to the conclusion that CRF can promote REM. Interestingly, there also is evidence that systemic administration of some CRF antagonists can inhibit spontaneous REM in adult (Ahnaou et al., 2012) and neonatal (Liu et al., 2010) rats, which can be considered supportive of this conclusion. However, studies that administered CRF prior to stress (Yang et al., 2009, 2011b) or during the recovery period after sleep deprivation (Schüssler et al., 2006) indicate that CRF can be inhibitory to REM. Under non-stress conditions, we also found that ICV administration of AST decreased wakefulness and increased REM in stress-responsive BALB/cJ mice, but not in less-responsive C57BL/6J mice, whereas CRF decreased REM in both strains (Sanford et al., 2008). Both of these studies can be considered consistent with the hypothesis that CRF has a suppressing effect on REM.

Resolving the discrepancies regarding the role of CRF in mediating the effects of stress on REM will likely require considering the relationship between the time course of the CRF stress response and that of stress-induced alterations in sleep and arousal. Many indices of stress including corticosterone (Veening et al., 2004; van Bogaert et al., 2006), SIH (Veening et al., 2004; van Bogaert et al., 2006), and activation of the hypothalamic paraventricular nucleus and other stress reactive regions in the brain (Grahn et al., 1999; Coco and Weiss, 2005; Liu et al., 2009) return to pre-stress levels relatively shortly after termination of an acute stressor. By comparison, alterations in sleep can occur several hours into the post-stress period. Studies administering

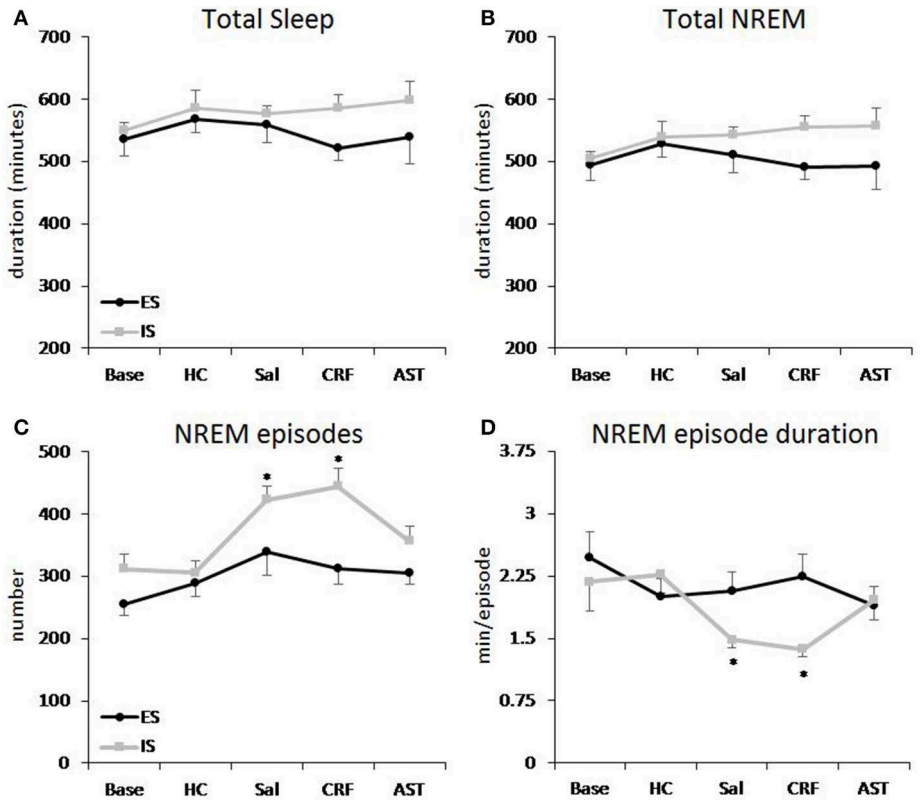

FIGURE 2 | Total sleep and amounts of NREM sleep plotted as 20 h totals for baseline (Base), handling control (HC) and for days on which the mice received ICV injections of saline (SAL), corticotropin releasing factor (CRF) or astressin (AST) prior to signaled, escapable shock (SES) or to signaled, inescapable shock (SIS) training. (A) Total sleep; **(B)** Total NREM sleep; **(C)** NREM episodes; **(D)** average NREM episode duration. Comparisons of SES ($n = 9$) and SIS ($n = 9$): $^*p < 0.05$. Values are means ± SEM.

an antagonist prior to presenting the stressor simply may have prevented or attenuated the initial CRF response which then altered the subsequent post-stress increase in REM that is seen with many stressors. Whether CRF is acting centrally or peripherally may also have consequences for its potential effects on REM in stress or non-stress conditions. This is suggested by findings that elevations of corticosterone after CRF is given ICV appear to be able to reduce REM even in CRF receptor 1 knockout mice (Romanowski et al., 2010).

The complexity of trying to assess the role of CRF in mediating sleep is also exemplified in work in humans. Four intravenous injections of human CRF at hourly intervals increased cortisol and reduced both REM and NREM in young healthy male volunteers (Holsboer et al., 1988). By comparison, hourly intravenous administration of cortisol decreased REM and increased NREM and plasma cortisol concentrations (Born et al., 1989; Friess et al., 1994). Together, these studies suggest that the decrease in REM after systemic CRF is mediated by cortisol, whereas the decrease in NREM may be a direct effect of CRF. CRF also appears to contribute to disinhibition of REM sleep and impaired NREM in depression, which is thought to be a stress related disorder. For example, administration of a CRF antagonist to patients with depression decreased REM density and awakenings and increased NREM (Held et al., 2004). Thus, the effects of CRF also may vary with route of administration, potentially with species, and across situations with altered neural functioning.

CRF and Stress-induced Hyperthermia

Stress-induced increases in core body temperature can begin within 10 s of the onset of stress induction (Clement et al., 1989; Krarup et al., 1999) and can be as much as 2°C in rats and mice for a variety of stressors including handling stress, exposure to novel environments, and restraint (Briese and De Quijada, 1970; Singer et al., 1986; Clement et al., 1989; Zethof et al., 1994). Anxiolytic drugs can decrease SIH (Vinckers et al., 2009). By comparison, some anxiogenic drugs have minimal effects on SIH possibly due to a maximum limit to the amount temperature may increase (Vinckers et al., 2009) whereas others may reduce or prevent SIH responses (Houtepen et al., 2011).

All of the stress conditions that we examined produced increases in average body temperature relative to time matched Base though there were differences in the time course of the response. Notably, the HC condition, putatively a milder stressor, returned to Base levels more rapidly whereas the conditions involving foot shock (both SES and SIS) had more persisting increases, regardless of whether the mice had received SAL, AST, or CRF.

TABLE 2 | Total sleep, NREM and wake parameters for the 8 h light period and 12 h dark period during baseline (Base), handling control (HC), and for days on which the mice received ICV injections of saline (Sal), corticotropin releasing factor (CRF) or astressin (AST) prior to training with signaled, escapable shock (SES) or signaled, inescapable shock (SIS).

		Base	HC	Sal	CRF	AST
LIGHT PERIOD						
Total sleep	SES	211.4 ± 19.7	269.3 ± 21.0	231.5 ± 19.2	182.8 ± 14.2#^	239.7 ± 24.1
	SIS	250.8 ± 20.6	259.9 ± 16.6	254.3 ± 10.9	241.1 ± 7.6#^	270 ± 16.9
NREM total	SES	198 ± 18.0	253.8 ± 19.7	218.2 ± 18.7	177.1 ± 13.1#	224.6 ± 20.8
	SIS	234.4 ± 19.3	243.7 ± 15.0	245.2 ± 10.0	234.9 ± 7.5#	258.3 ± 16.3
NREM episodes	SES	116.9 ± 16.3	1.22 ± 11.9	159.4 ± 14.8*	133.6 ± 18.4*	140.6 ± 11.2
	SIS	150.9 ± 13.8	143.6 ± 10.8	192.9 ± 13.4	198.7 ± 13.0	168.7 ± 12.8
NREM duration	SES	1.70 ± 0.18	1.86 ± 0.22	1.26 ± 0.15#	1.44 ± 0.24#	1.56 ± 0.22
	SIS	1.49 ± 0.19	1.53 ± 0.16	1.10 ± 0.06#	1.12 ± 0.10#	1.36 ± 0.12
Active wake	SES	132.8 ± 20.0	96.3 ± 17.0	111.6 ± 18.9	134.7 ± 21.2	130.5 ± 22.0
	SIS	89.1 ± 11.7	99.9 ± 11.7	85.8 ± 11.2	91.2 ± 6.5	89.9 ± 14.1
Quiet wake	SES	135.8 ± 9.7#^	114.5 ± 9.5	136.9 ± 12.8#^	162.6 ± 13.2#^	109.7 ± 7.1
	SIS	140.1 ± 14.6#^	120.3 ± 8.3	139.9 ± 4.7#^	147.7 ± 6.4#^	120.1 ± 7.9
DARK PERIOD						
Total sleep	SES	323.8 ± 26.9	298.7 ± 20.5	328.6 ± 18.0	339.6 ± 26.0	299.6 ± 25.4
	SIS	299.8 ± 21.2	326.2 ± 15.9	323 ± 9.5	344.9 ± 21.2	328.3 ± 16.6
NREM total	SES	296 ± 23.7	274.7 ± 18.7	292.2 ± 17.0	313.2 ± 23.8	268.7 ± 22.4
	SIS	271.2 ± 19.0	295 ± 14.8	298.5 ± 10.5	320.7 ± 17.4	298.9 ± 15.1
NREM episodes	SES	139.1 ± 9.9	166.8 ± 15.4	179.5 ± 24.4*	179.3 ± 12.8*	164.9 ± 18.2
	SIS	161.3 ± 15.4	162.3 ± 17.3	230.3 ± 12.2	245.8 ± 19.4	188.3 ± 15.0
NREM duration	SES	1.97 ± 0.31	1.23 ± 0.06	1.36 ± 0.16	1.36 ± 0.23	1.02 ± 0.18
	SIS	1.75 ± 0.22	1.33 ± 0.11	1.12 ± 0.13	1.23 ± 0.12	1.14 ± 0.22
Active wake	SES	197.8 ± 13.1	209.1 ± 25.9	203.2 ± 28.1	168.8 ± 20.1	233.2 ± 32.7
	SIS	212.3 ± 29.4	195.7 ± 16.9	182.3 ± 11.2	156.7 ± 13.8	189.1 ± 18.9
Quiet wake	SES	198.4 ± 17.3	212.2 ± 14.1	188.2 ± 13.7	211.6 ± 19.2	187.2 ± 17.9
	SIS	207.9 ± 16.7	198.1 ± 11.0	214.6 ± 7.8	218.4 ± 13.1	202.7 ± 11.5

*Comparisons between SES (n = 9) and SIS (n = 9): *p < 0.05. Comparisons to Base: +p < 0.05. Comparisons to HC: #p < 0.05. Comparisons to AST: ^p < 0.01. Values are means ± SEM. Total Sleep, NREM Total, NREM Duration, Active Wake, and Quiet Wake are reported in minutes. NREM Episodes are reported as number of episodes.*

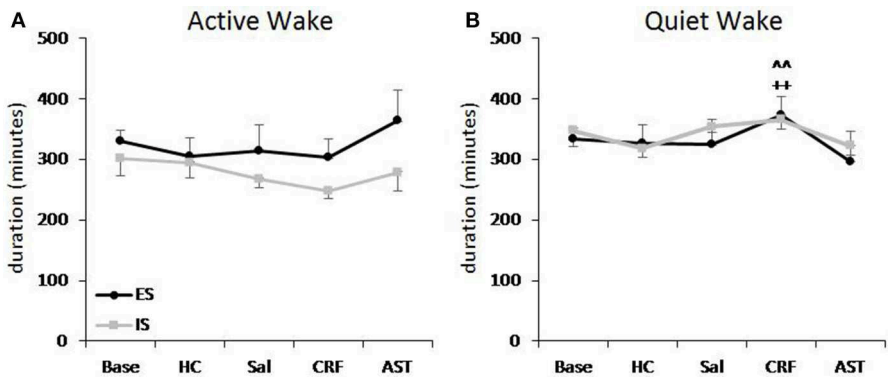

FIGURE 3 | Total amounts of active waking (A) and quiet waking (B) plotted as 20 h totals for baseline (Base), handling control (HC) and for days on which the mice received ICV injections of saline (SAL), corticotropin releasing factor (CRF), or astressin (AST) prior to signaled, escapable shock (SES, *n* = 9) or to signaled, inescapable shock (SIS, *n* = 9) training. Comparisons of CRF to HC: ++, $p < 0.01$. Comparisons of CRF to AST: ^^$p < 0.01$. Values are means ± SEM.

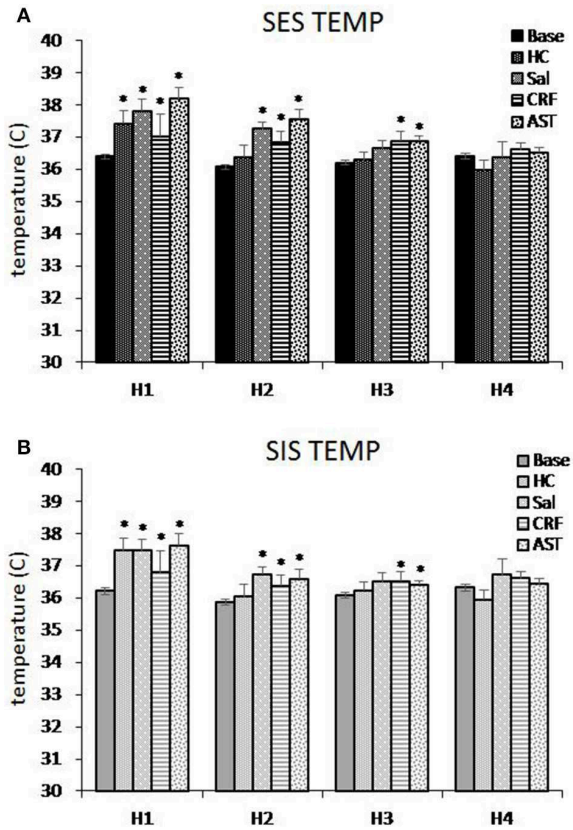

FIGURE 4 | Average core body temperature plotted hourly for the first 4 h of the 20 h recording period for mice trained with signaled, escapable shock (SES, A) or signaled, inescapable shock (SIS, B) training. Average temperature is shown for baseline (Base), handling control (HC), and for ICV microinjections of saline (SAL), corticotropin releasing factor (CRF), and astressin (AST) prior to SES ($n = 9$) or SIS ($n = 9$). Comparisons to Base: $^*p < 0.05$. Values are means ± SEM.

As we reported before for training with ES alone (Yang et al., 2011b), AST did not produce a significant attenuation of SIH for either SES or SIS trained mice. However, other studies have reported that CRF antagonists attenuate SIH. The CRF receptor 1 antagonists antalarmin and SSR125543A [4-(2-chloro-4-methoxy-5-methylphenyl]-*N*-[(1*S*)-2-cyclopropyl-1-(3-fluoro-4-methylphenyl)ethyl]5-methyl-*N*-(2-propynyl)-1,3-thiazol-2-aminehydrochloride) administered orally or intraperitoneally reduce SIH in rats subjected to isolation stress (Griebel et al., 2002). ICV administration of the broad CRF antagonist αHelCRF in rats also reduced body temperature after exposure to a cage change stressor (Morimoto et al., 1993; Nakamori et al., 1993). Interestingly, central administration of CRF in rats produces an increase in body temperature (Heinrichs et al., 2001; Figueiredo et al., 2010) that is attenuated by AST and antalarmin (Figueiredo et al., 2010).

The reason for the differences compared to our results is not clear, but both isolation and cage change likely are less intense stressors than the footshock paradigm used in our studies. They also likely involve only psychological stress whereas footshock involves both psychological and strong physiological stress which would activate additional neural pathways. Furthermore, though *in vitro* assays indicate that AST is more potent for both CRF1 and CRF2 receptors than is αHelCRF (Hauger et al., 2006), *in vivo* studies in rats suggest that AST may be less potent in preventing some CRF- and stress-induced and anxiety-related behaviors including CRF-induced locomotor activity (Spina et al., 2000). It is also possible that a higher dosage of AST than we used (1.0 μg) may be effective in reducing SIH. For example, a 5.0 μg, but not a 1.0 μg, dosage of AST blocked the reduction in the number of entries into the open arms of an elevated plus maze produced by central administration of 0.5 μg CRF (Spina et al., 2000). Strain or species differences in the central CRF system could also be involved. BALB/cJ mice, the strain used in this study, have differences in their CRF system (Blank et al., 2003) and reactivity to CRF and CRF antagonists compared to C57BL/6J mice (Sanford et al., 2008), thereby suggesting differences in the regulation of sleep by CRF.

CRF and the Functional Significance of Post-stress Sleep

SIH responses have a time course that parallels that of hypothalamic-pituitary-adrenal (HPA) axis activation (Veening et al., 2004; van Bogaert et al., 2006) and both SIH and corticosterone (Shors et al., 1989) are similarly enhanced by ES and IS which are followed by distinctly different alterations in sleep. Fear, as indicated by behavioral freezing, also is very similar in response to contextual reminders of SES and SIS (Machida et al., 2013) or ES and IS alone (Yang et al., 2011a). This suggests that the mere presence of a peripheral stress response and fearful behavior do not dictate, or even predict, the types of alterations in sleep that occur after acute stress. Instead, post-stress sleep appears to be a function of the context in which the stressor was encountered and the emotional and learning processes that were engaged as the animal evaluated and reacted to the situation. In line with the effects of waking experiences on sleep, various lines of evidence suggest that, in turn, sleep plays a significant role in adaptive responding to stress. For instance, sleep disturbances both before (Bryant et al., 2010) and after (Lavie, 2001) significant or traumatic events have been linked to the development of stress-related pathology.

The increases in REM that occur following controllable stress (Sanford et al., 2010) and following fear extinction (Wellman et al., 2008), and the decreases in REM that can occur without recovery following uncontrollable stress (Liu et al., 2003; Sanford et al., 2003c) and associated with learned helplessness (Adrien et al., 1991) suggest that post-stress REM may play a role in adaptive responses to stress. This is also consistent with findings in posttraumatic stress disorder (PTSD) patients that found, in sleep recordings soon after trauma, a more fragmented pattern of REM characterized by shorter average duration REM episodes before shifting stage or awakening in PTSD patients compared to patients without PTSD and a non-traumatized comparison group

(Mellman et al., 2002, 2007). There were also a greater number of REM episodes in the PTSD patients than in patients that experienced trauma without developing PTSD. These differences led to the suggestion that intact REM aids in the processing of memory for trauma (Mellman et al., 2002, 2007). Similar hypotheses emphasizing a positive role for REM in mediating the effects of stress have been put forth including suggestions that REM plays an important role in consolidating memories for aversive events and in "decoupling" those memories from their emotional charge (Nishida et al., 2009; Walker, 2009) and that it serves to weaken unwanted memory traces in the cortex (Crick and Mitchison, 1983). Experimental paradigms employing controllable and uncontrollable stressors that produce clear distinctions in post-stress sleep should be useful for determining the role that sleep may have in orchestrating neurobiological changes that promote adaptive behaviors and the role that impaired sleep may play in stress-induced pathologies.

