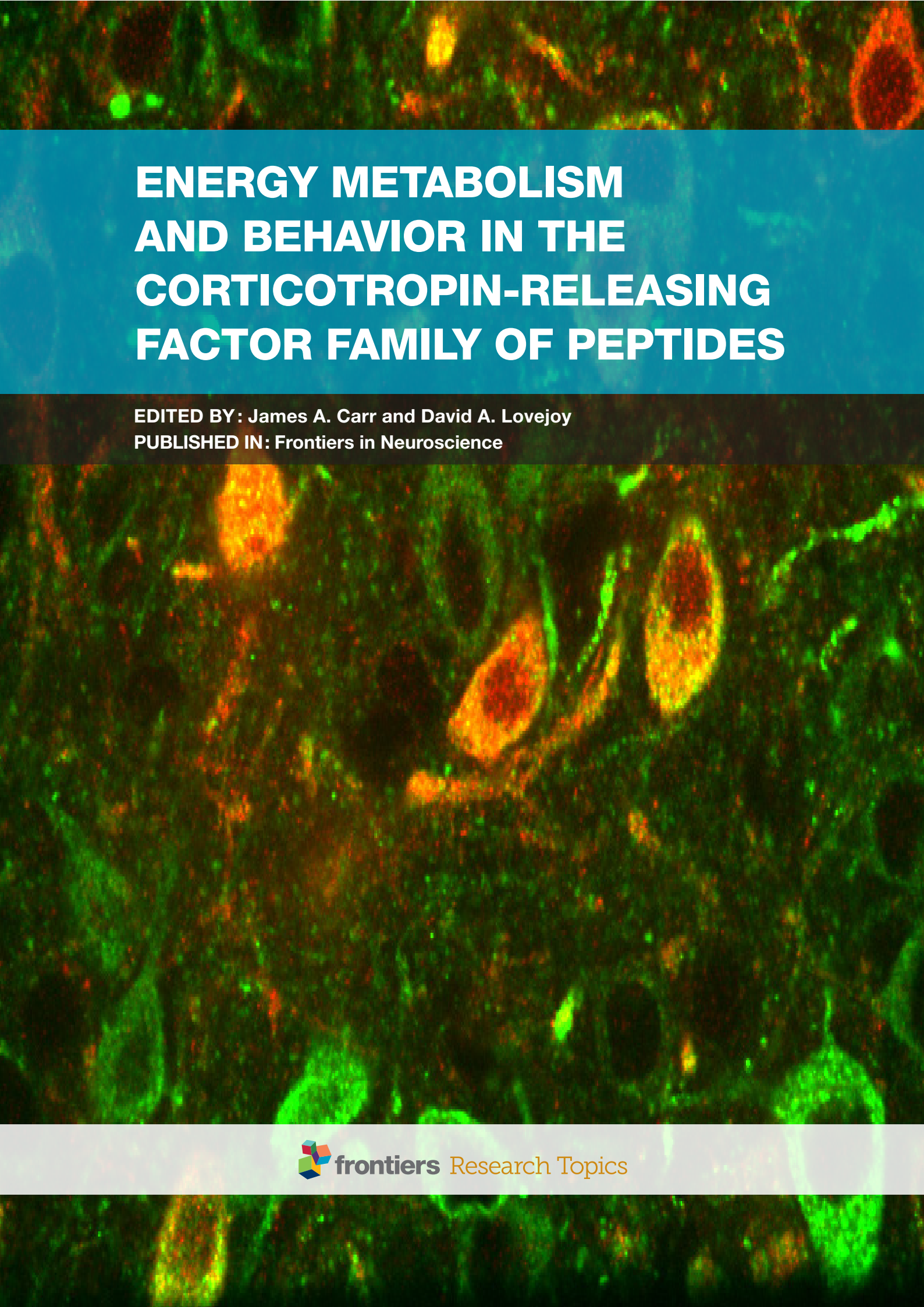

ENERGY METABOLISM AND BEHAVIOR IN THE CORTICOTROPIN-RELEASING FACTOR FAMILY OF PEPTIDES

EDITED BY: James A. Carr and David A. Lovejoy

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ENERGY METABOLISM AND BEHAVIOR IN THE CORTICOTROPIN-RELEASING FACTOR FAMILY OF PEPTIDES

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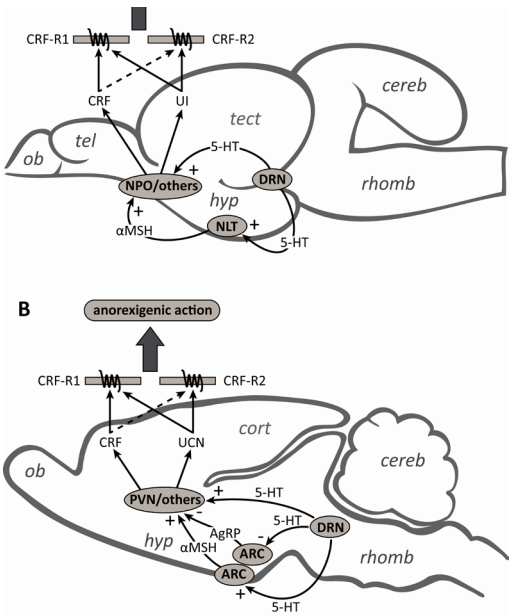

Diagrams of midsagittal sections through (A) the rainbow trout and (B) rat brains to summarize the contributions and interactions of the serotonergic and corticotropin-releasing factor(CRF) systems in the regulation of food intake. From Ortega, V.A., Lovejoy, D.A., and Bernier, N.J. (2013). Appetite-suppressing effects and interactions of centrally administered corticotropin-releasing factor, urotensin I and serotonin in rainbow trout (*Oncorhynchus mykiss*). Front Neurosci. 7:196. doi: 10.3389/fnins.2013.00196.

Cover image: Co-localization of CRF (red) and glutamic acid decarboxylase (green) in the bed nucleus of the stria terminalis of the rat. From Dabrowska, J., Hazra, R., Guo, J.D., Dewitt, S., and Rainnie, D.G. (2013). Central CRF neurons are not created equal: phenotypic differences in CRF-containing neurons of the rat paraventricular hypothalamus and the bed nucleus of the stria terminalis. Front. Neurosci. 7:156. doi: 10.3389/fnins.2013.00156

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EDITORIAL
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Energy metabolism and behavior in the corticotropin-releasing factor family of peptides

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Keywords: hypothalamus, stress, evolution, anorexia, anxiety, fishes, mammals, CRF

This year, 2015, will mark the sixtieth anniversary of the seminal work by Guillemin and Rosenberg (1955) and Schally and Saffran (1955) which, along with the earlier work from Geoffrey Harris' lab, initiated the search for an adrenocorticotropin releasing factor that culminated with the discovery of corticotropin-releasing factor (CRF) in 1981 by Wylie Vale's laboratory (Vale et al., 1981). Since the 1980s, the CRF story has had many twists and turns from the finding that CRF and its receptors are located in many extra-hypothalamic brain areas and extending to the discovery that two genome duplications expanded the CRF peptide family hundreds of millions of years ago. Because of the early metazoan ancestry of CRF, this peptide has become ensconced in a number physiological processes. We now know that stress in vertebrates is comprised of a complex set of physiological actions that regulate organismal metabolism to promote protection of the individual and progeny from life-threatening situations. This stress response likely evolved before the earliest multicellular organisms yet has been retained throughout metazoan evolution. Vertebrates, in particular, have developed some of the most complex stress-regulating mechanisms. Integral to this stress response is CRF, and its paralogue, urocortin (urotensin-I/sauvagine) and a parallel paralogous lineage consisting of urocortin 2 and 3.

Six review papers in this volume summarize some of the latest research on the role of CRF and urocortin (UCN) peptides in modulating behavior during stress. In addition, two new research studies present data supporting an ancient role for CRF/UCN receptors in social behavior and feeding, while two additional papers explore the role of extrahypothalamic CRF neurons in behavioral and endocrine responses.

A consistent theme running through this set of papers is that many of the effects of CRF/UCN peptides on physiology and behavior are evolutionarily conserved. In their contribution, Chen et al. (2013) examine the evolutionary origins of the tenuerin C-terminal associated peptides (TCAP), peptides which may have predated the origin of the CRF peptide family. Chen et al. (2013) review data on the neuromodulatory actions of TCAP-1 and both its direct and indirect effects as an indirect modulator of CRF intracellular signaling.

Feeding and intake of nutrients is essential for the success of any species. A stress-response, as regulated by the CRF family of peptides, generally has suppressive actions on appetite and digestion in order to shunt energy from a parasympathetic need to arousal and sympathetic requirements. From an evolutionary point of view this may be an adaptive response when an animal is threatened by a threat in its environment, however there are obvious energetic costs if anorexia is prolonged. As the papers by Matsuda (2013) and Ortega et al. (2013) indicate, CRF inhibition of appetite is a phylogenetically ancient response based on data in fishes. Matsuda (2013) summarizes the data on CRF and appetite in fish and points out the complexities of dissociating specific effects on appetite from the broader impact of CRF peptides on anxiety and locomotor behavior. Ortega et al. (2013) present new data in rainbow trout (*Oncorhyncus mykiss*) suggesting that the anorexic effects of

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serotonin may be mediated by CRF- and/or urotensin I receptors. Stengel and Taché (2014) provide an extensive and critical look at CRF/UCN peptide signaling via CRF R1/2 receptors in feeding behavior in mammals. These authors not only summarize the latest studies on the central actions of CRF peptides related to food intake in the context of stress adaptation but also look at the interesting possibility that peripheral receptors also may be involved. Stengel and Taché (2014) also summarize studies on CRF receptor single nucleotide polymorphisms (snps) that are associated with obesity.

Emerging evidence from comparative and preclinical studies indicates that CRF may play an adaptive role in modulating social behavior. In vertebrates, social stress is one of the greatest forms of stressors. Prolonged and intense social stress can affect nutrient acquisition and reproduction thus compromising both the health of the individual and the population. In their paper, Solomon-Lane et al. (2013) review the literature linking the hypothalamus-pituitary-interrenal axis to socially-mediated sex change in fishes and provide new data on the role of socially mediated sex change in the blue banded goby (*Lythyrpnus dalli*). Backström and Winberg (2013) take a broad comparative approach to examine the role of the HPI/HPA axis on social stress while Hostetler and Ryabinin (2013) provide an extensive review of the role (s) that central CRF pathways play in social and particularly, reproductive and courtship behavior. Interestingly, a number of authors have commented on the close relationship between the CRF system and the regulation of 5-HT, a neurotransmitter whose evolutionary origins predate multicellular organisms.

Elaboration of the central nervous system is a hallmark of vertebrate morphology and success. It is not surprising, therefore, that the CRF peptide family is associated with numerous neurological circuits regulating most behaviors. The role of CRF and its regulation of pituitary ACTH have been well covered in the past. However, several of the papers in this volume touch on the involvement of CRF/UCN peptides targeting receptors in extra hypothalamic brain areas. These studies collectively highlight the diverse phenotypes of cells producing CRF within the CNS. Fox and Lowry (2013) review the interaction between CRF- and 5-HT-producing neurons in the dorsal raphe nucleus, the major site for 5-HT production within the brain. Dabrowska et al. (2013) provide evidence that CRF neurons in the BNST and PVN are phenotypically different based upon their physiological response to local neurotransmitter release and whether they also synthesize GABA or glutamate and may have either inhibitory or excitatory actions, respectively. Callahan et al. (2013) use RNAi techniques to locally knockdown CRF expression in the central nucleus of the amygdala. Interestingly, disruption of CRF synthesis in this brain area interferes with stress-induced HPA activity and the expression of anxiety-like behaviors during stress.

In summary, papers within this compendium illustrate the long evolutionary history of the CRF family of peptides and their importance to organismal physiology and behavior. Despite the achievements over the last 60 years, there remains much to learn about the role of the CRF family of peptides in the physiology and pathology of vertebrates.

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REVIEW ARTICLE
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Teneurin C-terminal associated peptides (TCAP): modulators of corticotropin-releasing factor (CRF) physiology and behavior

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The existence of the teneurin C-terminal associated peptides (TCAP) was reported in 2004 after screening a rainbow trout hypothalamic cDNA for corticotropin-releasing factor (CRF)-related homologs. In vertebrates, there are four TCAP paralogs, where each peptide is associated with a teneurin transmembrane protein. The TCAPs are 40 or 41 amino acids in length and possess less than 20% residue identity with the CRF family of paralogs. Orthologs of TCAP are found in all metazoans with the possible exception of poriferans and cnidarians. Recent evidence indicates that TCAP and the teneurins may have been introduced into the Metazoa via horizontal gene transfer from prokaryotes into a basal protistan. Thus, the origin of the TCAPs likely predated that of the CRF family. In the mammalian brain, TCAP-1 is transcribed independently from teneurin-1. Moreover, TCAP-1 acts on neurons by a CRF-receptor independent signal transduction pathway to regulate cellular cytoskeletal function to stimulate cell activity. Administration of synthetic TCAP-1 to rodents inhibits a number of CRF- and stress-associated behaviors via a hypothalamic–pituitary–adrenal (HPA) axis-independent mechanism.

Keywords: stress, HPA axis, glucocorticoids, dystroglycan, ERK1/2, cytoskeleton, peptide evolution

INTRODUCTION

Corticotropin-releasing factor (CRF) is part of an evolutionary conserved peptide family integral to the regulation of stress-associated behavior and physiology in metazoans. The long period of time during which CRF-like peptides have persisted suggests that the CRF response has been evolutionarily advantageous. Early-evolving gene systems integral to the survival of an organism are evolutionarily selected for, and because of this, may give rise to additional paralogous lineages via gene, genomic, and chromosomal duplication events. In addition, such early evolving systems will likely become associated with newer evolving molecular and physiological systems. Thus, because of the evolutionary age of the CRF system, it is likely that it is modulated, in turn, by even earlier evolving systems.

One such candidate for an early evolving CRF-modulatory system may be represented by TCAP-1, a member of the teneurin C-terminal-associated peptides family. Highly conserved in all metazoans, TCAP-1 is active in the regulation of metabolism, stress, and reproduction and significantly inhibits a number of CRF-induced stress responses. Although TCAP was originally identified in a rainbow trout cDNA library screen for potential CRF homologs and share a number of structural similarities with the CRF family of peptides, they have less than 20% sequence similarity to the CRF family (Qian et al., 2004; Lovejoy et al., 2006). *In situ* experiments in Sprague-Dawley rats show that TCAP is highly expressed in all hippocampal subregions, central amygdala (CeA), basolateral amygdala (BLA), and various hypothalamic nuclei regions that are known to express CRF receptors (Wang et al., 2005). The TCAP-1 peptide also blocks both CRF-induced stress behaviors and c-fos activation within brain areas known to modulate behavioral responses to stress in Wistar rats (Tan et al., 2009). Recent studies show that TCAP exerts its neuromodulatory role on CRF elements independent of the hypothalamic–pituitary–adrenal (HPA) axis, harboring the idea that several accessory pathways may have evolved alongside the HPA axis-mediated signaling pathway.

CRF EVOLUTION AND PHYSIOLOGY

In vertebrates (Mammalia, Amphibia, Actinoptergyii, and Chondrichthyes), the CRF family consists of four paralogous peptides that are the result of two rounds of genome duplications in early evolution and has been previously described in detail (Lovejoy and Balment, 1999; Lovejoy and Jahan, 2006) (**Figure 1**). Orthologs of CRF in invertebrates occur as the diuretic hormones in arthropods, and diuretic hormone-like peptide in tunicates. These findings indicate that only a single CRF peptide was present in the genome of an ancestral protochordate. The first of the genome duplications led to the formation of two CRF-related peptides. After subsequent selection, both lineages were retained but modified during the accrual of mutations leading to a modified amino acid sequence. One peptide shares sequence similarities to both CRF and urotensin-1 (urocortin in mammals; Vaughan et al., 1995; Donaldson et al., 1996), whereas the second peptide possesses both urocortin 2 and 3 characteristics (Lovejoy and Jahan, 2006). The second genome duplication led to the formation of the four peptides that are found in extant vertebrates (Lewis et al., 2001; Reyes et al., 2001). However, the presence of CRF orthologs in arthropods indicates that the earliest CRF-like

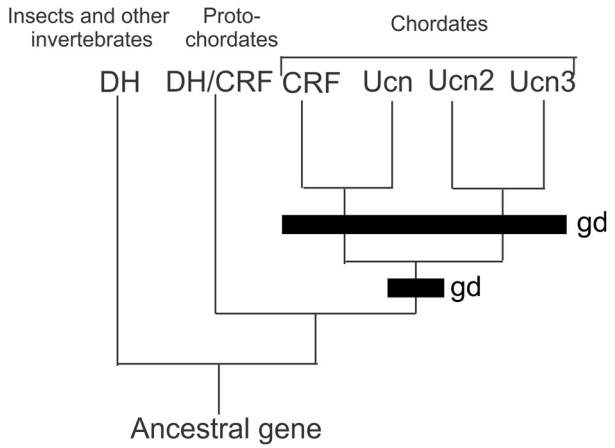

FIGURE 1 | Comparison of the CRF peptides family in chordates by genome duplication. Two rounds of genome duplications early in chordate ancestry gave rise to the four paralogous CRF related peptides. Abbreviations: DH, diuretic hormone; DH/CRF, diuretic hormone/corticotrophin releasing factor like peptide; CRF, corticotrophin releasing factor; UCN, urocortin; UCN2, urocortin 2; UCN3 urocortin 3; gd, genome duplication event.

peptides existed before the bifurcation of deuterostome and protostome animals (Lovejoy and Balment, 1999; Lovejoy and Jahan, 2006). Research in the last few decades has provided a basis for understanding the regulation of stress and metabolism by CRF. Currently, it is understood that in vertebrates, CRF activates the stress response through the HPA axis. It is also through this pathway that CRF is regulated by modulatory components of the central nervous system.

The ancient evolution of CRF and CRF-like peptides indicates that there is functional necessity of the actions of CRF on regulatory mechanisms in physiology. In many species, humans included, CRF induces behaviors and physiological adaptations that endeavor to cope with environmental stressors. Stressors that are associated with new environments, for example, generally bring about behaviors of increased arousal and decreased food intake. Changes in behaviors and physiology that are induced by CRF are mediated by interactions of CRF/Ucn with both the CRF_1 and CRF_2 receptors. Urocortin-2 and -3 mediate their functions through a separate CRF_2 receptor (Hauger et al., 2006).

In numerous vertebrates including Osteichthyes, Amphibia, Reptilia, Avia, and Mammalia species, exposure to stress leads to activation of the hypothalamic–pituitary–adrenocortical (HPA) axis (Barsyte et al., 1999; Lovejoy and Balment, 1999; Lovejoy and Jahan, 2006; Denver, 2009; Lovejoy, 2009). The HPA axis is a neuroendocrine feedback system that is activated when afferents from the sensory system and brainstem signal to the paraventricular nucleus (PVN) to secrete CRF and arginine vasopressin (AVP) hormones into the hypophyseal portal system (Tsigos and Chrousos, 2002; Ulrich-Lai and Herman, 2009). Upon reaching the anterior pituitary, CRF stimulates the corticotropes to secrete adrenocorticotropic hormone (ACTH) into the systemic circulation (Tsigos and Chrousos, 2002; Denver, 2009). This hormone in turn acts on the inner adrenal cortex to synthesize and release glucocorticoids, such as cortisol (Denver, 2009; Ulrich-Lai and Herman, 2009). Circulating glucocorticoids induce a multitude of different responses which all act to promote energy mobilization; these include gluconeogenesis in the liver, liberation of amino acids, inhibition of glucose uptake into muscle and adipose tissue, increased lipolysis and suppression of immune and reproductive functions (Ulrich-Lai and Herman, 2009). Regulation of the system occurs through negative feedback of glucocorticoids at the hypothalamus and anterior pituitary, inhibiting the release of CRF and ACTH respectively; in addition, the action of glucocorticoids on neuronal inputs to the PVN restricts activation of the HPA axis (Tsigos and Chrousos, 2002; Denver, 2009). However, the HPA system, as it is understood in vertebrates, does not appear to be present in non-vertebrates (Lovejoy, 2009). Thus, given the evolutionary age of CRF-like peptides, it has become well ensconced in numerous physiological processes predating the HPA axis. However, CRF itself has a number of non-HPA associated functions including feeding, diuresis, metamorphosis, locomotion, and vocalization (Lovejoy and Balment, 1999). These regulatory actions may have evolved to accompany stress regulation. Given the widespread actions of the CRF family of peptides, evolutionarily older physiological systems may be working alongside the CRF lineage or interacting with elements of the CRF lineage in a neuromodulatory manner in complex organisms.

TENEURIN AND THE TENEURIN C-TERMINAL ASSOCIATED PEPTIDES (TCAP)

The teneurins are a family of four type-II transmembrane proteins that are critical for morphogenesis and pattern formation in metazoans (Wang et al., 2005; Lovejoy et al., 2006, 2009; Tucker et al., 2007; Young and Leamey, 2009). Originally discovered in *Drosophila*, teneurins were later discovered in vertebrates to have four paralogs (teneurin-1 to -4) (Kenzelmann et al., 2007). Vertebrate teneurins are predominately expressed in the nervous system and have been implicated in neural development and maintenance (Lovejoy et al., 2006; Kenzelmann et al., 2007). They are typically about 2800 amino acids long with a structure that is highly conserved (Tucker et al., 2007). At the end of the C-terminus of teneurin-1 to -4 is a cleavable bioactive peptide termed the teneurin C-terminal-associated peptide (TCAP)-1 to -4. Recent studies, however, indicate that TCAP-1 is independently transcribed from teneurin-1, thus providing evidence for the independence of TCAPs from teneurins (Chand et al., 2012a).

Initial attempts to identify paralogous genes to the CRF family led to the discovery of this TCAP family (Qian et al., 2004). It was first discovered in a screening of the hypothalamic cDNA library of rainbow trout using a hamster urocortin probe (Barsyte et al., 1999; Qian et al., 2004). Sequence analyses revealed a putative peptide sequence on the 3′ region of teneurin-3 in trout and this peptide sequence was termed TCAP-3, by virtue of its association with teneurin-3 (Qian et al., 2004). Further analyses led to the discovery of the 3 other TCAP peptides on teneurins-1, -2, and -4 (Wang et al., 2005). The TCAP family is ubiquitously expressed among all metazoan species (Lovejoy et al., 2006; Tucker et al., 2012). The four vertebrate TCAP paralogs show about 80% identity with each other amongst metazoan species (Lovejoy et al.,

2006, 2009). This high level of conservation indicates that this family of peptides evolved before the protostome-deuterostome divergence and that its role implicates a functional necessity for organisms. The teneurin-TCAP system likely evolved by horizontal gene transfer from a prokaryote gene to a choanoflagellate (Zhang et al., 2012), where subsequently this gene integrated within the choanoflagellate genome and was passed into subsequent metazoan speciation (Tucker et al., 2012; Chand et al., 2013; Tucker, 2013). Moreover, there is 13–30% similarity with various peptides and the CRF family of peptides (Wang et al., 2005; Lovejoy et al., 2006) (**Figure 2**). This level of sequence similarity, along with the peptide and mRNA size suggests that there may be a common origin in evolutionary history with the CRF peptides, although the evolutionary relationship between the lineages is not understood (Chand et al., 2013; de Lannoy and Lovejoy, in press).

The TCAP family of peptides all have characteristics of a cleavable bioactive peptide; a cleavage motif on the N-terminus and an amidation motif on the C-terminus. The TCAP-1, -2, and -4 peptides are 41-amino residues in length and TCAP-3 is 40 amino residues in length (Lovejoy et al., 2006). Of the four, mouse TCAP-1 is the only peptide in the family confirmed to be expressed as a separate transcript from the teneurin gene in adults, although this may be the case for mouse TCAP-3 as well. Thus, in our model of TCAP release, we assume that TCAP-2 and -4 are released by ectodomain cleavage, whereas TCAPs -1 and -3 may be released by ectodomain cleavage and by independent transcription, translation, and subsequent release via cytosolic processes. Studies in zebrafish, chick, and rodents indicate that although expressed throughout the brain, TCAP-1 is particularly concentrated in the limbic regions including the hippocampus, hypothalamus, and amygdala (Wang et al., 2005; Tan et al., 2009, 2011). It is in the rat hippocampus and amygdala that TCAP-1 has been shown to exert its neuromodulatory effects on behavior and stress.