The current results suggest that centrally acting CRF is a significant regulator of post-stress sleep. It also has been linked to stress-related psychopathology. Elevated levels of CRF have been found in the cerebrospinal fluid (Bremner et al., 1997; Baker et al., 1999; Sautter et al., 2003) and plasma (de Kloet et al., 2007) of PTSD patients and elevated CRF coupled with either enhanced negative feedback or downregulated CRF receptors has been hypothesized to play a role in the reduced delta sleep found in patients with PTSD (Neylan et al., 2006). Stress also can be a significant factor in insomnia (Healey et al., 1981; Basta et al., 2007) which is associated with higher activity in the HPA axis and considered to be a disorder of hyperarousal in the central nervous system (Bonnet and Arand, 1997; Vgontzas et al., 2001; Basta et al., 2007). Increased CRF activity figures prominently in hypotheses regarding the pathogenesis of primary insomnia (Richardson and Roth, 2001). Thus, CRF may be a significant mediator of the stress and arousal/sleep systems and their interactions in regulating the outcomes of stressful experiences. This is also suggested by our recent findings that antagonizing CRF in the basolateral nucleus of the amygdala (BLA) prior to training with inescapable shock blocked the reductions in REM that normally occur (Wellman et al., 2013). It also blocked subsequent conditioned reductions in REM without reducing freezing, suggesting that fear memory had been altered, but not blocked. By comparison, inactivation of BLA with the GABAergic agonist, muscimol, blocked the reduction in REM, and reduced freezing (Wellman et al., 2014), suggesting that CRF was working in a region that mediates the memory linkage between fearful behaviors and sleep.

A Note on Experimental Design

We chose to complete the microinjections of CRF before we conducted studies with AST. This was based on the concern that antagonizing CRF early in the series might alter subsequent stress responses, or the impact of stress on sleep. For that reason, we conducted SAL controls for both CRF and AST to be able to determine whether there were potential carryover effects across the experiment. Based on these controls, the effects of SES and SIS were consistent across treatment days, with the exception of the relative effects of CRF and AST on stress-induced alterations in sleep.

Conclusion

Post-stress sleep appears to be determined by the types of information available in the stressful context, the animal's processing of that information, and the emotional responses that are induced. Post-stress sleep, in particular REM, also appears to play a role in mediating the positive or negative outcomes of stress, and behavioral experiences during wakefulness likely set in motion the underlying neurobiological processes that guide and enable sleep to mediate the effects of stress. The current results demonstrate that centrally acting CRF is a major regulator of post-stress alterations in sleep that may occur independently of the induction of the peripheral stress response. Work focused on understanding the role that central CRF has in regulating interactions between the stress and arousal/sleep systems should provide insight into the neural processes that are associated with adaptive and non-adaptive responding to stress.

Acknowledgments

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REVIEW
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Melanin-Concentrating Hormone (MCH): Role in REM Sleep and Depression

Pablo Torterolo[1], Cecilia Scorza[2], Patricia Lagos[1], Jessika Urbanavicius[2], Luciana Benedetto[1], Claudia Pascovich[1], Ximena López-Hill[2], Michael H. Chase[3] and Jaime M. Monti[4]*

[1] Department of Physiology, School of Medicine, Universidad de la República, Montevideo, Uruguay, [2] Department of Experimental Neuropharmacology, Instituto de Investigaciones Biológicas Clemente Estable, Montevideo, Uruguay, [3] WebSciences International and University of California, Los Angeles School of Medicine, Los Angeles, CA, USA, [4] Department of Pharmacology and Therapeutics, School of Medicine, Hospital de Clínicas, Universidad de la República, Montevideo, Uruguay

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***Correspondence:**
Pablo Torterolo
ptortero@fmed.edu.uy

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The melanin-concentrating hormone (MCH) is a peptidergic neuromodulator synthesized by neurons of the lateral sector of the posterior hypothalamus and zona incerta. MCHergic neurons project throughout the central nervous system, including areas such as the dorsal (DR) and median (MR) raphe nuclei, which are involved in the control of sleep and mood. Major Depression (MD) is a prevalent psychiatric disease diagnosed on the basis of symptomatic criteria such as sadness or melancholia, guilt, irritability, and anhedonia. A short REM sleep latency (i.e., the interval between sleep onset and the first REM sleep period), as well as an increase in the duration of REM sleep and the density of rapid-eye movements during this state, are considered important biological markers of depression. The fact that the greatest firing rate of MCHergic neurons occurs during REM sleep and that optogenetic stimulation of these neurons induces sleep, tends to indicate that MCH plays a critical role in the generation and maintenance of sleep, especially REM sleep. In addition, the acute microinjection of MCH into the DR promotes REM sleep, while immunoneutralization of this peptide within the DR decreases the time spent in this state. Moreover, microinjections of MCH into either the DR or MR promote a depressive-like behavior. In the DR, this effect is prevented by the systemic administration of antidepressant drugs (either fluoxetine or nortriptyline) and blocked by the intra-DR microinjection of a specific MCH receptor antagonist. Using electrophysiological and microdialysis techniques we demonstrated also that MCH decreases the activity of serotonergic DR neurons. Therefore, there are substantive experimental data suggesting that the MCHergic system plays a role in the control of REM sleep and, in addition, in the pathophysiology of depression. Consequently, in the present report, we summarize and evaluate the current data and hypotheses related to the role of MCH in REM sleep and MD.

Keywords: peptides, hypothalamus, serotonin, raphe, mood, paradoxical sleep, MCH

INTRODUCTION

The search for new pharmacological strategies to treat psychiatric disorders is a "hot topic" in neuroscience. There is also a large body of evidence suggesting that neuropeptides play a critical role in these pathologies (Kormos and Gaszner, 2013). In addition, neuropeptides are also involved in the control of wakefulness and sleep (Richter et al., 2014). A robust example of the results of translational neuroscience research was the discovery of the neuropeptides hypocretins 1 and 2 (also called orexins); the degeneration of hypocretins-containing neurons produces the sleep pathology called narcolepsy (Mignot, 2011). Hypocretins also play a role in mood disorders (Nollet and Leman, 2013).

Research on the neuropeptide melanin-concentrating hormone (MCH) is currently focused on the study of physiological and translational possibilities of the neuropeptide. In the present report, we review the role of the MCHergic system in the control of REM sleep and the pathophysiology of major depression (MD).

PATHOPHISIOLOGY OF DEPRESSION: ROLE OF THE SEROTONERGIC SYSTEM

The Diagnostic and Statistical Manual of Mental Disorders (DSM-5) lists several depressive disorders. The common features that characterize them are the presence of sadness, empty or irritable moods, which are accompanied by somatic and cognitive changes that significantly affect an individual's capacity to function. Major Depressive Disorder or MD is a distinctive condition that is diagnosed based on symptomatic criteria such as sadness or melancholia, guilt, irritability and anhedonia. It is accompanied by several symptoms including insomnia or hypersomnia, fatigue, alterations in body weight, thought and concentration impairments as well as recurrent suicidal thoughts (Fava and Kendler, 2000; American-Psychiatric-Association, 2013). MD is one of the most common psychiatric diseases with a prevalence of 5–12% in men and 10–25% in women, and it is considered to be a principal cause of disability, outnumbered only by cardiovascular diseases (Boyd and Weissman, 1981; Murray and Lopez, 1996, 1997a,b). In addition, a great deal of attention has been paid to MD due to its association with suicide; 15–20% of depressive patients end their lives by suicide (Miret et al., 2013). Affective disorders are also particularly disabling and among the most important contributors to the total burden of disease (Miret et al., 2013).

Several mechanisms have been involved in the neurobiology of depression, ranging from synaptic plasticity to epigenetic, and from postnatal neurogenesis to immunological processes (recently reviewed by Palazidou, 2012; Saveanu and Nemeroff, 2012; Palagini et al., 2013; Willner et al., 2013; Ménard et al., 2015). In the present report, we review the role of MCH in depression with an emphasis on its interaction with the serotonergic system. However, it is important to note that in addition to serotonin, other neurotransmitters such as dopamine, noradrenaline, and glutamate have been involved in the pathophysiology of depression; in fact ketamine, a N-methyl-d-aspartate (NMDA) glutamate receptor antagonist can alleviate depressive symptoms in patients within hours of administration (Saveanu and Nemeroff, 2012; Dutta et al., 2015).

The serotonergic system comprises one of the most widely distributed neurochemical systems in the central nervous system (CNS). The majority of the somata of serotonergic neurons are located within the dorsal raphe nucleus (DR); another important group of serotonergic neurons is located in the median raphe nucleus (MR). Due to its projections throughout the CNS, serotonergic neurons are capable of playing an important role in the regulation of emotional states and in several functions including motor activity and the control of sleep and wakefulness (Monti, 2010a,b; Olivier, 2015).

Numerous studies have shown that the serotonergic system is involved in the pathophysiology of MD. Low levels of serotonin and/or its principal metabolite (5- hydroxy-indol-acetic acid) have been found in the urine and cerebrospinal fluid (CSF) of MD patients (Praag, 1977; van Praag and de Haan, 1979; Young, 1993). In addition, the number of attempted suicides by MD patients and its lethality are correlated with a reduced CSF concentrations of serotonin (Roy et al., 1989; Träskman-Bendz et al., 1992; Nordström et al., 1994; Mann et al., 2001; Kalia, 2005; Berton and Nestler, 2006). Furthermore, abnormalities in serotonergic receptors, serotonin reuptake proteins and other alterations in serotonergic neurotransmission have been related to the susceptibility to commit suicide (Arango et al., 1995; Mann, 1998; Courtet et al., 2005; Hamet and Tremblay, 2005; Bondy et al., 2006). Depressive-suicide patients show significant differences in serotonergic markers in the DR compared to control individuals. Therefore, it has been suggested that disruption of the functioning of serotonergic neurons in the DR underlies MD (Underwood et al., 1999; Arango et al., 2001, 2002; Boldrini et al., 2005; Bach-Mizrachi et al., 2006).

The serotonergic system is also involved in the mechanisms of action of antidepressant drugs. The selective serotonin reuptake inhibitors (SSRI) such as fluoxetine or escitalopram, produce its therapeutic action upon the enhancement of central serotonergic neurotransmission (Keller et al., 1992; Holtzheimer and Nemeroff, 2006). A problem related with antidepressant drugs treatment is the delayed onset of the therapeutic effects, despite the fact that these drugs cause an immediate increase in extracellular levels of monoamines. This fact suggests that the acute biochemical effect does not directly determine the therapeutic effect, which is likely produced by slower neurobiological modulations, such as the desensitization of serotonergic receptors, the modulation of intracellular pathways, gene expression of growth factors such as brain-derived neurotrophic factor (BDNF) and the regulation of postnatal neurogenesis (Palazidou, 2012; Saveanu and Nemeroff, 2012; Willner et al., 2013). In spite of the previous statements, it is important to take into account that a meta-analyses study showed that the placebo effect for antidepressant treatment is exceptionally large, and antidepressant medications have reported only modest benefits over placebo treatment (Kirsch et al., 2008; Kirsch, 2014).

REM SLEEP

Sleep remains one of the great neurobiological mysteries. Humans spend one third of their life sleeping, without awareness of the outside world. However, during dreams, there is bizarre cognitive activity that is disconnected from reality and is ruled by internal stimuli (Pace-Schott, 2005).

In mammals, sleep is comprised of two different behavioral states: slow wave sleep, also called non-REM (NREM) sleep and REM (rapid eyes movements) sleep (Carskadon and Dement, 2005; Brown et al., 2012). Polysomnography is the basic tool to study behavioral states; it consists in the simultaneous recording of the electroencephalogram (EEG), electromyogram (EMG), and eye movements (electrooculogram). During wakefulness, there is an optimal interaction with the environment that enables to carry out different behaviors that optimize survival. An EEG consisting of high frequency rhythms and low amplitude waves characterizes wakefulness. In normal adults, NREM sleep occurs at sleep onset. During this period, there is a marked decrease in the interaction with the environment, the adoption of a suitable position to conserve heat, an increase in the threshold of reaction to external stimuli and a decrease in muscle activity and tone. During NREM sleep, the EEG exhibits low frequency (0.5–4 Hz), high amplitude waves and "sleep spindles." NREM sleep is accompanied by a tonic increase in parasympathetic activity, which results in a decrease in visceral activity (Parmeggiani, 1994). In the deepest stage of NREM sleep, cognitive processes (dreams) are absent or minimal (Pace-Schott, 2005).

During a typical night's sleep in a young adult, REM sleep, which occurs with an ultradian rhythm of approximately 90 min, is always preceded by NREM sleep. During REM sleep the EEG is similar to wakefulness (consequently, this state is also called paradoxical sleep). REM sleep is also characterized by the absence of muscle activity (muscle atonia), rapid eyes-movements, ponto-geniculo-occipital (PGO) waves, theta waves in the hippocampus electrogram, and phasic changes in autonomic activity (Carskadon and Dement, 2005; Siegel, 2011). The arousal threshold for sound stimulation in humans during tonic REM sleep is similar than NREM sleep stage 2, and increases during phasic REM sleep (when rapid eyes movements are present) to the same level as NREM sleep stage 4 (Ermis et al., 2010). Dreams are present mostly during REM sleep (Pace-Schott, 2005).

It is well established that the activating system, which is a neuronal network centered in the mesopontine reticular formation, the postero-lateral hypothalamus and basal forebrain, is critical for generating and maintaining wakefulness (Torterolo and Vanini, 2010). The activating system comprises various neurochemical groups of neurons including glutamatergic, serotonergic, dopaminergic, noradrenergic, histaminergic, cholinergic and hypocretinergic (Torterolo and Vanini, 2010; Monti, 2013). On the other hand, the preoptic area is essential for the generation of NREM sleep, while the thalamus is responsible for the generation of slow waves and sleep spindles (Steriade et al., 1993; Torterolo et al., 2009a; Torterolo and Vanini, 2010; Benedetto et al., 2012).

The neuronal network that is "necessary and sufficient" for the generation of REM sleep is located in the mesopontine reticular formation (Siegel, 2011). Within this region, cholinergic neurons of the latero-dorsal and pedunculo-pontine tegmental nucleus (LDT-PPT) as well as glutamatergic neurons of the nucleus pontis oralis (NPO, that is considered the executive area for REM sleep generation) are active during REM sleep (REM "on," or wake and REM "on" neurons), whereas noradrenergic neurons of the locus coeruleus (LC) as well as serotonergic neurons of the DR and MR suppress their firing (REM "off" neurons; Monti, 2010b; Siegel, 2011; Brown et al., 2012; Chase, 2013a; Boucetta et al., 2014). Neurons of the ventrolateral periaqueductal gray may also play a role in REM sleep generation (Vanini et al., 2007). Mutual interactive models that include REM "on" and REM "off" neurons have been presented in order to explain the generation of REM sleep (Lu et al., 2006; Luppi et al., 2007; Brown et al., 2012). The mesopontine REM sleep-generating neuronal network is strongly modulated by forebrain sites. This region receives important MCHergic and hypocretinergic projections from the hypothalamus (Torterolo et al., 2009b, 2013).

DEPRESSION AND REM SLEEP

A decrease in the latency to the first episode of REM sleep is a trait of MD, and is considered to be one of the most robust and specific biological markers of this condition (Adrien, 2002; Palagini et al., 2013). Furthermore, MD patients have an increase in the total time spent in REM sleep, in the length of the first episode of REM sleep and in the density of rapid eye movements during this state. The fact that most antidepressant drugs decrease or eliminate REM sleep and that selective REM sleep or total sleep deprivation are effective in the treatment of MD, highlights the relationship between MD and REM sleep (Adrien, 2002; Benca, 2005; Palagini et al., 2013). In relation with preceding concepts, serotonergic neuronal activity in the DR is reduced in animal models of depression, while the firing rate of such DR neurons is exacerbated during total sleep deprivation (which appears to be correlated with its antidepressant effect; Yavari et al., 1993; Gardner et al., 1997).

One of the most widely used preclinical paradigm for assessing antidepressant activity is the forced swimming test (FST) (Porsolt et al., 1977; Porsolt, 2000). When rodents are placed in an inescapable container of water, they swim or climb more following the systemic administration of antidepressant. Thus, these agents prevent an immobility state called "behavioral despair." Passive (immobility) or active (swimming and/or climbing) responses should be independent from alterations in locomotive activity that the drug may also induce. Strikingly, 24-h of sleep deprivation in rats results in a decrease in the immobility time, which is consistent with an antidepressant effect (Lopez-Rodriguez et al., 2004). Sleep deprivation also enhances the effect of antidepressant drugs treatment (van Luijtelaar and Coenen, 1985). The antidepressant effect of sleep deprivation has been linked to an increase in serotonergic activity (Lopez-Rodriguez et al., 2004).

In conclusion, it has been proposed that patients with MD have an increased "pressure" to generate REM sleep; in other

words, the generation of REM sleep is abnormally promoted or facilitated in these patients. The relationship between REM sleep and MD depends, at least in part, on the activity of serotonergic neurons of the DR. These neurons are involved both in the generation of REM sleep and in the pathophysiology of MD (Adrien, 2002; Palagini et al., 2013).

ROLE OF THE SEROTONERGIC SYSTEM IN REM SLEEP

Serotonergic neurons of the DR have a slow and regular firing during wakefulness, there is a decrease in their activity during NREM sleep, and an almost complete inactivation during REM sleep ("REM-off" neurons; Monti, 2010b). A decrease in the release of serotonin in brain areas during REM sleep correlates with the electrophysiological data of DR neuronal activity (Portas and McCarley, 1994). GABAergic neurons of the DR are involved in the inhibition of serotonergic neurons during REM sleep, and the consequent promotion of this behavioral state (Nitz and Siegel, 1997; Torterolo et al., 2000; Monti, 2010a). On the other hand, experimental activation of DR serotonergic neurons prevents the generation of REM sleep (Monti, 2010a). Hence, the generation of REM sleep depends on the inactivation of the serotonergic neurons; these neurons are considered "permissive" for the generation of this behavioral state.

THE MELANIN-CONCENTRATING HORMONE

MCH is a 19-aminoacids cyclic peptide, which was initially characterized as a circulating factor that mediated color changes in teleost fishes (Torterolo et al., 2011; Macneil, 2013; Monti et al., 2013). MCH was subsequently identified as a neuromodulator in mammals, including humans (ibid.). MCH is synthesized in neurons whose somata are located in the lateral sector of the posterior hypothalamus, dorsomedial hypothalamus and zona incerta; the location of the MCHergic neurons is shown in a coronal section of the hypothalamus of the cat in **Figure 1**. These neurons project to different regions of the CNS (Bittencourt et al., 1992; Torterolo et al., 2006, 2009b). A small number of MCHergic neurons have been also identified in the olfactory tubercle and the pontine reticular formation (Bittencourt et al., 1992). In addition, MCHergic neurons are present in the medial preoptic area of lactating rats and in the latero-dorsal tegmental nucleus of female rats (Rondini et al., 2007, 2010). MCH is also present in the gastrointestinal tract and pancreas (Pissios et al., 2007; Kokkotou et al., 2008).