NEUROMODULATION OF CRF-INDUCED BEHAVIOR BY TCAP-1

Gene expression studies on mouse brain revealed that TCAP-1 (as part of the teneurin-1 gene) was expressed mainly in the limbic system, hypothalamus and cerebellum (Wang et al., 2005), suggesting these areas as focal points of TCAP-1 action.

```
human CRF       SEEPPISLDLTFHLLREVLEMARAEQLAQQAHSNRKLMEII-NH2
Human UCN        DNPSLSIDLTFHLLRTLLELARTQSQRERAEQNRIIFDSV-NH2
Human UCN2         IVLSLDVPIGLLQILLEGARARAAREQATTNARILARV-NH2
Human UCN3         FTLSLDVPTNIMNLLFNIAKAKNLRAQAAANAHLMAQI-NH2

human TCAP 1      QQLLSTGRVQGYDGYFVLSVEQYLELSDSANNIHFMRQSEI-NH2
human TCAP 2      QQLLSTGRVQGYEGYYVLPVEQYPELADSSSNIQFLRQNEM-NH2
human TCAP 3       QLLSAGKVQGYDGYYVLSVEQYPELADSANNIQFLRQSEI-NH2
human TCAP 4      QQVLSTGRVQGYDGFFVISVEQYPELSDSANNIHFMRQSEM-NH2
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FIGURE 2 | Comparison of the primary structure between the TCAP and CRF families of peptides. Amino acid identity among CRF paralogs are shown as dark gray, and continued among TCAP peptides where they match. Conservative homologous substitutions are shown in light gray. Note that the identity among TCAP peptides is much greater than that of CRF peptides.

However, like other neuromodulatory peptide systems, behavioral studies in rodents indicate that TCAP-1 has differential effects on anxiety depending upon the test, the treatment regimen, and perhaps even the baseline anxiety level of rats (Rotzinger et al., 2010). Acute administration of synthetic TCAP-1 into the BLA significantly increases the acoustic startle response in low-anxiety rats and decreases the response in high-anxiety rats (Wang et al., 2005). Repeated administration of TCAP-1 at picomole concentrations into the lateral ventricles decreased acoustic startle in all rats. A 50% decrease in the acoustic startle response was apparent 15 days after the last TCAP-1 treatment (Wang et al., 2005). Moreover, using the same regimen, TCAP-1 treatment completely ablated the CRF-induced increase in the acoustic startle response (Tan et al., 2008). In contrast, the same pre-treatment potentiated the effects of a CRF ICV challenge in the elevated plus maze and open field tests, where, upon CRF challenge, rats showed decreased open arm entries in the elevated plus maze, and decreased locomotion, as well as time, entries and distance traveled in the center zone of the open field (Tan et al., 2008). However, IV injections of TCAP-1 resulted in differential behaviors in response to an IV or ICV CRF challenge (Al Chawaf et al., 2007b). Overall, rats treated with repeated IV doses of TCAP-1, followed by an acute ICV CRF challenge were less anxious than rats given an injection of CRF via the IV route (Al Chawaf et al., 2007b). Again, these behavioral effects only occurred upon co-administration with CRF; TCAP alone had little effect on behavior, further indicating that this peptide plays a neuromodulatory role. This stress-mediated action of TCAP-1 is further reflected by studies using a cocaine reinstatement model (Kupferschmidt et al., 2010). Using the number of active lever responses as tests for reinstatement of cocaine seeking, prior TCAP-1 treatment in rodents resulted in fewer lever responses after a CRF injection. However, TCAP-1 pre-treatment did not modulate reinstatement by footshock stress, or cocaine-induced reinstatement.

The difference in effects of TCAP-1 on IV- vs. ICV- injected CRF in rats also suggests that TCAP-1 may be involved in the feedback of glucocorticoids to the brain. A previous study in mice (Martins et al., 1996) found that ICV-administered CRF induces its effects by targeting sites in the CNS as well as peripheral sites along the HPA axis. However, IV-administered CRF does not cross the blood–brain barrier and thus will only activate the HPA axis at the level of the pituitary and adrenal gland (Martins et al., 1996). Thus, the difference in TCAP-1 effects on ICV- and IV-administered CRF may be due to a modulatory effect of the glucocorticoid receptors or signaling in limbic regions by TCAP-1. In particular, TCAP-1 may be acting on the hippocampus and amygdala to change the sensitivity of the glucocorticoid feedback on the PVN CRF secretion.

Further studies indicated the action of TCAP-1 occurs at a pre-transcription and cellular activation level. TCAP-1 attenuates expression of CRF-induced *c-fos* in the amygdala (Tan et al., 2009). Rats pre-treated with TCAP-1 showed lower c-Fos immunoreactivity in the amygdala, medial prefrontal cortex, hippocampus, and the dorsal raphe nucleus upon ICV CRF injections. Activation of CRF promotes *c-fos* synthesis and expression

through a PKA/cAMP/CamKII pathway that leads to CREB phosphorylation. Phosphorylated CREB then regulates *c-fos* gene transcription by binding to response elements CRE and AP-1 (Kovacs, 1998). Similarly to CRF, TCAP-1 leads to cAMP accumulation in vitro (Wang et al., 2005). Studies on Gn11 mouse immortalized cell lines show that TCAP-1 has a biphasic effect on cAMP such that low concentrations of TCAP-1 increased cAMP, and high concentrations decreased cAMP levels (Wang et al., 2005). Moreover, a CRF1 receptor antagonist addition failed to block the TCAP-1-induced increase in cAMP suggesting the presence of independent signaling pathways.

Administration of TCAP alone had a minor effect on *c-fos* activation *in vivo* in rats. However, TCAP administration after concomitant CRF administration produced significant behavioral changes (Tan et al., 2009). This strongly suggests that TCAP plays a neuromodulatory role on elements of CRF signaling systems. Evidence shows that glucocorticoids inhibit AP-1 and CRE responses (Kovacs, 1998). Thus, TCAP could regulate elements of CRF signaling through modulation of CRE and AP-1 activity. In addition, TCAP could inhibit CRF-induced *c-fos* through inhibition of CRF expression itself, as the promoter of the CRF gene contains a CRE (Hauger et al., 2006). Using luciferase reporter assays, we found that TCAP administration decreases AP-1 reporter activity in an immortalized N3 hypothalamic cell line (Nock et al., unpublished observations). Thus, TCAP's ability to attenuate CRF induced *c-fos* activation *in vivo* may be, in part, through interactions with the AP-1 response element. However, AP-1 is regulated by numerous elements, and TCAP-1 may regulate AP-1 activity by a CRF-independent mechanism.

In vitro, TCAP-1 administration did not alter CRF-induced cAMP increases in the hypothalamic cell line Gn11 (Wang et al., 2005), nor CRF-induced CRE activation in human embryonic kidney cells transfected with CRF_1 and CRF_2 receptors. *In vitro*, TCAP-1 does not modulate cellular distribution or total protein of CRF_1, or the GC receptors GR and MR in mouse embryonic hippocampal E14 cells. Administration of TCAP-1 also did not modulate the phosphorylation of a CRF_1 downstream transcription factor CREB. Repeated daily TCAP-1 administration in mice did not affect basal non-stressed HPA activity based on serum cortisol and ACTH levels. From these studies, it is apparent that under basal non-stressed conditions, TCAP-1 does not interact directly with HPA regulatory pathways involving CRF or GC receptors (De Almeida et al., unpublished studies).

The lack of direct interaction of TCAP-1 on CRF signal transduction systems led us to investigate alternative explanations for its mechanism. The similarity of the TCAP-1 primary structure to CRF and related peptides had led us to postulate that the peptide activated a G-protein coupled receptor (GPCR) similar to the family of GPCRs related to the CRF receptors. However,*in vitro* screening of most of the receptors in this family did not show any significant binding or activation (Lovejoy, unpublished studies). However, gene microarray profiling indicated a similarity to neurotrophic factor activation and a relationship with the dystroglycan complex (Chand et al., 2012b). Subsequent studies confirmed this relationship, thus TCAP-1 may be the first identified soluble ligand for the dystroglycan complex. The dystroglycan complex is hypothesized to be integral to the intracellular signaling of extracellular TCAP-1 due to a strong co-localization of TCAP-1 with β-dystroglycan, the plasma membrane bound subunit of the dystroglycan complex (Chand et al., 2012b). In essence, TCAP-1 mediates cytoskeletal re-organization in E14 cells by binding to the dystroglycan complex which then signals through a MEK- ERK1/2-dependent phosphorylation of stathmin and filamin A. This leads to f-actin polymerization, increased tubulin immunoreactivity, and increased filopodia development (Chand et al., 2012b). The ERK1/2 pathway is the proposed mechanism through which TCAP-1 modulates neurite and dendrite morphology in immortalized mouse hypothalamic cell lines, primary hippocampal cultures (Al Chawaf et al., 2007a), and Golgi-stained rat brains (Tan et al., 2011).

Within multiple areas of the mouse brain, CRF induces stress-related cytoskeletal changes. CRF_1 activation in the CA3 mediates dendritic retraction by inducing destabilization of f-actin and the cell adhesion molecule nectin-3 (Chen et al., 2008; Wang et al., 2011). Activation of CRF_1 in the locus coeruleus (LC) also mediates outgrowth of neuronal processes through the Rho GTPase Rac1 (Swinny and Valentino, 2006). In the hippocampus, this Rac1-dependent outgrowth of neuronal processes is counteracted by RhoA inhibition on dendritic spine formation and branching. Thus, TCAP-1 may exert its CRF modulating effects through countering CRF_1 mediated changes in cytoskeletal re-organization-like synaptic plasticity within stress modulating brain areas that express both CRF_1 and TCAP-1.

CONCLUSION

Current data suggests that TCAP-1 acts as a separate and distinct peptide modulating system, which is involved in cytoskeletal-associated synaptic plasticity. From an evolutionary perspective, it is perhaps not surprising that TCAP-1 acts independently of CRF signaling. The teneurins and TCAP evolved early in metazoan history, whereas the CRF family of peptides and their role in integrating energy metabolism and the perception of stressors evolved later after evolution of vertebrates (Lovejoy and Jahan, 2006; Lovejoy, 2009). Moreover, the later evolution of the HPA axis in stress signaling and the independence of TCAP-1 neuromodulatory actions from the HPA axis further support a possible parallel evolution. The TCAP-1 peptide acts on the dystroglycan complex in order to signal intracellularly. This signaling may be downstream of other effectors from the CRF signaling pathway downstream of the CRF_1 receptor. The observed TCAP-1 modulatory effects on CRF behaviors may then be mediated through TCAP-1 specific signaling that oppose CRF mediated effects on cellular cytoskeletal organization within brain areas that co-express TCAP-1 and CRF. This in turn manifests into modulation of CRF-mediated stress behaviors in brain regions that co-express CRF and TCAP-1.

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Regulation of feeding behavior and psychomotor activity by corticotropin-releasing hormone (CRH) in fish

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Corticotropin-releasing hormone (CRH) is a hypothalamic neuropeptide belonging to a family of neuropeptides that includes urocortins, urotensin I, and sauvagine in vertebrates. CRH and urocortin act as anorexigenic factors for satiety regulation in fish. In a goldfish model, intracerebroventricular (ICV) administration of CRH has been shown to affect not only food intake, but also locomotor and psychomotor activities. In particular, CRH elicits anxiety-like behavior as an anxiogenic neuropeptide in goldfish, as is the case in rodents. This paper reviews current knowledge of CRH and its related peptides derived from studies of teleost fish, as representative non-mammals, focusing particularly on the role of the CRH system, and examines its significance from a comparative viewpoint.

Keywords: goldfish, CRH, ICV injection, food intake, anorexigenic action, psychomotor activity, anxiogenic-like action

INTRODUCTION

Corticotropin-releasing hormone (CRH), a 41-amino-acid neuropeptide present in the brains of vertebrates, was first isolated and characterized from the ovine hypothalamus (Vale et al., 1981), and then subsequently identified in non-mammalian brains (Lovejoy and Balment, 1999). CRH is a member of a family of related peptides that includes urotensin-I (UI), sauvagine, and urocortin/stresscopin in vertebrates (Lovejoy and Balment, 1999; Boorse and Denver, 2006). In mammals, CRH is known to induce the release of adenohypophyseal hormones such as adrenocorticotropic hormone (ACTH), β-endorphin, and α-melanocyte-stimulating hormone (α-MSH) from the pituitary, and there is ample evidence that CRH and its related peptides play multiple roles in animal development and also in physiological and behavioral adaptation to environmental changes and energy balance (Tonon et al., 1986; Hauger et al., 1988, 2006; Lowry and Moore, 2006; Cooper and Huhman, 2007; Denver, 2009; Papadimitriou and Priftis, 2009; Chen et al., 2012; Kubota et al., 2012).

In non-mammalian vertebrates such as amphibians and teleosts, CRH acts as a potent stimulator of corticotropin, thyrotropin, and α-MSH release (Boorse and Denver, 2004, 2006; Calle et al., 2005; Ito et al., 2006; Okada et al., 2007). CRH and its related peptides also act as regulators of feeding behavior and stress responses in vertebrates including mammals, birds, amphibians, and fish (Kalra et al., 1999; Bernier and Peter, 2001; Ohgushi et al., 2001; Hillebrand et al., 2002; Tachibana et al., 2004; Saito et al., 2005; Lowry and Moore, 2006; Carr et al., 2010; Matsuda et al., 2010b; Morimoto et al., 2011; Khan et al., 2013). It has been reported that, in the goldfish, intracerebroventricular (ICV) administration of CRH or UI exerts an anorexigenic action (de Pedro et al., 1997; Bernier and Peter, 2001; Volkoff et al., 2005; Matsuda, 2009), which is blocked by treatment with a CRH 1/CRH 2 receptor antagonist, α-helical $CRH_{(9-41)}$ (de Pedro et al., 1997; Bernier and Peter, 2001; Bernier, 2006; Maruyama et al., 2006). In fish, ICV administration of CRH also affects locomotor activity (Clements and Schreck, 2004; Maruyama et al., 2006; Carpenter et al., 2007; Backström et al., 2011a; Ghisleni et al., 2012; Matsuda et al., 2013b), suggesting that CRH exerts psychophysiological effects in fish. Recent reports indicate that a fish's swimming pattern can be used to evaluate psychomotor activities, notably anxiety-like behavior (Faganello and Mattioli, 2007; Grossman et al., 2010; Maximino et al., 2010a,b; Matsuda et al., 2011a,b, 2013b; Blaser and Rosemberg, 2012; Maaswinkel et al., 2012). Therefore, the present mini-review summarizes recent advances in knowledge about the regulation of feeding behavior and locomotor or psychomotor activity by CRH and its related peptides in fish, especially with reference to the goldfish model.

CONTROL OF FOOD INTAKE BY CRH AND ITS RELATED PEPTIDES IN FISH

The effects of ICV administration of neuropeptides on food intake in goldfish have been extensively studied. For example, ICV-injected ghrelin, neuropeptide Y, and orexin increase food consumption whereas CRH, UI, proopiomelanocortin (POMC)-derived peptides such as α-MSH, pituitary adenylate cyclase-activating polypeptide (PACAP), cholecystokinin (CCK), neuromedin U (NMU), and diazepam-binding inhibitor-derived peptides such as octadecaneuropeptide (ODN) decrease food intake (Matsuda, 2009). These neuropeptides are not independently involved in the control of feeding behavior, but mutually interact with each other. The anorexigenic actions of PACAP and NMU are abolished by treatment with α-helical $CRH_{(9-41)}$, and CCK- and ODN-evoked anorexigenic actions are also attenuated by treatment with the melanocortin 4 receptor

(MC4R) antagonist HS024 (Maruyama et al., 2006, 2009; Kang et al., 2010; Matsuda et al., 2010a). These findings suggest that CRH and α-MSH mediate the actions of PACAP and NMU, and CCK and ODN, respectively. In goldfish, α-MSH-containing nerve fibers or endings lie in close apposition to CRH-containing neurons in a specific region of the hypothalamus, the nucleus posterioris periventricularis (NPPv). The anorexigenic action of the α-MSH agonist melanotan II (MT II) is abolished by treatment with α-helical $CRH_{(9-41)}$ whereas the anorexigenic action of CRH is not affected by treatment with HS024 (Matsuda et al., 2008a). These observations indicate that, in goldfish, α-MSH-induced anorexigenic action is mediated by the CRH-signaling pathway, and that CRH plays a crucial role in the regulation of feeding behavior as an integrated anorexigenic neuropeptide in this species.

The distribution of CRH in the brain of teleost fish including the goldfish, has been well-reported: CRH-containing neuronal cell bodies are localized in various hypothalamic regions, including the preopticus periventricularis (NPP), the nucleus preopticus (NPO), the lateral part of the nucleus lateralis tuberis (NLTl) and the NPPv, and CRH-containing fibers or endings are distributed throughout the brain, and in the neurohypophysis (Olivereau et al., 1984, 1988; Yulis et al., 1986; Yulis and Lederis, 1987). For example, in goldfish, neuronal cell bodies exhibiting CRH-like immunoreactivity are located mainly in the preoptic parvocellular areas comprising the NPP and NPO, the NLTl, and paraventricular organ areas such as the NPPv, and their fibers are distributed in the diencephalon, mesencephalon, and neurohypophysis. CRH-containing neurons that originate in the NPP and NPO parvocellular population seem to innervate the pituitary. As described above, studies of the effect of CRH on feeding behavior in goldfish have shown that it acts as a powerful hypothalamic anorexigenic peptide (de Pedro et al., 1993, 1997; Bernier et al., 1999, 2004; Bernier and Peter, 2001; Maruyama et al., 2006). Interestingly, we and others have found that ICV injection of gonadotropin-releasing hormone 2 (GnRH2, also known as chicken GnRH II) affects food consumption, and that GnRH2 decreases food intake (Hoskins et al., 2008; Matsuda et al., 2008b). Subsequently it has been indicated that the anorexigenic actions of CRH and α-MSH are blocked by treatment with the GnRH type I receptor antagonist Antide, suggesting that GnRH2 mediates the actions of other anorexigenic neuropeptides examined so far, and that GnRH2 acts as a key neuropeptide exerting satiety control (Kang et al., 2011).

PSYCHOPHYSIOLOGICAL EFFECT OF CRH IN FISH

Recent studies have shown that several neuropeptides such as CRH, GnRH2, ODN, PACAP, NPY, ghrelin, and orexin affect not only food intake but also locomotor activity in fish (**Table 1**): ICV injection of CRH enhances swimming distance, and stimulates locomotor activity (Maruyama et al., 2006; Carpenter et al., 2007; Backström et al., 2011a,b; Matsuda et al., 2013b). Psychophysiological compounds including diazepam, serotonin, a selective serotonin reuptake inhibitor Fluoxetin, a central-type benzodiazepine receptor inverse agonist FG-7142, and an N-methyl-d-aspartate receptor antagonist MK-801 also modify locomotor activity (Kang et al., 2010; Matsuda et al., 2011b, 2013b; Winder et al., 2012). Recent reports have indicated that the swimming pattern of a fish in a tank can be used to evaluate psychomotor activity (Faganello and Mattioli, 2007; Cachat et al., 2010; Grossman et al., 2010; Maximino et al., 2010a,b; Khor et al., 2011, 2013; Matsuda et al., 2011a; Piato et al., 2011). The scototaxis test (light/dark preference test) has been developed, and used for measuring psychomotor activity (Faganello and Mattioli, 2007; Blaser and Rosemberg, 2012). Intact animals usually prefer the dark area to the light area, and psychophysiological substances affect this preference: treatment with diazepam increases the time spent in the light area, and treatment with

Table 1 | Effects of neuropeptides and psychophysiological compounds on food intake, locomotor activity, and emotional action in fish.

Substances	Species	Food intake	Locomotor activity	Emotional action	References
CRH	Goldfish	Down	Up	Anxiogenic-like	Maruyama et al., 2006; Matsuda et al., 2013b
	Rainbow trout		Up	Anxiogenic-like	Carpenter et al., 2007; Backström et al., 2011a,b
GnRH2	Goldfish	Down	Up		Hoskins et al., 2008; Matsuda et al., 2008b
	Zebrafish	Down			Nishiguchi et al., 2012
ODN	Goldfish	Down	Up	Anxiogenic-like	Matsuda et al., 2007, 2011b
PACAP	Goldfish	Down	Up	Anxiogenic-like	Matsuda et al., 2006a, 2013a
NPY	Goldfish	Up	Down	Anxiolytic-like	Matsuda et al., 2011a, 2012b
	Zebrafish	Up			Yokobori et al., 2012
Ghrelin	Goldfish	Up	Up or Down		Matsuda et al., 2006b; Yahashi et al., 2012
ORX	Goldfish	Up	Up		Nakamachi et al., 2006; Matsuda et al., 2012a
	Zebrafish	Up	Up		Yokogawa et al., 2007; Yokobori et al., 2011
Diazepam	Goldfish		Down	Anxiolytic-like	Matsuda et al., 2011b
Fluoxetine	Sheepshead minnow		Down		Winder et al., 2012
	Chinook salmon		Down		Clements and Schreck, 2007
FG-7142	Goldfish		Up	Anxiogenic-like	Matsuda et al., 2011b
MK-801	Goldfish		Up		Kang et al., 2011

Abbreviations: CRH, corticotropin-releasing hormone; GnRH2, gonadotropin-releasing hormone 2; ODN, octadecaneuropeptide; PACAP, pituitary adenylate cyclase-activating polypeptide; NPY, neuropeptide Y; ORX, orexin; Fluoxetine, a selective serotonin reuptake inhibitor; FG-7142, a central-type benzodiazepine receptor inverse agonist; MK-801, an N-methyl-d-aspartate receptor antagonist.

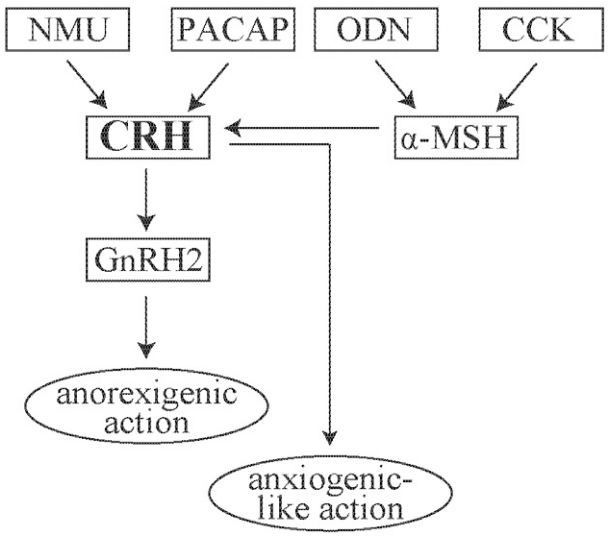

FIGURE 1 | Schematic drawings of the neuronal signaling pathways of anorexigenic and anxiogenic-like action in goldfish. ODN and CCK-induced anorexigenic actions are mediated by α-MSH-signaling pathway, and the anorexigenic actions of NMU, PACAP, and α-MSH are mediated by CRH- and subsequent GnRH2-signaling pathways. CRH also evokes anxiogenic-like action. Abbreviations: NMU, neuromedin U; PACAP, pituitary adenylate cyclase-activating polypeptide; ODN, octadecaneuropeptide; CCK, cholecystokinin; CRH, corticotropin-releasing hormone; α-MSH, α-melanocyte-stimulating hormone; GnRH2, gonadotropin-releasing hormone 2.

FG-7142 increases the time spent in the dark area, suggesting that the former and latter treatments induce anxiolytic- and anxiogenic-like actions, respectively (Matsuda et al., 2011b). Since intact goldfish and zebrafish prefer the lower to the upper area of a tank, another preference test has also been developed to evaluate the effect of CRH or other substances on psychomotor activity (Khor et al., 2013; Matsuda et al., 2013b). ICV administration of CRH and FG-7142 both increase the time taken to move from the lower to the upper area, and the anxiogenic-like action of CRH is blocked by treatment with α-helical $CRH_{(9-41)}$ (Matsuda et al., 2013b). Recent studies of other fish have also indicated that CRH induces behavioral changes including anxiety and suppression of aggressive behavior (Lastein et al., 2008; Carpenter et al., 2009; Backström et al., 2011a,b; Ghisleni et al., 2012). These studies suggest that CRH exerts psychophysiological effects as an anxiogenic factor in addition to satiety control in fish. **Figure 1** shows a schematic drawing of the anorexigenic signaling pathways mediated by CRH and other neuropeptides in the central nervous system of goldfish. As described above, CRH also evokes anxiogenic-like action in this species. Although it is unclear why regulation of food intake and the psychophysiological effects of CRH are closely linked, CRH appears to induce both anorexigenic- and anxiogenic-like actions in fish. Therefore, it is reasonable to suggest that the increased locomotor activity of fish in an experimental tank induced by CRH can be interpreted as escape behavior triggered by the anxiogenic-like action of CRH and subsequent stress response. Further study is warranted to clarify the function of CRH and its related peptides in the regulation of feeding and emotional activity in fish.

CONCLUSION

In fish, CRH exerts potential effects on food intake, as well as locomotor and psychomotor activities, providing an example of a neuropeptide that regulates both feeding behavior and psychophysiological activity such as anxiogenic- or anxiolytic-like action.

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Appetite-suppressing effects and interactions of centrally administered corticotropin-releasing factor, urotensin I and serotonin in rainbow trout (*Oncorhynchus mykiss*)

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Corticotropin-releasing factor (CRF), urotensin I (UI) and serotonin (5-HT) are generally recognized as key regulators of the anorexigenic stress response in vertebrates, yet the proximal effects and potential interactions of these central messengers on food intake in salmonids are not known. Moreover, no study to date in fishes has compared the appetite-suppressing effects of CRF and UI using species-specific peptides. Therefore, the objectives of this study were to (1) assess the individual effects of synthesized rainbow trout CRF (rtCRF), rtUI as well as 5-HT on food intake in rainbow trout, and (2) determine whether the CRF and serotonergic systems interact in the regulation of food intake in this species. Intracerebroventricular (icv) injections of rtCRF and rtUI both suppressed food intake in a dose-related manner but rtUI [ED_{50} = 17.4 ng/g body weight (BW)] was significantly more potent than rtCRF (ED_{50} = 105.9 ng/g BW). Co-injection of either rtCRF or rtUI with the CRF receptor antagonist α-$hCRF_{(9–41)}$ blocked the reduction in food intake induced by CRF-related peptides. Icv injections of 5-HT also inhibited feeding in a dose-related manner (ED_{50} = 14.7 ng/g BW) and these effects were blocked by the serotonergic receptor antagonist methysergide. While the anorexigenic effects of 5-HT were reversed by α-$hCRF_{(9–41)}$ co-injection, the appetite-suppressing effects of either rtCRF or rtUI were not affected by methysergide co-injection. These results identify CRF, UI and 5-HT as anorexigenic agents in rainbow trout, and suggest that 5-HT-induced anorexia may be at least partially mediated by CRF- and/or UI-secreting neurons.

Keywords: stress, food intake, anorexigenic actions, icv injections, fish

INTRODUCTION

A variety of homeostatic challenges activate signaling pathways in the brain that stimulate the hypothalamic-pituitary-adrenal (HPA) stress axis in mammals and disrupt food intake regulation (Bazhan and Zelena, 2013). In general, intense stressors that acutely activate the HPA axis and result in a transient increase in circulating glucocorticoid levels result in decreased food intake. For example, acute stressors such as immobilization/restraint, social defeat and immune challenges suppress food intake in rodents (Meerlo et al., 1996; Vallès et al., 2000; Calvez et al., 2011). Similarly, acute physical, environmental, social, and immune stressors lead to appetite suppression in fish (Bernier, 2006, 2010; Leal et al., 2011). In contrast, exposure to chronic psychological stress together with access to palatable food leads to hyperphagia (Adam and Epel, 2007; Tomiyama et al., 2011). In fact, chronic stress is recognized as an important risk factor for weight gain and obesity (Sinha and Jastreboff, 2013). Overall, while there is a basic understanding of the neural pathways that mediate the bidirectional effects of stress on food intake in mammals (Maniam and Morris, 2012), relatively little is known about the mechanisms by which stressors alter food intake in non-mammalian species.

Important players in the coordinated regulation of the stress axis and food intake are members of the corticotropin-releasing factor (CRF) family of neuropeptides (Richard et al., 2002; Kuperman and Chen, 2008). In fish, CRF and the related neuropeptide urotensin I (UI) are both potent hypophysiotropic factors of the hypothalamic-pituitary-interrenal (HPI) axis (the fish homolog to the HPA axis; Bernier et al., 2009) and key anorexigenic signals for the regulation of food intake (Volkoff et al., 2005; Matsuda, 2009). Central injections of ovine CRF (De Pedro et al., 1993; Matsuda et al., 2008), rat/human CRF (r/hCRF; Bernier and Peter, 2001) or carp/goldfish UI (c/gUI) in goldfish (*Carassius auratus*) suppress food intake in a dose-related manner, and UI is significantly more potent than CRF (Bernier and Peter, 2001). While these results are consistent with the known appetite-suppressing effects of CRF-related peptides in rats (Spina et al., 1996) and can be explained by the differential binding profile of UI and CRF for the mammalian CRF-R2 receptor (Vaughan et al., 1995), CRF receptor binding assays in fish do not support a preferential activation of CRF-R2 by UI (Arai et al., 2001; Pohl et al., 2001). To date, all experiments investigating the central effects of CRF on food intake in fish have been carried out on goldfish using heterologous CRF peptides with varying sequence identity (78–92%) to the native ligand.

Another key player for the coordinated regulation of the endocrine stress response and food intake is the central

serotonergic system (Leibowitz and Alexander, 1998; Jørgensen, 2007). Besides stimulating the activity of the HPI axis (Winberg et al., 1997), serotonin or 5-hydroxytryptamine (5-HT) is a potent suppressor of appetite in fish. In goldfish, central injections of 5-HT (De Pedro et al., 1998) or intraperitoneal (i.p.) treatment with the selective serotonin reuptake inhibitor fluoxetine (Mennigen et al., 2010) significantly reduces food intake. Similarly, oral administration of 5-HT reduces food intake in sea bass (*Dicentrarchus labrax*; Rubio et al., 2006) and i.p. injections of the 5-HT releasing agent fenfluramine temporarily suppressed food intake in rainbow trout (Ruibal et al., 2002).

Adding to the complexity of the stress-sensitive anorexigenic neurocircuitry is the neuroanatomical and physiological evidence for bidirectional regulatory relationships between the CRF and serotonergic systems (Liposits et al., 1987; Summers et al., 2003). In several fish species, serotonergic neurons from the raphe nuclei in the hindbrain innervate the hypothalamic preoptic area (POA) (Kah and Chambolle, 1983; Ekström and Van Veen, 1984; Lillesaar et al., 2009), an important site of CRF and UI expression in teleosts (Alderman and Bernier, 2007). While a previous study suggested that the anorexic actions of 5-HT are mediated by CRF in goldfish (De Pedro et al., 1998), studies in salmonids have also shown that CRF can modulate behavioral responses by interacting centrally with 5-HT (Clements et al., 2003; Carpenter et al., 2007).

In this context, we synthesized rainbow trout (*Oncorhynchus mykiss*) CRF (rtCRF) and rtUI from available cDNA sequences and investigated the central effects of a wide range of doses to determine the potency of the native peptides on food intake. In rainbow trout, while CRF has 73–75% residue conservation to ovine and rat/human CRF (Doyon et al., 2003), UI has 59–63% residue identity to rat and human urocortin (UCN; the mammalian ortholog to UI; Barsyte et al., 1999; Lovejoy and Balment, 1999). As such, using native peptides in a non-mammalian model such as rainbow trout may be a key factor for determining whether CRF and UI have a differential contribution to the regulation of food intake. Also, to further our understanding of the complex bidirectional regulatory relationships between the CRF and serotonergic systems and their contribution to the regulation of appetite, we examined the effects of central administration of 5-HT on food intake and used CRF and 5-HT receptor antagonists to determine whether the anorexigenic effects of CRF-related peptides and 5-HT result from interactions between the serotonergic and CRF systems.

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

Sexually immature rainbow trout of either sex (68.3 ± 1.4 g, mean $\pm$ sem; $n = 240$) were transported from Rainbow Springs trout farm (Thamesford, ON, Canada) to the University of Guelph (Guelph, ON, Canada). Prior to experimental use, fish were maintained in indoor tanks (650 l) supplied with aerated well-water (6 l/min) at 14.0 ± 0.5°C and kept on a 12:12-h light-dark photoperiod regime. In order to promote a conditioned feeding regiment, fish were hand fed commercially prepared trout chow (3PT Classic floating fish pellets, Martin Mills, Elmira, ON, Canada) at 10:30 am daily. Before the start of each experiment, fish were individually placed in flow-through 10 l tinted tanks (Aquatic Habitats, Apopka, Fl, USA). Fish were acclimated to these conditions for a minimum of 10 days prior to experimentation and were maintained on the same feeding regiment. All procedures were approved by the local Animal Care Committee and conform to the principles of the Canadian Council for Animal Care.

CRF AND UI PEPTIDE SYNTHESIS, PURIFICATION AND IDENTIFICATION

Deduced amino acid sequences of rtCRF (GenBank accession No. AF296672) and rtUI (GenBank accession No. AJ005264) from cloned cDNA sequences were used for peptide synthesis. Rainbow trout CRF (rtCRF) peptide was synthesized on an automated peptide synthesizer, model Novayn Crystal (Novabiochem, Nottingham, UK) on PEG-PS resin using continuous flow Fmoc chemistry. Following cleavage and deprotection, the final peptide was desalted on a sephadex G-10 column using an aqueous 0.1% triflouroacetic acid (TFA) solution and lyophilized. Confirmation of the homogeneity of the synthetic CRF peptide was determined by reverse-phase high performance liquid chromatography (HPLC). A Beckman model 126 HPLC system (Beckman, Palo Alto, CA) attached to a UV detector module 168 and C-18 column (3.5 μm particle size; Waters Ltd., Mississauga, ON, Canada) was used to purify the CRF peptide. The column flow rate was 0.1 ml/min with the mobile phase B increasing from 0 to 60% over 80 min. Finally, purified CRF peptide was identified on a Micromass Q-TOF (hybrid quadrupole time of flight) mass spectrophotometer (Micromass, Manchester, UK), and analyzed using MassLynx program (Micromass, Manchester, UK). Rainbow trout UI was synthesized as described in Barsyte et al. (1999).

REAGENT PREPARATION

The CRF/UI receptor antagonist α-helical $CRF_{(9–41)}$ (α-$hCRF_{(9–41)}$) was purchased from American Peptide Company (Sunnyvale, CA, USA). Serotonin hydrochloride (5-hydroxytryptamine; 5-HT) was purchased from Sigma-Aldrich (St Louis, MI, USA). The mixed 5-HT_1/5-HT_2 receptor antagonist, methysergide maleate, was purchased from Tocris (Ellisville, MO, USA). All reagents were dissolved in teleost Ringer's solution (0.2% $NaHCO_3$ in 0.6% NaCl solution), excluding methysergide, which was dissolved in a modified teleost Ringer's solution (30% methanol, 70% Ringer's). Working concentrations were prepared on the day of the experiment from frozen stock solutions and appropriately diluted in Ringer's solution.

INTRACEREBROVENTRICULAR INJECTIONS

Each animal was quickly netted and deeply anesthetized in a buffered ($NaHCO_3$; 0.25 g/l) solution of tricaine methanesulfonate (MS-222) (0.125 g/l, Syndel, Vancouver, BC, Canada). The fish were weighed and placed in an orbital bar restraint (Peter and Gill, 1975). The reagents were administered using a 10 μl glass syringe with a 26-gauge needle (Hamilton # 701, Reno, NV, USA) secured into a stereotactic apparatus and positioned directly over the cranium. Injections were performed midline, postorbitally and to a depth of 3 mm into the 3rd ventricle.

Although the success of each individual injection was not verified during the experiments, we validated our intracerebroventricular (icv) injection procedure in preliminary studies using two approaches. Initially, to determine the depth and position of the 3rd ventricle relative to surface structures, we serially sectioned fixed and decalcified whole heads from 3 fish (66.6 ± 8.4 g, mean ± sem) and quantified the distance from the epithelial surface of the cranium to the top and bottom of the 3rd ventricle. The measurements were made from the anterior opening of the 3rd ventricle in the telencephalon to its caudal end in the posterior hypothalamus. We also used methylene blue (Sigma-Aldrich) as a dye tracer to determine the depth and accuracy of the procedure. During the icv injection procedure, the reagents were administered slowly over a 5 s period. Following injections, each fish was immediately placed back to its respective tank for recovery from the anesthetic, which occurred within 5 min. The injection procedure, from anesthesia to injection, took an average of 6 min. Each fish was subjected to only one icv injection.

ASSESSMENT OF FOOD INTAKE

Fish were fed a pre-determined excess number of food pellets [5% of body weight (BW)] 10 min post recovery from the icv injection procedure. Uneaten food pellets were removed from the tank 2 h later and counted. The average weight of a food pellet was calculated and used to quantify food intake as mg food/g BW/h.

EXPERIMENTAL DESIGN

Experiment 1: effects of rtCRF and rtUI icv injections on food intake

Individual fish acclimated to separate tanks for a minimum of 10 days were icv injected with teleost Ringer's (Control), rtCRF or rtUI between 10:30 and 11:30 am ($n = 8$). Dosages of rtCRF and rtUI were 1, 5, 25, and 125 ng/g BW (0.2–25 pmol/g BW). Fish were returned to their respective tanks and food intake was assessed as above. In addition, food intake was determined in a non-anaesthetized non-handled group ($n = 8$).

Experiment 2: effects of α-hCRF$_{(9–41)}$ icv injections on rtCRF- and rtUI-induced changes in food intake

Individual fish acclimated to separate tanks for a minimum of 10 days were icv injected with teleost Ringer's (Control), rtCRF (25 ng/g BW; 5 pmol/g BW) or rtUI (25 ng/g BW; 5 pmol/g BW) alone, or in combination with α-hCRF$_{(9–41)}$ (250 ng/g BW; 65 pmol/g BW) between 10:30 and 11:30 am ($n = 8$). Fish were returned to their respective tanks and food intake was assessed as above.

Experiment 3: effects of 5-HT and methysergide icv injections on food intake

Individual fish acclimated to separate tanks for a minimum of 10 days were icv injected with 5-HT (0, 1, 10, 100 ng/g BW; 0–470 pmol/g BW) alone, or 5-HT (0, 10 ng/g BW; 0–47 pmol/g BW) in combination with methysergide (100 ng/g BW; 213 pmol/g BW) between 10:30 and 11:30 am ($n = 8$). Fish were returned to their respective tanks and food intake was assessed as above.

Experiment 4: effects of methysergide icv injections on rtCRF- and rtUI-induced changes in food intake

Individual fish acclimated to separate tanks for a minimum of 10 days were icv injected with teleost Ringer's (Control), rtCRF (5 ng/g BW; 1 pmol/g BW) or rtUI (5 ng/g BW; 1 pmol/g BW) alone, or in combination with methysergide (100 ng/g BW; 213 pmol/g BW) between 10:30 and 11:30 am ($n = 8$). Fish were returned to their respective tanks and food intake was assessed as above.

Experiment 5: effects of α-helical CRF$_{(9–41)}$ i.c.v. injections on 5-HT-induced changes in food intake

Individual fish acclimated to separate tanks for a minimum of 10 days were icv injected with 5-HT (0, 10 ng/g BW; 0–47 pmol/g BW) alone, or 5-HT (10 ng/g BW) in combination with α-hCRF$_{(9–41)}$ (250 ng/g BW; 65 pmol/g BW) between 10:30 and 11:30 am ($n = 8$). Fish were returned to their respective tanks and food intake was assessed as above.

STATISTICAL ANALYSIS

All results are presented as mean ± sem. In Experiment 1, differences between the Control and rtCRF treatments or between the Control and rtUI treatments were determined by a One-Way ANOVA and a pairwise Tukey's test. Differences between the rtCRF and rtUI treatments at a given dosage were evaluated with a Student's *t*-test. While differences between treatments in Experiment 2 were assessed by a Two-Way ANOVA, the results in Experiments 3–5 were assessed by a One-Way ANOVA. In each case, the ANOVAs were followed by a Tukey's test for all pair-wise multiple comparisons. Hill plots and linear regression analyses were used to determine the half-maximal effective dose (ED_{50}) value of rtCRF, rtUI, and 5-HT in suppressing food intake. The software SigmaStat 3.0 was used for statistical analysis (SPSS, Chicago, IL, USA). The significance level for all statistical tests is $P < 0.05$.

RESULTS

EXPERIMENT 1: EFFECTS OF rtCRF AND rtUI icv INJECTIONS ON FOOD INTAKE

Mean food intake in the fish injected with teleost Ringer (**Figure 1A**; Control treatment) and in the non-anaesthetized non-handled group did not differ from one another. In the latter treatment, mean food intake was 22.0 ± 0.4 mg/g BW/h. Relative to the Control treatment, icv injection of either rtCRF or rtUI inhibited food intake in a dose-dependent manner, with significant effects being observed at dosages of 5 ng/g BW and higher. With the 25 and 125 ng/g BW doses, rtUI icv injections elicited a larger reduction in food intake than rtCRF, and overall rtUI (ED_{50} = 17.4 ng/g BW) was significantly more potent than rtCRF (ED_{50} = 105.9 ng/g BW) in producing anorectic effects (**Figure 1B**).

EXPERIMENT 2: EFFECTS OF α-hCRF$_{(9–41)}$ icv INJECTIONS ON rtCRF- AND rtUI-INDUCED CHANGES IN FOOD INTAKE

Injection of the CRF receptor antagonist, α-hCRF$_{(9–41)}$ (250 ng/g BW), in combination with either rtCRF or rtUI (25 ng/g BW), effectively blocked the rtCRF- and rtUI-induced suppression

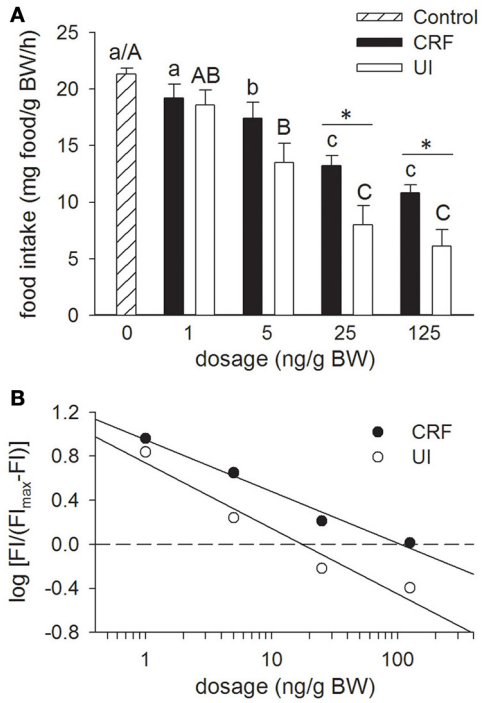

FIGURE 1 | Effects of teleost Ringer's (0.2% $NaHCO_3$ in 0.6% NaCl; Control treatment), rainbow trout CRF (rtCRF) or rtUI i.c.v. injections on food intake in rainbow trout. (A) Fish received food 10 min after i.c.v. injection and food intake was assessed over a 120 min period. Control and rtCRF that do not share a common lowercase letter, or Control and rtUI treatments that do not share a common uppercase letter, are significantly different from each other as determined by a One-Way ANOVA and a pairwise Tukey's test. An asterisk indicates a significant difference between the rtCRF and rtUI treatments at a given dosage as determined by a Student's *t*-test ($P < 0.05$). Values are means + sem ($n = 8$). **(B)** Hill plots demonstrating the half-maximal effective dose (ED_{50}) of rtCRF ($ED_{50} = 105.9$ ng/g BW) and rtUI ($ED_{50} = 17.4$ ng/g BW) i.c.v. injections on food intake (FI). Linear regression analysis and test for parallelism (analysis of covariance) indicates that rtUI is significantly more potent than rtCRF in suppressing food intake ($P < 0.05$).

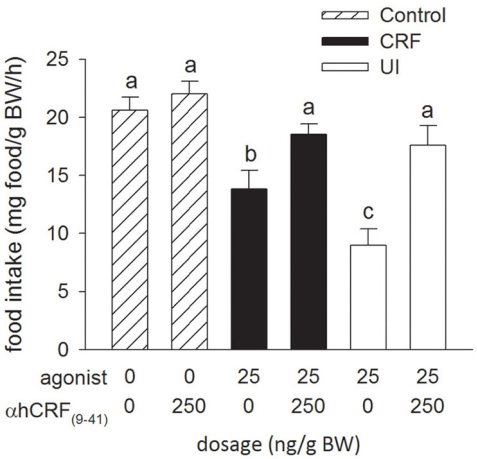

FIGURE 2 | Effects of i.c.v. injections of teleost Ringer's (0.2% $NaHCO_3$ in 0.6% NaCl; Control treatment), rainbow trout CRF (rtCRF) or rtUI alone, or in combination with the CRF receptor antagonist, α-helical $CRF_{(9-41)}$ ($\alpha hCRF_{(9-41)}$) on food intake in rainbow trout (agonist, $P < 0.001$; antagonist, $P < 0.001$; agonist × antagonist, $P = 0.034$). Assessment of food intake was carried out as in **Figure 1**. Bars that do not share a common letter are significantly different from each other as determined by a Two-Way ANOVA and by a pairwise Tukey's test ($P < 0.05$). Values are means + sem ($n = 8$).

of food intake (**Figure 2**). Central injections of α-$hCRF_{(9-41)}$ alone had no effect on food intake relative to the Control treatment.

EXPERIMENT 3: EFFECTS OF 5-HT AND METHYSERGIDE icv INJECTIONS ON FOOD INTAKE

Relative to the Control treatment (the 0 ng/g BW dosage), icv injection of 5-HT inhibited food intake in a dose-dependent manner with significant effects being observed at dosages of 10 ng/g BW and higher (**Figure 3A**). The icv injection of the 5-HT receptor antagonist, methysergide (100 ng/g BW), in combination with 5-HT (10 ng/g BW), reversed the decrease in food intake elicited by icv injection of 5-HT alone. Injection of methysergide alone had no effect on food intake relative to the Control treatment. Overall, the potency of 5-HT ($ED_{50} = 14.7$ ng/g BW) at producing anorectic effects was similar to the potency of rtUI and greater than that of rtCRF (**Figure 3B**).

EXPERIMENT 4: EFFECTS OF METHYSERGIDE icv INJECTIONS ON rtCRF- AND rtUI-INDUCED CHANGES IN FOOD INTAKE

Relative to the Control treatment, injection of the 5-HT receptor antagonist, methysergide (100 ng/g BW), in combination with either rtCRF or rtUI (5 ng/g BW), had no effect on the decrease in food intake elicited by icv injection of rtCRF and rtUI (**Figure 4**).

EXPERIMENT 5: EFFECTS OF α-$hCRF_{(9-41)}$ i.c.v. INJECTIONS ON 5-HT-INDUCED CHANGES IN FOOD INTAKE

Relative to the Control treatment, injection of the CRF receptor antagonist, α-$hCRF_{(9-41)}$ (250 ng/g BW), in combination with 5-HT (10 ng/g BW) injection, prevented the 5-HT-induced suppression of food intake (**Figure 5**).

DISCUSSION

Results from this study provide original evidence that CRF-related peptides have anorexigenic properties in a salmonid species, rainbow trout. Using native peptides, we demonstrate that doses as low as 5 ng/g BW are effective in decreasing food intake, and that UI is more potent than CRF at suppressing appetite. We also show that 5-HT has anorectic actions in rainbow trout that are at least partially mediated by CRF- and/or UI-secreting neurons. As such, given the known contribution of these signaling molecules to the regulation of the stress response, our results provide strong evidence for the hypothesis that central CRF-related peptides and serotonergic neural populations play a role in mediating stress hypophagia.

The appetite-suppressing effects of i.c.v. injections of CRF in rainbow trout are consistent with the results from similar

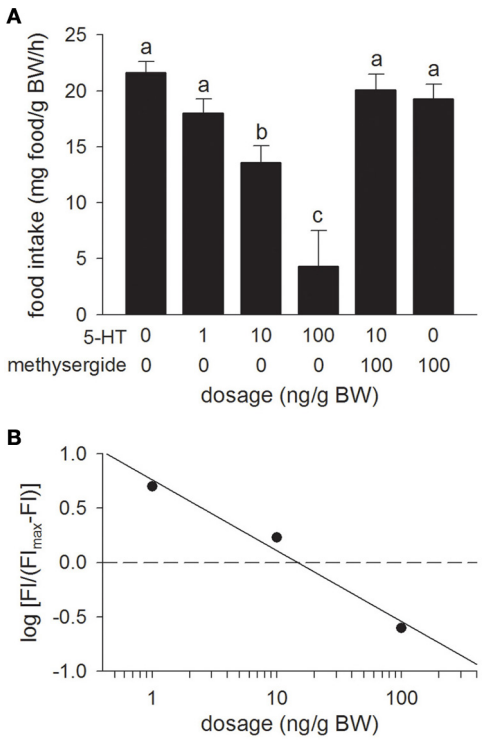

FIGURE 3 | (A) Effects of i.c.v. injections of modified teleost Ringer's (30% methanol, 70% teleost Ringer's) or serotonin (5-HT) alone, or in combination with the mixed 5-HT$_1$/5-HT$_2$ receptor antagonist, methysergide on food intake in rainbow trout. Assessment of food intake was carried out as in **Figure 1**. Bars that do not share a common letter are significantly different from each other as determined by a One-Way ANOVA and by a pairwise Tukey's test ($P < 0.05$). Values are means + sem ($n = 8$). **(B)** Hill plot demonstrating the half-maximal effective dose (ED_{50}) of 5-HT ($ED_{50} = 14.7$ ng/g BW) i.c.v. injections on food intake (FI).

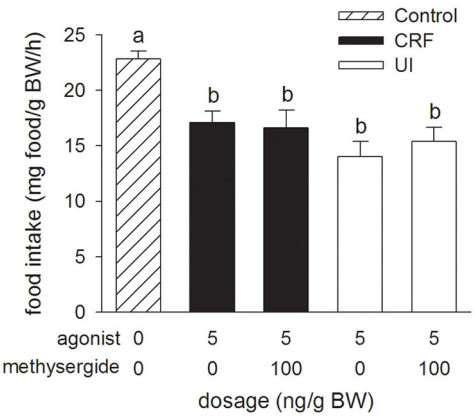

FIGURE 4 | Effects of i.c.v. injections of modified teleost Ringer's (30% methanol, 70%, teleost Ringer's), rainbow trout CRF (rtCRF) or rtUI alone, or in combination with the mixed 5-HT$_1$/5-HT$_2$ receptor antagonist, methysergide on food intake in rainbow trout. Assessment of food intake was carried out as in **Figure 1**. Bars that do not share a common letter are significantly different from each other as determined by a One-Way ANOVA and by a pairwise Tukey's test ($P < 0.05$). Values are means + sem ($n = 8$).

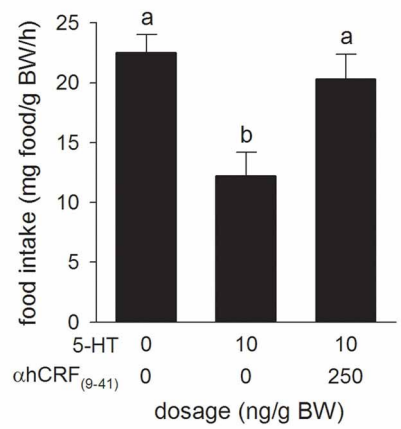

FIGURE 5 | Effects of i.c.v. injections of teleost Ringer's (0.2% $NaHCO_3$ in 0.6% NaCl) or serotonin (5-HT) alone, or in combination with the CRF receptor antagonist, α-helical CRF$_{(9–41)}$ (αhCRF$_{(9–41)}$) on food intake in rainbow trout. Assessment of food intake was carried out as in **Figure 1**. Bars that do not share a common letter are significantly different from each other as determined by a One-Way ANOVA and by a pairwise Tukey's test ($P < 0.05$). Values are means + sem ($n = 8$).

experiments in goldfish (De Pedro et al., 1993; Bernier and Peter, 2001; Matsuda et al., 2008), amphibians (Crespi et al., 2004; Morimoto et al., 2011), chickens (Denbow et al., 1999; Zhang et al., 2001) and rats (Britton et al., 1984; Negri et al., 1985). Similarly, i.c.v. injections of UI have previously been shown to inhibit food intake in goldfish (Bernier and Peter, 2001), chicken (Zhang et al., 2001), and rats (Spina et al., 1996). Overall, rtCRF and rtUI consistently suppressed food intake in a dose-related manner with potencies similar to those of r/hCRF and c/gUI in other species (Spina et al., 1996; Bernier and Peter, 2001). In addition, the stronger potency of rtUI over rtCRF in suppressing food intake corroborates earlier observations in mammals (Britton et al., 1984; Negri et al., 1985; Spina et al., 1996) and goldfish (Bernier and Peter, 2001). In mammals (Spina et al., 1996; Contarino et al., 2000) and *Xenopus* (Boorse et al., 2005), UCN is also a more potent inhibitor of appetite than CRF. The only exception appears to be in neonatal chicks where CRF has greater anorexic activity than UCN (Zhang et al., 2001). In general, the results of receptor binding studies suggest that the greater appetite-suppressing effects of UCN in mammals and *Xenopus* may be due to the higher affinity of this peptide for the CRF-R2 receptor than that of CRF (Vaughan et al., 1995; Zorrilla et al., 2003; Boorse et al., 2005). In contrast, the evidence from binding studies in chum salmon (*O. keta*) and catfish (*A. nebulosus*) suggest that neither the CRF-R1 or CRF-R2 receptors in fish are able to discriminate between CRF and UI (Arai et al., 2001; Pohl et al., 2001). In mammals, the pharmacological differences in the affinity of UCN and CRF for the CRF binding protein (CRF-BP) may also explain the greater anorexic activity of UCN (Fekete et al., 2011). Although, the binding properties of CRF-BP in fish are not known, CRF-BP is broadly expressed in the hypothalamus

of rainbow trout (Alderman et al., 2008). Moreover, in zebrafish (*Danio rerio*), the greater overlap between the expression pattern of CRF-BP and CRF than between CRF-BP and UI, suggest that CRF-BP may differentially regulate these peptides in fish (Alderman and Bernier, 2007). Therefore, although the results from this study and from our previous work in goldfish (Bernier and Peter, 2001) suggest that UI plays a more important role than CRF in the regulation of food intake, more investigations are needed to explain the rank order potency of CRF-related peptides in fish.

The reversal of CRF- and UI-induced appetite inhibition by α-helical $CRF_{(9–41)}$ in rainbow trout (this study) and goldfish (De Pedro et al., 1997; Bernier and Peter, 2001) suggests that the central effects of these peptides on appetite are mediated via CRF receptors. This conclusion is supported by *in situ* hybridization data showing that CRF-R1 and CRF-R2 are highly expressed in hypothalamic regions (Arai et al., 2001) that have been associated with the control of feeding in fish (Matsuda, 2009; Volkoff et al., 2009). Relative to non-injected and saline-injected fish, the lack of effect of i.c.v injections of α-helical $CRF_{(9–41)}$ on food intake in this study and in goldfish (Bernier and Peter, 2001) suggest that basal levels of CRF-related peptides do not exert an inhibitory tone on appetite in fish. Similarly, although both CRF receptor types have been implicated in mediating the anorectic actions of CRF and UCN in mammals (Fekete and Zorilla, 2007), knocking out the CRF-R1 or CRF-R2 receptor does not influence basal food intake in rats (Preil et al., 2001). In contrast, i.c.v. injection of α-helical $CRF_{(9–41)}$ increased food intake in *Xenopus laevis* (Crespi et al., 2004).

The dose-dependant reductions in appetite after i.c.v. administration of 5-HT concur with similar studies in goldfish (De Pedro et al., 1998), birds (Denbow et al., 1983; Steffens et al., 1997) and mammals (Dagnault et al., 1993; Gibson et al., 1993; Smith et al., 1999), and directly implicate 5-HT as an anorexigenic signal in rainbow trout. Similarly, rainbow trout treated with fenfluramine, a 5-HT releasing agent, are characterized by a dose-dependent reduction in food intake (Ruibal et al., 2002). While the 5-HT dose needed to suppress food intake by 50% in rainbow trout is similar to the potency of 5-HT in rats (Smith et al., 1999), methodological differences in the administration of the agonist may explain the relatively low anorectic potency of 5-HT in goldfish (De Pedro et al., 1998). The reversal of 5-HT-induced appetite inhibition by the relatively non-selective $5\text{-}HT_{1-2}$ antagonist methysergide provides original evidence that the anorexigenic effects of 5-HT in fish are likely mediated by $5\text{-}HT_1$ and/or $5\text{-}HT_2$ receptors. These results are consistent with the current view in mammals that $5\text{-}HT_{1B}$ and $5\text{-}HT_{2C}$ receptors are the prime mediators of serotonin's anorectic action (Tecott, 2007; Lam et al., 2010; Marston et al., 2011). Whether the same receptor subtypes play a dominant role in mediating the anorexigenic effects of 5-HT in fish remains to be determined.

CRF-related peptide-secreting neurons operate within a complex system of neuropeptides and neurotransmitters, where interactions with other substances are critical for proper functioning (Herman et al., 2003; Joëls and Baram, 2009). In keeping with this tenant, the reversal of 5-HT-induced anorexia by α-helical $CRF_{(9–41)}$ in rainbow trout, as previously observed in goldfish (De Pedro et al., 1997) and mammals (Grignaschi et al., 1996), suggests that the anorexic actions of 5-HT are at least partially mediated by CRF-secreting neurons. In mammals, the notion that 5-HT mediates its actions on food intake through CRF neurons is also supported by the fact that CRF antibodies block the anorexic effects of i.c.v. injections of 5-HT, its precursor 5-hydroxytryptophan and the 5-HT reuptake inhibitor, fenfluramine (Le Feuvre et al., 1991). In goldfish (Mennigen et al., 2010) and mammals (Laflamme et al., 1996; Choi et al., 2006), the appetite-suppressing effects of 5-HT reuptake inhibitors are associated with an increase in hypothalamic CRF gene expression, and in rats these effects are blocked by injection of the $5\text{-}HT_{1–2}$ receptor antagonist, metergoline (Boisvert et al., 2011). The fact that CRF neurons in the paraventricular nucleus (PVN) (Liposits et al., 1987) and the nucleus preoptic (NPO) (Lillesaar et al., 2009) receive serotonergic inputs, and that direct application of 5-HT into the PVN decreases food intake (Leibowitz, 1986), suggest that serotonergic fibers may directly engage CRF in the regulation of food intake (**Figure 6**). In contrast, although there is some evidence that the UCN neurons of the Edinger–Westphal (EW) nucleus (the principle site of UCN synthesis) are involved in the regulation of food intake (Weitemier and Ryabinin, 2005; Kozicz et al., 2011), the role of UCN-secreting neurons as mediators of the anorexic actions of 5-HT are not known. Similarly, it is not known whether the UI neurons of the nucleus of the medial longitudinal fascicle (NMLF) (the primary site of UI synthesis; Alderman and Bernier, 2007; Bräutigam et al., 2010), or those of the NPO and the hypothalamus, contribute to the anorexic actions of 5-HT in fish.

Mammalian studies have also shown that a functional melanocortin pathway is essential to exert the effects of 5-HT on food intake (Heisler et al., 2006; Lam et al., 2008). The evidence supports a model (**Figure 6**) in which 5-HT activates arcuate nucleus anorexigenic pro-opiomelanocortin (POMC) neurons to facilitate the release of the endogenous melanocortin 4 receptor (MC4R) agonist α-melanocyte-stimulating hormone (αMSH), and inhibits orexigenic agouti-related peptide (AgRP) neurons, the endogenous MC4R antagonist (Lam et al., 2010; Marston et al., 2011). The fact that CRF neurons are rapidly activated by MC4R agonists in rats (Lu et al., 2003) and that the appetite-suppressing effects of these agonists are abolished by pre-treatment with a non-selective CRF receptor antagonist (Kawashima et al., 2008), suggest that the actions of 5-HT on CRF neurons may also be indirect via the melanocortin system (Lam et al., 2010). Although the anorexigenic action of melanocortin receptor agonists also appear to be mediated by the CRF-signaling pathway in goldfish (Matsuda et al., 2008), to our knowledge the interactions between 5-HT and the melanocortin system in fish have yet to be investigated. Also unknown is whether the anorexigenic actions of the melanocortin system can be mediated through either UCN or UI neurons.

The melanocortin and CRF systems also appear to mediate the anorexic effects of 5-HT in chicken. For example, in broiler cockerels pre-treatment with melanocortin receptor

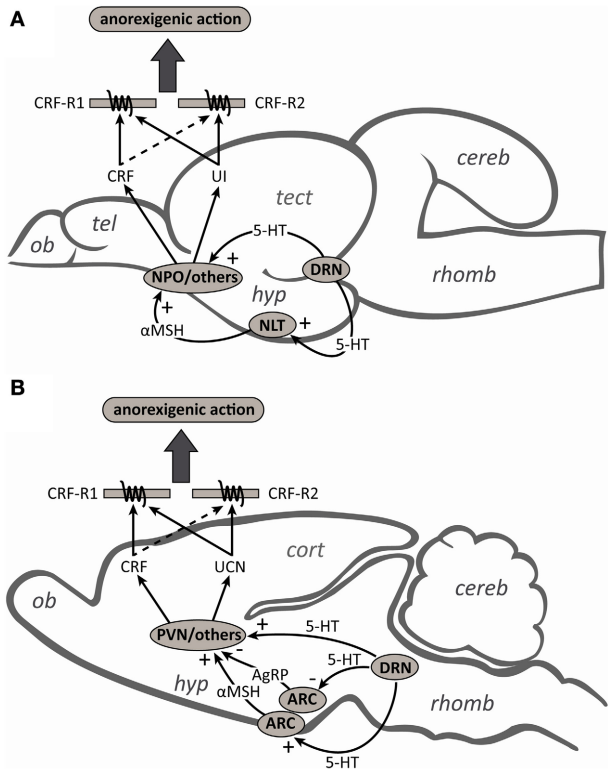

FIGURE 6 | Schematic diagrams of midsagittal sections through (A) the rainbow trout and (B) rat brains to summarize the contributions and interactions of the serotonergic and corticotropin-releasing factor (CRF) systems in the regulation of food intake. Serotonergic (5-HT) neurons from the dorsal raphe nucleus (DRN) can directly stimulate the release of CRF from the nucleus preoptic (NPO) and paraventricular nucleus (PVN) in fish and mammals, respectively. In mammals, 5-HT neurons also activate arcuate nucleus (ARC) neurons to facilitate the release of the melanocortin 4 receptor (MC4R) agonist α-melanocyte-stimulating hormone (αMSH) and inhibit the release of the MC4R antagonist agouti-related peptide (AgRP). CRF neurons in the PVN act as a downstream mediator of MC4R signaling and contribute to the regulation of food intake. In fish, while the anorexigenic actions of αMSH also appear to be mediated by the CRF-signaling pathway, the interactions between 5-HT and the melanocortin system have yet to be investigated. Also unknown are the roles of urotensin I (UI) and urocortin (UCN) secreting neurons as mediators of either the direct or indirect anorexigenic actions of 5-HT. Although CRF and UI/UCN have anorexigenic actions and both the CRF type 1 (CRF-R1) and type 2 (CRF-R2) receptors have been implicated in the regulation of food intake, the higher affinity of UI/UCN than CRF for CRF-R2 likely explains why UI/UCN more potently inhibits food intake than CRF (see text for further details). Abbreviations: cereb, cerebellum; cort, cerebral cortex; hyp, hypothalamus; NLT, nucleus lateralis tuberis; ob, olfactory bulb; rhomb, rhombencephalon; tect, optic tectum; tel, telencephalon.

antagonists attenuates the anorexic effects of icv injections of 5-HT (Zendehdel et al., 2012). In chicks, non-selective CRF receptor antagonists reverse the appetite-suppressing effects of α-MSH (Tachibana et al., 2007) and β-MSH (Kamisoyama et al., 2009). Moreover, central administration of α-MSH is associated with an increase CRF gene expression in the hypothalamus of chicken (Kamisoyama et al., 2009). In contrast, while it is currently not known whether 5-HT regulates feeding in amphibians via the melanocortin or CRF pathways, there is evidence that CRF can inhibit visually guided prey-catching behavior in toads (*Bufo speciosus*; Carr et al., 2002). In *Xenopus laevis*, CRF expressed by optic tectum neurons may regulate visually-guided feeding behavior by modulating the communication between the sensory and motor pathways that are involved in food intake (Carr et al., 2013).

In mammals, while CRF and UCN fibers can modulate the stress response and stress-related behaviors by regulating dorsal raphe serotonergic activity (Kozicz, 2010; Waselus et al., 2011), the anorexigenic effects of CRF-related peptides appear to be mediated through non-serotonergic targets such as the ventromedial hypothalamic nucleus, the basolateral amygdala, the lateral septum and the bed nucleus of the stria terminalis (Jochman et al., 2005; Kozicz et al., 2011; Ohata and Shibasaki, 2011). Similarly, while the effects of CRF-related peptides on locomotor activity, aggression and anxiety-like behaviors in salmonids may be at least partly mediated through the serotonergic system (Clements et al., 2003; Carpenter et al., 2007, 2009; Backström et al., 2011), the appetite-suppressing actions of i.c.v. CRF and UI in this study were not affected by co-injection of the 5-HT_{1-2} receptor antagonist, methysergide. Although we cannot exclude the possibility that the anorexigenic effects of i.c.v. injections of CRF-related peptides in fish are at least partly the result of non-specific behavioral changes such as an increase in locomotor activity and/or anxiogenic-like effects (Matsuda, 2013), overall our results suggest that CRF- and UI-induced anorexia in rainbow trout are not mediated by 5-HT_1 or 5-HT_2 receptors. Identifying the direct targets of CRF and UI within the feeding-related neurocircuitry in fish remains an important avenue for future investigation.

In summary, results from the present study show that exogenous CRF, UI and 5-HT are potent anorexigenic factors in rainbow trout. The low doses of native ligands required to inhibit food intake and the ability of i.c.v. co-injections of receptor antagonists to reverse these effects, suggest that central CRF-related peptide-expressing neurons and serotonergic fibers are involved in the regulation of food intake in this species. While many questions remain regarding the specific nature of the interactions between the CRF and serotonergic systems in fish, our study also demonstrates that the appetite-suppressing effects of 5-HT in rainbow trout are at least partially mediated by CRF-related peptide-secreting neurons. Given the roles of CRF, UI and 5-HT in the regulation of the HPI axis, our data suggest that these neurons are likely involved in the coordinated regulation of food intake and the stress response, and are important mediators of the appetite-suppressing effects of stressors in salmonids.

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CRF and urocortin peptides as modulators of energy balance and feeding behavior during stress

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Early on, corticotropin-releasing factor (CRF), a hallmark brain peptide mediating many components of the stress response, was shown to affect food intake inducing a robust anorexigenic response when injected into the rodent brain. Subsequently, other members of the CRF signaling family have been identified, namely urocortin (Ucn) 1, Ucn 2, and Ucn 3 which were also shown to decrease food intake upon central or peripheral injection. However, the kinetics of feeding suppression was different with an early decrease following intracerebroventricular injection of CRF and a delayed action of Ucns contrasting with the early onset after systemic injection. CRF and Ucns bind to two distinct G-protein coupled membrane receptors, the CRF_1 and CRF_2. New pharmacological tools such as highly selective peptide CRF_1 or CRF_2 agonists or antagonists along with genetic knock-in or knock-out models have allowed delineating the primary role of CRF_2 involved in the anorexic response to exogenous administration of CRF and Ucns. Several stressors trigger behavioral changes including suppression of feeding behavior which are mediated by brain CRF receptor activation. The present review will highlight the state-of-knowledge on the effects and mechanisms of action of CRF/Ucns-$CRF_{1/2}$ signaling under basal conditions and the role in the alterations of food intake in response to stress.

Keywords: body weight, CRF, food intake, stress, urocortin

INTRODUCTION

Hans Selye was the first to introduce the biological concept and term of stress based on similar macroscopic changes namely the development of gastric erosions, involution of the lymphatic organs and hypertrophy of the adrenal glands in response to the exposure to a variety of noxious chemical agents or physical constraint in rats (Selye, 1936, 1976). Fourteen years later, Geoffrey Harris showed that various stressors induced the release of adrenocorticotropic hormone (ACTH) and provided the evidence for the release of a hypothalamic factor acting *via* hypophyseal portal vessels (De Groot and Harris, 1950; Harris, 1950). This concept was further supported by the purification of a hypothalamic factor stimulating ACTH release from the rat pituitary gland in 1955 (Guillemin and Rosenberg, 1955; Saffran et al., 1955). Therefore, this factor—which eluded its identification up to 1981—was named corticotropin-releasing factor (CRF) (Guillemin and Rosenberg, 1955; Saffran et al., 1955). From the beginning and with great foresight, Selye assumed that CRF would be "the first mediator that integrates the adaptive bodily response to stress" (Selye, 1976). After its identification and characterization by Vale's group as a 41 amino acid (aa) peptide (Vale et al., 1981), numerous studies unraveled pleiotropic stress-like actions induced by central injection of CRF beyond the mere activation of the hypothalamus-pituitary-adrenal gland axis. This includes the modulation of autonomic (sympathetic and sacral parasympathetic activation), visceral and immune functions but also behaviors (anxiogenic, reproductive and feeding) (Dunn and Berridge, 1990; De Souza, 1995; Heinrichs et al., 1997; Habib et al., 2000; Bale and Vale, 2004; Stengel and Taché, 2010).

It is well known that acute exposure to various stressors such as visceral (physical) (Stengel et al., 2011), immune (Basa et al., 2003; Wang et al., 2006; Stengel et al., 2010) and cognitive (psychological) (Rybkin et al., 1997; Kinzig et al., 2008; Calvez et al., 2011) stressors results in the suppression of food intake in rodents. This inhibitory effect is largely mediated by the recruitment of CRF signaling pathways in the brain. This is supported by the activation of CRF-containing neurons and pituitary ACTH release (Calvez et al., 2011; Wang et al., 2011a), the mimicry by brain injection of CRF or related peptides, urocortins, and its prevention by central injection of CRF receptor antagonists in rats (Koob and Heinrichs, 1999). Interestingly, models of chronic stress can have a dual effect on feeding and food preference in experimental animals and humans (Dallman, 2010). Social defeat

stress led to an increase in daily food intake in mice giving rise to the recruitment of other/additional signaling systems under these conditions, while chronic exposure to a battery of physical/environmental stressors reduces food intake and body weight in rats (Lutter et al., 2008; Kumar et al., 2013).

The present review will describe first the state-of-knowledge on the distribution of the CRF signaling systems including CRF receptors, CRF, and urocortins in the brain and in the periphery and the effects and mechanisms of central and peripheral injection of CRF and Ucns on food intake. Lastly, the role of CRF receptor signaling pathways in the modulation of food intake under conditions of stress will be highlighted.

THE CRF SIGNALING SYSTEM

LIGANDS: CRF AND UROCORTINS