The biological functions of MCH are mediated by two G-protein coupled receptors known as MCHR-1 and MCHR-2. Of note, the MCHR-2 gene is a pseudo-gene in rodents but it is functional in carnivores and primates including humans. It has been determined that MCHR-1 activates Gi and Gq proteins and inhibits Ca^{2+} currents. MCH has mainly an inhibitory role, both at the presynaptic level where it decreases the release of GABA and glutamate, and at the post-synaptic level (Gao, 2009; Macneil, 2013).

MCH Regulates the Energy Homeostasis

The MCHergic system was traditionally related to the control of energy homeostasis; i.e., feeding and metabolic activity (reviewed by Macneil, 2013). In this regard, chronic infusion of a synthetic MCHR-1 receptor agonist induces obesity in mice, which is accompanied by hyperphagia, a reduction in body temperature and stimulation of lipogenic activity in the liver as well as white adipose tissue (ibid.). At the same time, genetically-modified animals with over-expression of MCH are obese, whereas animals lacking MCH are hypophagic and lean. These data suggest that by increasing food intake and promoting anabolism, MCH promotes the conservation of body energy.

The MCHergic System Promotes Sleep

Conservation of energy is one of the main functions of sleep (Siegel, 2005). Since MCHergic neurons are critical in the control of energy homeostasis, it is expected to be involved in sleep regulation. MCH, via regulation of the activating and somnogenic systems, promotes sleep, especially REM sleep (reviewed by Torterolo et al., 2011; Monti et al., 2013; Konadhode et al., 2015).

The arguments that support MCH as a sleep promoter are described below. The main experimental results are summarized in **Table 1**.

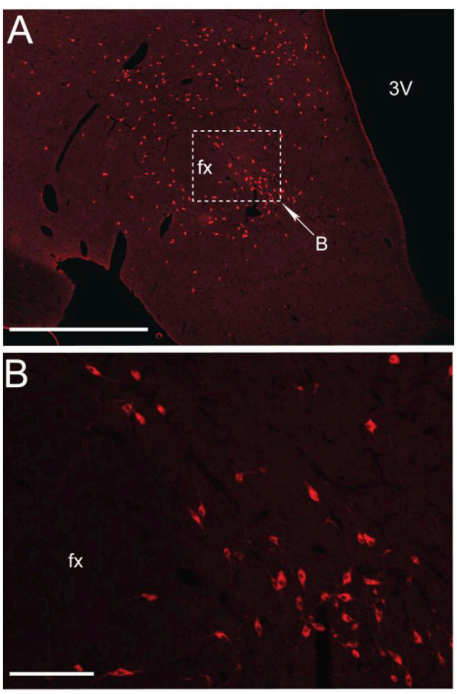

FIGURE 1 | MCHergic neurons are located in the hypothalamus. (A) Low magnification photomicrographs that exhibit MCHergic neurons at the tuberal level of the hypothalamus of the cat. **(B)** The inset in **(A)** is shown at higher magnification. This photomicrograph shows MCHergic neurons of the perifornical region. The photomicrographs were taken from 20 μm -thick sections that were processed for immunofluorescence. Fx, fornix; 3 V, third ventricle. Calibration bars: **(A)** 1 mm; **(B)** 100 μm. Original microphotographs taken from the data set of Torterolo et al. (2006).

TABLE 1 | MCH and sleep.

Site	Main effect	References
MCH MICROINJECTION		
Intracerebroventricular (rat)	Increases REM sleep. Moderate increase in NREM sleep	Verret et al., 2003
Dorsal raphe (rat)	Increases REM sleep. Moderate increase in NREM sleep	Lagos et al., 2009
Locus coeruleus (rat)	Increases REM sleep	Monti et al., 2015
Nucleus pontis oralis (cat)	Increases REM sleep	Torterolo et al., 2009b
Basal forebrain (rat)	Decreases wakefulness. Increases REM sleep in the first 2-h of the recordings	Lagos et al., 2012
Ventro-lateral preoptic nucleus (VLPO) (rat)	Increases NREM sleep	Benedetto et al., 2013
MCHR-1 ANTAGONIST MICROINJECTION		
Systemic (rat)	Decreases REM and NREM sleep. Increases wakefulness	Ahnaou et al., 2008
Type	**Main effect**	**References**
KNOCK-OUT ANIMALS		
Prepro-MCH (mice)	Sleep less in basal condition. Decreases REM sleep during fasting more than wild-type controls	Willie et al., 2008
MCHR-1 (mice)	Hypersomniac phenotype both in basal conditions and after total sleep deprivation	Adamantidis et al., 2008
MCHR-1 (mice)	Increases wakefulness and reduces NREM sleep. Restraint stress reduced both NREM and REM sleep more than wild-type controls	Ahnaou et al., 2011
Optogenetic	**Main effect**	**References**
STRATEGY		
Stimulation of MCHergic neurons (mice)	Increases NREM and REM sleep	Konadhode et al., 2013
Stimulation of MCHergic neurons at the onset of REM sleep (mice)	Increases REM sleep duration	Jego et al., 2013
Stimulation of MCHergic neurons (mice)	Induces transitions from NREM to REM sleep and increases REM sleep time	Tsunematsu et al., 2014
Inhibition of MCHergic neurons at the onset of REM sleep (mice)	Reduces the frequency and amplitude of hippocampal theta rhythm	Jego et al., 2013
Inhibition of MCHergic neurons (mice)	No effect	Tsunematsu et al., 2014
Electrophysiology	**Main effect**	**Reference**
***IN VIVO* RECORDINGS**		
Identified MCHergic neurons (rat)	Firing rate: REM >NREM sleep>W	Hassani et al., 2009
***In vivo* Microdialysis**	**Main effect**	**Reference**
SITE		
Amygdala (human)	MCH release increases during NREM sleep onset	Blouin et al., 2013

MCHergic Neurons Project to the Activating and Limbic Systems

Classical studies have linked the postero-lateral hypothalamus, where MCHergic neurons are located, with the control of sleep and wakefulness (Torterolo and Vanini, 2010). These neurons have a close anatomical relationship with hypocretin-containing neurons, whose somata are also located within the postero-lateral hypothalamus and project to comparable brain sites (Torterolo et al., 2006, 2009b, 2013; Torterolo and Chase, 2014). Hypocretinergic neurons are considered part of the activating system, and are essential for the maintenance of wakefulness (Torterolo and Vanini, 2003; Chase, 2013b; Torterolo and Chase, 2014). Hence, it is likely that MCHergic and hypocretinergic neurons interact, in a complementary mode, in order to regulate wakefulness and sleep (Torterolo and Chase, 2014).

MCHergic neurons send dense projections to activating and somnogenic regions (Monti et al., 2013). Using retrograde tracers, we have characterized the MCHergic neuronal projections to the NPO (Torterolo et al., 2009b). There is also a high density of MCHergic fibers in activating regions such as DR and LC (Torterolo et al., 2008; Lagos et al., 2011b; Yoon and Lee, 2013). MCHergic fibers in the DR and their anatomical relation with serotonergic neurons are shown in **Figure 2**. Regions of the limbic system involved in the control of emotional states including amygdala, nucleus accumbens, septum and hippocampus also receive MCHergic fibers and express MCH receptors (Bittencourt et al., 1992; Hervieu et al., 2000; Chee et al., 2013).

We have also documented that tanycytes in the DR of the cat exhibit immunoreactivity to MCH (Torterolo et al., 2008). Tanycytes are specialized cells whose somata lies in the ependymal or sub-ependymal region that present long basal processes that projected deeply into the subventricular parenchyma. These cells absorb substances present in the CSF

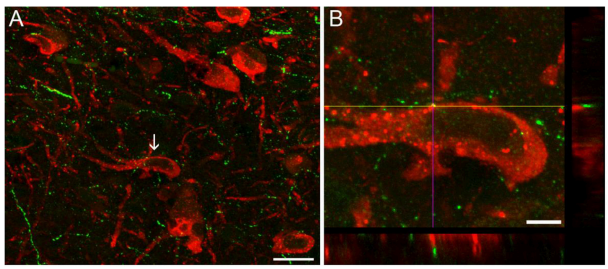

FIGURE 2 | Images of dual-immunostaining for 5-HT and MCH in the dorsal raphe nucleus. Coronal sections (30 μm thickness) were double-labeled to visualize 5-HT- (red) and MCH−immunoreactivity (green). **(A)** MCH+ fibers were observed as small beaded processes around 5−HT+ neurons, intermingled with 5−HT+ neurons located in the mid-rostral level of the DR of the rat, according to Paxinos and Watson (2005). Arrow indicates the neuron in **(B)** that is shown at a high magnification (100X). **(B)** Orthogonal views (xz and yz) reveal apposition between MCH+ fibers and 5-HTergic soma. Image in **(B)** is comprised of 45 optical sections of 0.1 μm. Scale bars **(A)** 20 μm; **(B)** 5 μm. Original microphotographs taken from the data set of Urbanavicius et al. (2015b).

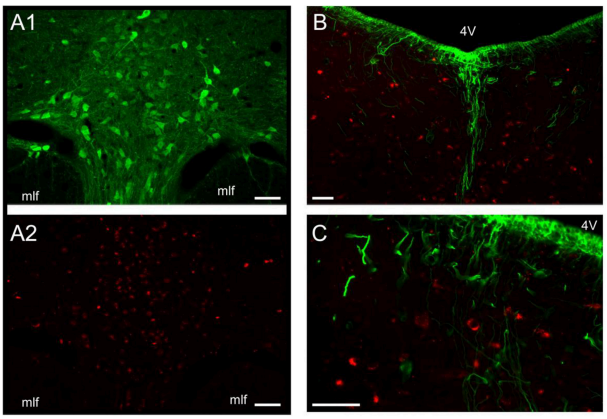

FIGURE 3 | MCH-rhodamine is internalized by DR neurons. (A1) Photomicrographs of the DR of the cat illustrating serotonin immunolabeled neurons. **(A2)** DR neurons of the same field as in **(A1)** that are labeled with rhodamine (these neurons internalized MCH-rhodamine). Note that these MCH-rhodamine labeled neurons are mainly located in the same area as serotonergic neurons. The internalization of MCH strongly suggests that these neurons present MCH receptors. **(B,C)** sections were immunolabeled to detect vimentin, a marker of tanycytes in the adult cat. These photomicrographs of the DR show rhodamine fluorescence within DR neurons (red). The rhodamine-labeled neurons indicate that these neurons internalized MCH-rhodamine. Note that these neurons are located in close relationship to tanycytes (green). 4 V, fourth ventricle; mlf, medial longitudinal fascicle. Calibration bars: 100 μm. Original microphotographs taken from the data set of Devera et al. (2015).

and transport them to the neuronal parenchyma (Rodríguez et al., 2005). These data, together with the presence of MCH in the CSF of the rat, sheep and humans (Peyron et al., 2011; Ungerfeld et al., 2011; Pelluru et al., 2013), suggest that the MCHergic system regulates the activity of the DR through a neurohumoral pathway (by volume conduction through the ventricular system) complementing its regulation via direct neuronal projections (Torterolo et al., 2008).

A wide distribution of MCHR-1 has been identified in the CNS of the rat, which coincides with the distribution of MCHergic fibers (Lembo et al., 1999; Saito et al., 2001). By a novel approach, utilizing intra-cerebro-ventricular administration of MCH labeled with a fluorescent tag (Rhodamine), Devera et al. (2015) have recently shown in cats and rats that neurons of the DR internalize MCH-rhodamine, indicating that they express receptors for MCH (**Figures 3A1,A2**). In the cat, there is a particularly high density of neurons with MCH receptors surrounding the basal processes of tanycytes within the DR (Devera et al., 2015; **Figures 3B,C**). MCHR-1 are present both in serotonergic and non-serotonergic neurons of the DR (**Figure 4**). Some of the non-serotonergic neurons were demonstrated to be GABAergic (Devera et al., 2015). Recently, MCHR-1 mRNA has been identified in serotonergic neurons of the ventro-medial and lateral wing areas of the DR of mice (Spaethling et al., 2014).

MCHergic Neurons are Active during Sleep

Using the Fos protein as a marker of neuronal activity, it has been shown that MCHergic neurons are active during REM sleep in the rat (Verret et al., 2003). Furthermore, Hassani et al. (2009) have recorded MCHergic neurons in non-anesthetized animals. These neurons have a very low frequency of discharge during wakefulness, their firing rate increases slightly during NREM sleep and reaches the maximum level of activation during REM sleep (Hassani et al., 2009). However, even during this state the average discharge rate was still quiet low (approximately 1 Hz) comparing with other neuronal groups (ibid.).

Quantification of MCH during Wakefulness and Sleep

The concentration of MCH in the CSF of rats increases during the light phase, when the animals are predominantly asleep, while it decreases during the dark period when rats are mainly awake (Pelluru et al., 2013). Utilizing the *in vivo* microdialysis technique, it has been shown that the release of MCH in the amygdala of patients with treatment-resistant epilepsy is minimal during active wakefulness with social interactions, increases after eating (consummatory behavior), and reaches a maximum level at sleep onset (Blouin et al., 2013).

Studies with Genetically Modified Animals

Studies of preproMCH and MCHR-1 knockout mice indicate that the sleep architecture of these animals is altered. Mice lacking MCH, sleep less than wild-type animals (Willie et al., 2008). Moreover, in response to fasting, MCH deficient mice became hyperactive and show a marked decrease in REM sleep.

A study in MCHR-1 knockout mice showed an unexpected hypersomniac-like phenotype, both in basal conditions and after total sleep deprivation, compared to wild-type mice (Adamantidis et al., 2008). According to the authors, these surprising effects might be produced by compensatory mechanisms that have been identified as potential limitations of the gene-targeting approach. In contrast, Ahnaou et al. (2011) described an increase of wakefulness and a reduction of NREM sleep in MCHR-1 knockout mice, which agrees with the currently proposed role of MCH in the regulation of sleep-wake

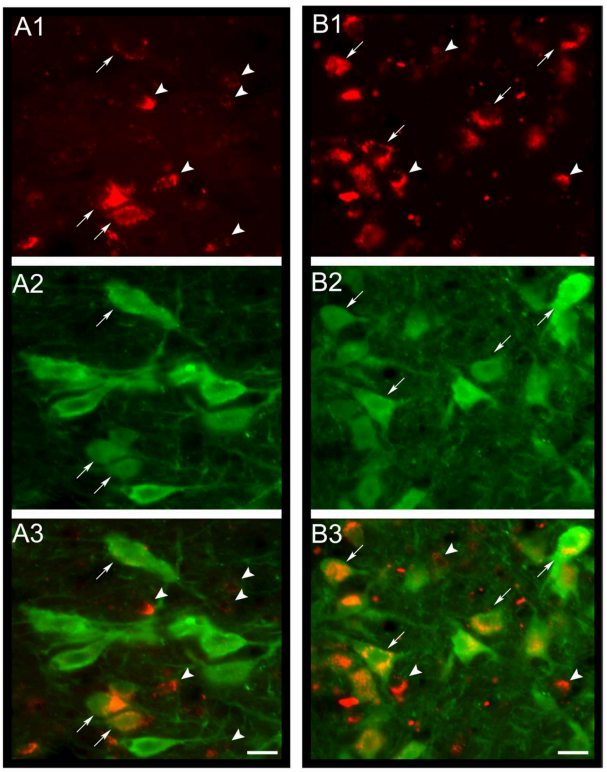

FIGURE 4 | MCH-rhodamine is internalized in DR serotonergic neurons. In **(A1,B1)**, the photomicrographs shows that MCH-rhodamine (red) was internalized by DR neurons in the cat. Photomicrographs in **(A2,B2)** depict neurons with serotonin immunoreactivity (green). Superimposition of both photomicrographs is shown in **(A3,B3)**. It is readily observed that MCH-rhodamine is present in serotonergic (arrows) and non-serotonergic neurons (arrowheads). Calibration bars: 20 μm. Orginal microphotographs taken from the data set of Devera et al. (2015).

states. Moreover, restraint stress further increases wakefulness and reduces both NREM and REM sleep in these mutant mice (Ahnaou et al., 2011).

Administration of MCH or MCHR-1 Receptor Antagonists

Intracerebroventricular administration of MCH in the rat produces a marked increase in REM sleep and a moderate enhancement in the time spent in NREM sleep (Verret et al., 2003). Furthermore, the systemic administration of MCHR-1 antagonists decreases both REM and NREM sleep and increases wakefulness (Ahnaou et al., 2008).

Microinjection of MCH into the DR of the rat facilitates the generation of REM sleep (Lagos et al., 2009). Conversely, the immunoneutralization of endogenous MCH within the DR (through the microinjection of anti-MCH antibodies) produces the opposite effect (Lagos et al., 2011a). Preliminary studies in cats (where the two types of MCH receptors are active) have also shown that MCH microinjections into the DR produced an increase in REM or NREM sleep depending on the exact location of the microinjection sites (Devera et al., 2007).

MCH also promotes REM sleep when microinjected into either the basal forebrain of the rat or the NPO of the cat, two areas related to the generation of this behavioral state (Torterolo et al., 2009b; Lagos et al., 2012). Noradrenergic "REM-off" neurons of the LC are critically involved in the generation of REM sleep and in the pathophysiology of MD (Itoi and Sugimoto, 2010; Brown et al., 2012). Interestingly, microinjections of MCH into this nucleus also produce a marked increase in REM sleep (Monti et al., 2015). In contrast, the administration of MCH into the ventro-lateral preoptic area (VLPO), a NREM sleep promoting area, induced NREM sleep (Benedetto et al., 2013).

Experimental Activation of MCHergic Neurons Induces Sleep

Recent optogenetic studies have confirmed the role of MCH in sleep generation (Jego et al., 2013; Konadhode et al., 2013; Tsunematsu et al., 2014). Konadhode et al. (2013) inserted the gene for the photosensitive rhodopsine-2 cation channel in MCHergic neurons of mice, and specifically stimulated MCHergic neurons. Stimulation induced a decrease in the latency to sleep, reduced the duration of wakefulness and increased the total time spent in NREM and REM sleep during the night, whereas it increased the depth of sleep during the day (ibid.). The authors hypothesized that MCHergic neurons are able to counteract the actions of the activating systems. Consequently, it was concluded that MCHergic agonists might be useful for the treatment of insomnia.

Jego et al. (2013) found that acute optogenetic activation of MCH neurons at the onset of REM sleep extended the duration of REM sleep episodes. In contrast, their acute optogenetic silencing reduced the frequency and amplitude of the hippocampal theta rhythm during REM sleep without affecting the duration of the episodes.