CRF was identified in 1981 as a 41-aa hypothalamic peptide stimulating the release of ACTH and β-endorphin from the anterior part of the pituitary gland (Vale et al., 1981). Subsequently, other members of the CRF family were identified (Vaughan et al., 1995; Hsu and Hsueh, 2001; Lewis et al., 2001; Reyes et al., 2001), namely Ucn 1, a 40-aa peptide sharing 45% sequence identity with rat/human (r/h) CRF, Ucn 2, a 39-aa peptide sharing 34% homology with r/h CRF and 42% with r/h Ucn 1 (Reyes et al., 2001; Vaughan et al., 2013), and Ucn 3, a 38-aa peptide sharing only 26% homology with r/h CRF and 21% with r/h Ucn 1, respectively (Lewis et al., 2001). These four peptides are all derived from distinct genes highly conserved across mammalian, non-mammalian, and invertebrate species consistent with the physiological importance of this signaling system (Lovejoy and de Lannoy, 2013).

CRF is widely distributed in the rodent brain with robust expression at the mRNA and peptide level in the cerebral cortex, amygdala, hippocampus, paraventricular nucleus of the hypothalamus (PVN) and the Barrington's nucleus (Valentino et al., 1994; Wang et al., 2011a; Beckerman et al., 2013). Despite the fact that urocortins show an extensive brain distribution, little neuroanatomical overlap exists between CRF and urocortins. Ucn 1 has limited brain distribution with one major expression site which is the Edinger-Westphal nucleus (EWN) (Bittencourt et al., 1999; Morin et al., 1999; Shah et al., 2013), and to a lesser extent, the olfactory bulb, supraoptic nucleus (SON), ventromedial hypothalamus (VMH), lateral hypothalamic area, lateral superior olive, ambiguus nucleus, dorsolateral tegmental nucleus, linear and dorsal raphe nuclei, substantia nigra and cranial nerve motor nuclei, namely facial and hypoglossal (Kozicz et al., 1998; Bittencourt et al., 1999). Ucn 2 mRNA is found in the parvo- and magnocellular part of the PVN, the arcuate nucleus of the hypothalamus, SON, locus coeruleus, in cranial nerve motor nuclei (trigeminal, facial and hypoglossal nuclei) and also in the ventral horn of the spinal cord (Reyes et al., 2001; Mano-Otagiri and Shibasaki, 2004). It is important to note that the knowledge on Ucn 2′s distribution at the peptide level has been limited by the lack of a specific Ucn 2 antibody. Lastly, Ucn 3 mRNA and peptide expression was detected in the amygdala, lateral septum, PVN, VMH, basomedial nucleus of the stria terminalis, dorsal raphe nucleus and in the area postrema (Lewis et al., 2001; Li et al., 2002; Mano-Otagiri and Shibasaki, 2004; Venihaki et al., 2004).

Besides the widespread expression of CRF and related peptides initially found in the central nervous system and thought to be restricted to this site, as commonly observed for other peptides, CRF and urocortins were also detected in visceral organs, namely the lung, heart, spleen, adipose tissue, gonads (Boorse and Denver, 2006; Wypior et al., 2011), pancreas (Li et al., 2003; van der Meulen et al., 2012) and the gastrointestinal tract (Kozicz and Arimura, 2002; Taché and Perdue, 2004; Taché and Bonaz, 2007; Yuan et al., 2012b) including the enteric nervous system (Liu et al., 2006; Kimura et al., 2007). The past decade witnessed an increasing knowledge on the peripheral expression and regulation of CRF and urocortin signaling systems and recognition of their implication in health and disease (Yuan et al., 2010; Buckinx et al., 2011; Overman et al., 2012; Diaz and Smani, 2013; Onorati et al., 2013).

CRF RECEPTORS

CRF ligands bind to CRF_1 and/or CRF_2 receptors consisting of 415 aa and 411 aa, respectively, that are encoded from two distinct genes belonging to the B1 subfamily of seven-transmembrane G-protein coupled receptors encompassing seven membrane-spanning α-helices and an extracellular domain (Perrin and Vale, 1999). Both receptors share 70% identity within their species homologue. The most variable component is the binding domain that encompasses the N-terminal and the three extracellular coils that share only 40% homology between the two receptor subtypes. In their N-terminal extracellular region, CRF receptors contain several potential points of N-glycosylation along with several Cys residues that form disulfide bonds closely associated with their functionality (Zmijewski and Slominski, 2010; Liapakis et al., 2011). Various partial or total exon deletions or insertions in the CRF gene - in some cases associated with a shift in the open reading frame - generate multiple isoforms of CRF_1 and CRF_2 receptors (Pisarchik and Slominski, 2001; Wu et al., 2007, 2011; Zmijewski and Slominski, 2010). Among the nine human CRF_{1a-i} variants, CRF_1, also named CRF_{1a}, is the main functional receptor. The CRF_{1b} isoform is also called pro-CRF_1, and this is the only variant coded by all 14 exons, leading to a 29-aa insertion into the first intracellular loop which impairs agonist binding and signaling activity compared to CRF_{1a} (Markovic and Grammatopoulos, 2010). The other isoforms CRF_{1c-i} have the exon 6 spliced out and in addition there are either cryptic exons (1h), or the skipping of single (1c, 1d, and 1f) or multiple exons (1e and 1g) (Pisarchik and Slominski, 2001, 2004; Wu et al., 2011). The latest splice variant identified, CRF_{1i} has a deletion of exon 4 and is functional. This was shown by the increased phosphorylation of the extracellular signal regulated kinase ½ (ERK1/2) in response to Ucn 1 assessed in CRF_{1i} transfected human embryonic kidney (HEK) cells (Wu et al., 2011). Other soluble isoforms such as CRF_{1e} and CRF_{1h} may play a modulatory role as their expression in transfected COS cells either decreased or amplified the $CRF_{1\alpha}$-coupled cAMP production induced by Ucn 1 (Pisarchik and Slominski, 2004).

With regards to the CRF_2, three functional splice variants 2a, 2b, and 2c (also originally named 2α, 2β, and 2γ) derived from alternative splicing exist in humans, while in other mammals only two isoforms, CRF_{2a} and CRF_{2b} are expressed (Hauger et al.,

2003; Hillhouse and Grammatopoulos, 2006). These isoforms differ structurally in their N-terminal extracellular domains (Hauger et al., 2003). The 34-aa N-terminal extracellular region of the CRF_{2a} is replaced by 61 aa in the CRF_{2b} and 20 aa in the CRF_{2c}, while the C-terminus is common to all CRF_2 receptor splice variants (Miyata et al., 2001; Dautzenberg et al., 2004; Wu et al., 2007). Sequence comparison indicated high homology between rat and mouse CRF_{2a} (94%) and mouse and human (92%) (Dautzenberg et al., 2004). The presence of CRF_{2a} in amphibian species suggests an earliest occurrence of this splice variant during vertebrate evolution, while the CRF_{2b} is less conserved and appears to be evolutionarily younger and is found only in mammals (Dautzenberg et al., 2001). Recently, Chen et al. reported a novel CRF_{2a} splice variant in the mouse brain that includes the first extracellular domain of the CRF_{2a} receptor and acts as a soluble binding protein ($sCRF_{2a}$), thereby modulating the accessibility and signaling (Chen et al., 2005). In the rat esophagus, CRF_{2b} wild-type transcript is predominantly expressed and in addition, several new CRF_2 splice variants including six CRF_{2a} isoforms were identified (Wu et al., 2007; Yuan et al., 2012b).

Similar to CRF, the CRF_1 receptor displays a wide distribution throughout the brain. In rats, the CRF_1 is densely and widely expressed in the forebrain, in the septal region and amygdala (Justice et al., 2008), whereas basal expression is low in the hypothalamus but up-regulated under conditions of stress and also by CRF as a feed-forward mechanism (Bonaz and Rivest, 1998; Imaki et al., 2001; Konishi et al., 2003). Contrasting with the wide distribution of the CRF_1, expression of the CRF_2 is mainly found in the lateral septum, amygdala and hypothalamic nuclei including the SON and VMH, dorsal raphe, area postrema and nucleus tractus solitarius (Bittencourt and Sawchenko, 2000; Chen et al., 2012). Both, CRF_1 (laminae III-VIII) and CRF_2 mRNA (laminae III-X) were also detected in the mouse spinal cord (Korosi et al., 2007). In the periphery, the CRF_1 was detected in the anterior and intermediate lobe of the pituitary gland (Turnbull and Rivier, 1997) and in the gastrointestinal tract more prominently in colonic endocrine, neuronal and immune cells (Chatzaki et al., 2004; Yuan et al., 2007, 2012a), while CRF_2 was found in the stomach including the luminal surface of the crypts and in blood vessels of the submucosal layer (Chatzaki et al., 2004; Yuan et al., 2012b).

Following the identification of CRF receptors, the characterization of binding affinities to endogenous ligands showed that the CRF_1 and CRF_2 receptors display distinct affinities to CRF and urocortins (Lewis et al., 2001; Hauger et al., 2003; Hillhouse and Grammatopoulos, 2006). CRF displays a 10- to 40-fold higher affinity for the CRF_1 than the CRF_2 receptor, whereas Ucn 1 binds with equal affinity to both CRF receptors thereby displaying a 100-fold higher affinity to the CRF_2 receptor compared to CRF (Grace et al., 2007). It is important to note that so far no endogenous selective ligand for the CRF_1 has been identified. In contrast to CRF and Ucn 1, Ucn 2, and Ucn 3 show high selectivity for the CRF_2 (Grace et al., 2007). In contrast to the CRF_1 receptor isoforms, binding characteristics of the CRF receptor splice variants, CRF_{2a}, CRF_{2b}, and CRF_{2c} are almost identical with high affinity for Ucn 1, Ucn 2, and Ucn 3, and lower affinity for r/hCRF (Kostich et al., 1998; Ardati et al., 1999; Suman-Chauhan et al., 1999; Lewis et al., 2001). However, the isoforms of CRF_{2a} show distinct pharmacological profiles; the mouse $sCRF_{2a}$ receptor has very low affinity for Ucn 2 and Ucn 3, while binding to Ucn 1 (Ki 6.6 nM), and, to a lesser extent, to CRF (23 nM), and inhibits the cAMP and ERK1/2-p42,44 responses to Ucn 1 and CRF (Chen et al., 2005). In contrast, rat CRF_{2a-tr} binds with low affinity to CRF (Kd 12.7 nM) and does not bind to Ucn 1 (Miyata et al., 1999).

FOOD INTAKE INHIBITORY ACTIONS OF BRAIN CRF AND Ucns

It is well documented that members of the CRF family and more prominently Ucns injected into the brain ventricle suppress food intake in various species (Zorrilla et al., 2003; Wang et al., 2011b) and increase energy expenditure (Richard et al., 2002). In *ad libitum* fed rats, intracerebroventricular (icv) injection of CRF (Spina et al., 1996), Ucn 1 (Spina et al., 1996; Yakabi et al., 2011), Ucn 2 (Ohata and Shibasaki, 2004), and Ucn 3 (Ohata and Shibasaki, 2004) decreases dark phase food intake. At the lowest icv doses, Ucn 1 and Ucn 2 decrease food intake without inducing conditioned taste aversion or visceral illness (Benoit et al., 2000; Inoue et al., 2003; Zorrilla et al., 2004). It is to note that icv Ucn 1 is more potent in suppressing food intake compared to CRF (Spina et al., 1996), and Ucn 2 is 10-fold more potent than Ucn 3 in reducing food intake (Pelleymounter et al., 2004). Kinetic studies also showed differences in the time course of food intake suppression induced by icv injection of the CRF-related peptides. CRF, a preferential CRF_1 agonist, induces a rapid onset and short-term reduction of the re-feeding response to an overnight food deprivation in mice and rats (Ohata and Shibasaki, 2004; Pelleymounter et al., 2004), while the dark phase food intake-reducing effect of icv Ucn 2 or Ucn 3 is delayed (onset >3 h) and long lasting in rats (Inoue et al., 2003; Zorrilla et al., 2004). Conversely, in CRF_1 deficient mice, icv Ucn 1 still induces a delayed onset suppression of food intake while the early phase is no longer observed (Bradbury et al., 2000). Similar to the acute effects, under chronic conditions observed in mice overexpressing CRF there is also a curtailing of the re-feeding response to a fast (Stengel et al., 2009).

Convergent studies established the primary role of CRF_2 in mediating the anorexigenic effect of acute or repeated icv injections of CRF or Ucns using complementary pharmacologic (selective CRF_1 or CRF_2 antagonists) (Smagin et al., 1998; Contarino et al., 2000; Pelleymounter et al., 2000; Cullen et al., 2001; Sekino et al., 2004) and gene deletion (CRF_1 or CRF_2 deficient mice) (Bradbury et al., 2000) approaches in addition to the selective CRF_2 agonists, Ucn 2, and Ucn 3 (Cullen et al., 2001; Richard et al., 2002; Zorrilla et al., 2003; Pelleymounter et al., 2004). To date, the role of CRF_1 in mediating the effects of icv CRF and Ucn 1 appears to be less specific and is confounded by competing behaviors (increased locomotion, grooming, anxiety- or fear-like) induced by activation of the brain CRF_1 signaling pathway. This contrasts with icv Ucn 2 that does not elicit behavioral arousal or anxiogenic effects (Pelleymounter et al., 2000; Inoue et al., 2003; Zorrilla et al., 2004; Jochman et al., 2005). Several brain sites expressing high concentrations of CRF_2 (Bittencourt and Sawchenko, 2000) have been identified to be

responsive to Ucns resulting in a CRF_2-mediated anorexigenic response, namely the lateral septum (Bakshi et al., 2007), PVN (Currie et al., 2001), VMH (Ohata et al., 2000; Fekete et al., 2007; Chen et al., 2012), medial amygdala (Fekete et al., 2007) and dorsal raphe (Weitemier and Ryabinin, 2006). In addition, hindbrain structures are involved based on the observation that Ucn 1 injected into the fourth brain ventricle is still able to reduce food intake in chronically decerebrated rats (Daniels et al., 2004). Among those, the nucleus tractus solitarius has been identified as a brainstem site responsive to Ucn 1 (Grill et al., 2000).

Analysis of changes in feeding patterns associated with the decreased food intake indicates that Ucn 2 injected icv reduced the size of the meal (increased satiation) and the rate of ingestion, whereas meal frequency was not altered in rats (Inoue et al., 2003). In addition, Ucn 3 injected icv, and even more potently, when microinjected into the PVN and VMH, increased inter-meal interval (induction of satiety), whereas meal size was reduced (induction of satiation) at the highest dose only (Fekete et al., 2007). In the medial amygdala, Ucn 3 was shown to promote nibbling (smaller but more frequent meal) (Fekete et al., 2007), indicating the distinct modulation of feeding patterns by Ucn 2 and Ucn 3 and the influence of brain sites of action.

The physiological relevance of the brain CRF_2 signaling pathways in the regulation of feeding pattern and body weight is supported by reports that CRF_2 knockout mice showed increased nocturnal food intake of normal chow (Tabarin et al., 2007) and consumed more high fat food compared to their wild type littermates (Bale et al., 2003). In addition, mice with a site specific knockdown of CRF_2 in the VMH using small hairpin RNA exhibit increased food intake under basal and stimulated conditions by an overnight fast (Chao et al., 2012). This supports a role of CRF_2 in the VMH to curtail the cessation of eating. There is also evidence that continuous icv infusion of the CRF_2 antagonist antisauvagine-30 over 13 days increased food intake by 5% in normal rats (Cullen et al., 2001), while chronic injection of a CRF_1 antagonist had no effect (Ohata et al., 2002). However mice lacking Ucn 1 (Vetter et al., 2002) or Ucn 2 (Chen et al., 2006) have a normal spontaneous food intake which may merely emphasize the compensatory mechanisms by other endogenous peptide members of the CRF family as genetic deficient Ucn 3 showed elevated basal feeding and increased nocturnal food intake after overnight fasting compared with the wild-type littermates (Chao et al., 2012).

Several potential mechanisms could participate in icv Ucns-induced anorexia. Central injection of CRF and Ucns potently suppressed gastric emptying (Stengel and Taché, 2010) and induces hyperglycemia (Brown et al., 1982; Chen et al., 2010). Both effects are known to reduce feeding. Delayed gastric emptying by slowing meal transit leads to accrual of food in the stomach and consecutively to gastric satiety signaling to the brain (Phillips and Powley, 1996) while elevated glucose exerts a direct action on glucose sensing of hypothalamic neurons regulating food intake (Levin, 2006; Cha et al., 2008). There is also evidence Ucn 1 icv suppressed circulating acyl ghrelin (Yakabi et al., 2011), the only known peripherally produced and centrally acting orexigenic hormone (Hosoda et al., 2002; Stengel and Taché, 2012). Moreover, Ucn 1 microinjected into the PVN increased plasma levels of leptin (Kotz et al., 2002), a potent appetite suppressant (Keen-Rhinehart et al., 2013). Future studies are needed to evaluate the relative influence of these hormonal and functional alterations.

Besides the reduction in food intake, accumulated evidence indicates that activation of brain CRF and Ucns signaling pathways increases energy expenditure (Richard et al., 2002; Kuperman and Chen, 2008). Sympathetically-regulated heat production in brown adipose tissue (BAT) and lipid metabolism contributes to the non-shivering thermogenic component of energy expenditure and body weight regulation in rodents (Landsberg et al., 1984). The icv injection of Ucns increases oxygen consumption in rats as assessed by indirect calorimetry and elevates body temperature resulting in increased energy expenditure (De Fanti and Martinez, 2002; Telegdy and Adamik, 2008). Microinjection studies showed that CRF sites of action to increase sympathetic nerve activity to interscapular BAT leading to BAT-mediated thermogenesis and energy expenditure are located in the medial preoptic area and dorsomedial hypothalamus unlike the PVN or VMH (Egawa et al., 1990; Cerri and Morrison, 2006; Chao et al., 2012). However, during food restriction, Ucn 1 microinjected into the PVN increases thermogenic capacity by elevating uncoupling protein-1 mRNA levels in BAT (Kotz et al., 2002). The peptide at this site also changes energy substrate utilization as shown by reduction of the respiratory quotient (QR) under basal or NPY- or ghrelin-stimulated conditions (Currie et al., 2001). The CRF_2 seems to play a major role in the effects on energy expenditure as mice lacking the CRF_1 showed a greater reduction in body weight following a 7-day icv infusion of Ucn 1 compared to their wild type littermates (Bradbury et al., 2000). Additionally, the hyperthermic response to icv Ucn 2 and Ucn 3 was blocked by selective CRF_2 receptor antagonists while CRF_1 blockade had no effect (Telegdy et al., 2006). Moreover, selective depletion of CRF_2 in the VMH reduced lipolysis and increased white fat (Chao et al., 2012).

FOOD INTAKE INHIBITORY ACTIONS OF PERIPHERAL CRF AND Ucns

In addition to the central actions of CRF and Ucns, peripheral (intraperitoneal, ip) injection of Ucns reduced food intake in several species (Asakawa et al., 1999; Weisinger et al., 2000; Wang et al., 2001; Tanaka et al., 2009) and repeated administration also lowered body weight gain in mice (Asakawa et al., 1999). The anorexigenic effect of Ucn 1 on re-feeding food intake was stronger than that of cholecystokinin (CCK), leptin, and also CRF in mice (Asakawa et al., 1999; Wang et al., 2001; Tanaka et al., 2009). Interestingly, a synergistic interaction between ip injected Ucn 1 and CCK-8 to reduce the feeding response to a fast and liquid gastric emptying has been reported in mice (Gourcerol et al., 2007). The demonstration that Ucn 1 injected peripherally displays a similar potency as after icv injection supports a peripherally initiated mode of action (Cullen et al., 2001; Sinnayah et al., 2003; Pelleymounter et al., 2004). However, it is to note that systemic doses at which the preferential CRF_1 agonist CRF, and potent CRF_1/CRF_2 agonist, Ucn 1 decrease food intake are associated with conditioned taste aversion and diarrhea (Fekete et al., 2011), which are not observed at the lower anorexigenic doses of

Ucn 1 given icv (Benoit et al., 2000; Inoue et al., 2003; Zorrilla et al., 2004).

The reduction of food intake by ip Ucn 1 is CRF_2 mediated as the selective CRF_2 antagonists, antisauvagine-30 and astressin$_2$-B blunted the reduction of food intake, whereas selective CRF_1 antagonists did not (Weisinger et al., 2000; Wang et al., 2001, 2011b). In line with this finding, fasted mice lacking the CRF_2 did not show a reduction of re-feeding food intake following ip injected Ucn 1 (Wang et al., 2011b). The mechanisms through which peripheral Ucn 1 exerts its anorexigenic effect do not involve capsaicin sensory afferents, unlike ip CCK-8 tested under the same conditions in mice (Wang et al., 2001). It was also shown that the slowing of gastric emptying associated with ip Ucn 1 accounts only for 35% of the reduction of food intake induced by Ucn 1 (Wang et al., 2001). It may be speculated that Ucn 1 acts through CRF_2 densely expressed in appetite-/taste aversion-regulating brain structures outside the blood brain barrier, namely the area postrema (Sakai and Yamamoto, 1997; Bittencourt and Sawchenko, 2000) shown to be activated by systemic injection of Ucn 1 (Wang et al., 2000). This contention will need to be further ascertained.

With regards to Ucn 2 and Ucn 3, both peptides induce a rapid in onset and CRF_2 mediated reduction of re-feeding food intake after a fast and also dampen *ad libitum* dark phase food intake with a potency of Ucn 2 > Ucn 3 in rodents (Wang et al., 2001, 2011b; Gourcerol et al., 2007; Tanaka et al., 2009; Fekete et al., 2011). In contrast to ip Ucn 1, Ucn 2 given ip at an anorexigenic dose did not induce signs of taste aversion or malaise (Fekete et al., 2011). Of interest, there is a synergistic interaction between ip CCK-8 and Ucn 2 to reduce the re-feeding response to a fast in mice which was also observed at the level of vagal activity recorded from the gastric afferents in an *in vitro* preparation (Gourcerol et al., 2007). This functional and electrophysiological evidence combined with the expression of CRF_2 in rat nodose ganglia (Mercer et al., 1992; Lawrence et al., 2002) point toward a role of vagal signaling in the mediation of ip Ucn 2 anorexic action that will need to be ascertained using vagal deafferentation.

Additional insight into the characterization of the food intake-reducing effects of peripheral administration of Ucns came from studies detailing changes in meal patterns. Using the micropellet technique, ip injection of Ucn 1 reduces the meal frequency (as a characteristic of satiety), whereas the size of the meal (as a characteristic of satiation) was less robustly altered during the re-feeding period following a 24-h fast in rats (Fekete et al., 2011). Under the same conditions, ip injection of Ucn 2 only reduced meal frequency while not altering meal size (Fekete et al., 2011). These data were recently extended to mice following ip injection of Ucn 2 using an automated episodic food intake monitoring system for solid food: Ucn 2 reduced meal size and duration (induction of satiation) but also increased meal frequency (reduction of satiety) in overnight fasted mice (Wang et al., 2011b). Interestingly, when injected ip in freely fed animals at the beginning of the dark phase, Ucn 2 only affected satiation (reduction of meal size), whereas satiety (indicated by the number of meals) was not altered (Wang et al., 2011b) giving rise to a differential modulation of light and dark phase feeding by Ucn 2.

INVOLVEMENT OF BRAIN CRF SIGNALING PATHWAYS IN THE ANOREXIGENIC RESPONSE TO STRESS

Accumulated evidence supports a role of brain activation of CRF signaling pathways in initiating the hypophagia and weight loss induced by various types of stressors. This was established using pharmacological approaches and to a lesser extent, genetic deletion of CRF ligand or receptors in rodents. Earlier studies showed that icv or 3rd ventricular injection of the non-selective CRF_1/CRF_2, antagonist, α-helical CRF_{9-41} before exposure to stressors prevented the decreased food intake observed after acute exposure to restraint (Krahn et al., 1986; Shibasaki et al., 1988; Smagin et al., 1999), 40 min forced exercise (Rivest and Richard, 1990) or emotional stress induced by a communication box paradigm (Hotta et al., 1999) while having no effect on food intake under non-stressful conditions (Hotta et al., 1999). The CRF antagonist injected into the 3rd ventricle also blocked repeated restraint-induced weight loss (Smagin et al., 1999). Subsequent studies to elucidate the importance of each CRF receptor subtype in the stress-related decrease in food intake indicated that either CRF receptor subtype alone or in combination can be involved in the hypophagia depending upon the modality of the stressors. Evidence so far supports that the relative ability of selective CRF receptor subtype antagonists to block stress-related anorexia is critically dependent upon specific brain sites activated and related CRF ligands and receptor subtypes recruited by different stressors. For instance, emotional stress triggered by the communication box paradigm, foot shock and 1-h restraint was reported to involve both CRF_1 and CRF_2 receptors in rats as shown by partial or complete reversal induced by either icv injection of CRF_2 antagonist, antisauvagine-30 or peripheral administration of the CRF_1 antagonist, CRA 1000 (Hotta et al., 1999; Sekino et al., 2004). In mice, novelty/group separation stress-induced reduction of food intake was curtailed by icv pretreatment with the CRF_1 antagonist, NBI- 127914 while the potent CRF_2 antagonist, astressin$_2$-B had no effect (Saegusa et al., 2011). Brain site specific targeting by CRF antagonists indicates that the CRF_1 antagonist, NBI-27914 microinjected into the basolateral amygdala, unlike central amygdala, prevented emotional stress (rat exposed to predator)-induced decreased food intake and increased grooming while astressin$_2$-B had no effect (Jochman et al., 2005). By contrast, the CRF_2 antagonist, antisauvagine-30 microinjected into the lateral septum or posterior division of the bed nucleus of the stria terminalis prevented acute restraint-induced anorexia while the selective CRF_1 antagonist, antalarmin had no effect in rats (Ohata and Shibasaki, 2011). There is also evidence that CRF_2 knockout mice showed an abbreviated inhibition of food intake induced by restraint stress while not being involved in the early orexigenic response (Tabarin et al., 2007).

Of (patho)physiological relevance is the demonstration that mutation in the CRF_2 gene (Val411Met) was associated with an early onset of severe obesity (Challis et al., 2004). Moreover, several studies observed an association with a portion of chromosome 7 also coding the CRF_2 gene (7p15–7p21) and body mass index (Wu et al., 2002), type 2 diabetes mellitus (Wiltshire et al., 2001) and also fat-free body mass (Chagnon et al., 2000). Future studies are needed to corroborate

these findings and investigate the consequences of these associations.

SUMMARY

In summary, activation of CRF_2 by Ucns reduced feeding after central as well as peripheral injection without provoking behavioral arousal or anxiogenic effects as observed for the anorexigenic action induced by the activation CRF-CRF_1 signaling pathways. Brain sites of action of Ucns involve the lateral septum, PVN, VMH, medial amygdala, dorsal raphe and nucleus tractus solitarius. Both, CRF_1 and CRF_2 receptor activation contributes to the reduction of food intake associated with exposure to various stressors. Their respective involvement is stressors and brain sites specific mostly in relation with endogenous CRF ligands and receptors recruited under the condition of stress. Still little is known whether peripheral Ucn-CRF_2 signaling plays a role in the food intake response to visceral stressors. More research is needed using site specific knockout or overexpression of CRF receptors in order to address this issue and investigate the impact on food intake and body weight.

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Stress and serial adult metamorphosis: multiple roles for the stress axis in socially regulated sex change

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Socially regulated sex change in teleost fishes is a striking example of social status information regulating biological function in the service of reproductive success. The establishment of social dominance in sex changing species is translated into a cascade of changes in behavior, physiology, neuroendocrine function, and morphology that transforms a female into a male, or vice versa. The hypothalamic-pituitary-interrenal axis (HPI, homologous to HP-adrenal axis in mammals and birds) has been hypothesized to play a mechanistic role linking status to sex change. The HPA/I axis responds to environmental stressors by integrating relevant external and internal cues and coordinating biological responses including changes in behavior, energetics, physiology, and morphology (i.e., metamorphosis). Through actions of both corticotropin-releasing factor and glucocorticoids, the HPA/I axis has been implicated in processes central to sex change, including the regulation of agonistic behavior, social status, energetic investment, and life history transitions. In this paper, we review the hypothesized roles of the HPA/I axis in the regulation of sex change and how those hypotheses have been tested to date. We include original data on sex change in the bluebanded goby (*Lythyrpnus dalli*), a highly social fish capable of bidirectional sex change. We then propose a model for HPA/I involvement in sex change and discuss how these ideas might be tested in the future. Understanding the regulation of sex change has the potential to elucidate evolutionarily conserved mechanisms responsible for translating pertinent information about the environment into coordinated biological changes along multiple body axes.

Keywords: hypothalamic-pituitary-adrenal/interrenal axis, sex change, corticotropin-releasing factor, cortisol, metamorphosis, stress

INTRODUCTION

The mechanisms underlying the initiation of teleost sex change remain of great scientific interest in part because this life history transition is often socially regulated. Following the removal of the dominant fish in the social group, the individual that establishes and maintains dominance over the group changes sex (Godwin, 2009). Across species, the social environment exerts a powerful influence over individual phenotype, behavior, neuroendocrine state, reproductive success, and survival (Wilson, 1980; Ellis, 1995; Adkins-Regan, 2009). Here, we highlight sex change as a unique opportunity to understand the dramatic and diverse biological processes regulated by the social environment.

In addition, sex change has important fitness consequences. Dominance in sex changing species offers a significant reproductive advantage, as it does in many non-sex changing species (Smuts and Smuts, 1993). Social groups often have a heavily skewed sex ratio, and those dominant individuals of the underrepresented sex reproduce with multiple subordinate group members. The ability to transition from a subordinate of one sex to the dominant of the other sex allows individuals to reproduce as female when young and small, for example, and as a male when older, larger, and able to defend a territory. Sex change thus results in an exponential increase in reproductive success (Ghiselin, 1969; Warner, 1975). Therefore, in understanding sex change, there is the potential to uncover mechanistic links connecting social information to the biological state of an individual to lifetime fitness.

In this paper, we review what is known about sex change in fishes, articulate the hypothesized roles for the hypothalamic-pituitary-adrenal axis (HP-interrenal in fish and amphibians; HPA/I) in sex change and how those hypotheses have been tested, and present original data that support roles for both cortisol and corticotropin-releasing factor (CRF) in both social hierarchy establishment/maintenance and sex change in the bluebanded goby (*Lythrypnus dalli*), a highly social, bidirectionally sex changing fish. We then synthesize our findings and propose future research directions that will more clearly elucidate the role(s) of the HPI axis in sex change.

SEX CHANGE IN TELEOST FISHES

Teleost fishes display a remarkable amount of sexual plasticity, including the ability to sexually reorganize in adulthood (reviewed in Devlin and Nagahama, 2002; Godwin et al., 2003; Sadovy de Mitcheson and Liu, 2008; Kobayashi et al., 2013).

Here, we focus on sex change in sequential hermaphrodites. Protogynous sex changing fish (e.g., wrasses, Labridae) can transition from a functional female (i.e., producing female gametes) to a functional male (i.e., producing male gametes). Protandrous species (e.g., clownfish, Pomacentridae) change from male to female, and bidirectionally sex changing species (e.g., gobies, Gobiidae) can change back and forth multiple times (i.e., serial adult metamorphosis). For each type of sequential hermaphrodite, individuals reproduce as male or female but not both simultaneously (see simultaneous hermaphrodite). Sex change involves coordinated biological changes along multiple body axes. Behavioral sex change is the earliest observable transition, often occurring within minutes in a permissive environment. During this phase, individuals adopt patterns of agonistic, courtship, and even reproductive behavior typical of the sex to which they are transitioning (Reavis and Grober, 1999; Godwin, 2009), changes that can occur independently of the gonad (Godwin et al., 1996). Physiologically, the most critical changes occur within the hypothalamic-pituitary-gonadal axis and result in the growth of new, and regression of old, gonadal tissue (Bass and Grober, 2001; Frisch, 2004; Godwin, 2009, 2010; Guiguen et al., 2010). Gonadal steroid hormones drive morphological sex change (Bass and Grober, 2001), which can range from relatively subtle changes in external genitalia for species that are not sexually dimorphic (St Mary, 1993) to dramatic changes in coloration and size (Godwin, 2009). Together, this coordinated cascade of behavioral, physiological, and morphological changes results in a functional female becoming a functional male, or vice versa.

Although previous research has elucidated many of the biological changes occurring during the different phases of sex change, particularly within the hypothalamic-pituitary-gondal axis (reviewed in Godwin, 2010), the biological signaling that interprets a permissive social environment and translates that information into the initiation of sex change has yet to be identified. A number of neuromodulators have been investigated as a potential biological switch important for the initiation of sex change, including neural steroid hormones (i.e., estradiol, 11-ketotestosterone, testosterone, cortisol) (Godwin, 2010; Lorenzi et al., 2012), gonadotropin-releasing hormone, arginine vasotocin (Reavis and Grober, 1999; Godwin et al., 2000; Bass and Grober, 2001), aromatase (Black et al., 2011), and serotonin (Lorenzi et al., 2009), with an increasing interest in kisspeptin (Godwin, 2010). Despite this focus, the biological link between social environment and sex change has not been resolved. Here, we address the roles that the HPA/I axis might play in the initiation or elaboration of adult sex change.

HYPOTHESES FOR HYPOTHALAMIC-PITUITARY-ADRENAL/INTERRENAL AXIS REGULATION OF SEX CHANGE

The HPA/I axis has been implicated on multiple levels in the mechanistic control of sex change (e.g., Perry and Grober, 2003) because of its unique biological position translating environmental cues into biological responses (Lowry and Moore, 2006; Denver, 2009). In all vertebrates, the HPA/I axis integrates important internal and external information in response to environmental stressors, or external conditions that disrupt or threaten to disrupt homeostasis, and coordinates responses such as changes in behavior and physiology. In fish, CRF released from the hypothalamus signals the release of adrenocorticotropic hormone, which then initiates the release of glucocorticoids (GCs, e.g., cortisol) from the interrenal gland (Wendelaar Bonga, 1997; Mommsen et al., 1999). Previous research supports a role for the HPI axis in the regulation of three, non-mutually exclusive functions related to sex change: (1) social status, (2) agonistic behavior, and (3) life history transitions.

First, HPA/I axis activity plays a role in the establishment and maintenance of social status. In a range of social vertebrates, there are consistent differences in basal GC levels between dominant and subordinate social group members. Across species, dominants are almost equally likely as subordinates to have elevated GCs, and factors such as the distribution of resources, social stability, reproduction, and the nature of agonistic interactions among group members largely determine which status class is more socially "stressed" (Creel, 2001; Sapolsky, 2005). In cooperative breeders, for example, basal GCs are typically higher in dominant individuals (Creel, 2001). Correlations between social status and GCs have been reported in a number of teleosts, including rainbow trout (Øverli et al., 2004; Gilmour et al., 2005; Bernier et al., 2008), cichlids (Mileva et al., 2009), zebrafish (Filby et al., 2010), protandrous anemonefish (Iwata et al., 2012), and protogynous sandperch (Frisch et al., 2007). There are also status differences in brain CRF activity. In zebrafish, for example, CRF is more highly expressed in dominant hypothalamus but subordinate telencephalon (Filby et al., 2010). In rainbow trout, social subordination increases CRF expression in the preoptic area (Bernier et al., 2008), and in the cichlid *Astatotilapia burtoni*, transitioning from dominant to subordinate status results in a transient decrease in CRF, CRF receptor 2, and CRF binding protein (Chen and Fernald, 2011). Together, these data support HPI axis activity as an indicator of status that could be utilized in sex changing species to distinguish dominant from subordinate. A change in social status concurrent with a change in HPI function could play a role in the initiation of sex change.

Second, the HPA/I axis is implicated in the control of agonistic behavior. In mammals, fish, amphibians, and reptiles, neurons that express CRF are found throughout the brain in a conserved distribution (Lovejoy and Balment, 1999; Denver, 2009), and CRF signaling has highly conserved effects on arousal and anxiety-related behaviors, locomotion, exploration, and feeding (Koob and Heinrichs, 1999; Bale and Vale, 2004; Lowry and Moore, 2006). Exogenous manipulation of CRF signaling can also affect agonistic behavior and social status, although the direction of the effect is not fully resolved (Carpenter et al., 2009; Backström et al., 2011). The behavioral effects of CRF are likely mediated, in part, by monoamine signaling (e.g., serotonin, dopamine) (Summers and Winberg, 2006; Carpenter et al., 2009; Backström et al., 2011). At the level of GCs, fish that release greater amounts of cortisol in response to a stressor (high responsive) are consistently subordinate to low stress responders (Pottinger and Carrick, 2001), and individual variation in the amount of cortisol released in response to a stressor can be used to predict dominance outcome in a novel pair of fish (Øverli et al., 2004). Because agonistic

behavior is critical during status establishment, and there are persistent behavioral differences among statuses in stable groups (Drews, 1993; e.g., Smuts and Smuts, 1993; Clarke and Faulkes, 2001; Graham and Herberholz, 2008), the role of the HPI/A axis in the control of agonistic behavior may be closely related to its role as a correlate of social status.

Third, the HPA/I axis could serve in an evolutionarily conserved role as a mediator of vertebrate life history transitions (Denver, 1999). Importantly, this role as a regulator of developmental plasticity seems to be independent of HPA/I-mediated responses to "unpredictable" environmental stressors, responses that might include the mobilization of energy reserves or altering behavior. Just prior to major transitions including birth/hatching (mammals, reptiles, birds, "large egg" fish), fledging (birds), dispersal (mammals, reptiles, birds), metamorphosis (amphibians), and smoltification (anadromous fish), both CRF and GCs have been shown to naturally increase (reviewed in Wada, 2008; Crespi et al., 2013). Exogenous elevation of GCs has also been used to initiate life history transitions (e.g., partuition in sheep), increase the success of the transition (e.g., hatching success in turkeys), and facilitate behavior (e.g., dispersal behavior in ground squirrels). In amphibians and fish, CRF stimulates the secretion of both thyroid hormone and GCs, which promote developmental transitions between life history stages and regulate developmental plasticity (e.g., amphibians: Denver, 1997; Boorse and Denver, 2004; Okada et al., 2007; fish: Larsen et al., 1998; Ebbesson et al., 2011) through both individual and synergistic actions (Hayes, 1997; Krain and Denver, 2004; Bonett et al., 2010; Kulkarni and Buchholz, 2012). Increases in thyroid hormone also precede reproductive maturation in fishes (also in mammals, Mann and Plant, 2010), and it has been hypothesized that serial adult sex change is simply the reoccurrence of these maturation processes (Dufour and Rousseau, 2007). Although there has been limited research on the thyroid axis and sex change (An et al., 2010; Park et al., 2010), sex change shares many characteristics of "classical" metamorphoses that are largely facilitated by actions of thyroid hormone and GCs. For example, smoltification in salmon, amphibian metamorphosis, and sex change all involve environmentally triggered morphological, physiological, and behavioral transformations in post-embryonic animals (Laudet, 2011). Therefore, we hypothesize that evolutionary conserved hormonal systems that mediate developmental plasticity, such as the HPA/I axis, are also acting during sex change in fishes.

It is important to note that for each hypothesized role for the HPI axis in sex change, HPA/I axis regulation of energy may also be relevant. Basal GCs are indicative of an individual's energetic demands and may be affected by time of day (e.g., appetite/foraging patterns), season (e.g., reproductive state), and life history stage. Stress-induced GC levels indicate the response of the HPA/I axis to an environmental challenge that requires energy mobilization to fuel behavioral and/or physiological responses (Sapolsky et al., 2000). In the case of social stressors, energetic demands may be elevated simply by the perception of dominant individuals (Sapolsky, 2005), and status differences in HPA/I activity could serve to facilitate differences in rates of behavior and/or reproductive demands. During life history transitions, changes in behavior, physiology, and morphology dramatically increase energetic requirements (Wada, 2008). For a transition like sex change, the exponential increase in reproductive success clearly outweighs energetic costs of sexual reorganization (Warner et al., 1975; Warner, 1984; Schreck, 2010). Finally, following a life history transition, HPA/I activity may be set to a new baseline because the energetic demands of the pre- and post-transition animal differ. In protogynous sex change, for example, reproductive investment might decrease because the energy required to produce sperm is traditionally considered lower than the energy to produce eggs. For some species, however, this difference may not be as sexually dimorphic as predicted (Yong and Grober, 2013), particularly for externally fertilizing species (Warner, 1997).

Together, these multiple lines of evidence strongly suggest a role for the HPI axis as a critical, proximate regulator of sex change. Here, we present original data that elucidates the roles of cortisol and CRF in a sex changing fish. All experiments were conducted in accordance with IACUC regulations and standards (Georgia State University, Atlanta, GA).

CORTISOL, SOCIAL HIERARCHIES, AND SEX CHANGE

Cortisol has been implicated in environmentally controlled sex determination in both gonochoristic (e.g., Hattori et al., 2009) and sex changing fish (Perry and Grober, 2003). For example, in gonochoristic fish with temperature-dependent sex determination, cortisol plays a critical mechanistic role in masculinization. At high water temperatures that normally cause testes to develop, pejerrey have elevated cortisol compared to fish at female-producing temperatures (Hattori et al., 2009). Exogenous cortisol administration can induce masculinization in the absence of high water temperatures in pejerrey (Hattori et al., 2009), Japanese flounder (Yamaguchi et al., 2010), and medaka, and an antagonist can prevent this masculinization (Hayashi et al., 2010). Cortisol seems to induce these changes through effects on enzymes involved in androgen pathways (Yamaguchi et al., 2010; Fernandino et al., 2012), and there is evidence in medaka that coritsol can also suppress feminization (Hayashi et al., 2010).

In sex changing fish, cortisol could serve a similar role, linking environmental conditions to sexual differentiation. In one of the first mechanistic hypotheses, Perry and Grober (2003) suggested that dominant males of protogynous species prevent sex change in subordinate females via aggressive interactions that cause an increase cortisol levels. They hypothesized that this chronically elevated female cortisol is responsible for inhibiting sex change. If the dominant male were removed from the social group, then the most dominant female would be released from social subordination stress and her cortisol levels would decrease to male-typical levels. This release from social stress could trigger the initiation of sex change (**Figure 1A**). The remaining females in the social group would not change sex despite the permissive environment because aggression from the dominant female/sex changer would keep their cortisol elevated (Perry and Grober, 2003). Frisch et al. (2007) tested this hypothesis with the protogynous sandperch (*Parapercis cylindrica*) by inserting cortisol implants into the dominant female of a social group to prevent sex change once the male was removed. While the implants successfully elevated cortisol levels, they did not inhibit sex change

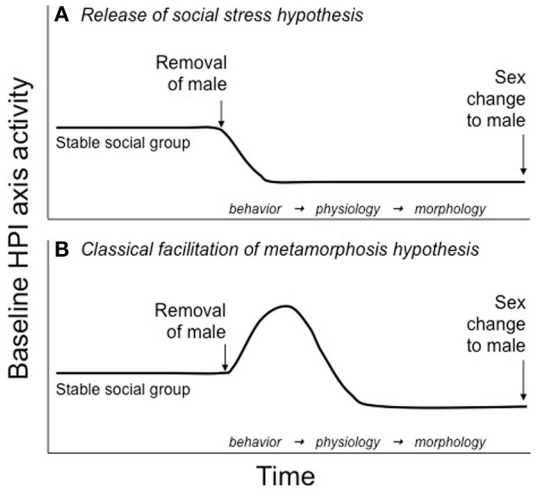

FIGURE 1 | Hypotheses for HPI axis involvement in teleost sex change. **(A)** The release of social stress hypotheses, originally put forth by Perry and Grober (2003), suggests that baseline HPI axis activity is maintained at relatively constant, elevated levels in females of protogynous species because of the stress of subordination imposed by the dominant male. After male removal from the social group, HPI axis activity *decreases* in the most dominant female as she is released from the social stress of subordination. It is this decrease in HPI activity that allows for the initiation of sex change. Lower ranking females do not change sex because the dominant female/sex-changer maintains their subordinate status and, subsequently, their elevated baseline HPI axis activity. **(B)** The classical facilitation of metamorphosis hypothesis, discussed here for the first time, suggests that following male removal, baseline HPI axis activity *increases* in the dominant female/sex changer. Elevated HPI axis activity could be used to fuel the energetically costly changes in behavior, physiology, and morphology that occur during sex change and/or activate the thyroid axis, which could regulate cellular differentiation and apoptosis associated with sex change. This hypothesis is consistent with an evolutionarily conserved role for the HPA/I axis in the regulation of life history transitions.

(Frisch et al., 2007). If elevated CRF associated with social stress is involved in sex change, however, this manipulation would not recapitulate that condition. Interestingly, although designed to test the Release from Social Stress hypothesis (**Figure 1A**), the cortisol manipulation in Frisch et al. actually mimics the action of GCs in the competing hypothesis we present in this paper, the Classical Facilitation of Metamorphosis hypothesis (**Figure 1B**, discussed below). It cannot be determined from the data presented in Frisch et al. whether elevated cortisol facilitated or accelerated sex change, which could provide support for this alternative hypothesis.

We took a different approach to testing the Release from Social Stress hypothesis (**Figure 1A**) and measured endogenous cortisol levels in experimental social groups of another sex changing fish, the bluebanded goby (*L. dalli*). This small [standard length (SL) 18–45 mm] marine goby is highly social and lives on rocky reefs in the Pacific Ocean, from Morro Bay, California to as far south as the Galapagos Islands, Ecuador (Miller and Lea, 1976; Béarez et al., 2007). Mixed-sex social groups of *L. dalli* vary from small and isolated (3–10 fish) to aggregations of 120 fish/m^2 (Steele, 1996) and are comprised of a dominant, territorial male and multiple subordinate females (St Mary, 1993). On the reef, *L. dalli* primarily undergoes protogynous sex change, and this could occur when a male is eliminated from his territory by predation or when multiple females converge on a territory without a male. In the laboratory, *L. dalli* is capable of both protogynous and protandrous sex change (e.g., bidirectional sex change, Rodgers et al., 2007).

The fish used in the following experiments were collected during the reproductive season from reefs offshore of Santa Catalina Island, CA using hand nets and SCUBA diving. For experiments described in **Figures 2A,B**, fish were then shipped to Georgia State University (Atlanta, GA) and housed communally before being placed into individual social groups (38 l aquaria). These tanks were maintained with artificial salt water and exposed to a 12:12 light-dark cycle. All other experiments took place at the Wrigley Institute for Environmental Studies (University of Southern California) on Catalina Island where water tables were continuously supplied with ocean water and exposed to a natural light cycle. To form social groups of specific sizes and sex ratios, fish were briefly anesthetized in tricaine methanesulfonate (MS-222), and we measured SL and determined sex based on genital papilla morphology (St Mary, 1993).

We conducted 3 experiments to determine whether elevated cortisol levels could be responsible for the chronic inhibition of sex change in female *L. dalli*. First, we tested whether cortisol levels were elevated in females compared with males by forming social groups ($n = 39$) of 1 large dominant male, 1 large dominant female (alpha; smaller and subordinate to the male), and 2 smaller females (beta and gamma). Hierarchies in these social groups were allowed to establish and be maintained for 21 days, and 10 min behavioral observations were conducted multiple times to verify hierarchy stability. On day 22, water-borne hormones were collected, a measure of systemic hormones closely related to hormone levels in circulation (i.e., plasma, Kidd et al., 2010). Fish of each social status were placed individually in a beaker of salt water for 1 h. Steroids were extracted from the water using C18 columns and measured using cortisol enzyme immunoassay kits (Cayman Chemical, Ann Arbor, Michigan) as in Lorenzi et al. (2008). The hormone pellet was resuspended in enzyme immunoassay buffer (5% EtOH), and we completed the assay according to the supplied instructions. All samples were assayed in duplicate.

We found that water-borne cortisol levels differed significantly among social statuses in stable *L. dalli* hierarchies. Cortisol levels were highest in the alpha females and lowest in males. Beta and gamma cortisol levels were intermediate between the males and alphas (**Figure 2A**). These results are consistent with the hypothesis of Perry and Grober (2003) that protogynous sex change could be inhibited in females via elevated cortisol, especially in the alpha female. Elevated alpha cortisol levels could result from aggression received from the male, the hybrid social position of being subordinate to the male yet needing to maintain dominance over the beta and gamma females, and/or increased energetic demands to fuel higher rates of agonistic interaction.

Next, we tested whether these status differences in cortisol were due to intrinsic differences rather than a consequence of the social hierarchy, as hypothesized. To determine whether social

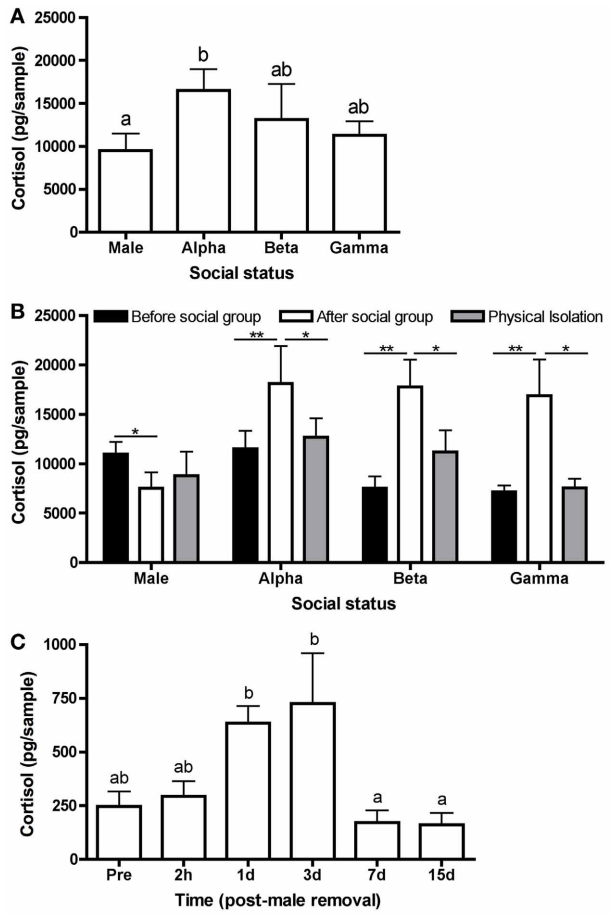

FIGURE 2 | Variation in water-borne cortisol levels. (A) Mean (± s.e.m.) cortisol levels in each status class from stable social groups. Cortisol levels differed significantly among males, alpha females, beta females, and gamma females after 21 days in a stable social group ($n = 39$) (one-way ANOVA following a natural log transformation: $F_{(3,\ 149)} = 3.64$, $p = 0.014$). **(B)** Mean water-borne cortisol in males, alpha females, beta females, and gamma females prior to being placed in a social group ("before") ($n = 15$), after 21 days in a social group ("after") ($n = 15$), and following at least 1 day in a social isolation chamber ("physical isolation") ($n = 15$). There were no differences in cortisol levels within statuses following 1, 2 or 3 days in the isolation chamber ($p > 0.05$); therefore, cortisol levels were pooled and presented in one column ("physical isolation"). Male cortisol "before" was significantly higher than "after" [paired t-test: $t_{(14)} = 2.06$, $p = 0.05$], whereas female cortisol was significantly higher "after" than "before" [paired t-tests: $t_{(14)} > 2.8$, $p < 0.01$]. Following isolation, cortisol levels were not significantly different from "before" for any social status [paired t-test: $t_{(14)} < 1.8$, $p > 0.09$]. For alpha, beta, and gamma females, "after" cortisol levels were significantly higher than isolation (paired t-tests: $t_{(14)} > 1.9$, $p < 0.07$); however, there was no difference for males [paired t-test: $t_{(14)} = 0.52$, $p = 0.60$]. **(C)** Mean water-borne cortisol differed significantly over time in alpha females in a stable social hierarchy ("pre") ($n = 12$) and 2 h, 1, 3, 7, and 15 days ($n = 12$ each time point) following the removal of the male [one-way ANOVA: $F_{(5,\ 64)} = 7.77$, $p < 0.0001$]. Asterisks and different letters ($p = 0.05$) indicate significant differences.

status drives differences in cortisol, we identified fish from the communal holding tank to form an additional 15 social groups and measured water-borne cortisol in those individuals prior to being placed in a social group, after 21 days in a social group, and then following 1 ($n = 5$), 2 ($n = 5$), or 3 ($n = 5$) days in isolation. The isolation chamber consisted of four compartments separated by glass partitions with 5 cm of space between each compartment. In this apparatus, the male and 3 females from a social group remained in visual and olfactory contact but could not physically interact. Fish were allowed to maintain some sensory contact because total isolation has been shown to independently increase cortisol in some individuals, particularly males (Earley and Grober, unpublished data). We found that before being placed into a social group, males had higher cortisol levels than after 21 days in a social group. Interestingly, females showed the opposite pattern and had higher cortisol levels after being in the social group. Following isolation, female cortisol decreased significantly to levels comparable to before being in the social group. Male cortisol levels did not change following isolation (**Figure 2B**). These data strongly suggest that the status- and sex-dependent cortisol differences after 21 days of interaction (**Figure 2A**) emerge as a consequence of the social environment rather than being reflective of intrinsic variation.

Finally, we tested whether cortisol decreases in the alpha female following the removal of the male. These data would provide support for the release of social stress initiating sex change (**Figure 1A**). To quantify cortisol over the course of sex change, we formed social groups ($n = 60$) of 1 large dominant male, 1 large dominant female, and 3 smaller females. Eight days after the groups were established, the male was removed to facilitate sex change in the alpha female. Water-borne cortisol was collected from the alpha female when the male was still present ($n = 12$) and then subsequently from alphas in different social groups ($n = 12$ each) 2 h, 1 day, 3 days, 7 days, and 15 days following male removal. Cortisol levels differed significantly over time, peaking 1–3 days after male removal (**Figure 2C**). These data show that contrary to the Perry and Grober (2003) hypothesis, cortisol in the alpha female/sex changer increased following the removal of the male. Interestingly, in the protandrous anemonefish (*Amphiprion melanopus*), cortisol levels do not differ between males in females but increases in the sex changer following the removal of the dominant female (Godwin and Thomas, 1993). This elevation in cortisol may be more consistent with the classical facilitation of metamorphosis hypothesis in which the HPA/I axis acts in a conserved role to facilitate vertebrate life history transitions (**Figure 1B**).

These data, in combination with Frisch et al. (2007), strongly indicate that there is no simple relationship between cortisol and sex change such that removing the male (the assumed source of elevated alpha cortisol) removes social subordination stress and leads to the initiation of sex change. Instead, cortisol may increase in the first few days of sex change, indicating increased CRF signaling and HPI axis activity. This increase could be necessary to meet the increased energetic demands involved with sex change and/or activate the thyroid axis, which could regulate cellular differentiation and apoptosis associated with sex change, similar to the gene programs activated by these axes during amphibian metamorphosis (**Figure 1B**). Interestingly, environmental cues such as increased density, reduced water volume, and reduced food availability activate the HPI axis in amphibian tadpoles and facilitate "stress-induced" metamorphosis (reviewed in Crespi

and Denver, 2005). There could be a similar role for the HPI axis during sex change whereby a change in the environment (i.e., the removal of a social cue), or the behavioral changes induced by the environmental change, facilitates an important life history transition.

CORTICOTROPIN-RELEASING FACTOR, SOCIAL STATUS, AND SEX CHANGE

To further investigate the increase in HPI axis activity during sex change (**Figure 2C**), we focused on a role for CRF, the signal that drives the increase in cortisol. We hypothesized that a change in social environment, from a stable social group to an environment permissive for sex change, leads to an increase in CRF that could be involved in the establishment of dominance, agonistic behavior, and/or the metamorphic process of sex change (**Figure 1B**). To test these hypotheses, we exogenously elevated CRF using intracerebroventricular (icv) injection that was timed to coincide with a permissive environment: 2 size-matched female *L. dalli* in the absence of a male.

We collected females from reefs offshore of Catalina Island, CA and housed them in water tables at the Wrigley Institute for Environmental Studies. One day prior to pairing females, we briefly anesthetized fish (MS-222) to measure SL and mass. Paired females were size matched and differed in SL by an average of 0.19 ± 0.028 (s.e.m.) mm and differed in mass by 0.033 ± 0.0042 g. Females were then held in isolation overnight. The next morning, immediately prior to pairing, we used an established protocol for icv injection (Solomon-Lane and Grober, 2012) to acutely elevate CRF (Sigma-Aldrich, St. Louis, MO). Corticotropin-releasing factor-injected fish received 500 ng CRF/50.6 nL 0.1 M sterile phosphate buffer solution. All CRF-injected fish ($n = 15$) were paired with a size-matched female that received an injection of vehicle only (50.6 nL phosphate buffer) to control for the effects of injection during dominance establishment [similar design to Carpenter et al. (2009), Backström et al. (2011)]. Pairs of injected fish were compared to non-anesthetized, non-injected control pairs ($n = 14$). After both females in a pair recovered from the injection (described below), they were transferred simultaneously into a novel tank. Control females were not anesthetized and were transferred into the novel tank directly from their isolated housing.

Injections were performed using a Nanoject II Auto-Nanoliter Injector (Drummond Scientific Company, Broomall, PA, USA). Anesthetized fish were gently held under a dissecting microscope, and the pulled capillary tube needle attached to the Nanoject was lowered into position using a micromanipulator. The external anatomy of the head was used to correctly position the needle, and after penetrating the skull at the midline of the brain, the solution was injected into the third ventricle. Following injection, the needle was held in place for 5 s to reduce leakage, and after the needle was removed entirely from the fish, we performed a test injection to ensure that the needle was not clogged and to validate proper Nanoject function. Between injections, the needle was wiped with ethanol and allowed to dry, and the needle was changed between injections of CRF and vehicle. This technique has a success rate of at least 85% (Solomon-Lane and Grober, 2012) and has been used successfully in *L. dalli* to manipulate enzyme activity in the brain (Pradhan, Solomon-Lane, Willis, and Grober, in review).

Following injection, fish recovered in a 200 mL beaker of fresh salt water. Observing recovery provides independent verification that the injection procedure does not compromise an individual's locomotion or capacity for social interaction. Recovery from anesthesia is stereotyped and involves first initiating ventilation, indicated by movement of the opercula, and then regaining equilibrium, when the dorsal fin of the fish first reoriented to a vertical position. Observers were blind to the treatment of the recovering fish. Vehicle-injected fish did not differ from CRF-injected fish in the time required to initiate ventilation (Mann–Whitney U-test: $U = 76.50$, $n_{CRF} = 15$, $n_{veh} = 15$, $p = 0.14$) or regain equilibrium (independent t-test: $t = 1.23$, $d.f. = 28$, $p = 0.23$) following anesthetization and injection, suggesting that CRF did not negatively affect basal physiology or behavior. We also recorded ventilation rate for the first 300 s following the initiation of ventilation. This serves as a bioassay for injection efficacy because CRF has an evolutionarily conserved role in elevating ventilation rate. As previously shown in *L. dalli* (Solomon-Lane and Grober, 2012), CRF injection significantly increased ventilation rate compared to vehicle-injected fish (**Figure 3**), indicating the successful elevation of central CRF.

Injected females were paired as soon as both members of the pair had recovered fully (i.e., regained equilibrium) (CRF-injected: 16.2 ± 1.5 min post-injection; vehicle-injected: 22.8 ± 1.7 min post-injection). To pair females, we gently transferred each fish into a novel tank simultaneously. Tanks were supplied with a PVC tube (15.2 cm length, 1.9 cm diameter) that dominant *L. dalli* establish as their territory and males use as a nest. Following a 1 min acclimation period, we began behavioral observations (10 min each) and recorded approaches, when one fish swims directly toward any part of another fish's body, within 2 body lengths, and displacements, in which the approached fish retreats or swims away. We also recorded lateral displays, an escalated aggressive interaction. We conducted up to 3, rolling behavioral observations (maximum 30 min). If dominance was established and one fish displaced the other 5 times without being displaced itself, we did not conduct additional morning observations.

Overall, significantly fewer injected pairs than control pairs had an established dominant fish based on our original criteria (5 uninterrupted displacements) within the first 30 min of pairing (injected: 3 of 15 pairs; control: 12 of 14 pairs) (Chi-square: $\chi^2 = 12.54$, $d.f. = 1$, $p < 0.001$). Using a broader definition of dominance that included occupation of the nest territory and high agonistic efficiency, the proportion of approaches that lead to a displacement, a ratio that is substantially higher in dominants, there were still significantly fewer injected pairs with a clear dominant fish (injected: 7 of 15 pairs; control: 13 of 14 pairs) (Chi-square: $\chi^2 = 7.24$, $d.f. = 1$, $p = 0.01$). To determine if CRF facilitates dominance establishment in *L. dalli*, we compared the number of pairs with a CRF-injected and a vehicle-injected dominant fish. Of the 7 injected pairs with a clear dominant, only 1 dominant fish had been injected with CRF, which was not a significant difference from random (Chi-square: $\chi^2 = 2.28$, $d.f. = 1$, $p = 0.13$). During the afternoon observation, approximately

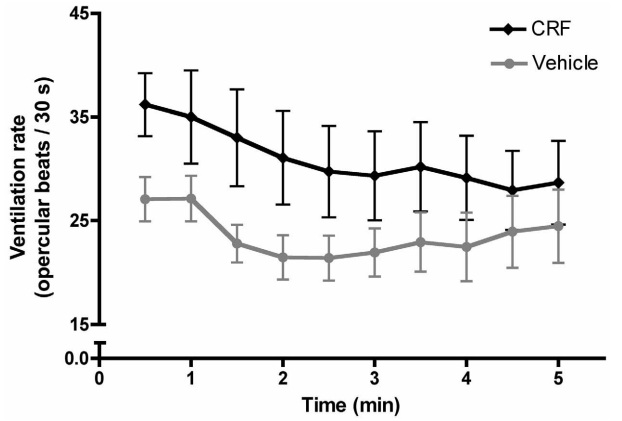

FIGURE 3 | Mean (± s.e.m.) ventilation rate, measured as the number of opercular beats recorded in 30 s intervals during the first 300 s following the initiation of ventilation. A two-way mixed factorial ANOVA with time interval as the within subjects factor and treatment as the between subject factor demonstrated a significant effect of treatment [$F_{(1,\ 280)} = 88.11$, $p < 0.0001$] and time [$F_{(9,\ 280)} = 3.187$, $p = 0.0011$] on ventilation rate. *Post-hoc* analysis revealed that CRF-injected fish had a significantly higher ventilation rate in the first 300 s following the initiation of ventilation (independent *t*-test: $t = 4.83$, $d.f. = 298$, $p < 0.0001$), indicating the successful injection of CRF. *Post-hoc* testing showed no significant effect of time on ventilation rate (one-way ANOVA: $F_{(9,\ 290)} = 0.78$, $p = 0.635$).

3 h after pairing, all 14 control pairs had an established dominant fish, defined by occupation of the nest territory and/or the consistent displacement of the subordinate fish. For the injected pairs, 13 of 15 pairs had an established dominant fish, and there was no difference between the number of CRF-injected (5) and vehicle-injected (8) dominants (Chi-square: $\chi^2 = 0.3$, $d.f. = 1$, $p = 0.58$).