Tsunematsu et al. (2014) showed that acute optogenetic activation of MCH neurons at 10 Hz induced transitions from NREM to REM sleep and increased REM sleep time. Acute optogenetic silencing of MCHergic neurons had no effect on any vigilance state. On the contrary, temporally-controlled ablation of MCH neurons by cell-specific expression of diphtheria toxin A increased wakefulness and decreased NREM sleep duration without affecting REM sleep (Jego et al., 2013; Konadhode et al., 2013; Tsunematsu et al., 2014). The authors concluded that acute activation of MCHergic neurons is sufficient, but not necessary, to trigger the transition from NREM to REM sleep and that MCHergic neurons also play a role in the initiation and maintenance of NREM sleep.

Effect of MCH on Serotonergic Neurons of the Dorsal Raphe

Utilizing *in vivo* extracellular recordings, we determined that the intracerebroventricular or juxtacellular application of MCH inhibits the discharge of the majority of DR neurons (Devera et al., 2015); some of these neurons were presumed to be serotonergic, according to their electrophysiological and pharmacological characteristics. **Figure 5** presents an example of the inhibitory effect of the juxtacellular application of MCH

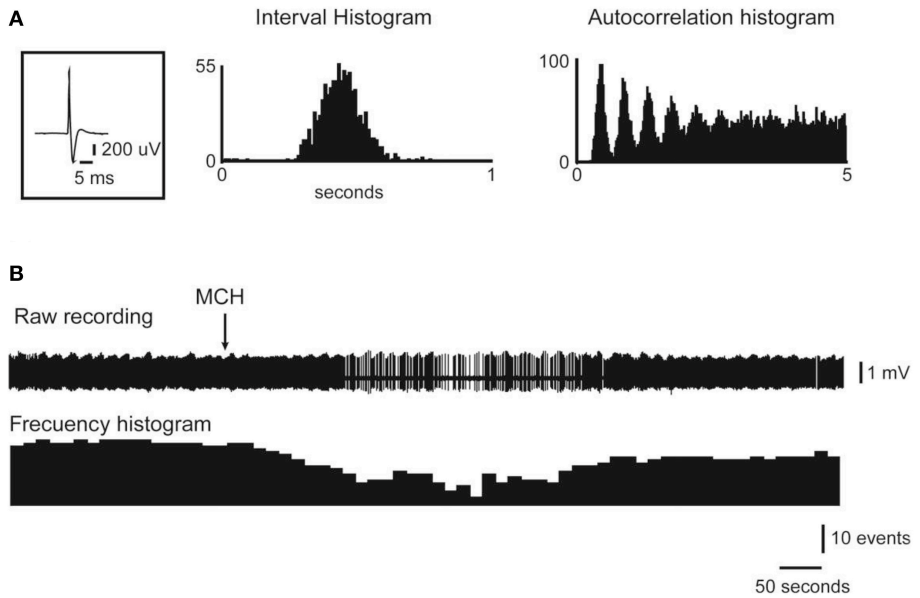

FIGURE 5 | Juxtacellular administration of MCH reduces the activity of DRN neurons. The action potential average, the interval histogram and the autocorrelation histogram of a representative DR neuron are presented in **(A)**. The raw recording and frequency histogram are shown in **(B)**. Note that the application of MCH (indicated by the arrow) produced a decrease in the firing rate. Original Figure taken from the data set of Devera et al. (2015).

on a representative DR neuron. In agreement with these electrophysiological results, *in vivo* microdialysis studies have shown that the perfusion of low concentrations (30 μM) of MCH into the DR elicited a significant decrease in extracellular serotonin levels within this region (Urbanavicius et al., 2013, 2015b).

ROLE OF THE MCHergic SYSTEM IN THE PATHOPHYSIOLOGY OF DEPRESSION

As mentioned above, the large density of MCHergic fibers in the DR, the expression of MCHR-1 in serotonergic neurons, as well as MCHergic projections toward the limbic system, suggest a relevant role of MCH in the control of emotional states. At the same time, MCH facilitates REM sleep (REM sleep is increased in MD) and stimulates the hypothalamus-pituitary-adrenal axis (which is over-activated in MD). These data also suggest that hyperactivity of the MCHergic system is related to certain aspects of MD.

There are several evidences that relate MCH and MD (see below); however, most of the data are from preclinical studies. Hence, it is important to be cautious, because the results from animal models of depression may not correspond with clinical findings (recently reviewed by Belzung, 2014).

Borowsky et al. (2002) demonstrated that the MCHR-1 antagonist, SNAP-7941, possesses antidepressant and anxiolytic effects in animal models of MD (Borowsky et al., 2002). Similar results have been presented by other authors (Shimazaki et al., 2006; David et al., 2007; Chung et al., 2010). In addition, following a 5-week exposure to repeated chronic mild stress (an ethologically relevant animal model of depression), in C57Bl/6J mice there is an increase in the hippocampal gene expression of MCHR-1. This increased gene expression was reversed by chronic fluoxetine treatment (Roy et al., 2007).

The importance of the MCHergic system in MD is emphasized in a recent study which suggests that an increase in the expression of preproMCH and consequent MCH receptor down-regulation could be a biomarker of the severity of depressive disorders (García-Fuster et al., 2012).

Because MCHergic neurons regulate energy homeostasis, it is expected that this function would be altered during MD; in fact, changes in body weight are characteristic of patients with MD (American-Psychiatric-Association, 2013). At the same time, preclinical studies have demonstrated that MCHR-1 antagonists are not only antidepressants, but also have strong anti-obesity effect (Shimazaki et al., 2006; Chung et al., 2010).

As mentioned before, MCH is expressed in neurons of the medial preoptic nucleus of the female rat, which is a critical area in the control of maternal behavior, but only during the post-partum period (Rondini et al., 2010). Recently, Benedetto et al. (2014) have shown that microinjections of MCH into this preoptic nucleus decrease active maternal behaviors during the early post-partum period. Hence, it would be important to know whether a dysfunction of MCH-containing neurons, which are exclusively present during the post-partum period is related to emotional imbalances that take place in 75–80% of mothers between 3 and 5 days after delivery (Lee, 1998).

MCH and Raphe Nuclei: Role in Depression

In **Table 2**, we summarized the effects of MCH agents applied into the DR and MR. In direct relation with MD, we explored the role of MCH within the rat DR in the generation of depressive-like behaviors (Lagos et al., 2011b; Urbanavicius et al., 2015a). MCH microinjections into the DR induce

TABLE 2 | MCH and the raphe nuclei.

Substance	Site	Strategies	Main effect	References
MCH	DR	Microinjection and sleep recording	Increases REM sleep. Moderate increases in NREM sleep	Lagos et al., 2009
Anti-MCH antibody	DR	Microinjection and sleep recording	Decreases REM sleep. Increases wakefulness	Lagos et al., 2011a
MCH	DR	Microinjection, FST	Increases immobility time. This effect is blocked by systemic administration of fluoxetine and nortriptyline	Lagos et al., 2011b; Urbanavicius et al., 2015a
Anti-MCH antibody	DR	Microinjection, FST	Decreases immobility time	Lagos et al., 2011b
MCHR1 antagonist (ATC0175)	DR	Microinjection, FST	Reverts the pro-depressive effect of microinjections of MCH into the DR	Urbanavicius et al., 2015a
MCH	MR	Microinjection, FST	Increases immobility time	López Hill et al., 2013
MCH	DR	Unit recording, intraventricular and juxtacellular administration of MCH	Inhibit serotonergic and non-serotonergic neurons	Devera et al., 2015
MCH	DR	Microdialysis of serotonin, local perfusion of MCH	At low doses decreases serotonin release	Urbanavicius et al., 2013, 2015b

All the experiments were performed in rats. FST, forced swimming test.

a pro-depressive response evaluated in the FST. MCH produced a significant increase in immobility time without affecting locomotor activity; this response is opposite to the prototypical antidepressant effect. The response was blocked when the animals were pretreated systemically with fluoxetine or nortriptyline, which are selective serotonin and noradrenergic reuptake inhibitors antidepressant, respectively (Lagos et al., 2011b; Urbanavicius et al., 2015a). Furthermore, the pro-depressive effect was also suppressed by the intra-DR microinjection of ATC-0175, a selective MCHR-1 antagonist. Additionally, immunoneutralization of endogenous MCH produced an antidepressant effect, since a significant reduction of immobility time was observed (Lagos et al., 2011b). This response was accompanied by an increase in the swim time; this effect is associated with an increase in serotonergic neurotransmission (Lagos et al., 2011b).

Considering the electrophysiological and *in vivo* microdialysis results described above, the pro-depressive effect induced by MCH could be generated by the inhibition of DR serotonergic activity elicited by this neuropeptide.

Recent studies have also shown that the MR is also involved in the pro-depressive effect induced by MCH (López Hill et al., 2013). Serotonergic neurons of the MR express are also inhibited by the intracerebroventricular or juxtacellular administration of MCH (Pascovich et al., 2011).

In this regard, Roy et al. (2006) demonstrated that the chronic deletion of MCHR-1 have altered serotonergic neurotransmission in the prefrontal cortex, one of the main target structures of the serotonergic system and highly associated with the control of emotional processes (Roy et al., 2006). Of note, other areas such as the nucleus accumbens and the basolateral amygdala, have been also proposed to be involved in the pro-depressive effect of MCH (Georgescu et al., 2005; Kim et al., 2015).

Our working hypothesis is that MD is associated with an increase in the activity of MCHergic neurons. In accord with this hypothesis, the antagonism of MCH would be effective in treating MD (Shimazaki et al., 2006; Chung et al., 2010). If our hypothesis is correct, we would expect that antidepressants would decrease the activity of these neurons. Interestingly, it has been observed that the acute treatment with escitalopram (an antidepressant of the SSRI group) inhibits REM sleep rebound that follows a sleep deprivation protocol and promotes a reduction in the activity of MCHergic neurons (Katái et al., 2013). Both set of data support our hypothesis. In addition, in electrophysiological recordings of identified MCHergic neurons *in vivo*, we observed that the juxtacellular application of fluoxetine decreases MCHergic neuronal activity (Pascovich et al., 2014). Interestingly, in accordance with our results, Kim et al. (2015) showed that the antidepressant effect of exercise is associated with the suppression of MCHergic activity within the basolateral amygdala (Kim et al., 2015).

CONCLUSIONS AND FUTURE DIRECTIONS

Preclinical studies suggest that the MCHergic system is involved in the control of REM sleep and depression. The role of MCHergic system in the regulation of sleep, especially REM sleep is well-established. In fact, Luppi et al. (2013) introduced a new model of REM sleep in which the MCHergic neurons plays a REM sleep promoting effect. However, the electrophysiological effect of MCH on the mesopontine areas critical for REM sleep generation (according to this model), such as the ventro-lateral periaqueductal gray and the sublaterodorsal nucleus are still to be tested. Another important issue in the research agenda is to know the role of the other neurotransmitters and neuromodulators (such as neuropeptide E-I, neuropeptide G-E, GABA) that are co-localized with MCH (Macneil, 2013).

Our working hypothesis is that an abnormal increase in the activity of MCHergic neurons is involved in the pathophysiology

of depression. Hence, it would be important to determine whether depressive patients have higher levels of MCH in the CSF as compared to normal subjects. In this respect, Schmidt et al. (2015) have shown that MCH serum levels decrease in major depressive disorder following 4 weeks of antidepressant treatment (Schmidt et al., 2015). However, measurements of MCH within the CSF are of rule.

Finally, preclinical studies have demonstrated that MCHR-1 antagonists have an enormous potential as antidepressant drugs (Shimazaki et al., 2006; Chung et al., 2010). Their efficacy, rapid start of action as well as apparent absence of severe adverse events tends to support the proposal.

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Actions of Prolactin in the Brain: From Physiological Adaptations to Stress and Neurogenesis to Psychopathology

*Luz Torner**

Neuroendocrinología, Departamento de Neurociencias, Centro de Investigación Biomédica de Michoacán, Instituto Mexicano del Seguro Social, Morelia, Mexico

Prolactin (PRL) is one of the most versatile hormones known. It is considered an adaptive hormone due to the key roles it plays in the modulation of the stress response and during pregnancy and lactation. Within the brain, PRL acts as a neuropeptide to promote physiological responses related to reproduction, stress adaptation, neurogenesis, and neuroprotection. The action of PRL on the nervous system contributes to the wide array of changes that occur in the female brain during pregnancy and result in the attenuation of the hypothalamic–pituitary–adrenal axis. Together, all these changes promote behavioral and physiological adaptations of the new mother to enable reproductive success. Brain adaptations driven by PRL are also important for the regulation of maternal emotionality and well-being. PRL also affects the male brain during the stress response, but its effects have been less studied. PRL regulates neurogenesis both in the subventricular zone and in the hippocampus. Therefore, alterations in the PRL system due to stress or exposure to substances that reduce neurogenesis or other conditions, could contribute to maladaptive responses and pathological behavioral outcomes. Here, we review the PRL system and the role it plays in the modulation of stress response and emotion regulation. We discuss the effects of PRL on neurogenesis and neuroprotection, the putative neuronal mechanisms underlying these effects, and their contribution to the onset of psychopathological states such as depression.

Keywords: anxiety, depression, prolactin receptors, mood disorders, neurogenesis

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***Correspondence:**
Luz Torner
luz_torner@yahoo.com

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INTRODUCTION

Prolactin (PRL) is a pleiotropic pituitary hormone with more than 300 known physiological effects. This protein hormone has a regulatory control on reproduction, immunomodulation, angiogenesis, energy metabolism, osmotic balance, and development. In addition to its peripheral functions, PRL also plays many important roles as a neuropeptide. PRL crosses the blood–brain barrier, and local hypothalamic production contributes to reach several brain regions producing strong modulatory effects. In particular, PRL contributes to the regulation of the stress responses through the inhibition of the hypothalamic–pituitary–adrenal (HPA) axis. Moreover, PRL modulates anxiety and depressive-like behaviors. PRL also regulates neurogenesis, the generation of new neurons, in both the subventricular zone (SVZ) and the hippocampus. PRL levels and environmental conditions may induce changes in neurogenesis, potentially leading to an impact on emotional behavior. Indeed, reduction of PRL levels during early pregnancy affects olfactory bulb neurogenesis and increases

postpartum anxiety in the female rat. In addition, PRL may exert neuroprotective effects in the hippocampus of adult animals exposed to chronic stress or subjected to hippocampal infusions of kainic acid. However, PRL reduces neurogenesis, when administered, during the early developmental stages and promotes depressive-like behavior in adulthood. Some of these effects are mediated by the activation of different neuronal signaling systems and ion channels. Here, we will summarize some aspects of PRL actions within the brain and their contribution to the onset of psychopathological states like depression.

THE PROLACTIN SYSTEM

PRL Synthesis

Prolactin is produced mainly by the lactotroph cells in the anterior pituitary. PRL is under the inhibitory control by dopamine released from the tuberoinfundibular dopaminergic (TIDA) neurons (1). Despite the abundant efforts to map PRL in cerebral regions using several techniques, the synthesis of PRL within the brain has been brought into question. PRL positive neurons were localized by immunocytochemistry in males and females of different species (2). PRL mRNA was detected by PCR in paraventricular nucleus (PVN) and supraoptic nucleus (3) and by qPCR in the hypothalamus of female rats (4). *In situ* hybridization showed PRL expression in the medial preoptic area (MPOA), periventricular preoptic nucleus, the bed nucleus of the stria terminalis, the PVN, and the lateral septum in the domestic turkey and in both the fetal and adult sheep (5, 6). Recent studies using microarray techniques detected the expression of PRL in the hippocampus of resilient mice (7).

Peripheral PRL is considered the major effector within the brain. It has been hypothesized that PRL may cross the blood–brain barrier through a receptor-mediated mechanism occurring in the cells of the choroid plexus (8). However, recent studies using the PRL receptor (PRL-R) knockout mice showed that PRL transport does not depend on the PRL-R but rather on another, yet to be identified, mechanism (9). Thirty minutes after its peripheral administration, PRL activates brain neurons through the induction of the phosphorylation of Signal Transducer and Activator of Transcription 5 [pSTAT5 (9)]. PRL peaks in the cerebrospinal fluid (CSF) 30 min (10) or 90 min (9) after its peripheral administration. These last studies also suggest that PRL does not access the brain after entering the CSF (9). Since immunoreactive PRL was observed in the choroid plexus and ependymal cells of the cerebral ventricles, it has been hypothesized that PRL may be transported from these sites to reach its target regions (11, 12). However, it is still unknown how PRL accesses its distant target regions within the brain. Whether the ependymal cells, or the neurons, take up PRL and release it in the vicinity of its target cells, or if PRL is released in the extracellular space to diffuse until it reaches its receptors is still a matter of debate.

PRL Receptors

Prolactin receptors belong to the class 1 cytokine receptor superfamily (1). PRL-R is encoded by a single gene, but alternative splicing produces several PRL-R isoforms. In both humans and rats, three isoforms (long, intermediate, and short – e.g., the size of the intracellular domain) have been identified. The response is triggered after the dimerization of the receptors to form homodimers. Long–long dimers are able to activate second messenger pathways, particularly the JAK-signal transducer and activator of transcription (JAK-STAT) signaling cascade, through the activation of STAT3 and STAT5. The short PRL-R isoform activates the mitogen-activated protein kinase (MAPK) pathway (13). Both long and short receptor heterodimers inhibit PRL signaling (14) and contribute to modulate PRL actions.

STRESS RESPONSE AND PRL

Stress is a critical factor that may lead to depressive disorders. Stress exposure activates the HPA axis, triggering the release of corticotrophin releasing hormone (CRH) in the PVN, which promotes the secretion of adrenocorticotrophin (ACTH) from the pituitary. In turn, ACTH triggers the release of glucocorticoids from the adrenal glands. PRL is also secreted from the pituitary in response to a number of stressors. Initial studies suggested that PRL may counteract glucocorticoid actions on the immune system during the stress response (15). However, more recent studies showed that preventing PRL-R expression in the brain *via* an antisense probe strongly increases the stress-induced ACTH secretion in virgin and lactating rats, suggesting that PRL plays an inhibitory role on HPA axis reactivity (16, 17). It has been hypothesized that PRL modulates the activity of the HPA axis through a reduction of neural inputs to the PVN. Both acute and chronic PRL intracerebroventricular administrations in virgin female rats reduce neuronal activation in the amygdala and CRH hypothalamic expression in response to stress (18). Additionally, PRL is locally released from the PVN and MPOA in response to physiological stimuli, including stress (4).

Prolactin has been recently associated with resilience in a model of chronic mild stress (CMS). Adult rats, previously subjected to CMS, were selected as responders and non-responders, according to their reaction to stress. The resilient animals (i.e., non-responders) presented higher plasma levels of PRL, and higher PRL-R mRNA in the choroid plexus than their vulnerable (i.e., responders) counterpart (19). Accordingly, a microarray study performed on heterozygous 5-Htt-deficient offspring subjected to prenatal stress showed that PRL, growth hormone, and galanin receptor 3 were differentially expressed in the hippocampus of resilient mice when compared to controls (7). These results suggest a role for PRL in stress regulation at hippocampal level.