These data demonstrate that contrary to our hypothesis, acute elevation of central CRF in this context did not facilitate dominance establishment. In fact, within the first 30 min of pairing, CRF-injected fish tended to become subordinate. In two similarly designed studies using juvenile rainbow trout (*Oncorhynchus mykiss*), CRF had conflicting effects on dominance establishment. Carpenter et al. (2009) showed that icv CRF at the same dose used in this study positively affected dominance establishment (Carpenter et al., 2009); however, Backström et al. (2011) report the same status outcomes after 60 min as we do for *L. dalli* during the afternoon observation: 5 CRF-injected and 8 vehicle-injected rainbow trout became dominant. Interestingly, at a higher dose, they show a negative effect of CRF on dominance (Backström et al., 2011). These data suggest that CRF may facilitate subordinate status, which could be confirmed for *L. dalli* with a larger sample size and/or by increasing the dose of CRF.

To investigate why status establishment was significantly delayed in injected pairs, we compared rates of agonistic behavior during the morning observations. Fish that were injected, including both CRF-injected and vehicle-injected fish, approached (**Figure 4A**) and displaced (**Figure 4B**) less than controls and engaged in fewer lateral displays (**Figure 4C**). These differences in agonistic behavior were driven specifically by dominants. Injected dominant fish (1 CRF, 6 vehicle) approached (**Figure 4D**) and displaced (**Figure 4E**) significantly less than control dominants, yet there were no differences in subordinate approaches (**Figure 4F**) or displacements (**Figure 4G**). These data demonstrate that the injection procedure, independent of substance injected, depressed behavior, which is critical to the establishment of dominance. Interestingly, this effect was mediated by social context: rates of behavior were depressed only in dominant fish.

Although there was no significant difference in the number of CRF-injected vs. vehicle-injected dominants, vehicle-injected fish tended to become dominant in the first 30 min. To determine whether behavioral differences explain this skew and/or whether CRF affected agonistic behavior, we compared rates of approaches and displacements between CRF-injected and vehicle-injected fish, independent of status outcome. There were no differences in approaches (**Figure 5A**) or displacements (**Figure 5B**) in the first 30 min of pairing. Because there was only 1 CRF-injected dominant and only 1 vehicle-injected subordinate, we could not analyze whether agonistic behavior differed between CRF dominants and subordinates or vehicle dominants and subordinates. During the afternoon observation, when 5 CRF-injected and 8 vehicle-injected fish were dominant, there were no significant differences between CRF- and vehicle-injected dominants in approaches (**Figure 5C**) or displacements (**Figure 5D**). Although rates of behavior were lower in CRF-injected dominants, CRF did not seem to reduce agonistic efficiency: nearly all dominant approaches lead to a successful displacement. Among subordinates, there were no differences in approaches (**Figure 5E**) or displacements. Despite the low rates of subordinate behavior in the afternoon observation, CRF-injected subordinates interacted more than vehicle-injected subordinates, showing that CRF did not consistently decrease behavior further than the vehicle alone. Contrary to our hypothesis, therefore, exogenous elevation of central CRF did not affect agonistic behavior during status establishment and initiation of sex change. The lower rates of behavior due to injection, an effect also observed in rainbow trout injected with icv CRF (Carpenter et al., 2009), could also have limited our ability to detect differences.

To test for an effect on HPI activity, resulting from the exogenous CRF and/or stress of the injection, on status establishment and agonistic behavior, we collected water-borne cortisol following the afternoon behavioral observation. Afterwards the pair was returned to their home tank. Injected fish had significantly higher cortisol levels than control fish, but there was no significant difference between CRF-injected and vehicle-injected fish (**Figure 6A**). This indicates that the injection rather than the exogenous CRF activated the HPI axis. For both dominants (**Figure 6B**) and subordinates (**Figure 6C**), CRF-injected and vehicle-injected fish had significantly higher cortisol than control fish but did not differ from each other. Despite similar HPI axis activation between injected groups, these data provide additional support for our ability to manipulate CRF centrally because the treatment difference in ventilation rate was not driven by a CRF effect on cortisol. Overall, there was no effect of social status on cortisol (**Figure 6D**).

Both the injection and the novel social environment, which was designed to be competitive by pairing size-matched females,

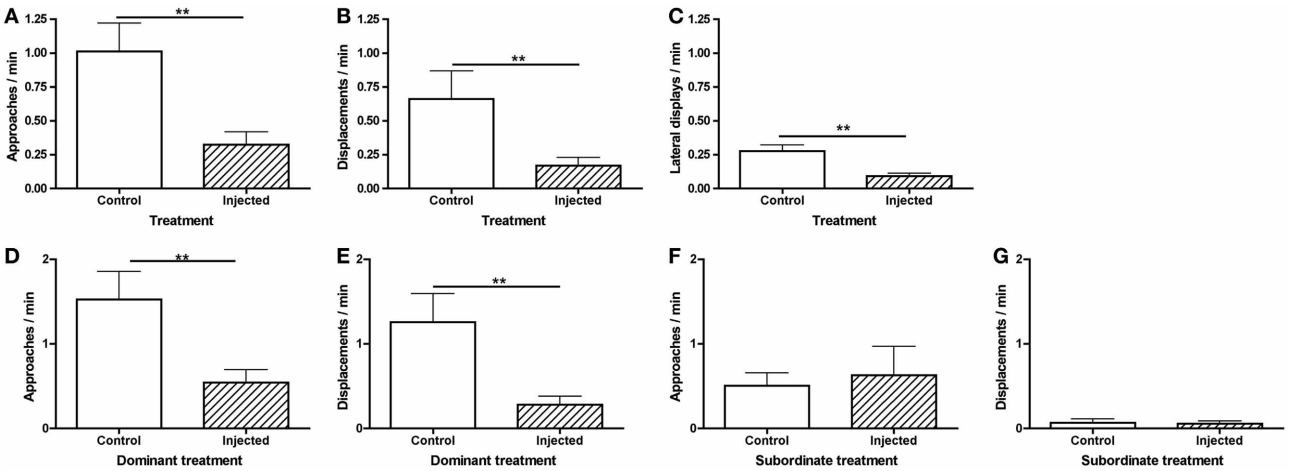

FIGURE 4 | Mean (± s.e.m.) agonistic behaviors within the first 30 min of pairing. Injected fish **(A)** approached (independent *t*-test following natural log transformation: $t = -3.09$, $d.f. = 44$, $p = 0.0035$) and **(B)** displaced (Mann–Whitney *U*-test: $U = 214.5$, $n_{Inj} = 30$, $n_{Con} = 26$, $p = 0.0039$) significantly less than control fish. **(C)** Injected fish also engaged in significantly fewer lateral displays (independent *t*-test following natural log transformation: $t = -3.53$, $d.f. = 24$, $p = 0.0017$). Injected dominants **(D)** approached (independent *t*-test following natural log transformation: $t = -3.22$, $d.f. = 18$, $p = 0.0079$) and **(E)** displaced (Mann–Whitney *U*-test: $U = 9.0$, $n_{Inj} = 7$, $n_{Con} = 13$, $p = 0.0043$) significantly less than control dominants. There were no significant differences in subordinate **(F)** approaches (Mann–Whitney *U*-test: $U = 38.5$, $n_{Inj} = 7$, $n_{Con} = 13$, $p = 0.61$) or **(G)** displacements (Mann–Whitney *U*-test: $U = 36.5$, $n_{Inj} = 7$, $n_{Con} = 13$, $p = 0.50$). $^{**}p < 0.01$.

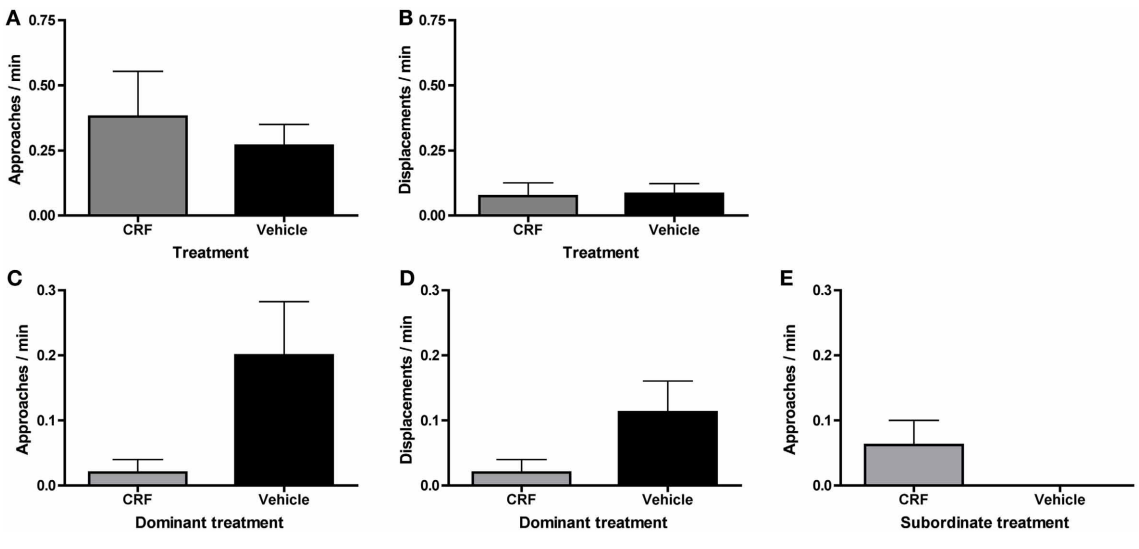

FIGURE 5 | Mean (± s.e.m.) agonistic behaviors in injected fish on day 1 of pairing. There were no differences in **(A)** approaches (independent *t*-test following natural log transformation: $t = 0.052$, $d.f. = 24$, $p = 0.95$) or **(B)** displacements (Mann–Whitney *U*-test: $U = 92.5$, $n_{CRF} = 15$, $n_{Veh} = 15$, $p = 0.41$) by CRF-injected and vehicle-injected fish, independent of social status, within the first 30 min of pairing. In the afternoon observation, dominant CRF-injected fish did not **(C)** approach (Mann–Whitney *U*-test: $U = 9.5$, $n_{CRF} = 5$, $n_{Veh} = 8$, $p = 0.13$) or **(D)** displace (Mann–Whitney *U*-test: $U = 12.5$, $n_{CRF} = 5$, $n_{Veh} = 8$, $p = 0.28$) significantly more than vehicle-injected dominants. **(E)** There were not a sufficient number of subordinate approaches to analyze statistically, and subordinate displacements are not shown because all values are 0.

likely contributed to the elevated cortisol levels, as both of these factors have been shown to activate the HPA/I axis. Interestingly, in a previous *L. dalli* study, CRF injected icv in the absence of a social manipulation did not affect cortisol levels when compared to anesthetized controls that were not injected (Solomon-Lane and Grober, 2012). This suggests a possible synergistic effect of the stressors. Elevated levels of CRF or cortisol also have been associated with suppression of behaviors, such as foraging, reproductive behaviors, and aggressive behaviors (Tokarz, 1987; Moore and Mason, 2001; Crespi and Denver, 2004). This is relevant to the present study because rates of behavior were depressed and cortisol levels were elevated in injected fish. Our inability here to distinguish between HPI activity in CRF-injected and vehicle-injected fish suggests that future tests of HPI involvement in

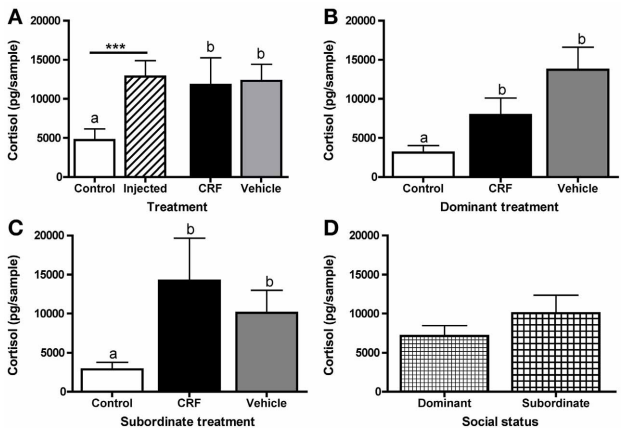

FIGURE 6 | Mean (± s.e.m.) water-borne cortisol levels (pg/sample). **(A)** Injected fish had significantly higher cortisol levels than control fish (independent *t*-test following natural log transformation: $t = 5.03$, $d.f. = 56$, $p < 0.0001$). Control, CRF-injected, and vehicle-injected fish cortisol also differed significantly (one-way ANOVA following natural log transformation: $F_{(2,\ 55)} = 12.68$, $p < 0.0001$). **(B)** Cortisol levels differed significantly among dominant (one-way ANOVA following natural log transformation: $F_{(2,\ 21)} = 10.30$, $p = 0.0008$) and **(C)** subordinate (one-way ANOVA following natural log transformation: $F_{(2,\ 21)} = 8.98$, $p = 0.0015$) fish. **(D)** There were no differences between all (control, CRF-injected, and vehicle-injected) dominant and all subordinate fish (independent *t*-test following natural log transformation: $t = -0.18$, $d.f. = 46$, $p = 0.86$). Asterisks and different letters ($p < 0.05$) indicate significant differences.

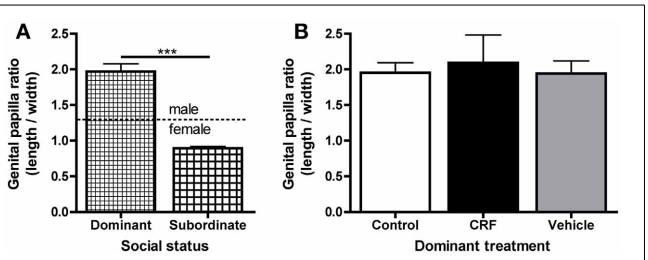

FIGURE 7 | Mean (± s.e.m.) genital papilla length-to-width ratio. (A) Dominant fish (control, CRF-injected, and vehicle-injected) had significantly higher genital papilla ratios than subordinate fish (Mann–Whitney *U*-test: $U = 12.0$, $n_{dom} = 25$, $n_{sub} = 27$, $p < 0.0001$). Dominant fish had male-typical length-to-width ratios (average 1.99 ± 0.11) and subordinates had female-typical ratios (average 0.89 ± 0.023). **(B)** There were no differences in genital papilla ratio among dominant control, CRF-injected, or vehicle-injected fish [one-way ANOVA: $F_{(2,\ 22)} = 0.099$, $p = 0.91$]. Similarly, there was no difference among subordinates (data not shown, one-way ANOVA: $F_{(2,\ 24)} = 0.22$, $p = 0.81$). Asterisks indicate significant differences ($p < 0.05$).

L. dalli sex change may be limited by the current techniques. The challenge of manipulating the HPA/I axis without independently activating it via handling or administration procedure is frequently encountered by researchers, and we discuss potential future directions below.

Finally, to test whether CRF affected sex change, we allowed females to remain in their pairs for 12 days, a sufficient time for *L. dalli* to change sex (Reavis and Grober, 1999). By day 6, all control ($n = 14$) and injected ($n = 15$) pairs had a clear dominant fish, and except for status reversals in two control pairs, dominance remained stable through day 12. On day 6, CRF-injected fish had established and maintained dominance in 5 of 15 pairs, which was not significantly different from random (Chi-square: $\chi^2 = 1.06$, $d.f. = 1$, $p = 0.30$). We evaluated sex change from digital images of the sexually dimorphic genital papilla (St Mary, 1993) and gonad morphology. Female *L. dalli* have a rounded genital papilla with a length-to-width ratio less than 1.4, whereas the male papilla is pointed with a ratio greater than 1.4 (St Mary, 1993; Carlisle et al., 2000). In all pairs, the dominant fish changed sex, indicated by the significantly higher genital papilla ratio in dominant compared to subordinate fish (**Figure 7A**), which is consistent with many past studies in this species (Reavis and Grober, 1999; Rodgers et al., 2007). Visual inspection of the gonads also confirmed that all dominants had transitional or male-typical gonads. Among dominants, there was no effect of treatment on the genital papilla ratio (**Figure 7B**) suggesting that neither a single icv injection of CRF nor the injection procedure, both of which delayed status establishment and elevated cortisol, affected the rate of sex change.

SUMMARY AND FUTURE RESEARCH DIRECTIONS

In this paper, we have discussed multiple hypotheses about HPI axis involvement in the regulation of teleost sex change and presented original data from our initial experiments with *L. dalli* that test those hypotheses. We have shown that in *L. dalli*, variation in HPI axis activity (measured as cortisol) was associated with social status in the hierarchy, and these differences were socially mediated. We also showed that cortisol increased during early stages of sex change, suggesting that activation of the HPI axis may be involved with stimulating the process of sex change similar to the ways in which HPA/I axis activation is involved in other life history transitions (Wada, 2008). Additional experiments suggested below could elucidate whether the HPI axis is acting in this evolutionarily conserved manner during sex change.

Corticotropin-releasing factor, either through its hypophysiotopic actions or its own actions as a neurotransmitter, also could facilitate sex change through two possible scenarios. First, naturally occurring sex change is always coupled with social dominance; therefore, if CRF facilitated dominance, CRF could indirectly mediate sex change. We showed that this is not the case for *L. dalli*. Acute elevation of CRF in the brains (through icv injection) of fish in a permissive environment was not associated with dominance establishment or the expression of agonistic behavior, although there may be a role for CRF (and cortisol) in subordinate status and/or behavior. Instead, both icv CRF and vehicle reduced agonistic behaviors and delayed dominance establishment. Acute increases in CRF resulting from environmental stressors (e.g., predation threat, threatening abiotic conditions) could inhibit aggression because this switch in behavior favors survival in such conditions, as it does in other vertebrates (e.g., Tokarz, 1987), independent of its role in the regulation of behaviors that maintain hierarchies. More research is needed to examine both of these hypotheses.

Second, if CRF was the biological signal for the initiation of sex change, then CRF could directly regulate this process, possibly even in the absence of social dominance. It is important to note that since sex change is socially regulated by nuanced interactions

among several individuals, there may not be one agonist or antagonist, within or outside of the HPI axis, capable of overriding the effects of social interactions and context. While we showed that a single icv injection of CRF prior to status establishment in a permissive environment did not trigger sex change, this does not necessarily rule out a role for CRF in its initiation. For example, it is likely that a prolonged elevation of hypothalamic CRF is necessary to activate the physiological hormonal cascade involved in sex change, as we detected elevated cortisol in alpha females days after removal of a dominant male. Indeed, Denver (1997) used repeated intraperitoneal (ip) injections of CRF to initiate precocious metamorphosis in tadpoles beyond a certain stage of development, and conversely, used repeated ip injections of a CRF antagonist to prevent metamorphosis.

Similar experiments could be conducted in *L. dalli* to test whether CRF administered to a dominant female in a permissive environment for sex change can accelerate the transition. This could be accomplished using multiple ip injections, which would allow for CRF to have hypophysiotropic effects (Denver, 1997) but may be less stressful than icv injections because less handling is required and anesthesia may not be necessary. Alternatively, icv injection of CRF in a viral vector could activate CRF over a longer period of time, thereby allowing fish to more fully recovery from the injection procedure before being exposed to a social challenge or an environment permissive to sex change. Repeated icv injections via indwelling cannula would also be possible in larger species of sex changing fish (e.g., wrasses, Labridae; parrotfishes, Scaridae). Another critical experiment would involve chronically inhibiting CRF in an environment permissive to sex change via administration of an antagonist (e.g., alpha-helical CRF), *vivo* morpholino (e.g., Ferguson et al., 2013), siRNA, or shRNA.

Overall, we encourage further investigation into the mechanisms underlying sex change in order to broadly elucidate social regulation of metamorphic processes, and, more specifically, identify a potentially evolutionarily conserved role for the HPI axis in this dramatic life history transition.

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Central corticotropin releasing factor and social stress

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Social interactions are a main source of stress in vertebrates. Social stressors, as well as other stressors, activate the hypothalamic–pituitary–adrenal (HPA) axis resulting in glucocorticoid release. One of the main components of the HPA axis is corticotropin releasing factor (CRF). The neuropeptide CRF is part of a peptide family including CRF, urocortin 1–3, urotensin 1–3, and sauvagine. The actions of the CRF family are mediated by at least two different receptors with different anatomical distribution and affinities for the peptides. The CRF peptides affect several behavioral and physiological responses to stress including aggression, feeding, and locomotor activity. This review will summarize recent research in vertebrates concerning how social stress interacts with components of the CRF system. Consideration will be taken to the different models used for social stress ranging from social isolation, dyadic interactions, to group dominance hierarchies. Further, the temporal effect of social stressor from acute, intermittent, to chronic will be considered. Finally, strains selected for specific behavior or physiology linked to social stress will also be discussed.

Keywords: corticotropin releasing factor (CRF), CRF-receptors, dominance hierarchies, hypothalamic–pituitary–adrenal axis, social defeat, social isolation, social stress

INTRODUCTION

The stress response elicited by social stressors does not differ from the response to other challenges. A stressor, which can be a real or perceived threat, causes a physiological response aimed at counteracting a homeostatic disruption. The immediate effect of the stress response is to prepare the animal for a quick and energetic reaction, often referred to as "the fight-or-flight response" (Cannon, 1915). "The fight-or-flight response" involves many different adaptations mainly by increasing energy availability and inhibiting processes not necessary for the immediate survival (Johnson et al., 1992; Carrasco and Van De Kar, 2003). The primary physiological stress response is mediated by the sympathetic nervous system and hypothalamic–pituitary–adrenal (HPA) axis in mammals (see **Figure 1**) and the hypothalamic–pituitary–interrenal (HPI) axis in teleost fish (Wendelaar Bonga, 1997; Carrasco and Van De Kar, 2003). During a stress response, the hypothalamus is activated and releases corticotropin releasing factor (CRF; also known as corticotropin releasing hormone, CRH), which stimulates the release of adrenocorticotropic hormone (ACTH) from the pituitary gland. ACTH induces glucocorticoid synthesis and release from the adrenal/interrenal tissue into the blood. The HPA/HPI axis is self-regulated by an array of feedback loops (Carrasco and Van De Kar, 2003).

One of the main mediators of the stress response is CRF and here we will briefly detail the distribution and main effectors of the CRF system [for a more detailed description, see review by Ronan and Summers (2011)]. CRF was initially discovered in 1955 when factors in the hypothalamus were found to induce ACTH release from the pituitary gland (Guillemin and Rosenberg, 1955; Saffran and Schally, 1955). In 1981, CRF was characterized as a neuropeptide of 41 amino acids (Vale et al., 1981) inducing ACTH release from the pituitary gland (Lederis et al., 1985). Cells synthesizing CRF are primarily found in the hypothalamus, more precisely in the parvocellular neurons of the paraventricular nucleus (PVN) in mammals (Bloom et al., 1982; Pelletier et al., 1983; Swanson et al., 1983; Raadsheer et al., 1993). CRF is also found in other brain areas including the magnocellular cells of the PVN and supraoptic nucleus (Paull and Gibbs, 1983; Delville et al., 1992; Luo et al., 1994). The CRF system seems to be well conserved since CRF synthesizing cells are found in the parvocellular neurons in the preoptic area (POA) in teleost fish (Matz and Hofeldt, 1999; Pepels et al., 2002), the teleostean POA being homologues to the mammalian PVN. A recent study suggests that CRF is also present in other areas in the teleost brain, outside the POA such as the dorsal telencephalon and suprachiasmatic nucleus (Alderman and Bernier, 2007).

The effects of CRF are mediated by at least two different receptor types, namely corticotropin releasing factor receptor 1 (CRF-R1) and corticotropin releasing factor receptor 2 (CRF-R2), and these appear to be present in both mammals (Chang et al., 1993; Chen et al., 1993; Perrin et al., 1993, 1995; Vita et al., 1993; Kishimoto et al., 1995; Lovenberg et al., 1995; Stenzel et al., 1995) and teleost fish (Arai et al., 2001; Pohl et al., 2001). The CRF-R1 have been reported to mediate the activation of the HPA/HPI axis (Timpl et al., 1998; Bale et al., 2002b; Huising et al., 2004) and to induce anxiety-like behavior (Britton et al., 1986; Heinrichs et al., 1997; Bale et al., 2002b), whereas the CRF-R2 have been reported to affect several other behavioral and physiological responses to stress (Liebsch et al., 1999; Coste et al., 2000; Bale et al., 2002b), and also to be involved in anxiety control (Bale et al., 2000, 2002a). In addition to the receptors, there is a CRF binding protein (CRF-BP), which has been reported in mammals (Potter et al., 1991; Cortright et al., 1995; Behan et al., 1996) and the teleost Mozambique tilapia (*Tilapia mossambicus*) (Seasholtz

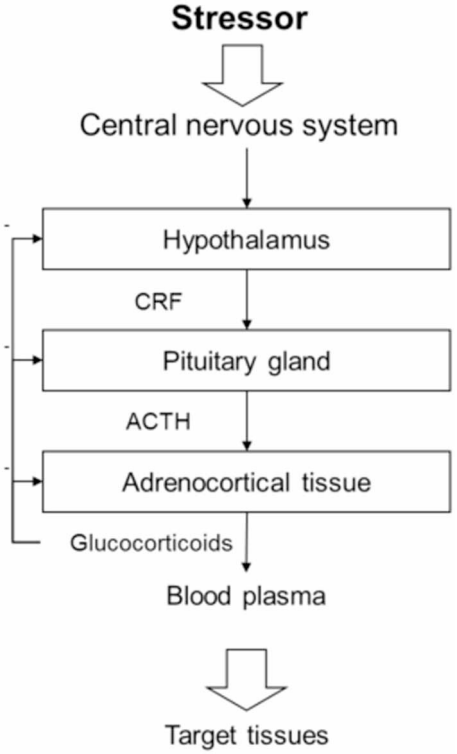

FIGURE 1 | Schematic drawing of the hypothalamic pituitary adrenocortical axis. The primary physiological stress response is partly mediated by the hypothalamic–pituitary–adrenocortical (HPA) axis in mammals. A stressor activates the central nervous system and the hypothalamus then releases corticotropin releasing factor (CRF) unto the pituitary gland. The pituitary gland will in its turn release adrenocorticotropic hormone (ACTH), which induces glucocorticoid synthesis and release from the adrenal tissue into the blood. The glucocorticoids will then affect target tissues throughout the body mainly to increase energy availability and inhibit processes not necessary for the immediate survival. Each step of the HPA axis is self-regulated by an array of feedback loops.

et al., 2002). The CRF-BP has been suggested to bind CRF with high affinity and thereby decrease CRF effects (Potter et al., 1991; Woods et al., 1994; Cortright et al., 1995).

Several neuropeptides closely related to CRF have been identified including urocortin-1, urocortin-2, and urocortin-3 in mammals (Vaughan et al., 1995; Lewis et al., 2001; Reyes et al., 2001), sauvagine in anurans (Montecucchi et al., 1979), and urotensin-I (UI) in teleost fish (Lederis et al., 1982; Ishida et al., 1986; Bernier et al., 1999). The CRF-R1 binds both CRF and urocortin 1 with similar affinity whereas the CRF-R2 has a higher affinity for urocortin-1 (Vaughan et al., 1995; Donaldson et al., 1996; Ozawa et al., 1998). Urocortin-2, urocortin-3, UI, and sauvagine are mainly bound by CRF-R2 (Hauger et al., 2003). Thus, CRF-R1 main ligands are CRF and urocortin-1, whereas CRF-R2 main ligands are urocortin-2, urocortin-3, UI, and sauvagine.

Several behaviors in mammals are affected by CRF, and CRF has been reported to increase locomotor activity, elevate anxiety-like responses, and reduce feeding in vertebrates [reviewed by Heinrichs and Koob (2004) and Lowry and Moore (2006)]. Further, the CRF system is an important mediator of several behavioral stress responses [see reviews by Bale and Vale (2004), Heinrichs and Koob (2004), and Lowry and Moore (2006)]. These and several other excellent reviews describe how CRF, behavior, and physiology are interacting. However, the specifics of how social stressors and the CRF system interact have not been reviewed. Therefore, the focus of this review is on how social stressors and CRF are interacting in modulating behavior and physiology.

SOCIAL STRESSORS

Social systems and social stressors differ between species (Blanchard et al., 2001), but generally social animals form dominance-based social hierarchies. In these hierarchies, each individual has a social rank position. For example, dominance hierarchies can be linear, which means that the alpha individual, having the highest rank, dominates all other group members, whereas the beta individual being subordinate to the alpha individual dominates the rest of the group members, and so on until the omega individual, which has the lowest rank and is subordinate to all group members (Huntingford and Turner, 1987). Dominance hierarchies are often formed by agonistic interactions. These agonistic interactions are performed by two or more individuals competing for the higher social position. Initially, a phase of mutual displays and threats escalates into a phase of overt aggressive behavior including violent attacks (Huntingford and Turner, 1987). The overt aggressive behavior continues until inter-relational rank is firmly established and the subordinate individuals signal defeat and retreat. However, even after the hierarchy is established subordinate animals are subjected to social stress and show signs of chronic stress (Greenberg et al., 1984; Sapolsky, 1990; Winberg et al., 1991; Blanchard et al., 1993), especially in captivity or other situations where escape is not possible. Experimentally, the effect of social stress is often studied using dyads and thus creating one dominant and one subordinate individual. Similar pairing is also used for the resident–intruder test, where the resident typically is an experienced and aggressive fighter and thus predestined to become dominant. Intruders from these pairings are suffering social defeat, which is another common social stress model.

Social isolation can be stressful as well in several vertebrates, usually depending on social organization. In several species, either isolation or grouping would be stressful and some gender differences could also be apparent (Blanchard et al., 2001). Rodent models are often used for both social isolation and dominance hierarchies, whereas teleost fish has mainly been used in dominance hierarchical studies such as dyadic interactions. Several experiments have shown that the CRF system is important in mediating effects of social stress on aggression and anxiety-like behaviors. Early studies were mainly done using exogenous administration of CRF and have continued with specific antagonist as well as site-specific administration. Further studies have been using the expression of CRF mRNA, as well as the expression of other genes related to the CRF system, to monitor the effects of acute and social stressors. Recent studies have also been using genetic engineering and thus creating deficient or knockout strains for elucidating the effects of various components of

the CRF system. In the following sections, these studies will be reviewed.

ACUTE EFFECTS OF EXOGENOUS CRF

Aggressive behavior is crucial for establishing dominance, upholding territories, and protecting offspring. Several studies using exogenous CRF have reported modulating effects on various types of aggressive behavior in various vertebrates. However, the function of the CRF system in the control of aggressive behavior is not consistent across species. It seems that it can both induce and reduce aggression. For instance, in male mice intracerebroventricular (icv) injection of CRF as well as sauvagine reduces aggression (Mele et al., 1987). Similarly, in female mice icv injections of either CRF, urocortin-1, or urocortin-3 inhibit maternal aggression (Gammie et al., 2004; D'anna et al., 2005). Further, these responses seems to be mediated by CRF-R1, since the specific CRF-R1 antagonist antalarmin injected into the amygdala reduces defensive posture in socially defeated mice (Robison et al., 2004). In contrast, in male rats injections of CRF into the amygdala can increase aggression (Elkabir et al., 1990). Further, icv injections of the CRF non-selective antagonist α-helical CRH_{1-41} decrease aggressive behavior in male rats (Aloisi et al., 1999). Similarly, the CRF-R1 antagonist SSR125543A delayed the latency to attack intruders in Syrian hamsters (*Mesocricetus auratus*) (Farrokhi et al., 2004). It also seems as if CRF-R antagonists reduce the behavioral effects of aggression and defeat as a social stressor. For instance, the CRF-R1 antagonist NBI-30775 increased time in defensive posture and latency to submission, and reduced the defeat stress induced immobility in rats (Wood et al., 2012). Further, the CRF non-selective antagonist d-Phe $CRF_{(12-41)}$ injected into the dorsal raphe nucleus of Syrian hamster reduced both the acquisition and expression of conditioned defeat as seen by reduced submissive and defensive behavior, whereas the CRF-R2 specific antagonist anti-Svg-30 only reduced the expression of conditioned defeat (Cooper and Huhman, 2007). In a follow-up experiment, it was found that an injection of a non-selective CRF antagonist into the lateral ventricle reduced submissive and defensive behavior, and similarly a selective CRF-R2 antagonist also reduced submissive and defensive behavior, but a CRF-R1 antagonist had no effect on these behaviors (Cooper and Huhman, 2010). Thus, there is evidence for the involvement of both CRF-R1 and CRF-R2 in modulation of aggressive behavior. However, since CRF by itself elicits divergent responses in aggressive behavior, species-specific patterns could be present.

Interestingly, this ambiguity concerning the effect of CRF on aggression can be seen in a single species of teleost fish. In rainbow trout (*Onchorhynchus mykiss*), two studies using exogenous CRF have reached two different conclusions. Carpenter et al. (2009) found that an icv injection of CRF increased victory chances in dyadic interactions and decreased latency to attack in winners but also reduced aggressive interactions. However, Backström et al. (2011a) found, using similar injection and doses, that CRF induced subordinance during dyadic interactions but saw no effect on number of attacks or latency to attack. However, the longevity of fights might be involved since Carpenter et al. used 15 min and Backström et al. used 60 min of social interactions. Further, no modulating effects on aggression could be seen either by UI, the CRF-R antagonists α-helical CRH_{1-41}, or antalarmin (Backström et al., 2011a).

Studies applying administration of exogenous CRF have also shown that CRF is involved in anxiety-like behavior during social interactions (Arregi et al., 2006). In an early study on rats, icv injection of CRF reduced the number of social interactions indicating an anxiogenic effect (Dunn and File, 1987). Anxiety-like behavior in socially defeated male rats is reduced by injection of antisense oligodeoxynucleotide for CRF-R1 (Liebsch et al., 1995). Similarly, CRF and sauvagine reduces sociability in male mice (Mele et al., 1987). Urocortin-2 intraperitoneally injected is anxiogenic in prairie voles (*Microtus ochrogaster*) as indicated by increasing spontaneous parental behavior following injection (Samuel et al., 2008). In addition, icv administration of urocortin-3 as well as the CRF-R1 agonist, $stressin_1$-A, in rats also induces anxiety-like behavior (Zhao et al., 2007). However, the sodium lactate-induced panic-like behavior in male rats during social interaction test can be blocked by the CRF-R1 antagonist antalarmin injected intraperitoneally (Shekhar et al., 2011). Further, injections of CRF and urocortin-1 into the basolateral amygdala reduces social interactions and thus indicating an anxiety-like behavior (Rainnie et al., 2004; Spiga et al., 2006), and the CRF-R1 specific antagonist NBI3b1996 injected into the basolateral nucleus of the amygdala attenuates the anxiety-like behavior (Gehlert et al., 2005). Single and repeated injections of urocortin-1 into the bed nucleus of the stria terminalis induce anxiety-like behavior during social interaction test in male rats (Lee et al., 2008), indicating a difference in function between basolateral amygdala and bed nucleus of the stria terminalis. Thus, there is evidence of involvement of both CRF-R1 and CRF-R2 in anxiety-like behavior in mammals, but CRF-R1 seems to be most important for this effect. It seems as if the anxiety-like behavior expressed in vertebrates is well conserved since it has been reported in teleost fish as well. In rainbow trout, icv injections of CRF or UI have been shown to induce an anxiety-like behavior similar to a non-ambulatory motor activity in rodents both in isolation and in dyadic interactions (Carpenter et al., 2007, 2009; Backström et al., 2011a). However, no effects of CRF antagonists could be seen, including antalarmin, which has been shown to reduce avoidance behavior in crucian carp (*Carassius carassius*) (Lastein et al., 2008).

INTERMITTENT EFFECTS OF CRF—EXPRESSION STUDIES