PRL AND EMOTIONAL RESPONSIVENESS

The effects of PRL on anxiety and depressive behavior have been studied, but they differ depending on the species used and the physiological state.

PRL and Anxiety

Intracerebroventricular or intravenous administrations of PRL exert dose-dependent anxiolytic effects in both male and virgin female rats (16). Anxiety levels in pregnant and lactating rats undergo a progressive reduction (20, 21). These effects are likely

to be mediated by PRL, as suggested by the increased anxiety displayed by lactating female rats in the elevated plus maze after blockade of PRL-R brain expression (17). Chronic ICV administration of PRL to ovariectomized E2-replaced female rats (used to simulate the endocrine status during pregnancy) was shown to reduce anxiety levels in the elevated plus maze (18). Taken together, these results strongly suggest that PRL has an anxiolytic effect in rodents.

Studies on inbred lines of rodents have shown an association between elevated levels of PRL and increased anxiety. Increased basal and stress-induced levels of PRL were reported in male rats bred for high anxiety behavior (HAB) as compared to low anxiety behavior (LAB) rats (22). The Roman low-avoidance (RLA/Verh) line of rats (i.e., rats selected for low performance in a two-way active avoidance test) displays increased stress responses, higher levels of PRL, and a passive coping style compared to the Roman high-avoidance (RHA/Verh) line of rats (i.e., rats selected for high performance in a two-way active avoidance test) counterpart (23). The elevated levels of PRL found in this animal line could stem from an imbalance in one or more secretagogues of PRL (serotonin, GABA, etc.). Alternatively, high PRL concentrations could help to improve the excessive reactivity of the HPA axis observed in these animals. In contrast, clinical studies in humans have indicated a correlation between high PRL levels and psychological distress. Female patients with hyperprolactinemia usually report more symptoms of anxiety and hostility than control female subjects (24).

PRL and Depressive-Like States

Artificially inducing hyperprolactinemia in adult male rats by placing pituitary grafts of a donor rat in the kidney of the receptor animal was shown to exert antidepressive-like effects in the forced swimming test (25).

In humans, some patients with hyperprolactinemia exhibit depressive symptoms. However, no differences were observed in the prevalence of depression in hyperprolactinemic patients compared to controls (24, 26). Remarkably, few studies have made a distinction of the origin of hyperprolactinemia (e.g., a pituitary tumor, or dysregulation of neural pathways due to neurotransmitter alterations) and its relationship with anxiety and depression. Since PRL modulates the expression of its own receptors, it has been hypothesized that hyperprolactinemic patients may express more PRL-R in the brain. However, it is unknown if the receptors are functionally activated. Excessive levels of PRL may prevent the formation of the homodimers necessary for the physiological functions of PRL. Additionally, long-term hyperprolactinemia reduces the ability of the tuberoinfundibular neurons to synthesize dopamine (27). High PRL concentrations generate elevated levels of the 16K PRL fragment called vasoinhibin. This fragment exerts effects opposite to those of the native hormone (28, 29).

Depressive-Like States and Motherhood

Several animal studies report a causal link between reduced maternal care and depressive-like state or alterations of emotional behavior in the offspring. Stress exposure during pregnancy alters the neural mechanisms that prepare the female to her maternal role and contributes to the development of psychiatric diseases such as depression or anxiety (30, 31). Female rats subjected to chronic psychosocial stress during pregnancy exhibit a postpartum depressive-like state (32–34). The activation of oxytocin and PRL systems during lactation contributes to the attenuation of the HPA axis activation and triggers a positive mood (35). Therefore, alterations in these systems could contribute to the development of the affective disorders observed in the postpartum period. Indeed, oxytocin expression is reduced in the hypothalamus of postpartum female rats subjected to chronic stress during pregnancy (34). Postpartum levels of circulating oxytocin are decreased in women showing depressive symptoms during pregnancy (36). Low concentrations of oxytocin could increase the risk to develop postpartum depression (37).

Dysregulation of PRL-R responsivity could be associated with postpartum disorders (38). Indeed, low levels of PRL were found in women suffering from postpartum depressive disorder (39). Administration of bromocriptine in the early stages of pregnancy reduces PRL levels, induces a depressive-like state, and impairs maternal behavior in female rats, suggesting that PRL may play a key role in the onset of postpartum depression (40, 41). Moreover, breastfeeding-induced raise in PRL levels results in positive effects on both the mother's health and the mother–infant bonding. A correlation was found between stress, dysphoric moods, and reduced levels of interferon gamma in mothers using bottle feeding (42). On the contrary, breastfeeding mothers scored less in anxiety, depression, and anger tests (43).

NEUROGENESIS AND NEUROPROTECTION

Chronic stress exposure and depressive states are known to affect neurogenesis. Neurogenesis is the process that produces new neurons throughout life. New neurons are thought to transiently increase neuronal communication. Two neurogenic niches have been recognized in the adult brain: the hippocampus and the SVZ. The hippocampus exerts a negative control on the HPA axis activity and it is involved in emotional modulation. Antidepressive treatments increase hippocampal neurogenesis and show a correlation with mood improvement (44). Olfactory bulb neurogenesis and olfactory dysfunction are also decreased by CMS exposure, a procedure that is known to induce depressive-like states (45).

Prolactin is a regulator of neurogenesis. PRL receptors are expressed in the SVZ and the hippocampus (46–48). Initial evidence of the relationship between PRL and neurogenesis has been reported by studies showing an increase in neurogenesis in the SVZ of pregnant females. This increase was found to be mediated by PRL (47), and it was hypothesized that the olfactory discrimination of odor cues related to pups is critical for maternal success. Other olfactory signals are important cues that also induce neurogenesis in the SVZ and in the hippocampus. Exposure to pheromones from a dominant male induces cell proliferation in both the olfactory bulb and the hippocampus of female mice. These effects are mediated by PRL in the olfactory bulb and luteinizing hormone in the hippocampus, and both hormones contribute to the regulation of female reproduction (49). Exposure to male pheromones increases SVZ neurogenesis and promotes maternal behavior in virgin and postpartum

females (50). Injections of bromocriptine, a dopamine agonist, in female rats during the first days of pregnancy to lower PRL levels, reduces olfactory neurogenesis and induces behavioral alterations postpartum (41). These reports clearly suggest that PRL may regulate SVZ neurogenesis and play a key role in mood regulation.

The reduction of hippocampal neurogenesis due to chronic stress exposure was prevented by daily PRL administration in male mice. PRL was shown to promote neuronal fate and to exert neuroprotective actions in the hippocampus (51). PRL effects were initially thought to counteract glucocorticoid reduction of neurosphere proliferation and survival. However, incubation of PRL together with dexamethasone was unable to inhibit dexamethasone effects on neurospheres, despite the induction of ERK1/2. Therefore, it was suggested that PRL may affect hippocampal neurogenesis through an indirect mechanism (52). Following studies showed both *in vitro* (in primary adult hippocampal cells) and *in vivo* (direct injection into the dentate gyrus of adult mice) that PRL administration increases the neurosphere number, suggesting a direct effect of this neuropeptide on the hippocampus (53). Furthermore, these authors reported behavioral deficits in the PRL null mice trained in hippocampus-dependent learning tasks, and suggested a role of PRL in the learning and memory processes (53). In contrast, daily injections of PRL in rats during the first two postnatal weeks reduces neurogenesis in both the dentate gyrus and the olfactory bulb and promotes a depressive-like state in both adult male and female rats (54). These results suggest that PRL exerts different actions on neurogenesis depending on the age of the animals and it could be associated with emotional regulation.

During pregnancy and lactation, PRL protects the hippocampus against the high concentrations of glucocorticoids (55). Administration of ovine PRL to the cerebral ventricle of ovariectomized virgin females buffers the neurodegenerative process induced by kainic acid infusion in CA1, CA3, and CA4 areas of the hippocampus (56, 57). These results suggest a neuroprotective role for PRL in the hippocampus.

NEURAL MECHANISMS OF PRL ACTIONS

Prolactin effects on neurogenesis are mediated by its activation of the extracellular signal-regulated kinase 5 (ERK5). ERK5 is expressed in the neurogenic niches of the brain (58). Moreover, PRL increases both the expression and protein levels of Nestin and microtubule-associated protein 2 (MAP2) in neuroblastoma (SK-N-SH) cells. This suggests that PRL regulates cytoskeletal protein synthesis and therefore contributes to neuronal differentiation (59). Furthermore, PRL modulates Ca^{2+}-dependent K^+ channels, which could affect the release of neurotransmitters in different cerebral regions (60). In the hypothalamus, PRL stimulates the ERK/MAPK pathway in CRH, vasopressin, and oxytocin neurons (61) and promotes EGR-1 expression in magnocellular neurons (62). Activation of these pathways by PRL could contribute to the plastic changes observed in the brain during pregnancy. PRL stimulation of CRH transcription in hypothalamic cultured cells has been interpreted as an indirect inhibitory control of PRL on HPA axis activity (62). However, it is still unknown how PRL affects the HPA axis.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Prolactin alters neural circuits to help the individual to cope with stress. Reduced activation of neural inputs, activation of ionic channels, or the modulation of several signaling pathways are some of the putative mechanisms of action underlying the effects of PRL on brain circuits. It is unknown how PRL regulates the HPA axis function during the stress response. PRL could affect hypothalamic and/or hippocampal activity to regulate emotionality. The contribution of PRL to the onset of postpartum depression is still unknown. Low PRL levels in nursing women have been associated with postpartum depression. Inhibition of PRL reduces neurogenesis in the olfactory bulb, but the mechanisms that result in a postpartum depressive state are unknown. Besides the pathways affected by PRL to regulate anxiety are not yet known, and whether the PRL effects on hippocampal neurogenesis contribute to modulate anxiety is still uncertain. Administration of PRL in the early postnatal stages reduces neurogenesis in the hippocampus and the olfactory bulb and promotes a depressive-like state in adulthood. This suggests that PRL affects a critical window during brain development and that PRL actions are dependent on the age during which it is administrated. Further studies are needed to understand the mechanisms of action of PRL in the brain, and how this hormone modulates emotionality and anxiety at different developmental stages.

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Oxytocin and estrogen receptor β in the brain: an overview

*Alexandra Acevedo-Rodriguez[1,2], Shaila K. Mani[1,2,3] and Robert J. Handa[4]**

[1] Department of Neuroscience, Baylor College of Medicine, Houston, TX, USA, [2] Memory and Brain Research Center, Baylor College of Medicine, Houston, TX, USA, [3] Department Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX, USA, [4] Department of Biomedical Sciences, Colorado State University, Fort Collins, CO, USA

Oxytocin (OT) is a neuropeptide synthesized primarily by neurons of the paraventricular and supraoptic nuclei of the hypothalamus. These neurons have axons that project into the posterior pituitary and release OT into the bloodstream to promote labor and lactation; however, OT neurons also project to other brain areas where it plays a role in numerous brain functions. OT binds to the widely expressed OT receptor (OTR), and, in doing so, it regulates homeostatic processes, social recognition, and fear conditioning. In addition to these functions, OT decreases neuroendocrine stress signaling and anxiety-related and depression-like behaviors. Steroid hormones differentially modulate stress responses and alter OTR expression. In particular, estrogen receptor β activation has been found to both reduce anxiety-related behaviors and increase OT peptide transcription, suggesting a role for OT in this estrogen receptor β-mediated anxiolytic effect. Further research is needed to identify modulators of OT signaling and the pathways utilized and to elucidate molecular mechanisms controlling OT expression to allow better therapeutic manipulations of this system in patient populations.

Keywords: estradiol, oxytocin, hypothalamus, pituitary, adrenal, behavior, anxiety

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****Correspondence:***
Robert J. Handa
bhanda@colostate.edu

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INTRODUCTION

The nonapeptide hormone, oxytocin (OT), has gained widespread attention as a potential therapeutic agent in a myriad of disorders, including autism spectrum disorder, schizophrenia, and addiction (1). OT is produced primarily in the neurons of the hypothalamic paraventricular nucleus (PVN) and supraoptic nucleus [SON (2)], is released into systemic circulation, and plays an important role in lactation, parturition, maternal behavior, and pair-bond formation (3, 4). Additionally, OT is released from the PVN axon terminals that project throughout the brain into regions, such as the hippocampus, striatum, and amygdala (5), and has been implicated in the regulation of memory, stress, and social behaviors (1). In addition to traditional release across a synapse, OT is also released from neuron somas and dendrites and can reach nearby brain regions via volume transmission by

Abbreviations: ACTH, adrenocorticotropic hormone; BNST, bed nucleus of the stria terminalis; CBP, cAMP response element-binding protein; CeA, central nucleus of the amygdala; CORT, corticosterone; CRF, corticotropin-releasing factor; DPN, diarylpropionitrile; ERα, estrogen receptor α; ERβ, estrogen receptor β; ERβKO, ERβ knockout; FST, forced swim test; GABA, γ-aminobutyric acid; HPA, hypothalamic–pituitary–adrenal; ICV, intracerebroventricular; LS, lateral septum; MPOA, medial preoptic area; OT, oxytocin; OTA, oxytocin receptor antagonist; OTKO, oxytocin knockout; OTR, oxytocin receptor; OTRKO, oxytocin receptor knockout; PVN, paraventricular nucleus of the hypothalamus; SRC, steroid receptor coactivator; VMH, ventromedial nucleus of the hypothalamus.

diffusing across neural tissue (6). In this review, we focus on the function of OT in the brain and its modulation by estrogens.

OXYTOCIN RECEPTORS: BRAIN DISTRIBUTION AND FUNCTION

Oxytocin signals through OT receptors (OTRs), which are G protein-coupled receptors that, upon binding to OT, activate the Gq protein subunit and ultimately excite the cell. Autoradiographic studies have identified OTR expression in several regions of the rat brain, including the olfactory system, basal ganglia, hippocampus, central amygdala, and hypothalamus (7). The generation of a knock-in mouse strain where Venus, a variant of yellow fluorescent protein, is under the regulation of the *Otr* promoter sequence, facilitated the identification of OTR expressing cells in additional brain regions, e.g., the median raphe nucleus and the lateral hypothalamus. This mouse model has been valuable in the identification of the phenotype of the cells expressing OTRs. For instance, OTRs have been found in serotonergic neurons (8), implicating serotonergic involvement in OTs anxiolytic effects in depression and anxiety.

Central OT is important in homeostatic processes, such as thermoregulation (9), food intake (10), and mating (11, 12). OT also plays an important role in maternal behavior. Female rats that received and exhibited high maternal care showed higher levels of OTRs in various limbic regions of the brain, including the bed nucleus of the stria terminalis (BNST), central nucleus of the amygdala (CeA), lateral septum (LS), PVN, and medial preoptic area (MPOA). Additionally, central administration of an OTR antagonist (OTA) completely eliminated the elevated licking and grooming behaviors seen in the high maternal behavior animals, suggesting that OTRs mediate maternal behaviors (13).

Additional insights into the function of OT and the OTR are gained from the examination of genetically engineered mouse models. Female OT knockout (OTKO) mice show normal parturition and maternal behavior but are unable to nurse their pups demonstrating that in the mouse OT is not necessary for maternal behavior or labor but is essential for milk ejection (14). Compared to the normal maternal behavior observed in OTKOs, OTR knockout (OTRKO) mice show deficits in maternal behaviors demonstrated by their longer latency for pup retrieval (15). OT signaling is also implicated in social behavior, and the OTKOs and OTRKOs both showed deficits in social memory. Wild-type animals investigate a novel conspecific for a longer period of time than a familiar animal, whereas OTKOs and OTRKOs show similar investigation times for both novel and familiar animals (15, 16). Although OTR levels remain unaltered in OTKOs (14, 16, 17), OTKOs demonstrated increased OTR sensitivity as measured by increased grooming following central OT administration (17).

Unlike OTKOs, OTRKO males display increased aggression in the resident-intruder task (15). It is possible that this elevated aggressive behavior in the OTRKOs is mediated by a lack of OT signaling in the CeA, since administration of OT into the CeA of male rats decreased aggressive behavior (18). Interestingly, OTKO offspring generated from a homozygous breeding scheme demonstrated an increased aggression phenotype as compared to those bred from heterozygous parents, suggesting that OT from the heterozygous dam can prevent the aggressive phenotype in the OTKO pups (15). Although these changes in behavior may relate to the absence of OT or the OTR, these phenotypic changes could be due to compensatory mechanisms that occur during development to overcome the absence of OT signaling.

Furthermore, selective knockout of OTRs in the LS showed that OT plays a bi-directional role in fear regulation dependent on social context. Animals exposed to a non-fearful conspecific [positive social encounter; (19)] or to social defeat [negative social encounter; (20)] during contextual fear conditioning showed reduced or increased fear, respectively, compared to controls. Intra-LS administration of OTA or of a virally linked Cre-recombinase to knockdown OTR expression prevented the altered fear response mediated by the social stimulus (19, 20). These data demonstrate that the OT/OTR system enhances memory of social interactions, reducing fear after positive and enhancing fear after negative social interactions.

Various factors influence OT signaling. OTRs are largely expressed centrally but their regional localization varies across species. For instance, mice and rats both express OTRs in the ventromedial nucleus of the hypothalamus [VMH (7, 8)], but OTRs are not expressed in this region in rabbits (7). These species-specific differences in localization may account for different responses to OT, for example, mice and rats respond differently to OT administration (21). Additionally, mice and humans show different OTR localization. For example, OTR-Venus immunoreactivity was seen in the mouse hippocampus (8) but, in humans, OTRs were not localized to this area (22). OTR signaling also changes during development in rats with transient developmental patterns displayed postnatally, an adult-like expression pattern seen around postnatal day 21, and increased OTR quantities into adulthood (23). Additionally, OT signaling differs between males and females. Female rats were found to have fewer OTRs in the BNST, VMH, and medial amygdala compared to males (24), and in humans, men and women were found to respond differently to intranasal OT administration (25, 26). These differences between males and females may relate to hormone differences, which alter OT signaling and are discussed in more detail in a later section.

OXYTOCIN REGULATION OF HYPOTHALAMIC–PITUITARY–ADRENAL AXIS

Oxytocin release from neurons of the PVN and the presence of OTRs within the PVN suggests the possibility that OT can directly modulate the stress reactive hypothalamic–pituitary–adrenal (HPA) axis. The HPA axis responds to stressors and activates neurons residing in the PVN causing increased synthesis and secretion of corticotropin-releasing factor (CRF). The release of CRF into the hypophyseal portal system enhances synthesis and release of adrenocorticotropic hormone (ACTH) from the anterior pituitary. In turn, ACTH acts on the adrenal cortex to stimulate release of glucocorticoids [cortisol in humans and

corticosterone (CORT) in rats and mice]. Increased levels of circulating glucocorticoids can further inhibit HPA axis activity via glucocorticoid and mineralocorticoid receptors in the brain as well as acting upon specific brain sites to modulate behaviors (27).