Social stressors affect gene expression in the CRF system. Because of the involvement in the HPA-axis an up-regulation of CRF expression would be expected. This can also be seen in several studies using social isolation. For instance, socially isolated male rats have more CRF immuno-reactive cells expressed in the median eminence of the hypothalamus following an acute stressor compared to controls (Sánchez et al., 1998). Similarly, in prairie voles (*Microtus ochrogaster*), social stressors lead to an up-regulation of the CRF cell number and mRNA expressions in the PVN. Socially isolated females had more CRF immuno-reactive cells in the PVN after social defeat (Grippo et al., 2007), socially isolated males had higher CRF mRNA expression in the PVN

compared to paired males (Pan et al., 2009), and in both genders social isolation led to higher density of CRF immuno-reactive cells in the PVN compared to voles paired with a con-specific (Ruscio et al., 2007). Similarly, isolation stress for at least 24 h and other stressors elevates CRF mRNA expression in the POA of rainbow trout (Doyon et al., 2005), and isolation for 24 h and 96 h lead to up-regulation of both CRF and UI mRNA expression in the POA of rainbow trout (Bernier et al., 2008). Thus, an activation of the PVN/POA CRF cell population is apparent after isolation stress. A similar up-regulation of CRF expression can be seen in dominance hierarchies as well. In a 14 days exposure to a visible burrow system in male rats (groups of five males and two females), subordinate males had higher CRF mRNA expression in the central amygdala compared to dominants and controls, and a subset of subordinate males (subordinate responders) had higher CRF mRNA expression in the PVN (Albeck et al., 1997). The subordinate responders were individuals responding with a higher corticosterone response compared to controls and dominants. However, 35 days of social defeat in tree shrews (*Tupaia belangeri*) also lead to fewer urocortin 1 immuno-reactive cells in the neuron population of the Edinger-Westphal nucleus and fewer CRF immuno-reactive cells in the parvocellular PVN and central amygdala (CeA) (Kozicz et al., 2008). Therefore, it seems that social defeat affects CRF expression in the PVN differently over time, and a similar pattern can be seen in teleost fish. In subordinate rainbow trout, CRF expression is up-regulated in the POA following 8 h interactions but not after 24 h interactions (Bernier et al., 2008). However, the time course for effects on CRF expression seems to be complex, since in another experiment rainbow trout being socially subordinate for 72 h show an up-regulation of CRF expression in the POA (Doyon et al., 2003). Further, after 5 days of social interactions, there were no differences in telencephalic or POA, CRF, or CRF-BP expression between dominant and subordinate rainbow trout (Jeffrey et al., 2012).

Differences between interacting individuals can also be seen in other regions of the brain. For instance, socially defeated male rats have lower CRF levels in the hippocampus (Panksepp et al., 2007). Further, in zebrafish (*Danio rerio*) the CRF mRNA levels are higher in the telencephalon of subordinate males (but not females) on day 1 of social interactions (Filby et al., 2010b). Similarly, CRF was up-regulated in the telencephalon of male subordinates, but CRF was also down-regulated in the hypothalamus of subordinate males and females following one day of social interactions (Filby et al., 2010a). However, after 5 days of social interactions, no difference in brain CRF mRNA could be seen in zebrafish (Filby et al., 2010b; Pavlidis et al., 2011). In *Astatotilapia burtoni* interacting for 4 weeks establishing territorial and non-territorial males, very similar to dominants and subordinates, respectively, whole brain CRF as well as pituitary CRF-R1 are down-regulated whereas CRF-BP is up-regulated in non-territorial males (Chen and Fernald, 2008). Further, visual contact for 3 days, but not 1 or 7 days, kept CRF, CRF-R2, and CRF-BP up-regulated whereas CRF-R1 was down-regulated in the brain of non-territorial males compared to controls (Chen and Fernald, 2011). Finally, Senegalese sole (*Solea senegalensis*) kept at high density for 33 days increase brain CRF mRNA expression as well as plasma cortisol levels compared to fish kept at low density, but no differences were seen in CRF-BP (Wunderink et al., 2011). All these differences are difficult to interpret, but are based on different brain parts. However, it is clear, as seen in zebrafish, rainbow trout, and *Astatotilapia burtoni*, that the effects of social interaction on CRF expression changes over time. This could be due to several different mechanisms, but aggressive behavior has been shown to be reduced over time in dyadic interacting zebrafish (Pavlidis et al., 2011), suggesting that social stress may decrease over time in interacting fish. Thus, the expression pattern of CRF in the brain is not universal and could be species-, context-, and time-dependent. It also seems that social stressors also sensitize the HPA axis. Icv injections of CRF into isolated rats lead to a higher increase of plasma corticosterone compared to controls (Serra et al., 2005). Similarly, rats socially defeated 2 days consecutively respond with more ACTH release after intravenous CRF injections after 7 days (Buwalda et al., 1999). However, this effect was not apparent at 21 days after social defeat (Buwalda et al., 1999). Thus, it seems as if the sensitization is only occurring during acute stressors and is probably down-regulated by the negative feedback system of the HPA axis.

The modulating effects of CRF are mediated by the CRF-receptors, which are responsible for separate responses. For instance, in socially defeated rats, injection with antisense CRF-R1 reduced anxiety-like behavior in the elevated plus maze, whereas antisense CRF-R2 increased immobility in forced swim test (Liebsch et al., 1999). Further, socially defeated CRF-R1 deficient mice had less impaired spatial memory compared to wild type mice (Wang et al., 2011), indicating that CRF-R1 promotes deleterious effects during social stress. In CRF-R2 knockout mice, aggression is increased, as indicated by a shorter latency to first attack and a higher number of attacks performed (Coste et al., 2006). Similarly, aggression in urocortin-2 knockout male mice is lower than in wild-type mice as measured by higher latency to first attack and more time in passive social contact (Breu et al., 2012). This indicates that CRF-R2 would be involved in the modulation of aggression. However, since socially isolated female prairie voles down-regulates CRF-R2 expression in the hypothalamus and up-regulates CRF-R2 expression in the hippocampus (Pournajafi-Nazarloo et al., 2009), and socially defeated rats seem to have up-regulated CRF-R2 mRNA in the posterior medial amygdala (Fekete et al., 2009), these effects could be site specific.

CHRONIC EFFECTS OF CRF—EARLY LIFE STRESS MODULATING ADULT BEHAVIOR

Exposure to early life stress can have long-term effects on the development of neuroendocrine systems. These effects have been shown to increase risk for anxiety-like and depressive-like disorders in adulthood in humans [see Veenema (2009) and references therein]. Since CRF has been implied as being involved in anxiety-like behaviors, studies concerning its involvement in early life stress effects have been performed. One of the common models in studying early life stress is using the social stressor of maternal separation in different regimes. For instance, male rats suffering maternal separation and isolation through pre-adolescence and then re-socialized until early adulthood express several different anxiety-like behaviors, such as shorter duration of social interaction and longer duration of freezing (Lukkes

et al., 2009a). Similarly, maternal separation for 3 h a day during pre-adolescence and then being re-socialized in groups of three induces the anxiety-like behaviors shown in elevated plus maze in male rats (Babygirija et al., 2012). The maternal separation and then re-socialization also induce a higher expression of CRF cells in the PVN as well as in the parvocellular division of PVN following a restraint stressor for 5 consecutive days (Babygirija et al., 2012), indicating a more sensitized HPA axis in these rats. However, most of these effects could be ameliorated if the animal is re-socialized with naïve rats. Further, in male prairie voles, social isolation for 6 weeks post-weaning increased anxiety-like behavior in elevated plus maze and increased CRF mRNA levels in the PVN (Pan et al., 2009). Thus, it seems that early life stress increases anxiety-like behaviors as well as sensitizes the HPA axis in adulthood. The anxiety-like behavior seems to be controlled by the CRF-R2. For instance, in male rats going through the maternal separation and then re-socialization, the anxiety-like behavior is reduced by injection of a general CRF antagonist (D-Phe-$CRF_{(12-41)}$) into the dorsal raphe nucleus (Lukkes et al., 2009a) and the CRF-R2 expression is up-regulated in the dorsal raphe nucleus (Lukkes et al., 2009b). Further studies showed that the specific CRF-R1 antagonist antalarmin and the specific CRF-R2 antagonist anti-sauvagine injected into the dorsal raphe nucleus both modulated the induced anxiety-like behaviors. However, anti-sauvagine reversed all anxiety-like behavior whereas antalarmin only had minor effects (Bledsoe et al., 2011). These studies all indicate that CRF-R2 in the dorsal raphe nucleus is involved in the anxiety-like behavior modulation following early life stress. However, in a study using wild-type and CRF-R2 knockout mice in a context fear memory study, maternal separation and isolation post-weaning induced more fear responses in wild-type and knockouts as compared to controls reared in groups of three (Gresack et al., 2010), thus indicating other pathways inducing fear and anxiety.

STRAIN DIFFERENCES—THE IMPACT OF CRF ON STRESS COPING STYLES

In mammals, divergent stress responses can be consistent over context and time. The behavioral and physiological responses to stress can generally be divided into two coping styles, namely proactive and reactive stress coping (Koolhaas et al., 1999, 2007). Proactive animals are more active, behave more aggressively, and readily form behavioral routines as compared to reactive animals (see **Table 1**). Moreover, proactive animals show lower HPA axis reactivity but higher sympathetic reactivity than reactive animals. Since divergent coping styles appear related to differences HPA-axis reactivity and locomotory activity, which are both under the control of CRF [see reviews by Heinrichs and Koob (2004) and Lowry and Moore (2006)], differences in the CRF system should be apparent.

Few studies in mammals have addressed this possible link. However, rodents with similar differences in stress responsiveness have been studied. For instance, rats bred for high anxiety behavior (HAB) or low anxiety behavior (LAB) also seem to differ in stress coping style (Landgraf and Wigger, 2002). The HAB rats seem to be more passive and have a higher HPA axis reactivity, thus fitting nicely into the reactive stress coping style. Interestingly, there seems to be differences in the CRF system between HAB and LAB rats. During basal conditions, HAB rats display lower CRF mRNA in the bed nucleus of the stria terminalis, but higher CRF-R2 expression in the PVN and the ventromedial hypothalamus than LAB rats (Wigger et al., 2004). Following stress, HAB rats show higher CRF-R2 expression in the ventromedial hypothalamus and the central amygdala (Wigger et al., 2004) as compared to LAB rats. Thus, differences in stress coping style seem to be reflected in differences in the CRF system. Similarly, in rats subjected to social defeat for 7 days, the behavioral reactivity could be divided into short latency (SL) or long latency (LL) to assume submissive posture (Wood et al., 2010). These two groups also fit nicely with reactive and proactive stress coping styles, respectively. CRF mRNA densities in the PVN and CRF-R1 levels in the pituitary were decreased in the SL rats compared to controls 24 h after last social defeat (Wood et al., 2010). Further, in mice strains selected for aggressiveness based on long attack latency (LAL) or short attack latency (SAL), no difference in CRF mRNA in PVN was noted during basal conditions, but 24 h after a swim stress LAL mice had higher CRF expression in the PVN than basal LAL and stressed SAL (Veenema et al., 2003). Socially defeated mice could be divided into active (proactive) or passive (reactive) coping, and the active coping mice had higher CRF mRNA levels in the hypothalamus than passive or control mice 1 h after social stress (De Miguel et al., 2011). Several of these differences in the CRF system also make sense concerning the stress coping styles, although differences once again are dependent on time. The reactive stress coping style having a more sensitized CRF system, including elevated CRF and CRF-R1 expression in the PVN during acute social stressor, leading to a higher HPA-axis reactivity. Similarly, high CRF expression in mice reduces aggression.

In recent years, studies on teleost fish have established the existence of stress coping styles in several different species such as halibut (*Hippoglossus hippoglossus*) (Kristiansen and Fernö, 2007), Nile tilapia (*Oreochromis niloticus*) (Barreto and Volpato, 2011), and Senegalese sole (Silva et al., 2010). Further, salmonid species have been examined thoroughly. For instance, in brown trout (*Salmo trutta*) individuals clustered into two separate stress coping styles based on the plasma levels of noradrenaline

Table 1 | Summary of the behavioral and physiological differences between proactive and reactive animals.

	Proactive	Reactive
BEHAVIORAL CHARACTERISTICS		
Aggression	High	Low
Conditioned immobility	Low	High
Routine formation	High	Low
PHYSIOLOGICAL CHARACTERISTICS		
HPA axis reactivity	Low	High
Parasympathetic reactivity	Low	High
Sympathetic reactivity	High	Low

Modified from Koolhaas et al. (1999, 2007) and Øverli et al. (2007) and references therein.

and adrenaline post-confinement and behavior during hypoxia (Brelin et al., 2005). Similarly, in rainbow trout aggression, dominance and post-stress plasma levels of cortisol differed between individuals and resulted in two distinct coping styles (Schjolden et al., 2005b). Further, two selected strains of rainbow trout representing high responders (HR) and low responders (LR) with respect to plasma cortisol concentrations following a standardized confinement stress were studied by Pottinger and Carrick (1999). Over a series of experiments, these strains were shown to correspond to reactive and proactive stress coping strategy, respectively [see review by Øverli et al. (2007)]. A recent study presented results suggesting divergent effects of stress on the CRF system in HR and LR trout (Backström et al., 2011b). HR trout subjected to confinement stress for 180 min showed higher CRF mRNA levels than LR trout exposed to the same stressor, and following a 30 min confinement HR trout displayed higher CRF-R1 and lower CRF-R2 mRNA levels than LR trout (Backström et al., 2011b). Most likely, these differences are related to the divergent stress coping styles expressed by HR and LR trout. For instance, CRF has been reported to reduce feed intake in teleost fish (De Pedro et al., 1993; Bernier and Peter, 2001), and following a stressful challenge LR fish regain feed intake faster than the HR fish (Øverli et al., 2002). Thus, the higher expression of CRF mRNA in the HR strain during stress could mediate an anorexic effect in the HR strain. Further, CRF has been reported to increase locomotor activity in teleost fish (Clements et al., 2002; Clements and Schreck, 2004). Previous reports have shown diverging activity between the HR and LR fish. The HR fish has been reported to have lower or higher activity than the LR fish (Øverli et al., 2002; Schjolden et al., 2005a, 2006; Backström et al., 2011b). These effects appear to be inconsistent and could be context based. It appears as if LR fish show higher activity during non-stressful, whereas HR fish show higher activity during stressful conditions. The HR fish displayed lower activity when kept in groups in large tanks, in open field, and during isolation (Øverli et al., 2002; Schjolden et al., 2005a, 2006) but showed higher activity during resident-intruder tests (Øverli et al., 2002) and when in confinement (Backström et al., 2011b). This divergence between strains concerning activity could be due to the differences in the stress-induced effects on CRF release. Furthermore, CRF is involved in the control of aggressive behavior and has been reported to suppress aggressive behavior in rainbow trout (Backström et al., 2011a). This means that the diverging neuroendocrine stress responses between strains could explain strain related differences in aggression. The HR strain has been proposed to be less aggressive. For instance, Pottinger and Carrick (2001) reported that when interacting in size matched pairs, HR fish became subordinate significantly more often than LR fish. In addition, the higher locomotor activity shown by LR fish when reared in larger groups was suggested to be caused by high levels of agonistic interaction (Schjolden et al., 2006).

CONCLUDING REMARKS

CRF and its related peptides are involved in several behavioral and physiological responses to social stressors. These modulating effects are likely to be mediated through several different mechanisms and neuronal structures. The PVN/POA is involved in the HPA/HPI axis regulation through the CRF-R1. Both CRF-R1 and CRF-R2 seems to modulate the behavioral responses to social stress, such as aggression and anxiety-like behavior. Specifically, CRF-R1 seems to be more directly involved in modulating anxiety-like behavior, whereas CRF-R2 has more pronounced effects on aggressive behavior. However, some studies have also seen modulating effects of CRF-R1 on aggressive behavior. The effects of early life stress on stress responsiveness and anxiety-like behavior in adulthood appear to be at least in part mediated by CRF-R2 expressed in the dorsal raphe nucleus. Finally, differences in the CRF system is probably one factor responsible for the differences observed in behavioral profiles and stress responses of individuals displaying divergent stress coping styles. Since most of these studies have been performed on juvenile teleost fish and male rodents, further studies are needed to elucidate if there are also gender differences in the CRF system. Furthermore, the links between CRF and several other pathways including the vasopressinergic and monoaminergic systems are needed for a better understanding of the effects of social stress on behavior and physiology.

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The CRF system and social behavior: a review

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The corticotropin-releasing factor (CRF) system plays a key role in a diversity of behaviors accompanying stress, anxiety and depression. There is also substantial research on relationships between social behaviors and the CRF system in a variety of taxa including fish, birds, rodents, and primates. Some of these relationships are due to the broad role of CRF and urocortins in stress and anxiety, but these peptides also modulate social behavior specifically. For example, the social interaction (SI) test is often used to measure anxiety-like behavior. Many components of the CRF system including CRF, urocortin1, and the R1 receptor have been implicated in SI, via general effects on anxiety as well as specific effects depending on the brain region. The CRF system is also highly responsive to chronic social stressors such as social defeat and isolation. Animals exposed to these stressors display a number of anxiety- and stress-related behaviors, accompanied by changes in specific components the CRF system. Although the primary focus of CRF research on social behavior has been on the deleterious effects of social stress, there are also insights on a role for CRF and urocortins in prosocial and affiliative behaviors. The CRF system has been implicated in parental care, maternal defense, sexual behavior, and pair bonding. Species differences in the ligands and CRF receptors have been observed in vole and bird species differing in social behavior. Exogenous administration of CRF facilitates partner preference formation in monogamous male prairie voles, and these effects are dependent on both the CRF R1 and R2 receptors. These findings are particularly interesting as studies have also implicated the CRF and urocortins in social memory. With the rapid progress of social neuroscience and in understanding the complex structure of the CRF system, the next challenge is in parsing the exact contribution of individual components of this system to specific social behaviors.

Keywords: CRF, urocortins, social behavior, stress, psychological, isolation, maternal behavior

INTRODUCTION

Many converging lines of evidence implicate the corticotropin-releasing factor (CRF) system in social behavior. The CRF system may affect social behaviors via a broader role in stress, anxiety, depression, and adaptation (Dunn and Berridge, 1990; Heinrichs et al., 1995; Arborelius et al., 1999; Koob and Heinrichs, 1999; Radulovic et al., 1999; Bale and Vale, 2004; Ryabinin et al., 2012), but there is also support that the CRF system specifically modulates social behaviors. This paper will provide a comprehensive review of existing research on this interaction, and aims to identify key areas for future research. This is particularly timely as social models are becoming increasingly sophisticated, our understanding of the complexity of the CRF system has evolved substantially, and the contribution of the CRF system to these behaviors has gained significant appreciation.

Abbreviations: BLA, basolateral amygdala; BNST, bed nucleus of the stria terminalis; CeA, central amygdala; CRF, corticotrophin-releasing factor; CSD, chronic social defeat; DRN, dorsal raphe nucleus; HPA, hypothalamic-pituitary-adrenal; HPI, hypothalamic-pituitary-interrenal; -ir, immunoreactivity; KO, knock out; LC/PBN, locus coruleus/parabrachial nucleus; MS, maternal separation; PND, postnatal day; PVN, paraventricular nucleus of the hypothalamus; SDS, social defeat stress; SHRP, stress hyporesponsive period; SI, social interaction; SNP, single nucleotide polymorphism; Ucn, urocortin; VBS, visible burrow system.

Although many of the effects of CRF on behavior are due to its role in initiating the hypothalamic-pituitary-adrenal (HPA) response to stressors, the CRF system throughout the rest of the brain is quite complex. This review will focus on the role of central CRF systems. Studies examining CRF's role in HPA functioning will primarily be limited to those that included brain measures and behavioral roles of the CRF system. The CRF system consists of two receptor subtypes, R1 and R2, and five ligands: CRF, Urocortin1 (Ucn1), Ucn2, Ucn3, and CRF-binding protein (CRFBP) (Vale et al., 1981; Behan et al., 1995; Steckler and Holsboer, 1999; Ryabinin et al., 2002; Fekete and Zorrilla, 2007). CRF and each of the urocortins have distinct distributions and binding affinities for each receptor and CRFBP, which lends this system to a high degree of complexity and behavioral specificity. Because of this complexity in receptors and ligands, an important caveat to the interpretation of pharmacological studies is that the effects of administration of agonists or antagonists do not implicate just one endogenous ligand or receptor.

In addition to a high concentration in the paraventricular nucleus (PVN) of the hypothalamus, CRF is abundant in the central amygdala (CeA) and hindbrain in mammals (Merchenthaler et al., 1982). CRF has a high affinity for the R1 receptor and CRFBP. Ucn1 is concentrated primarily in the

centrally-projecting Edinger–Westphal nucleus, and to a lesser extent in supraoptic nucleus and dorsal lateral lemniscus, and binds with high affinity to R1 and R2 receptors, as well as CRFBP (Vaughan et al., 1995; Bittencourt et al., 1999; Fekete and Zorrilla, 2007). Ucn2 is produced in the PVN, other hypothalamic nuclei and the locus coeruleus, and binds primarily to the R2 receptor (Reyes et al., 2001; Yamauchi et al., 2005; Fekete and Zorrilla, 2007). Ucn3 is primarily found in hypothalamic and amygdalar regions, and has exclusive affinity for the R2 receptor (Lewis et al., 2001; Li et al., 2002; Fekete and Zorrilla, 2007). The R1 receptor is more abundant in the central nervous system than the R2 receptor, and is found in regions throughout the brain, including the olfactory bulb, cortex, septum, hippocampus, amygdala, and cerebellum (Bale and Vale, 2004; Fekete and Zorrilla, 2007). The R2 receptor is more restricted, concentrated primarily in the lateral septum, hypothalamus, dorsal raphe (DRN) and few other areas (Bale and Vale, 2004; Fekete and Zorrilla, 2007). The glycoprotein CRFBP is located throughout the brain, particularly in cortical regions, the amygdala, the bed nucleus of the stria terminalis (BNST), hypothalamus, and raphe nuclei (Potter et al., 1992). This distribution across many brain areas clearly indicates that the CRF system functions outside the classical HPA axis.

Understanding the role of this system outside of the stress response can be aided by examining how the CRF system has evolved. Despite the frequent assumption that glucocorticoid release is the primary outcome of CRF system activation, this response to stress emerged only during vertebrate evolution (Campbell et al., 2004b). In contrast, CRF/Ucn-like peptides appeared as early as mollusks and insects. Presumed functions for these homologous peptides in invertebrates include osmoregulation and feeding. The divergence of CRF/Ucn-like peptides occurred during vertebrate evolution, such that only one homologous peptide was found in tunicates (early vertebrates). Only thereafter did two gene duplications occur, first giving rise to segregation into CRF/Ucn1 and Ucn2/Ucn3 paralogs, and then into CRF, Ucn1, Ucn2, and Ucn3. Most likely, an additional duplication of Ucn1 occurred in amphibians, leading to sauvagine (Lovejoy, 2009; Lovejoy and Barsyte-Lovejoy, 2010).

Interestingly, the vertebrate CRF appears to be most distant from the ancestral peptides. Through its specialization for regulation of the HPA axis, it has lost many of its early characteristics (Coast, 1998; Lovejoy, 2009). Therefore, considering CRF as the prototypical CRF receptor ligand can be misleading. In parallel to the CRF/Ucn peptides, the genes homologous to the two CRF receptors and CRFBPs are also found in insects (Chang and Hsu, 2004; Huising and Flik, 2005). The link between CRF/Ucns and the stress response emerged only in vertebrates. Yet even in vertebrates, these peptides are involved in non-overlapping functions between taxa, such as metamorphosis in amphibians, and osmoregulation (via the urophysis) in fish. In this regard, it is worth noting that genetic deletion of either Ucn2 or Ucn3 in mice does not produce overt effects on stress reactivity, but does alter social behaviors, suggesting that mammals have adapted some of the CRF-related peptides to specific regulation of social interactions (SIs) (Deussing et al., 2003; Breu et al., 2012).

The CRF system has been studied in social contexts in a variety of taxa including fish, birds, rodents, and primates. The majority of research on the CRF system and social behaviors is in mammals, particularly rodents, therefore this review will focus on them. Short excursions into other taxa will be included where comparisons are illuminating.

DEVELOPMENTAL AND ADULT EFFECTS OF SOCIAL HOUSING CONDITIONS ON CRF

IMMEDIATE EFFECTS OF CRF ON ISOLATION RESPONSE

It has been demonstrated in many species that separation from the mother leads to an immediate increase in distress vocalizations from infants. Endogenous increases in central CRF following isolation (Walker et al., 1991) are thought to inhibit vocalizations. Cortisol (or corticosterone) response tends to peak during the same time period that vocalizations decline, ∼1 h after separation onset. Exogenous administration of CRF inhibits separation-induced vocalizations in rats (Insel and Harbaugh, 1989; Harvey and Hennessy, 1995), guinea pigs (Hennessy et al., 1991; Hennessy, 1997), and Japanese quail (Launay et al., 1993). These vocalizations are blocked or reversed with pre-treatment with a CRF-receptor antagonist (Hennessy et al., 1992; Harvey and Hennessy, 1995; McInturf and Hennessy, 1996), indicating that separation-induced vocalizations are mediated by CRF receptors. Although the site of action is unknown, these effects are independent of CRF's actions on HPA activity (Hennessy, 1997).

RESPONSE OF CRF SYSTEM TO EARLY ISOLATION AND SEPARATION

The early neonatal period in rodents is characterized by a "stress hyporesponsive period" (SHRP), during which pups show blunted HPA response to stressors (Levine, 2001). The SHRP is dynamically regulated by the dam, with different aspects of her care acting on specific aspects of the stress response. For example, tactile stimulation regulates changes in the brain associated with maternal deprivation, feeding regulates adrenal responsivity, and passive physical contact suppresses the stress response itself [as reviewed in Levine (2001)]. The SHRP is absent in maternally deprived pups. Loss of this regulating maternal influence early in life has significant consequences for the development of the HPA axis, including CRF gene expression in the hypothalamus, and related behaviors.

The handling procedure examines the effects of short-term (15 min) repeated maternal separations (MSs) on offspring. In the wild, dams must periodically leave the nest for short periods (for example, to forage for food). Therefore, handling is thought to better model naturalistic conditions than typical laboratory rearing under which pups have continuous access to the dam. Indeed, un-disturbed control groups tend to show HPA physiology and (hyper-) responsivity more similar to maternally deprived rats (see below) than handled animals (Plotsky and Meaney, 1993; Plotsky et al., 2005). Compared to un-handled rats, animals receiving early handling experience show reduced novelty-induced fear (Bodnoff et al., 1987), stress-induced anxiety (Meerlo et al., 1999), predator-induced behavioral inhibition (Padoin et al., 2001), and increased activity in the open field (Levine, 1967). Handled rats also show reduced HPA response to stress, as evidence by a lower endocrine response (plasma ACTH and corticosterone) to stressors, as well as a more rapid return to baseline at the end of the stressor (Plotsky and Meaney, 1993).

The effects of handling are observed within only a few days. Compared to undisturbed controls, increases in CeA CRF mRNA emerge only after 4 days of handling (Fenoglio et al., 2004), and with 1 week of handling CRF mRNA is elevated in the CeA and BNST, and decreased in the PVN (Fenoglio et al., 2004). When tested as 3–4-month-old adults, male rats that had received neonatal handling had lower levels of CRF mRNA in the PVN, CeA, BNST, and locus coruleus/parabrachial nucleus (LC/PBN), as well as reduced CRF protein in the PVN, CeA, BNST, LC/PBN, and median eminence than undisturbed animals (Plotsky and Meaney, 1993; Viau et al., 1993; Plotsky et al., 2005). Clearly, this modest but naturalistic procedure has profound effects on the CRF system.

Short-term separation (i.e., handling) produces less stress-reactive offspring, but longer term repeated separations (of many hours) can have the opposite effect. This effect of MS has been studied in a diversity of species and across many developmental timepoints. The stress of separation itself likely has direct effects on the pup, but the effects of MS are also mediated by changes in maternal care due to separation. When dams are given access to a foster litter during separation (rather than isolation, as is the common method), the effects of the MS procedure are attenuated or even prevented entirely (Huot et al., 2004). Although often not considered, this study demonstrates that even a simple procedure may have multiple effects on the social environment that each contribute to the end phenotype.

As with handling, the effects of MS are rapid. Maternal deprivation in preweanling female sheep decreases CRF-immunoreactivity (-ir) nerve terminals in the median eminence after only 3 days (Wankowska et al., 2006). Daily MS over the first 2 weeks of life increases CRF-R1 in the PVN of female juvenile rats (Rees et al., 2008). Interestingly, early isolation in domestic piglets may have very delayed consequences on CRF. In one study (Kanitz et al., 2004), animals were isolated for 2 h daily from postnatal day (PND) 3–11, and CRF-ir was investigated using radioimmunoassay from animals shortly after isolation (PND 12) and after weaning (PND 56). MS had no effect of on CRF-ir in the PND 12 animals, but at 56 days old, animals that had received early repeated isolation had higher CRF-ir in the amygdala, and reduced CRF-ir in the hypothalamus. This finding highlights the need for studies on long-term consequences of early social stressors, as immediate effects of even repeated social stressors may provide an incomplete picture. The effects of deprivation persist well into adulthood, as daily MS during the pre-weaning period increases CRF receptor binding in the raphe nucleus and CRF-ir in the median eminence and PBN of 108-day-old male rats, but no difference in other cortical or limbic regions examined (Ladd et al., 1996).