Oxytocin can putatively impact several sites within the HPA axis. PVN neurons that project to the median eminence release OT into the hypophyseal portal vasculature to stimulate adrenal glucocorticoid release by potentiating the actions of CRF at the anterior pituitary level in a similar fashion to the closely related neuropeptide vasopressin (28). By contrast, OT neurons in the PVN that project into the forebrain and release OT in response to stressors (29) exert anxiolytic actions (5). Intracerebroventricular (ICV) administration of OT decreases not only circulating CORT levels but also ACTH levels following exposure to stressors in rats (30, 31) and mice (32, 33), and central infusion of OT into the PVN inhibits HPA axis reactivity, via modulation of CRF neuronal activity (34). Using the restraint stress paradigm in association with OT administration (ICV), Windle et al. (31) demonstrated the presence of an OT-sensitive forebrain stress circuit involving the dorsal hippocampus, ventrolateral septum, and PVN (31).

Endogenous OT levels are also sufficient to alter HPA axis reactivity. ICV injection of OTA showed elevated ACTH and CORT levels prior to behavioral testing suggesting that endogenous OT levels can suppress HPA axis reactivity (34, 35). Additionally, administration of OTA via retrodialysis into the PVN resulted in increased ACTH and CORT release indicating that endogenous OT can inhibit PVN neurons (35). Female OTKO mice show elevated CORT levels following acute and repeated shaker stress compared to wild-type littermates (33), demonstrating a definitive role for OT in regulating HPA axis reactivity to stress.

Interestingly, OT also promotes social buffering in response to stress, similar to the effect seen with fear (19). Female prairie voles subjected to restraint stress demonstrated an increase in anxiety-like behaviors and CORT levels when recovering alone but not when recovering with a male partner, which also corresponded to an increase in OT release in the PVN of these females. Intra-PVN OT injections reduced CORT and anxiety-related behaviors when animals recovered alone, whereas intra-PVA OTA administration prevented social buffering. These observations suggest that PVN OT signaling is necessary and sufficient for social buffering effects in response to stress in prairie voles (36).

OXYTOCIN REGULATION OF ANXIETY AND DEPRESSIVE BEHAVIORS

Oxytocin is strongly implicated in social bond formation and social behavior [for review see Ref. (37)], but may also play a role in psychiatric disorders, such as anxiety and depression. The effect of OT in these disorders may be related to abnormal social behavior, but OT may also independently impact these disorders via regulation of the HPA axis. Dysregulation of the HPA axis and increased response to stressors are commonly seen in anxiety and mood disorders (38). In a clinical study with pediatric and adult participants, cerebrospinal fluid and plasma OT levels were found to be higher in participants that had lower anxiety (39). However, severe anxiety symptoms may be related to over-activation of the OT system as women with elevated OT levels were more likely to report being anxious on a daily basis (40). Reduced nocturnal levels of OT have been reported in depressed individuals; however, numerous studies have also reported no differences compared to healthy controls (41). This variability across studies for anxiety and depression may relate to OT levels corresponding more to personality traits rather than symptoms of depression or anxiety (42). Despite these inconsistencies in data concerning psychiatric disorder OT levels, a recent meta-analysis suggests that OT may be beneficial in the treatment of anxiety and depression (43).

Oxytocin signaling during early development may contribute to later anxiety. Prairie vole pups exposed to a single injection of OT on postnatal day 1 demonstrated an increase in serotonergic axon density in the anterior hypothalamus, cortical amygdala, and VMH but not in the PVN or medial amygdala. Such effects on serotonergic neurons could be a mechanism by which OT affects emotional behaviors, since serotonin is strongly linked to mood, and serotonin dysregulation is seen in depression and anxiety disorders (44).

In adult animals, OT administration reduces anxiety-related behaviors in the elevated plus maze (30, 45, 46) and open field assay (8, 11). OT administration centrally (30), to the medial prefrontal cortex (46), to the CeA (11), and to the PVN (45) was sufficient to reduce anxiety-related behaviors. Chronic central OT administration reduced anxiety in rats bred for high levels of anxiety-related behaviors (47). Further support for the role of OT in reducing anxiety comes from studies of OTKO mice with OTKO females showing increased anxiety-related behaviors compared to their wild-type counterparts (32, 33). The effect of OT was sex dependent as male OTKOs showed reduced anxiety-related behaviors (32, 48).

Oxytocin also reduces measures of depression in the forced swim test (FST) and tail suspension test. In FST, rats treated with an OT analog spent less time immobile and more time swimming and climbing the walls of the chamber than saline-treated animals indicating an antidepressant effect (49). Similarly, ICV OT or OTA administration showed a dose-dependent decrease or increase in immobility in both assays, respectively (50, 51). Interestingly, the antidepressant effect of OT was not blocked by a selective OTA, suggesting that OTs antidepressant effects are not OTR mediated (50).

REGULATION OF OXYTOCIN FUNCTION BY STEROID HORMONES

Steroid hormones are a broad family of hormones that include the estrogens, androgens, progestins, mineralocorticoids, and glucocorticoids. These hormones can readily cross the cell membrane where they bind and activate their respective intracellular receptors. Steroid receptor proteins have DNA and ligand-binding domains, and unliganded steroid receptors are maintained in an inactive state by a complex of chaperone proteins (52). Upon ligand binding, the receptors dimerize and translocate into the nucleus and bind DNA promoters and recruit cofactors and transcription machinery to promote gene transcription (53). Steroid hormones have been found to alter OT signaling. Estrogens can act in a

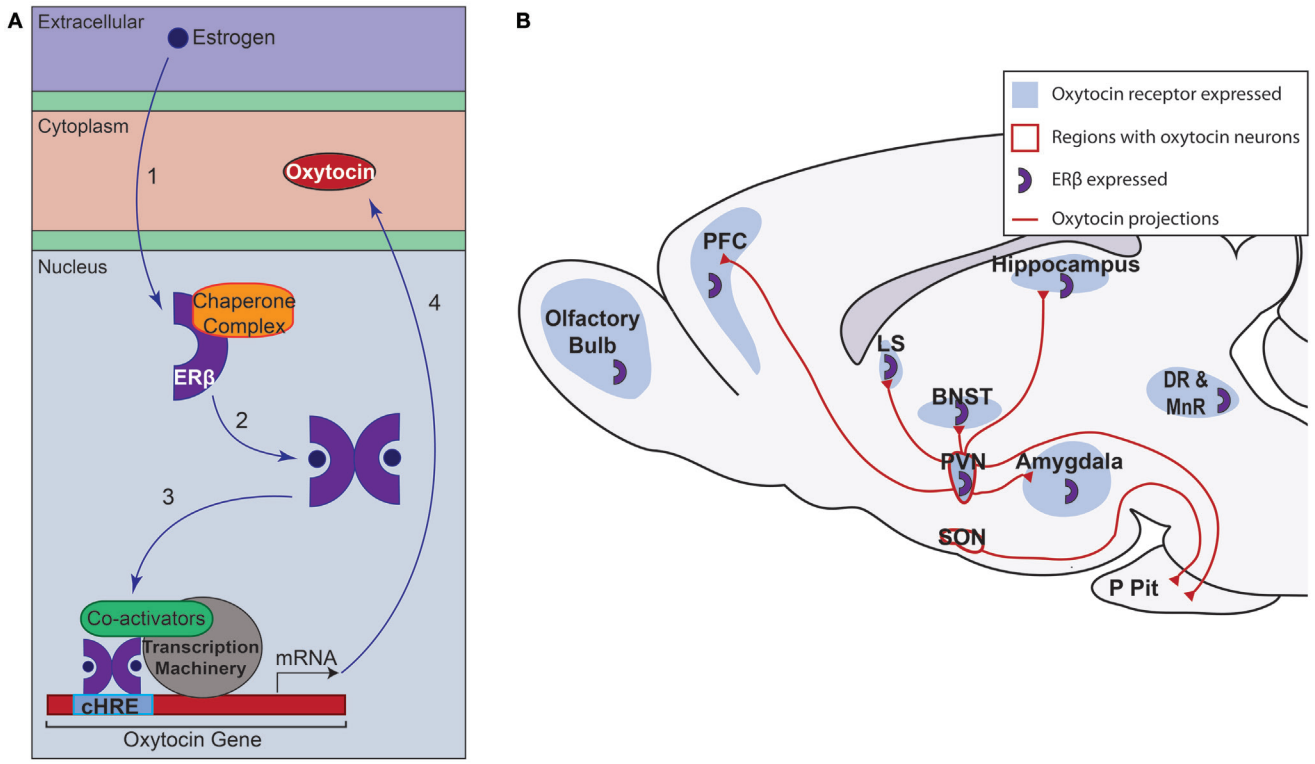

FIGURE 1 | Overview of estrogen receptor β (ERβ) action in oxytocin neurons of the paraventricular nucleus (PVN) and oxytocin signaling. (A) ERβ signaling in PVN oxytocinergic neurons. (1) Estrogen enters the cell and binds to inactive ERβ. In its inactive form, ERβ is bound to a complex of chaperone proteins. (2) Upon binding its ligand, ERβ dimerizes. (3) The dimerized receptor binds to the composite hormone response element (cHRE) of the oxytocin promoter. Co-activators, such are SRC-1 and CBP, are recruited along with the transcription machinery to promote transcription. (4) Ultimately, the oxytocin peptide is produced. **(B)** Oxytocin signaling in the brain. Oxytocin is produced in neurons of the PVN and supraoptic nucleus (SON). Oxytocin neurons from both regions project to the posterior pituitary (P Pit). In addition to this release, the PVN also sends oxytocin projections throughout the brain (5). ERβ is expressed in approximately 85% of neurons in the rodent PVN but not in the SON (72). This suggests that ERβ could play a role in increasing oxytocin production in regions important in responding to stress and can subsequently influence brain areas that express oxytocin receptors [shown in blue; (8, 13)]. BNST, bed nucleus of the stria terminalis; DR, dorsal raphe nucleus; LS, lateral septum; MnR, median raphe nucleus; PFC, prefrontal cortex.

synergistic manner with OT, not only by enhancing its anxiolytic effects (54) but also by increasing OTR levels in the mouse brain (55). In humans, a single dose of estradiol was sufficient to increase plasma OT levels in women (56). Similarly, testosterone alters OTR expression differently depending on brain region (21). Progesterone is important in pregnancy maintenance and *in vitro* studies found that progesterone could inhibit OT binding to the OTR (57). Also, treatment with a synthetic glucocorticoid significantly altered OTR expression in various brain regions, such as the amygdala, BNST, and VMH (58).

Understanding OT regulation by sex steroids is important since anxiety and depressive disorders show a larger gender disparity (38), which may be related to circulating steroid hormone levels. Testosterone has been shown to decrease HPA axis activity (59, 60), whereas estrogens can both increase (60, 61) or decrease (62, 63) HPA axis activity, and these alterations may in part be through modulations of OT activity. The differences in the observed effects of estrogens on behavior and neuroendocrine responses to stress may relate to its differential activity on ERα and ERβ. Activation of ERα can increase HPA axis activity, whereas activation of ERβ has the opposite effect (61, 64). Although ERα-mediated activity modulates OTR transcription, ERβ-mediated activity has been found to alter *Ot* mRNA levels (65, 66). Moreover, androgen modulation of OT appears to be mediated in part by the testosterone metabolite 3β-diol, which activates ERβ to allow binding to the *Ot* promoter to increases *Ot* mRNA (67).

ESTROGEN RECEPTOR β AND OXYTOCIN INTERACTIONS IN REGULATION HPA AXIS AND ANXIETY-RELATED BEHAVIORS

Activation of ERβ reduces HPA axis activity, as seen by reductions in ACTH levels and CORT levels, in mice (68) and rats (60, 69, 70) following a stressor. ERβ receptors are expressed widely throughout the brain and often overlap with ERα expression (71), except in the PVN of rats where only ERβ is expressed (72). Interestingly, approximately 85% of OT neurons in the PVN co-express ERβ (72), and activation of ERβ within the PVN, with the ERβ-specific agonist diarylpropionitrile (DPN) or testosterone metabolite 3β-diol, reduces HPA axis activity following

restraint stress in rats (61, 73). Treatment with estradiol increases *Ot* mRNA expression in the brains of wild-type mice, but not in ERβ knockout (ERβKO) mice in both males (65) and females (66). This ERβ-mediated increase in *Ot* mRNA was specific to the PVN and not seen in the MPOA, SON (65), medial amygdala, or VMH (66).

The substantial overlap in the distribution of ERβ and OT in the PVN suggests a potential interaction between the two in the regulation of HPA axis activity. As previously discussed, activation of ERβ reduced HPA axis reactivity and anxiety-like behaviors in rats and mice (64, 68, 70). ICV treatment with OTA, however, blocked the ERβ agonist-mediated reduction of anxiety-related behaviors and CORT secretion (70), suggesting interaction between ERβ signaling pathways and OTergic pathways in the control of anxiety-related behaviors and HPA axis reactivity in stress. Currently, the mechanisms involved in the crosstalk between these two pathways are not completely understood.

Recent studies have begun to investigate the complex interaction between ERβ and the *Ot* promoter. Using a mouse hypothalamic cell line expressing ERβ and OT, Sharma et al. (74) demonstrated *Ot* promoter occupancy by ERβ. The *Ot* promoter has a composite hormone response element, which allows for steroid receptor binding and *Ot* gene transcription regulation by ERs and other members of the nuclear receptor family but not the other steroid hormone receptors (75). Treatment of a neuronal cell line with the ERβ agonists, 3β-diol, DPN, or estradiol, elicited increases in *Ot* mRNA levels and *Ot* promoter occupancy (67, 74). In tandem with ERβ occupancy of the *Ot* promoter, cAMP response element-binding protein (CBP) and steroid receptor coactivator (SRC)-1 were found to occupy the *Ot* promoter, leading to increased acetylation of histone H4 in the presence of 3β-diol. Taken together, the data suggest that in the presence of 3β-diol, ERβ binds the *Ot* promoter and recruits ligand-dependent coactivator SRC-1, which binds CBP, and forms a functional complex that acetylates histone H4 to drive *Ot* gene expression (74). The role of ERβ related to OT signaling at the molecular level and its larger role in OT signaling throughout the brain are summarized in **Figure 1**. Further studies are needed to determine the extent of the binding of ERβ to the *Ot* promoter, the co-activators recruited, and how this interaction modulates HPA axis function *in vivo*.

CONCLUSION

Oxytocin has a wide range of roles in the brain and allows interesting and important directions for research. Current data suggest that the OT neurons of the PVN provide the principal OTergic innervation of the forebrain. The function of OT, through OTRs, is regionally specific; however, the localization of OTRs varies across species, age, and sex, so separating the effect of these variables is necessary to determine how animal studies translate to humans. Modulators of the OT system, particularly the steroid hormones, also provide additional regulatory targets since OT modulates HPA axis reactivity and participates in many diverse functions. In particular, ERβ is expressed by many neurons of the PVN, and ERβ activation increases OT synthesis and reduces anxiety and neuroendocrine responses in animals. Hence, such targets may be fruitful directions for future focus.

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Protein Kinase A and Anxiety-Related Behaviors: A Mini-Review

Margaret F. Keil[1], George Briassoulis[1,2], Constantine A. Stratakis[1] and T. John Wu[3]**

[1] Section on Endocrinology and Genetics, Eunice Kennedy Shriver National Institute of Child Health and Human Development (NICHD), National Institutes of Health (NIH), Bethesda, MD, USA, [2] Department of Pediatric Intensive Care, University of Crete, Heraklion, Greece, [3] Department of Obstetrics and Gynecology, Center for Neuroscience and Regenerative Medicine, Uniformed Services University of the Health Sciences, Bethesda, MD, USA

This review focuses on the anxiety related to cyclic AMP/protein kinase A (PKA) signaling pathway that regulates stress responses. PKA regulates an array of diverse signals that interact with various neurotransmitter systems associated with alertness, mood, and acute and social anxiety-like states. Recent mouse studies support the involvement of the PKA pathway in common neuropsychiatric disorders characterized by heightened activation of the amygdala. The amygdala is critical for adaptive responses leading to fear learning and aberrant fear memory and its heightened activation is widely thought to underpin various anxiety disorders. Stress-induced plasticity within the amygdala is involved in the transition from normal vigilance responses to emotional reactivity, fear over-generalization, and deficits in fear inhibition resulting in pathological anxiety and conditions, such as panic and depression. Human studies of PKA signaling defects also report an increased incidence of psychiatric disorders, including anxiety, depression, bipolar disorder, learning disorders, and attention deficit hyperactivity disorder. We speculate that the PKA system is uniquely suited for selective, molecularly targeted intervention that may be proven effective in anxiolytic therapy.

Keywords: protein kinase A, anxiety, knockout mice, regulatory subunit, catalytic subunit

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****Correspondence:***
Margaret F. Keil
keilm@mail.nih.gov;
T. John Wu
twu@usuhs.edu

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OVERVIEW OF THE PKA PATHWAY

Protein kinase A (PKA) is an inactive tetrameric holoenzyme consisting of two catalytic (C) subunits each bound to a regulatory (R) subunit dimer. The four R subunits RIα, RIβ, RIIα, and RIIβ, coded by different genes, characterize the subtypes PKA-I and -II (1). Different genes also code for the four catalytic (C) subunits Cα, Cβ, Cγ, and protein kinase X (PRKX), which are expressed (like the R subunits) in a cell- and tissue-specific manner (2). When the R and C subunits form the PKA tetramer (R2C2), there is no PKA catalytic activity. Additionally, R2C2 is bound and, thus, compartmentalized within the cell by A-kinase anchoring proteins (AKAPs) that direct PKA-signaling to specific cell regions and/or organelles (3).

Protein kinase A is considered the primary target for cyclic AMP (cAMP) in the cell, is widely distributed, and serves as the principal effector mechanism for G-protein-coupled receptors (GPCRs) linked to adenylate cyclase (4). The seven-transmembrane domain GPCRs sense molecules outside the cell and activate inside signal transduction pathways to induce cellular responses. The majority of receptors for proteins, biogenic amines, protons, hormones, neurotransmitters, and neuromodulators elicit their responses through guanine nucleotide-binding proteins (G-proteins). The G-protein α subunits, encoded by 16 distinct genes, confer receptor–effector specificity to G-proteins. The γ subunits, encoded by 12 genes, have G protein-specific recognition sites and bind tightly to β

subunits. The β subunits, encoded by five genes, also contain a common binding site for α subunit recognition. In their inactive state, the α and βγ subunits are bound to guanosine diphosphate (GDP) and can interact with effectors (**Figure 1**). This interaction releases GDP in an exchange with guanosine triphosphate (GTP) leading to the generation of α-GTP and a βγ subunit dimer (5, 6). Of the various Gα isoforms, the activation of $G_s\alpha$ stimulates adenylyl cyclase resulting in the production of cAMP. By contrast, the Giα mediates the inhibition of adenylyl cyclase (7, 8).

The increased intracellular cAMP, serving as a second messenger, binds to the regulatory PKA subunits, leading to the disassociation of the tetrameric PKA holoenzyme into an R2-cAMP 4 dimer and two monomers of free catalytic subunits (9). Then, the PKA C subunits catalyze the transfer of phosphates from ATP to serine and threonine residues of targeted intracellular proteins modifying hormonal and neurotransmitter responses, desensitizing receptors related to cortisol biosynthesis, cell differentiation, and synaptic plasticity, and activating or repressing gene transcription (10–12). The principal target of C subunits that translocate to the nucleus is phosphorylation of cAMP-responsive nuclear factors (13) that regulate the expression of genes containing cAMP-responsive elements binding proteins (CREBs) (14) (**Figure 1**). Phosphorylated CREB binds to CRE nucleotide sequences in DNA as a dimer, recruiting CREB-binding protein (CBP) and p300 cofactors to form larger transcriptional complexes, and catalyzes histone acetylation regulating target genes. Activated CREBs coordinate various neuronal functions, development, and synaptic plasticity (15). Through a feedback mechanism, activated by RIα, cytosolic phosphodiesterases (PDEs) terminate the signals generated by cAMP by hydrolyzing cAMP into 5′AMP (16). As shown in knockout (KO) mouse studies, these four genes function in a

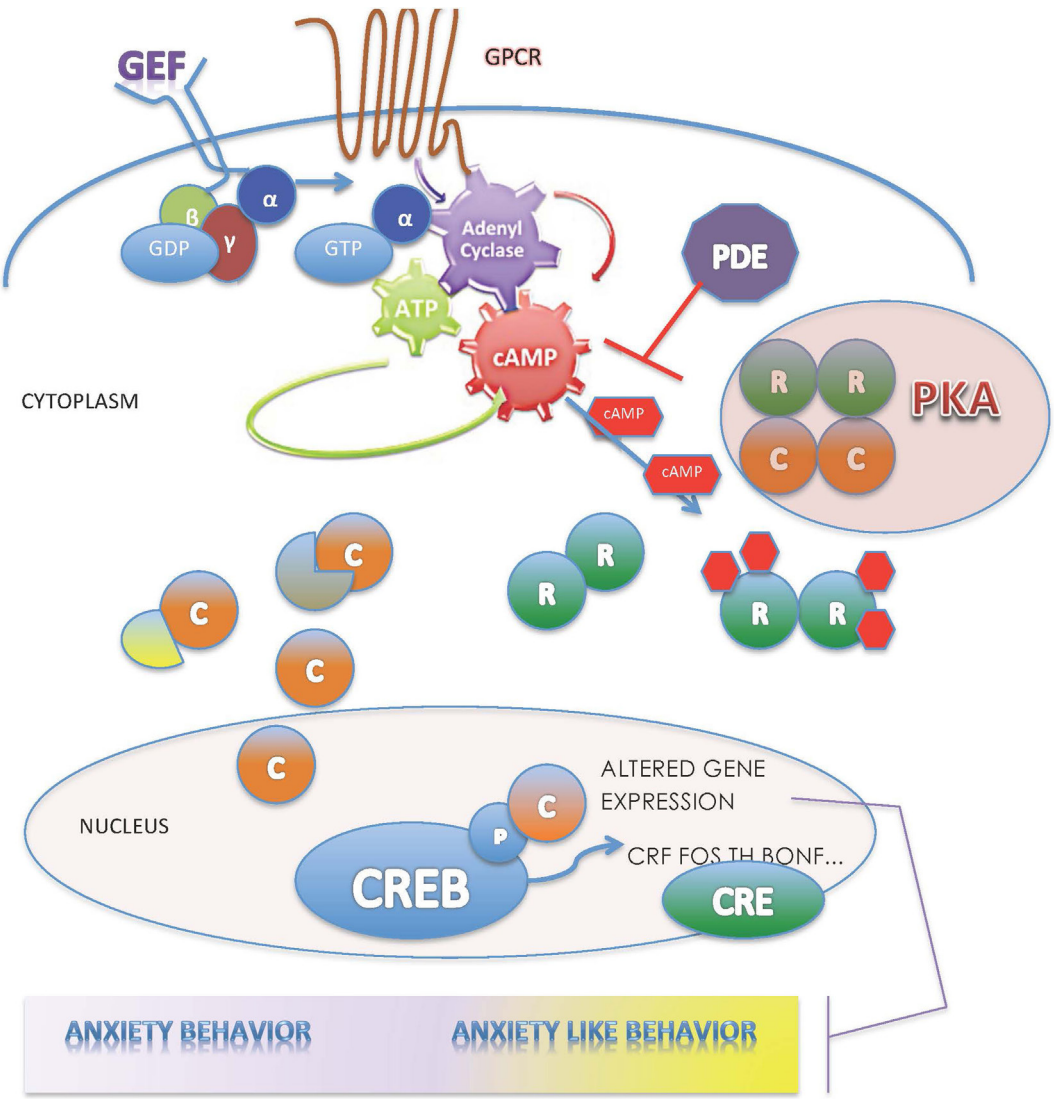

FIGURE 1 | The PKA enzyme is central in the regulation of the cAMP-signaling pathway; for description, please see the text.

tissue- and cell-type-specific manner to regulate accurately the activity of the C subunits (2).

CORTICOTROPIN-RELEASING HORMONE WITH PKA

Corticotropin (ACTH)-releasing hormone (CRH)-induced intracellular increase in calcium and cAMP (and PKA activation) through binding to its seven-transmembrane receptor activates several transcription factors including CREB, c-fos, and JunB, which subsequently activate the pro-opiomelanocortin promoter (17). ınnervation to CRH neurons is provided by fibers containing the pituitary adenylate cyclase-activating polypeptide (PACAP) (18). It is possible that stress induces secretion of PACAP in the paraventricular nucleus (PVN), thereby stimulating CRH gene expression via activation of the cAMP/PKA system (19). Especially, amygdala and lower brainstem contributions to the augmentation of the stress response have been identified as sites of pituitary PACAP innervation (19). Thus, CRH, released by stress signals, stimulates a pulsatile secretion of adrenocorticotropic hormone (ACTH) with peak levels seen before waking and declining at night.

Adrenocorticotropic hormone exerts its effects on the adrenal cortex by binding to a specific receptor (ACTHR) that is the melanocortin-2 receptor (MC2R, coded by the *MC2R* gene). MC2R is a GPCR linked to $G_s\alpha$ and, thus, ACTH binding triggers activation of adenylate cyclase that catalyzes the conversion of adenosine triphosphate to cAMP (20). The elevation of cAMP is followed by increased PKA activity at the adrenal cortex that results in increased steroidogenesis and the production of glucocorticoids (GCs). Negative feedback on pituitary ACTH secretion is exerted by cortisol at both the hypothalamic and anterior pituitary levels. Importantly, hypothalamic cAMP-inducing CRH might also counterbalance excessive stimulatory stress effects on the hypothalamic–pituitary–adrenal (HPA) axis and maintain immuno-neuroendocrine homeostasis (21).

Other peptide hormones that are secreted and function in an endocrine manner also act as ligands and signal via a wide range of GPCRs. Such peptide hormones, mostly bind within the transmembrane domain, include the growth-regulating hormones somatostatins, parathyroid hormone, angiotensin, HCRTR2, oxytocin, calcitonin, C5a anaphylatoxin, cannabinoids, follicle-stimulating hormone, gonadotropin-releasing hormone, neurokinin, thyrotropin-releasing hormone, and the cholecystokinin peptide hormone system. GPCRs are responsive to hormones, calcium, and neurotransmitters allowing them to form the largest family of validated drug targets (22).

ROLE OF PKA IN ANXIETY AND FEAR LEARNING

This review focuses on the role of the cAMP-signaling pathway and its mediator, PKA, in the pathogenesis of disorders related to fear learning and anxiety. Anxiety disorders are associated with abnormalities in neural processing of threat-related stimuli (fear learning), which is regulated by cAMP/PKA pathway.

Preclinical studies provide evidence that the pathogenesis of mood disorders, such as anxiety and depression, involves alterations in the plasticity of neuronal pathways (23, 24). Also, clinical studies demonstrate that chronic stress and depression alter brain structures (i.e., cell number and density, cell body size, neuronal and glial density in frontal cortical and hypothalamus) and that result in functional changes (25–29). In addition, studies of suicide subjects report alterations in levels of serotonin and norepinephrine, their metabolites, and receptors, in the brain and peripheral tissues, as well as intracellular signaling pathways (30, 31). Post-mortem studies demonstrate disruptions in cAMP/PKA/CREB/Rap1/BDNF in the brains of suicide subjects that are modulated by stress and GCs (32). There is a paucity of data about PKA activity in brain areas other than the frontal cortex from post-mortem brains of depressed and suicidal subjects. The cAMP/PKA signaling pathway in the central nervous system is well-characterized and has a crucial role for various physiological responses that are important for cell survival, synaptic plasticity, and gene expression (33, 34). Alterations in synaptic and structural plasticity are associated with mood disorders, including generalized anxiety disorder, depression, and suicide.

PKA and Fear Learning and Memory

There is substantial evidence from different species (fruit fly, mouse, chick, and rat) to support the role of cAMP/PKA signaling in the molecular pathways related to fear and fear memory. The seminal work of Schacher, Kandel, and Abel demonstrated the essential role of the cAMP/PKA pathway in the response to fear and consolidation of fear memory (15, 35, 36). Consistent with the evolutionarily conserved role of the cAMP/PKA pathway, anxiety and fear responses are essential to survival. The mechanisms of PKA in fear memory are well established and include a wide range of cellular processes, including activation of cAMP–CREB and various other transcription factors involved in the regulation of *de novo* protein synthesis that is required for long-term memory formation. Signaling activity in neural circuits pre- or post-stimuli may influence PKA activity and long-term potentiation (LTP), affecting fear learning and memory of the event (37–39). Also, there is a time-dependent activation profile in the kinase pathways involved in fear memory formation. PKA has two peaks of activity in the process of long-term memory formation, with the first occurring a few minutes after the event, and the second occurring 2 to 3 h after the event (requires both transcription and protein synthesis). The PKA pathway is also an important component of short-term memory within the first hour after the event. The phosphorylated form of CREB also increases at these same time periods as PKA and contributes to the synthesis of new proteins that are essential for long-term memory formation (40).

The cAMP–CREB element is ubiquitous in genes and functions as a promoter in many brain areas that respond to environmental stimuli. PKA signaling has been described as a "central hub" that interacts with varied other signaling pathways in neuroendocrine cells (41). PKA mediates and communicates cAMP effects to mitogen-activated protein kinases (MAPK) and protein kinase C (PKC) and B pathways. Signal transduction pathways, such as PKA and PKC, have important roles in the regulation of the HPA and autonomic nervous system (ANS) and, therefore, may

have a role in the expression of genes that contain cAMP in their promoters, which include key proteins that regulate the neuroendocrine stress response (i.e., brain-derived neurotrophic factor and GC receptor) (42).

Types of traumatic stress, which have been associated with maladaptive responses or psychopathology, include mass trauma, war, terrorism, natural or technological disasters, violent personal assaults, child abuse (physical, sexual, emotional), life-threatening illness, and accidents. However, not every person who is exposed to traumatic stress will develop long-lasting psychological morbidity, such as depression, anxiety, or post traumatic stress disorder. The development and/or severity of these conditions depends on multiple factors, including genetic pre-disposition to vulnerability, exposure to adverse environmental factors, and the timing of the stress exposure (43, 44).

Typically, the stress response has been identified as a "fight or flight" reaction, but may also include an increased state of vigilance, which is often accompanied by increased anxiety. The response to an environmental stressor involves the individual's interpretation of the threat, which is regulated by the brain. The brain and nervous system demonstrate adaptive plasticity through local neurotransmitters and systemic hormones, which interact to produce structural and functional changes (45).

The brain is also a target for the actions of stress hormones, in particular, GCs. With stress exposure, the PVN in the hypothalamus releases CRH and arginine vasopressin, which stimulate the anterior pituitary to release ACTH, which stimulates the adrenal cortex to release GCs. GCs exert a negative feedback to the hypothalamus and anterior pituitary to downregulate the stress response through their receptor (GR) which is found expressed highly in the hippocampus, amygdala, and prefrontal cortex. This facilitates the formation of memories associated with strong emotions particularly during stress.

PKA and Anxiety

Anxiety is an adaptive response to a potential threat that serves a protective function. However, pathological anxiety is associated with abnormalities in fear learning or threat detection (46–49) and a bias to interpret ambiguous situations as threatening with corresponding behavioral responses of avoidance or exaggerated reactions to potential threats (50). Fear memories can form quickly and be difficult to eliminate (51, 52). Evidence from experimental and preclinical studies provides support that anxiety disorders are associated with abnormal neural processing of threat-related stimuli, which is mediated by the PKA pathway.

The amygdala, located in the temporal lobe of the brain, has a crucial role in the processing and expression of emotional stimuli (53, 54). Prior studies with humans and laboratory animals provide evidence that novelty and fear-related stimuli are both processed by the amygdala (55–58). Hyperactivity of the amygdala as demonstrated by functional neuroimaging studies in humans, has been identified as a neural correlate for clinical symptoms seen in post traumatic stress disorder (59, 60), which suggests that amygdala dysfunction may be a risk factor for development of affective stress-related disorders (61). The basolateral amygdala is identified as a hub through which sensory information is relayed either directly or indirectly via the basal nucleus to the central amygdala (CEA), which is the major efferent source that directs fear-related behavioral response (51, 62).

The amygdala has a crucial role in the modulation of attention orientation to potential threats (63, 64). Activation of β-adrenoreceptors in the BLA enhances memory consolidation associated with fear via the stimulation of the cAMP/PKA pathway (65). Lesion and agonist/antagonist studies demonstrate the critical role of the BLA in mediating the effects of stress hormones on memory consolidation of fear-related stimuli. During threat processing, the prefrontal cortex is also engaged, although more gradually than the brisk response of the amygdala, which allows flexible modulation of amygdala-based processing by providing a more detailed representation of threat attributes (63, 66). Ghosh and Chattarji (67) recently reported that targeted activation of cAMP–PKA signaling in the lateral amygdala led to generalized fear, which provides novel insight of the cellular basis in the amygdala for the alteration of emotional states from normal to pathological fear.

Naturally, the endogenous PKA inhibitor (PKI) peptide participates in the regulation of PKA by binding to the free catalytic PKA subunit, thus preventing phosphorylation of PKA targets in various tissues and cell types. In addition, PKA signaling has been investigated using pharmacological PKIs, such as the H89 and KT5720 (68). These compounds, readily crossing the cell membranes, block PKA actions through competitive inhibition of the ATP site on the PKA catalytic subunit. Also, the introduction of a non-functioning PKA mutant, such as a dominant negative version of PKA into cells has allowed researchers to perturb specific signaling through PKA and to examine PKA's role in cell anchorage and protein expression in epithelial cells (69). Transfection of cDNA prevents binding of the R subunits to AKAPs also preventing PKA signaling and its localization to specific cellular organelles. Finally, among a number of other existing methods, PKA activity and signaling has been investigated in mice with genetic manipulation of the PKA system. These studies have allowed for particular investigation of various aspects of PKA signaling in organic systems and areas, focusing on altered physiology in intact animals. It has been anticipated that, by producing physiological changes, these mouse models might profitably be modulated for therapeutic purposes.

EFFECT OF INHIBITION OF PKA PATHWAY ON ANXIETY BEHAVIOR

Studies using inhibitors or activators of PKA helped to elucidate its role in memory formation. Inhibition of protein synthesis or PKA activity blocks LTP in the hippocampus and interferes with memory consolidation for fear in the amygdala (70–72). Also, infusion of PKIs into basolateral amygdala immediately following fear-conditioning training dose-dependently blocked consolidation of fear memory (24-h post training) but not short-term memory (4-h) (73). Infusion of inhibitor Rp-cAMP into the CEA decreased CREB function and decreased neuropeptide

Y expression and provoked anxiety-like behavior and alcohol intake in non-preferring rats (74).

In addition, studies using PDE4 inhibitors help to elucidate the molecular mechanisms involved with the behavioral response (anxiolytic-like) to inhibitors of PKA, which depend in part on neurogenic action and activation of GC receptor in the hippocampus (75, 76). There is ample data to support the role of the cAMP/PKA pathway in the mediation of antidepressant/anxiolytic activity (i.e., rolipram, fluoxetine, and clozapine increase cAMP and pCREB expression) in the hippocampus; however, our understanding of the specific effects on neurogenesis is evolving.

Studies with $G_s\alpha$ (*Gnas*) transgenic mice have shown that increased cAMP signaling is associated with an anxiety-like phenotype (77). Zhang et al. (78) reported that mice with reduced PDE 4B activity, the enzyme that degrades cAMP and interrupts the negative feedback of PKA pathway resulting in increased PKA activity, displayed anxiogenic behavior. In addition, transgenic mice with overexpression of the striatially enriched cAMP-producing adenyl cyclase 5 showed increased anxiety-related behavior (79). Results of the studies reviewed above indicate that increased cAMP signaling is associated with an anxiety-like phenotype, and provide indirect evidence that an increase in PKA activity may be associated with an increased risk for anxiety. Also, studies of mice with genetic deletion of specific PDE4 subtypes have reported anxiogenic behavior, suggesting that PDE4 may be involved in the regulation of anxiety (78, 80, 81).

EFFECT OF PKA DEFECTS ON ANXIETY-LIKE BEHAVIOR

Prior studies in our lab showed that transgenic mice with a downregulated *Prkar1a* gene (*tTA/X2AS*, antisense transgene) (82) exhibited behavioral abnormalities, including anxiety (83) and depression. A KO mouse heterozygous for a null allele of *Prkar1a* was developed in our lab as a model to investigate Carney complex that is caused by heterozygous inactivating *PRKAR1A* mutations (84), which results in increased PKA signaling in all cells where this gene is expressed. We hypothesized that a transgenic mouse model with downregulation of *Prkar1a* would provide a research tool to evaluate the effect of altered PKA expression on anxiety-like behaviors.

In support of our hypothesis, we found that downregulation of the regulatory subunit of PKA in mice led to an augmentation of anxiety-like behavior supporting the role of PKA in modulating anxiety-like behaviors. Compared with WT mice, *Prkar1a*$^{+/-}$ mice had higher basal and stimulated (cAMP) PKA activity levels in the central and basolateral amygdala, brain areas known to have a critical role in the processing of sensory information related to anxiety and emotion as well as regulation of arousal level (85).

Since activity in neural circuits prior to or immediately after stimuli may influence PKA activity and LTP and, therefore, may affect fear learning; we then investigated the rodent defensive response in the *Prkar1a*$^{+/-}$ mouse hypothesizing that *Prkar1a*$^{+/-}$ mice would exhibit an atypical response to threat detection (37, 39). As predicted, we found that in contrast to the response of WT mice, *Prkar1a*$^{+/-}$ mice failed to exhibit behavioral changes (exploratory or defensive) to distinguish between predator versus control odor. The behavioral changes paralleled significant differences found in PKA activity between WT and *Prkar1a*$^{+/-}$ mice in the amygdala, prefrontal cortex, and ventromedial hypothalamus (86). Our findings are consistent with results of electrophysiological studies showing that changes in amygdala circuitry and dendritic morphology affect fearful responses and correlate with BLA transmission and that the degree of anxiogenic effect of predator stress is positively associated with the degree of potentiation of amygdala circuitry (48, 87, 88). Also, since the function of the prefrontal cortex is to inhibit prepotent behavioral and promote task relevant behaviors, alterations in PKA activity in the prefrontal cortex may also have contributed to the atypical response to threat detection in the *Prkar1a*$^{+/-}$ mice (86).

We introduced half-null alleles of *Prkaca*$^{+/-}$ into the *Prkar1a*$^{+/-}$ mice, hypothesizing abrogation of the excess Cα activity caused by R1α haploinsufficiency. The phenotype of *Prkaca*$^{+/-}$ mice was characterized by attenuation but not elimination of the anxiety phenotype noted in *Prkar1a* heterozygote mice. Measurement of PKA activity in various brain areas showed increased PKA activity in the amygdala in *Prkar1a*$^{+/-}$ compared with *Prkaca*$^{+/-}$ or *WT*, and in part compared with *Prkar1a*$^{+/-}$/*Prkaca*$^{+/-}$ mice. The alteration of PKA activity in these transgenic mice was not a ubiquitous effect, since PKA activity was found to be similar between heterozygotes and WT mice in some brain areas (e.g., prefrontal cortex, hippocampus, paraventricular hypothalamus, cerebellum, and neural sensory areas). These findings highlight the importance of even modest changes in PKA activity in modulating anxiety-like behaviors and also that catalytic subunit activity is not the sole determinant of PKA's cAMP-signaling effects (89, 90). It is also possible that compensatory mechanisms in remaining PKA subunits and PDE4 may be a factor in areas not showing any differences in PKA activity between *Prkar1a*$^{+/-}$ and *Prkar1a*$^{+/-}$/*Prkaca*$^{+/-}$ and *WT* or *Prkaca*$^{+/-}$ mice.

SUMMARY/CONCLUSION

In this review, we highlighted the association of abnormal neural processing of threat-related stimuli and anxiety disorders, which is significantly influenced by the cAMP/PKA pathway, among others. Animal models have helped to elucidate the molecular pathways that have an important role in anxiety; however, there are limitations, so cautious interpretation is appropriate. A recently developed mouse of R1a deficiency provides a unique model to investigate the direct effect of increased PKA activity on the acquisition and expression of learned fear.

Results of clinical studies support the finding that alterations in PKA and some of its substrates are associated with various psychiatric disorders, including anxiety, depression, obsessive–compulsive and bipolar disorders, schizophrenia, and panic disorder (91–97). Also, in adult patients with *PRKAR1A* mutations, we reported an increased incidence of psychiatric disorders, including anxiety, depression, and bipolar disorder (in that order), and for children with *PRKAR1A* mutations

an increased incidence of learning disorders, attention deficit hyperactivity disorder, anxiety, and depression (in that order) (98). Recent animal studies support the hypothesis that selective gene intervention in the cAMP/PKA system may constitute a promising anxiolytic target.

AUTHOR CONTRIBUTIONS

All authors contributed equally to the writing (MK and GB) and editing (MK, GB, CS, and TW) of this manuscript.

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Brain circuits mediating the orexigenic action of peripheral ghrelin: narrow gates for a vast kingdom

Agustina Cabral, Pablo N. De Francesco and Mario Perello*

Laboratory of Neurophysiology, Multidisciplinary Institute of Cell Biology [Argentine Research Council (CONICET) and Scientific Research Commission, Province of Buenos Aires (CIC-PBA)], Buenos Aires, Argentina
**Correspondence: marioperello@yahoo.com; mperello@imbice.gov.ar*

Edited by:
Carol F. Elias, University of Michigan, USA

Reviewed by:
Jacques Epelbaum, INSERM, France
Sebastien G. Bouret, University of Southern California, USA

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The nervous and endocrine systems act together to regulate all physiological processes essential for the body homeostasis control. Given the strict communication restrictions that the brain–blood barrier (BBB) imposes, the interplay between these two systems requires a variety of delicate anatomical interfaces and physiological mechanisms that guarantee the precise function of the neuroendocrine system as a whole. The study of the mechanisms by which hormones act in the brain in order to regulate specific neuronal populations is a research topic rather neglected. Our group studies the neuronal circuitries and molecular mechanisms by which the stomach-produced hormone ghrelin regulates appetite and other physiological functions. A clear notion of the brain targets of peripheral ghrelin is essential for the comprehensive understanding of the physiological role of this hormone. Ghrelin is called "the hunger hormone" since it is the only known orexigenic peptide hormone. The target for ghrelin orexigenic actions is the brain, which contains a variety of ghrelin-responsive nuclei; however, several evidences suggest that the accessibility of peripheral ghrelin to the brain is strikingly low. Here, we briefly summarize the current knowledge in this topic and discuss this intriguing neuroendocrinological issue.

Ghrelin is a 28-amino acid octanoylated peptide predominantly secreted from endocrine cells located within the gastric mucosa (1). Ghrelin acts via the growth hormone secretagogue receptor 1A (GHSR-1A), a G-protein-coupled receptor highly expressed in the brain (2, 3). Ghrelin's central actions include modulation of the growth hormone secretion, blood glucose homeostasis, stress responses, and gastrointestinal tract motility, among others (4). Notably, ghrelin is recognized as the only mammalian peptide hormone able to increase appetite (5, 6). Ghrelin orexigenic effects are rapid, since food intake increases within 5–10 min after its systemic administration (7). Thus, the ghrelin brain's accessibility must be relevant for this unique role of the hormone. Direct ghrelin micro-injections, ranging from 10 to 800 pmol, in several brain areas potently increase food intake (6). Ghrelin-induced food intake recruits neuronal circuits, located in the hypothalamus and the brainstem, which regulate appetite depending on the energy store levels (6). In particular, ghrelin orexigenic actions depends on the hypothalamic arcuate nucleus (ARC), which highly expresses GHSR-1A and is located in close apposition to the median eminence (ME), an important circumventricular organ (3, 8). Ghrelin-induced food intake also occurs at other GHSR-1A-expressing hypothalamic areas that lack obvious access to circulating ghrelin, such as the paraventricular nucleus, the lateral hypothalamus, and the ventromedial nucleus (9–11). Ghrelin orexigenic actions also take place at the dorsal–vagal complex, which expresses GHSR-1A and includes the nucleus of the solitary tract, the dorsal motor nucleus, and the area postrema (AP), another important circumventricular organ (3, 12). In addition, ghrelin regulates appetite and some rewarding aspects of eating by directly acting on the ventral tegmental area and other centers of the mesolimbic pathway, which also express GHSR-1A (13, 14). Thus, the ghrelin-induced food intake depends on the ability of peripheral ghrelin to impact on these distributed brain targets.

Peptide hormones cannot freely enter the brain. The BBB displays specific transport mechanisms that can serve for particular peptides to gain access into the brain through an otherwise impermeable boundary. This transport mechanism can include (a) receptor-mediated transcytosis, (b) non-specific binding of positively charged peptides to the cell membrane and further transcytosis, (c) diffusion of molecules through the so-called extracellular pathway, or (d) free transmembrane diffusion, as seen for some small peptides (15). The fenestrations of the specialized capillaries of circumventricular organs allow for small peptides to penetrate the intercellular space and eventually diffuse toward neighboring brain areas (16). Notably, the ventromedial ARC represents an exceptional case of privileged permeability to blood-borne factors due to presence of a fenestrated vasculature branching from the ME (17). Peptide hormones can also reach the brain via the cerebrospinal fluid (CSF) after crossing the blood–CSF barrier at either the choroids plexus, a specialized layer of cuboidal ependymal cells that surround a core of capillaries in some brain ventricles and produce the CSF, and/or the hypothalamic tanycytes, a specialized layer of bipolar ependymal cells that line the floor of the third ventricle and bridge the CSF and the capillaries of the ME (18, 19). Additionally, some hormones signal to the brain by acting on sensory circumventricular

organs, such as the subfornical organ or the AP, which detect plasma hormone levels and transmit such information into specific brain regions (20). Currently, little is known in terms of which of these mechanisms underlie central orexigenic ghrelin actions.

In order to clarify the brain areas mediating orexigenic effects of peripheral ghrelin, we have recently performed a detailed neuroanatomical analysis in mice of both the distribution of ghrelin-induced increase of the marker of cellular activation c-Fos and the brain areas accessible to fluorescent ghrelin (8). We used a high (0.6 nmol/g BW) and a low (0.06 nmol/g BW) doses of ghrelin that induce a ~17- and 2-fold increase of plasma ghrelin concentrations 30 min after treatment, respectively. Interestingly, we found that the smaller increment of circulating ghrelin, which is sufficient to increase food intake, exclusively impacts the ARC/ME while the higher increment of circulating ghrelin increases c-Fos expression in and access not only the ARC but also the AP. In addition, the higher increment of circulating ghrelin accesses the periventricular hypothalamic regions and induces c-Fos expression in a few extra-brain areas such as the paraventricular nucleus and the nucleus of the solitary tract. Notably, fluorescent ghrelin was detected in tanycyte-like cells of the ME in all mice peripherally treated with the tracer. Ghrelin internalization in tanycytes has been recently confirmed *in vivo* and shown to also occur *in vitro* (21). Importantly, we showed that centrally administered ghrelin reaches and increases c-Fos levels in most of the GHSR-1A-expressing brain areas (8, 22). In addition, we found that ARC-ablated mice fail to eat in response to peripherally administered ghrelin but fully respond to the orexigenic effects of the centrally administered hormone. Thus, our data support the notion that the ARC is the main target of the orexigenic effects of peripheral ghrelin in mice.

Our study stresses the fact that the accessibility of peripheral ghrelin to the brain is surprisingly limited and restricted to specific brain areas. **Figure 1** summarizes our observations in terms of the brain accessibility of peripherally administered fluorescent ghrelin, and describes the potential mechanisms mediating this phenomenon. A limited ghrelin brain accessibility is in line with a seminal study showing that radioactive ghrelin is transported across the BBB in the brain-to-blood direction by a saturable system while blood-to-brain influx is extremely low (23). In addition, very limited amounts of peripherally injected ghrelin can be detected in the CSF of ewes (24). Our study adds neuroanatomical insights to these reports by showing that the ghrelin accessibility mainly occurs at the ARC/ME and, to a lesser extent, the AP. Thus, peripheral ghrelin seems to mainly reach and activate brain areas located close to some circumventricular organs, which would allow the access of the circulating hormone to the brain. In this regard, a recent study has shown that ghrelin passively and rapidly extravasates through fenestrated capillaries of the ME and reaches nearby brain regions (16). A higher accessibility at the ARC/ME has been also shown for other peptide hormones, including insulin and leptin (25, 26). Peripheral ghrelin could impact on specific neuronal circuits by acting at the subfornical organ, which expresses GHSR-1A; however, this pathway is not related to food intake regulation (20). The presence of fluorescent ghrelin in both the tanycytes and the choroids plexus (unpublished observation) and the hypothalamic periventricular regions of peripherally injected mice suggest a potential ghrelin transport from the periphery to the CSF that could impact on food intake regulation. Interestingly, it has been proposed that tanycytic could take up circulating leptin from the ME and transport it toward the apical cell pole in contact with the CSF (27). Further studies are required in order to test if ghrelin can access the brain via this mechanism. Notably, the different profile of c-Fos induction and fluorescent ghrelin distribution found for the lower and higher doses of ghrelin may indicate that different mechanisms of entry to the brain can take place depending on plasma hormone levels. The observation that very high increases in plasma ghrelin level are not sufficient to access and/or activate deeper brain areas known to express GHSR-1A support the notion that the transport of ghrelin through the BBB in a blood-to-brain direction is extremely limited in mice. Importantly, our study was performed using a single bolus of fluorescent ghrelin and 15 min after injection. Thus, our data do not invalidate that ghrelin may act on other brain areas through slower mechanisms either after a sustained increase of the hormone concentration or over prolonged time periods. However, these potential slow ghrelin-responsive pathways unlikely mediate the rapid orexigenic effects of the hormone. Interestingly, a recent article reported that mice fail to increase food intake in response to 0.0075 nmol/g BW of ghrelin, which increases ~14-fold plasma ghrelin concentrations 10 min after treatment, and concluded that supraphysiologic plasma ghrelin levels are required in order to stimulate appetite (28). The lack of an orexigenic response may be related to the transient nature of the ghrelin peak induced in these conditions, given the short half-life of the hormone (29). Still, these data also stress the limited brain response, in terms of food intake, to huge increases in plasma ghrelin levels.

Overall, it seems clear that most of the neuronal circuits known to regulate food intake are sensitive to ghrelin. However, the physiological relevance of the peripheral ghrelin signaling on these targets is unclear given the limited brain accessibility of hormone. The possibility that some of these neuronal circuits are engaged by centrally produced ghrelin has been disregarded since it seems now clear that ghrelin is not synthesized in the brain (30, 31). One possibility is that the ghrelin brain accessibility could be regulated under particular physiological states. Indeed, the extent to which the diffusion of molecules in the ARC/ME occurs can be regulated in circumstances, such as fasting, by modulating the amount of vascular fenestrations as well as by reorganizing the structure of the tight-junctions between tanycytes and limiting the diffusion of molecules to the CSF (32). In terms of ghrelin, it has been shown that the rate at which this hormone is transported into the brain is reduced in physiological states, such as obesity or neonatal overnutrition (21, 33). It has been also proposed that GHSR-1A can act in a ghrelin-independent manner since this receptor is able to both signal in the absence of its ligand (34) and heterodimerize with other G-protein-coupled receptors in order to allosterically modulate their

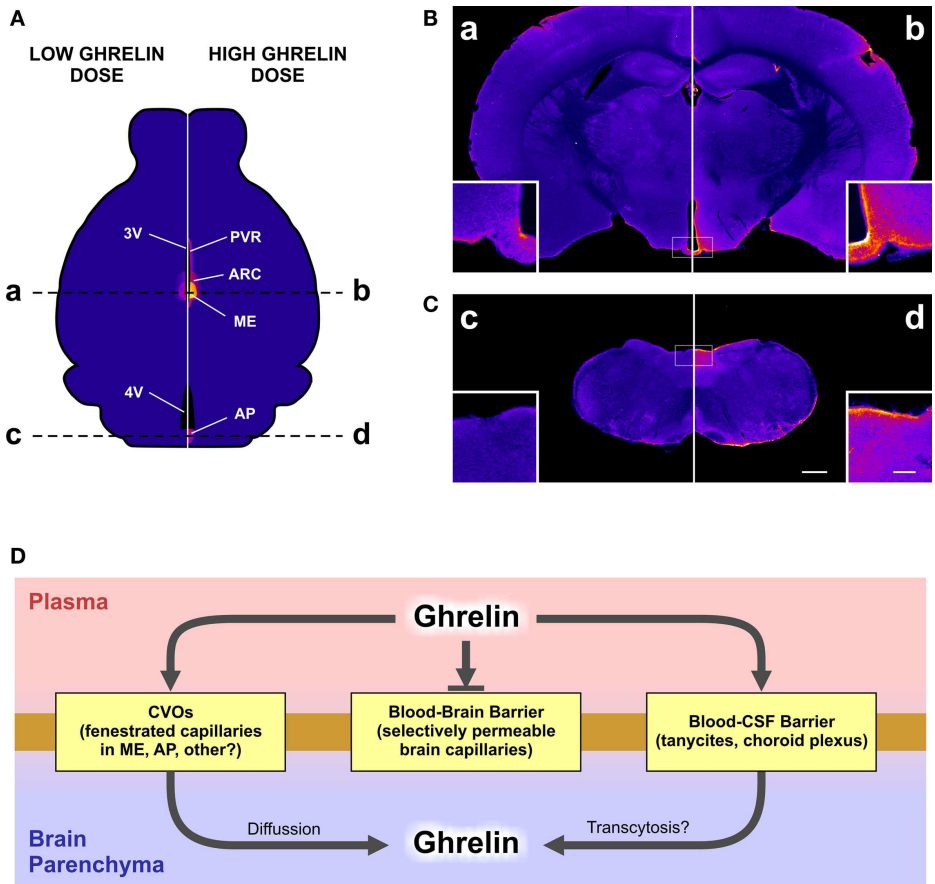

FIGURE 1 | The figure depicts the differential brain accessibility of a high or a low dose of peripherally administered ghrelin and the potential mechanisms mediating ghrelin brain accessibility. In our study, a set of mice were subcutaneously injected with a high (0.6 nmol/g BW) or a low (0.06 nmol/g BW) dose of fluorescent ghrelin and perfused 15 min later. Brains were removed, post-fixed, cryoprotected, and coronally cut. The neuroanatomical mapping of the presence of the ghrelin tracer was performed by the analysis of the amplified fluorescein-immunoreactive signal achieved by an anti-fluorescein antibody followed by biotinylated secondary antibody, streptavidin–peroxidase, and a chromogenic reaction. Stained brain sections were mounted and bright-field images were acquired with a digital camera at 16 bit/pixel. Finally, pixel intensity values were converted into optical density data by taking the negative decimal logarithm of the original value divided by the white flat field value and pseudocolored. Yellow to blue coloring represents higher to lower levels of fluorescein-immunoreactive signal. **(A)** displays a schematic illustration representing the distribution of fluorescein signal in a mouse brain after the peripheral administration of the low (left) or the high (right) dose of the ghrelin tracer. AP, area postrema; ARC, arcuate nucleus; ME, median eminence; PVR, periventricular regions; 3V, third ventricle; 4V, fourth ventricle. **(B,C)** show the actual images of mouse coronal brain sections, at the rostro-caudal levels of the brain labeled with dashed lines in the **(A)**. Letters a, b, c, and d label corresponding brain levels and ghrelin doses between **(A)** and **(B,C)**. Insets in each microphotography show higher magnification images. Scale bars: 500 μm in low magnification, 100 μm in high magnification. **(D)** displays a schematic diagram of the potential mechanisms mediating the ghrelin brain accessibility. CVOs, circumventricular organs; CSF, cerebrospinal fluid.

activity (35). Still, the impact of the constitutive GHSR-1A signaling on the orexigenic actions of the peripheral ghrelin is unclear. As usual, the more we know, the more we realize how much we do not know. Our knowledge about the ghrelin system has notably increased lately; however, many questions remain open: which are the mechanisms governing the ghrelin brain accessibility? Can the ghrelin brain accessibility be regulated? What is the physiological role of the ghrelin-responsive neuronal circuits without obvious access to peripheral ghrelin? Given its potent orexigenic effect, the ghrelin system has been perceived as a potential pharmacological target for compounds aimed to regulate appetite. Thus, we think that research efforts should be intensified in order to solve these particular issues about the ghrelin physiology.

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