Babygirija et al. (2012) examined the post weaning social environment on adult behavior and physiology of rats exposed to MS from PND 2–14. These rats were placed in post-weaning social housing either with other MS-reared rats or control (non-MS reared) rats. As expected, MS increased CRF mRNA in the PVN of adult rats, but this was attenuated when rats were housed with control animals. Therefore, the effects of early isolation in male rats are strongly influenced by later social experience (Babygirija et al., 2012). Seemingly paradoxically, these same MS rats housed with control partners have higher CRF cell counts in the PVN following a restraint stress than animals housed with similarly treated peers.

Traditional laboratory species, like most mammals, rely primarily on maternal care during postnatal development. Studies in biparental species, such as degus and prairie voles, indicate that paternal influences are also critical for the development of the CRF system. The degu (*Octodon degu*) is a caviomorph rodent that differs from typical laboratory rodents in a number of ways. Although the weaning age of degus is quite similar to other laboratory rodent species (∼PND 21), degus are born highly precocial following a 90–100 day gestation. The infant degu brain is more developed at birth and postnatal brain development is slower than a rat or mouse. In this species, 1 h of daily parental deprivation increases CRF-ir in the BLA, but decreases CRF-ir in the CeA, hippocampus, and somatosensory cortex, without altering PVN CRF-ir (Becker et al., 2007). Moreover, complete removal of only the father is associated with more CRF positive cells in the orbitofrontal cortex and BLA, but lower numbers of CRF positive cells in hippocampal regions at weaning (Seidel et al., 2011). However, by adulthood the only observed difference was a decrease in CRF positive cells in the stratum pyramidale of the CA1 region (Seidel et al., 2011).

Prairie voles (*Microtus ochrogaster*) are a social monogamous rodent that typically rears offspring biparentally. In a study of adult voles that had been reared only by their mother vs. both parents, Ahern and Young (2009) found that R2 binding in the DRN was increased in mother-reared offspring compared to biparentally-reared offspring. Further, the R2 binding in this area was positively correlated with the amount of licking and grooming in the natal nest, suggesting a direct link between parental behavior and the R2 receptor in DRN.

RESPONSE OF CRF SYSTEM TO ISOLATION OVER ADOLESCENCE

McCormick et al. (2007) looked at both short- and long-term responses of the PVN and CeA to different social stressors in adolescent rats. Brief (1 h) exposure to isolation induces a typical acute stress response: CRF mRNA is increased in the PVN but remains unchanged in the CeA. In males, this increase in the PVN is maintained if they are subsequently housed with a novel social partner, but is attenuated when they are returned to a familiar cagemate. Housing with a novel social partner after isolation also increases CRF mRNA in the CeA of males only. Isolation-induced changes in females were not affected by the post-isolation social environment, consistent with other research demonstrating greater sensitivity of the CRF system of males to many social stressors.

To investigate long-term changes in CRF mRNA to different social stressors, juvenile rats were isolated for 1 h daily over 2 weeks (McCormick et al., 2007). Animals were then either returned to their homecage with a familiar cagemate, or were housed with a different unfamiliar cagemate each day. Control rats were continuously housed with a familiar cagemate. Repeatedly-isolated animals had higher levels of CRF mRNA in the PVN, and showed habituation to an additional isolation (i.e., no change in CRF). Control males showed an increase in CRF mRNA in the CeA following acute isolation stress, but not the

other groups. Animals that were briefly isolated and exposed to a new partner every day had overall higher levels of CRF mRNA in this region than animals that were only isolated daily (McCormick et al., 2007). Similar to a lack of effects of acute social stress, female rats did not differ on either baseline or isolation-induced CRF in the CeA.

Juvenile rats that are housed in isolation following weaning have an increase in R2 in the DRN, and this is associated with decreased sensitivity to CRF-induced release of 5HT into the nucleus accumbens (Lukkes et al., 2009b). This increase in R2 in the DRN may promote isolation-induced changes in social anxiety (Lukkes et al., 2009a). Blockade of R2, but not R1 receptors in the DRN reversed the anxiogenic effects of isolation on plus maze behavior (Bledsoe et al., 2011). It should be noted that similar increases in R2 were observed in prairie voles reared without a father (Ahern and Young, 2009), as discussed above.

Male and female prairie voles that have been each short-term (4 day) and long-term (21 day) isolated following weaning show an increase in CRF-ir in the PVN and an accompanying increase in peripheral corticosterone (Ruscio et al., 2007). It is interesting to note that there is no difference between animals that are housed with a familiar same-sex sibling compared to those that are placed with a novel same-sex partner (Ruscio et al., 2007). Similarly, 6 weeks of post-weaning isolation in male prairie voles increases CRF mRNA in the PVN (Pan et al., 2009).

Male R2 knock out (KO) mice show greater behavioral sensitivity to post-weaning isolation housing than their wild-type littermates (Gresack et al., 2010). Isolated animals show increased exploratory rearing when initially placed in a novel environment (a locomotor activity chamber), and this is independent of genotype. However, isolated R2 KO's demonstrate poor habituation to the chamber, characterized by elevated activity and hole-poking compared to controls (Gresack et al., 2010). There are no behavioral differences in the locomotor activity between socially-housed KOs and wild-type mice. This supports the idea that R2 receptors are important for habituation to stressors, including social stressors such as isolation.

Domestic chickens (*Gallus gallus*) are a precocial bird species that do not receive postnatal care. Daily social isolation from conspecifics over PND 4–26 does not affect basal expression of R1 mRNA, but exposure to a novel restraint stress increases R1 mRNA in the thalamus/hypothalamus of male chickens that have undergone isolation (Goerlich et al., 2012). Females showed no upregulation of R1 mRNA, regardless of early experience, which is in agreement with rodent findings that social isolation effects are more robust in males. Unfortunately, this study did not examine CRF R2 levels, which would have allowed further comparisons with rodent studies.

RESPONSE OF CRF SYSTEM TO ADULT ISOLATION

There are limited available data on the role of the CRF system in response to acute isolation in adults. Oral administration of the R1 antagonist antalarmin in marmosets increased arousal response to acute separation from a pairmate (French et al., 2007). Studies examining the effects of acute, repeated, and chronic social isolation in adult prairie voles have focused on alterations of hypothalamic and hippocampal CRF measures. Specifically, exposure to either short-term (1 h) acute or daily repeated isolation increases CRF mRNA in the hypothalamus and hippocampus, but not in animals that are chronically isolated for 4 weeks (Pournajafi-Nazarloo et al., 2009, 2011). In contrast, chronic, but not acute or repeated, isolation leads to an increase in R2 mRNA in the hippocampus. All isolation stressors decreased R2 in the hypothalamus, and none had any effect on R1 receptors in either region (Pournajafi-Nazarloo et al., 2009, 2011). No sex differences were observed in any measures.

In another study in prairie voles, 4 weeks of isolation does not affect basal expression of CRF-ir in the PVN (Grippo et al., 2007b), however, there is an interesting sex difference in this region following resident-intruder challenge. Isolated females show a greater percentage of CRF cells in the PVN co-labeled with c-fos, but males show no difference based on housing history (Grippo et al., 2007b). Isolated females also have more CRF-positive cells in the PVN following resident-intruder stress [although this study did not examine males (Grippo et al., 2007a)]. These findings suggest that female prairie voles may show greater HPA sensitivity to isolation than males. This contrasts with studies of early isolation stress, which show higher sensitivity of male animals.

In a study of male voles, Bosch et al. (2009) looked at behavioral and physiological consequences of isolation from different types of social partners. Isolation from a female partner, but not a male sibling, increased passive stress-coping behavior in each the forced swim and tail suspension test. Interestingly, males that were housed with females had increased CRF mRNA in the medial BNST, and this was independent of whether they were subsequently isolated from their female partner (Bosch et al., 2009). The behavioral effects were blocked by central administration of a general CRF antagonist (d-Phe-CRF), as well as antagonists specific for each the R1 (CP154526) and R2 (Astressin-2B) receptors (Bosch et al., 2009). Lack of an effect of chronic isolation on CRF-ir in the PVN in voles (Grippo et al., 2007b) is contrasted by a study of California mice (*Peromyscus californicus*) in which long-term isolation in adulthood is associated with a reduction in basal CRF-ir in the PVN in males (Chauke et al., 2012). Similar to voles, these male California mice also show heightened expression of CRF and reduced neuronal activation in the CeA and PVN following a stressor (Chauke et al., 2012).

Although site-specific manipulation of CRF receptor activity in adult isolates has not been performed, we can speculate on candidate regions. Ehlers et al. (1993) examined changes in binding of ovine CRF (a R1 ligand with low affinity to CRF-BP) throughout the brain of adult rats that had been isolated for 6 weeks. Although no changes were observed in the hypothalamus, there was a decrease in the prefrontal cortex, and an increase in CRF binding in the cingulate cortex, piriform cortex, and cerebellum (Ehlers et al., 1993). Not surprisingly, these alterations were more pronounced in males.

It is pertinent to note that many studies in behavioral neuroscience use isolated subjects due to methodological simplicity, without addressing the direct and confounding effects this housing condition may have on results. Many of the common laboratory models are highly social species (rats, mice, primates), and group housing is often more naturalistic. As reviewed here,

chronic and repeated isolation are significant stressors for these animals, with long-term consequences on the CRF system and we caution against over-interpretation of studies involving only isolated subjects. For example, Lodge and Lawrence (2003) investigated the effects of the R1 antagonist antalarmin on alcohol self-administration in rats, but all subjects were reared in isolation. Therefore, it is difficult to know if the ability of antalarmin to reduce ethanol self-administration is significantly influenced by social stress effects on central CRF systems. As the CRF system has been proposed as a therapeutic target for a number of stress- and anxiety-related disorders (Kehne and Cain, 2010; Edwards et al., 2011; Paez-Pereda et al., 2011), it is critical that our behavioral models address or control for such isolation effects.

ANXIETY/SOCIAL INTERACTION

The CRF system is broadly implicated in anxiety across a number of behavioral paradigms, including open field, elevated plus maze, light-dark box, defensive withdrawal, and the SI test (File and Hyde, 1978). In the SI test, an animal is exposed to a novel conspecific and the time spent engaging in active social contact is recorded. Although this may differ based on laboratory, the active social contact measure includes all SIs, including sniffing, grooming, following, mounting, kicking, boxing, wrestling, and crawling, as a single measure of SI. Passive social contact, in which animals are touching but not interacting, is a separate measure. Using this metric, SI is inversely associated with anxiety, such that decreased levels of SI are indicative of anxiogenic state. Although rarely considered, the SI test may also measure direct effects on social behavior, as suggested by studies showing dissociated behavioral effects of CRF manipulations on SI and other anxiety measures (Zhao et al., 2007; Lee et al., 2008; Breu et al., 2012).

Central administration of CRF, Ucn1, or the R1 agonist stressin1-A decreases SI in rats (Dunn and File, 1987; Sajdyk et al., 1999; Campbell et al., 2004a; Gehlert et al., 2005; Zhao et al., 2007). A general CRF receptor or R1 specific antagonist has no direct effects on SI, but each are effective at blocking the anxiogenic effects of Ucn1 and CRF when administered as a pre-treatment (Sajdyk and Gehlert, 2000; Campbell et al., 2004a; Gehlert et al., 2005). Priming with a sub threshold dose of either Ucn1 (BLA: Sajdyk et al., 1999; Donner et al., 2012); (BNST: Lee et al., 2008) or CRF (BLA: Sajdyk et al., 1999) decreases SI and these behavioral effects can be observed for up to 4 weeks post-injection (Lee et al., 2008). These priming effects can be blocked by co-administration of an R1 antagonist (Sajdyk and Gehlert, 2000; Lee et al., 2008).

Each Ucn1 and CRF decreases SI when injected directly into the BLA (Sajdyk et al., 1999; Gehlert et al., 2005; Spiga et al., 2006). The behavioral specificity of Ucn1 on social anxiety is site-specific as administration into the BLA is anxiogenic across different behavioral and physiological markers (Sajdyk et al., 1999), however, it is highly specific for social anxiety in the BNST (Lee et al., 2008), and has no effect on any measure of anxiety when administered into the nucleus accumbens (Lee et al., 2008). Overall, these lines of evidence strongly implicate the R1 receptor and CRF or/and Ucn1 as modulating SI, consistent with their anxiogenic role in other tests.

There are limited data regarding the roles of Ucn2 and Ucn3 on SI. Male Ucn2 KO mice spend more time in passive social contact than wild-types, but do not differ in active SI (Breu et al., 2012). These KO males are also less aggressive. Females, however, do not differ in either measure in the SI test. Urocortin 3 has no effect on SI when administered i.c.v. (Zhao et al., 2007). At present, the role of R2 and its selective ligands (Ucn2 and Ucn3) in SI remain underexplored.

SOCIAL DEFEAT STRESS

Exposure to an aggressive conspecific induces rapid behavioral and neural adaptations. This social defeat stress (SDS) is an ethologically relevant model of stress, and demonstrates strong translational and construct validity (Wood et al., 2012). In this procedure, individuals are placed into the home cage of a larger, dominant, aggressive male. The resident male will attack and defeat the intruder male within 2 min, after which the subject is usually placed behind a mesh screen to protect from further attack, while maintaining the social stressor. The effects of the SDS on the CRF system are immediately apparent. In response to a single exposure, SDS induces an increase of CRF mRNA in the BNST and CeA, but not the PVN in rats (Funk et al., 2006), and activates CRF R2-positive neurons in the medial amygdala (Fekete et al., 2009). Six hours following defeat, CRF-ir is decreased in the hippocampus (Panksepp et al., 2007).

A single social defeat leads to an immediate anxiogenic effect, as demonstrated by a reduction in open arm time on the elevated plus maze (Heinrichs et al., 1992). The expression of SDS-induced anxiety is blocked by administration of the general CRF antagonist α-helical CRF into the CeA prior to elevated plus maze testing (Heinrichs et al., 1992). Liebsch et al. (1995) examined the effects of an antisense oligodeoxynucleotide blocking R1 mRNA in the CeA. Animals treated with the antisense oligodeoxynucleotide for 4 days prior to SDS testing showed greater open arm activity following the SDS compared to controls, indicating anxiolytic effect of this treatment (Liebsch et al., 1995).

SDS also leads to a rapid development of submissive and defensive behaviors in response to non-threatening social stimuli. This has been best characterized in male Syrian hamsters (*Mesocricetus auratus*), a species which typically demonstrates high levels of territorial aggression but switches to a defensive strategy following a single SDS (Huhman et al., 2003). The role of the CRF system in each the acquisition and expression of this conditioned social defeat (CSD) behavior has been examined. In order to study the role of the CRF system in the acquisition of CSD, Cooper and Huhman (2007, 2010) examined the effects of CRF antagonists administered prior to SDS on later CSD behavior. The non-selective antagonist D-Phe CRF and R2 specific antagonist anti-SVG-30 both reduced submissive/defensive behavior when administered i.c.v. (Cooper and Huhman, 2010), but the R1 antagonist CP154526 had no effect on later behavior. When administered directly into the DRN, D-Phe CRF reduced both submissive/defensive and social behaviors following SDS, but had no effect on controls (Cooper and Huhman, 2007). Intra-DRN administration of anti-SVG-30 also increased social behavior, but had no effect on submissive/defensive behavior. Taken together, these findings implicate the R2 receptor and urocortins

in the acquisition of conditioned defeat, with R1 receptors playing a negligible role. However, intra-amygdalar antalarmin, an R1 antagonist, prevents increases in submissive/defensive behaviors when administered immediately following SDS in mice (Robison et al., 2004).

The R2 receptor is also implicated in the expression of CSD in hamsters. The non-specific CRF receptor antagonist D-Phe CRF selectively decreases the expression of submissive/defensive behavior when administered into the lateral ventricles, BNST, and DRN, but not the CeA (Jasnow et al., 1999, 2004; Cooper and Huhman, 2007). CRF receptor blockade in the DRN reduces both the acquisition and expression of CSD (Cooper and Huhman, 2007). Similarly, the R2 antagonist anti-SVG-30 reduces CSD of animals treated directly into the ventricles, BNST, or DRN (Cooper and Huhman, 2005, 2007). In contrast to a general antagonist, anti-SVG-30 in the DRN is specifically reduces expression, but not acquisition, of CSD (Cooper and Huhman, 2007). Peripheral (i.p.) or central (i.c.v.) blockade of R1 receptors with the antagonist CP154526 has no effect on CSD (Jasnow et al., 1999; Cooper and Huhman, 2005).

Repeated or chronic SDS has even more profound effects, including reduction in body and adrenal weight, increases in depressive-like behavior, and changes in autonomic function (Wood et al., 2012). These effects are blocked by daily treatment with the R1 antagonist NBI-30775 over the course of chronic social defeat in rats (Wood et al., 2012). Additionally, NBI-30775 treated animals demonstrated a more active and less defensive response during the SDS, suggesting that R1 receptors may play a role in acute response to this social stressor (Wood et al., 2012). This is further supported with a study of transgenic mice expressing conditional, postnatal forebrain R1 deficiency (Wang et al., 2011). These mice are protected against chronic social defeat-induced impairments in object recognition, spatial memory, and hippocampal remodeling (Wang et al., 2011).

A naturalistic model of chronic social defeat has been examined by placing a mixed-sex group of rats in a visible burrow system (VBS). Dominance hierarchies are quickly established, with a single dominant male and three subordinate males. Subordinates display a number of behavioral and physiological characteristics typical of chronically stressed animals. Interestingly, a subgroup of subordinates fail to show a corticosterone increase following a novel stressor, and there is evidence that these non-responders also differ in CRF regulation of the HPA axis. Specifically, although dominant rats and typical subordinate rats show similar levels of CRF mRNA and cell numbers in the PVN to each other (Albeck et al., 1997; Choi et al., 2006), non-responding subordinates have reduced levels (Albeck et al., 1997). However, CRF mRNA is increased in the CeA of subordinates, regardless of their stress response (Albeck et al., 1997). It must be noted that another study found no difference in CeA CRF mRNA between subordinate and dominant animals (Choi et al., 2006), although this may reflect methodological differences between the two studies. Relative to both control and dominant rats, subordinates also have elevated CRF mRNA in BNST (Choi et al., 2006). Although there are inconsistencies between studies, both implicate the extended amygdala as a target of VBS stress on the CRF system.

These studies on social stress and the CRF system warrant some methodological considerations. All of the experiments discussed in this section specifically examine males. Although isolation and SDS are not as stressful for females, females do show a robust chronic stress phenotype in response to social instability (Haller et al., 1999; Schmidt et al., 2010). Examining the effects of an appropriate social stressor, such as group instability, in females is a critical avenue for this field. Additionally, many studies use animals that are housed in isolation, which may influence CRF systems (see above). Some studies even neglect to describe housing conditions at all. Finally, stress control conditions also vary, ranging from no stress, handling, or exposure to a novel but non-aggressive male. These different behavioral conditions likely have their own effects on the CRF system. A summary of these experimental conditions, and others, are described in **Table 1**.

SOCIAL STRESS IN FISH

The involvement of the CRF system in social stress in mammals (reviewed above), warrants comparison to evolutionary earlier taxa, such as fish. The CRF system of fish is quite similar to that of mammals, including CRF, Ucn1, Ucn2, Ucn3, CRFBP and both receptor subtypes. However, cortisol release is stimulated via two mechanisms: the hypothalamic-pituitary-interrenal (HPI) axis and the caudal neurosecretory system (CNSS). The HPI axis is similar to the mammalian HPA axis: CRF is released from the preoptic nucleus (POA) of the hypothalamus (analogous to the mammalian PVN) to stimulate ACTH release from the pituitary. ACTH then stimulates cortisol release from interrenal cells. The CNSS, which lacks a mammalian homologue, is characterized by magnocellular Dahlgren cells located along terminal segments of the spinal cord (Lu et al., 2004; McCrohan et al., 2007). Dahlgren cells both produce and release CRF and Urotensin-I (UI; orthologous to mammalian Ucn1) and projects axons directly to the urophysis, a neurosecretory organ. The CNSS also lacks cortisol-driven negative feedback that is characteristic of the HPA and HPI axis. Besides areas involved in regulation of these axes, CRF and urocortins are also expressed in extrahypothalamic areas (Lovejoy and Balment, 1999; Alderman and Bernier, 2007).

Many teleost fish form dominance hierarchies, with dominant and subordinate individuals displaying different behavioral, endocrine, neurophysiological, and metabolic phenotypes. Specifically, dominant animals display higher levels of aggression, and subordinates show blunted growth, decreased access to food, and increased metabolic rate. In order to examine these phenotypes in the laboratory two size-matched, unfamiliar fish are placed in a tank. The animals are separated from each other by an opaque divider for a 1- to 3-day acclimation period. The divider is then removed and animals can directly interact. Dominance is established rapidly, and is determined by which animal in the dyad displays the most aggressive attacks, patrolling the water column, and feeding access.

Subordinate rainbow trout (*Oncorhynchus mykiss*) show hyperactive HPI activity, as measured by chronically elevated cortisol and ACTH (Sloman et al., 2001; Doyon et al., 2003; Alderman et al., 2008; Bernier et al., 2008; Jeffrey et al., 2012). This hyperactivation may be supported in part through elevated levels

Table 1 | Experimental conditions for studies on the role of the CRF system in social defeat stress.

Study	Species (strain)	Sex	Housing	Control condition	Duration of stressor	Measure/ manipulation	Site/ route
EFFECTS OF SOCIAL DEFEAT STRESS ON CENTRAL CRF SYSTEM							
Funk et al., 2006	Rat (Wistar)	Male	Not spec.	No stress	30 min	CRF mRNA (*in situ*)	
Panksepp et al., 2007	Rat (Long–Evans)	Male	Group	Novel non-aggressive	30 min	CRF-like peptide (RIA)	
Fekete et al., 2009	Rat (Wistar)	Male	Single	Handling only	75 min	CRF R2 mRNA *in situ* and c-fos ICC	
PHARMACOLOGICAL EFFECTS ON THE EXPRESSION AND ACQUISITION OF CONDITIONED DEFEAT STRESS							
Robison et al., 2004	Mice (C57BL/6)	Not spec.	Single	Antalarmin, non-stress	10 min	Antalarmin	BLA
Jasnow et al., 1999	Syrian hamsters	Male	Single	None	4 × 5 min over 1 day	CP-154,526	i.p.
	Syrian hamsters	Male	Single	None	4 × 5 min over 1 day	D-Phe CRF	i.c.v.
Jasnow et al., 2004	Syrian hamsters	Male	Single	None	15 min	D-Phe CRF	BNST
	Syrian hamsters	Male	Single	None	15 min	D-Phe CRF	CeA
Cooper and Huhman, 2005	Syrian hamsters	Male	Single	Empty resident cage	15 min	anti-SVG-30	i.c.v.
	Syrian hamsters	Male	Single	Empty resident cage	15 min	CP-154,526	i.c.v.
	Syrian hamsters	Male	Single	Empty resident cage	15 min	anti-SVG-30	BNST
Cooper and Huhman, 2007	Syrian hamsters	Male	Single	Empty resident cage	15 min	D-Phe CRF	DRN
	Syrian hamsters	Male	Single	Empty resident cage	15 min	anti-SVG-30	DRN
	Syrian hamsters	Male	Single	Empty resident cage	15 min	D-Phe CRF	DRN
	Syrian hamsters	Male	Single	Empty resident cage	15 min	anti-SVG-30	DRN
Cooper and Huhman, 2010	Syrian hamsters	Male	Single	None	15 min	D-Phe CRF	i.c.v.
	Syrian hamsters	Male	Single	None	15 min	anti-SVG-30	i.c.v.
	Syrian hamsters	Male	Single	None	15 min	CP-154,526	i.c.v.
PHARMACOLOGICAL EFFECTS ON OTHER BEHAVIORAL CONSEQUENCES OF SOCIAL DEFEAT STRESS							
Heinrichs et al., 1992	Rat (Wistar)	Male	Not spec.	No stress	30 min	α-helical CRF	i.c.v.
	Rat (Wistar)	Male	Not spec.	No stress	30 min	α-helical CRF	CeA
Liebsch et al., 1995	Rat (Wistar)	Male	SINGLE	None	10 min	Antisense oligonucleotide	CeA
Wood et al., 2012	Rat (Sprague Dawley)	Male	SINGLE	Novel cage	30 min × 7 days	NBI-30775	s.c.
Wang et al., 2011	Mice	Male	SINGLE	No stress	24 h × 21 days	CRH R1 deficiency	Forebrain

Housing indicates how animals were housed prior to any SDS testing ("not spec." indicates that pre-testing housing was not indicated, and is presumed to be isolation). Control condition refers specifically to any behavioral controls ("none" indicates all animals were tested for response to stress).

of CRF mRNA in the POA that are observed within a few hours of subordination (Bernier et al., 2008) and stay elevated for many days (Doyon et al., 2003; Bernier et al., 2008). Elevated cortisol levels are observed out to 7 days (Sloman et al., 2001), but CRF mRNA in the POA does not differ between dominant and subordinate animals after 5 days (Jeffrey et al., 2012). Similarly, whole brain CRF mRNA levels do not differ based on social status after 5 days of dyadic interaction in zebrafish (Pavlidis et al., 2011). It appears that heightened levels of cortisol do not habituate to subordination stress, whereas CRF mRNA in the POA habituates within a few days to basal levels (**Table 2**).

Visual cues from a dominant male are sufficient to cause transient changes in stress-related gene expression in an African cichlid, *Astatotilapia burtoni* (Chen and Fernald, 2011). In a variation of the social dominance test described above, male fish were housed with a female on one side of a divided tank. On the other side were a female and a larger, dominant male fish. Following

Table 2 | Relative cortisol and mRNA levels of subordinate trout to dominant or control animals (n.d. = no difference).

Stressor	Time	Cortisol	POA			CNSS	
			CRF	UI	CRFBP	CRF	UI
Subordination	8 h	↑[a]	↑[b]	n.d.[b]	n.d.[a]	n.d.[b]	n.d.[b]
	24 h	↑[a,b]	↑[b]	n.d.[b]	n.d.[a]	n.d.[b]	n.d.[b]
	3 d	↑[d]	↑[d]				
	5 d	↑[e]	n.d.[e]		n.d.[e]		
Isolation	4 h	↑[c]	n.d.[c]			n.d.[b]	n.d.[b]
	24 h	↑[b,c]	↑[b,c]	—[b]			
	3 d	n.d.[c]	n.d.[c]				
	4 d	n.d.[b]				↓[b]	↓[b]

[a] *Alderman et al., 2008;* [b] *Bernier et al., 2008;* [c] *Doyon et al., 2005;* [d] *Doyon et al., 2003;* [e] *Jeffrey et al., 2012.*

a 2 day habituation period, the opaque divider was removed, and animals remained separated via a clear divider that allowed visual, but not physical or olfactory contact. Relative to control males, whole brain CRF and CRFBP were increased at 3 days of visual contact with a dominant male (Chen and Fernald, 2011). Similarly, CRF R2 mRNA was increased, but CRF R1 mRNA decreased, following 3 days of visual social stress. However, no differences were observed in any CRF system-related measures at either 1 day or 1 week of this social stress. These findings suggest that visual information during social stress is sufficient to induce broad changes in the CRF system, but may show a delayed response compared to physical interaction.

Likewise, exogenous administration of CRF may influence dyadic behavior in trout by influencing behavior directly. The effects of CRF on behaviors in a size-matched dyadic encounter are unclear. Fish treated with higher doses of i.c.v. CRF are more likely to become subordinate than controls (Backstrom et al., 2011). Central administration of CRF paradoxically decreases the number of both attacks and retreats, as well as the latency to attack (Carpenter et al., 2009). However, administration of UI, a general CRF antagonist (α-helical CRF) or the CRF R1 specific antagonist antalarmin had no effect on dyadic aggression (Backstrom et al., 2011). There is also evidence for differential neural response to different types of social stressors. Whereas cortisol remains elevated with prolonged subordination, isolation-induced levels of cortisol return to baseline within a few days (Doyon et al., 2005). Similarly, CRF mRNA in the POA seems to have both a delayed and more transient response to isolation (Doyon et al., 2005; Bernier et al., 2008; **Table 2**). The CNSS does not appear to be immediately responsive to subordination stress, but each CRF and UI mRNA is elevated in the CNSS following long-term isolation (Bernier et al., 2008). While these studies clearly show the greater involvement of the CRF than UI in regulation of social behavior in fish, the sparse analyses of other CRF R2 ligands makes it difficult to assess the role of urocortins in social behaviors.

INDIVIDUAL DIFFERENCES IN RESPONSE TO SOCIAL STRESS

Individual differences in the response of the CRF system to social stress have been observed in a number of rodent models (Albeck et al., 1997; Elliott et al., 2010; Wood et al., 2010; De et al., 2011). There is also some evidence that individuals may be genetically susceptible to effects of early social environment on later behavior. Barr et al. (2009) identified a single nucleotide polymorphism (SNP) in the promoter region of the *crh* gene (which encodes CRF protein) of rhesus macaques. A gene-environment interaction was found between this SNP and rearing experience. Specifically, peer-reared monkeys that were heterozygous for the SNP showed significant decreased environmental exploration and increased voluntary ethanol consumption compared to mother-reared animals and homozygous peer-reared monkeys (Barr et al., 2009). Similarly, in human patients with a history of child sexual abuse, a haplotype of the CRF R1 gene protects against the development of alcohol abuse (Nelson et al., 2010). Understanding how genetic polymorphisms and epigenetic regulation of the CRF system affect response to social stress and social behavior directly is an exciting direction for future study.

OTHER SOCIAL STRESSORS

Pheromones are by definition social signals, as release of a pheromone from one individual is specifically meant to elicit a response in another individual of the same species. The CRF system has been implicated in behavioral responses to alarm pheromone, which is released following footshock (Kikusui et al., 2001) and can increase stress- and anxiety-sensitive behaviors in exposed rats (Kiyokawa et al., 2005, 2006, 2007; Inagaki et al., 2008). Exposure to alarm pheromone affects male, but not female, sexual behavior in rats (Kobayashi et al., 2011, 2013). However, pre-treatment with the R1 antagonist CP154526 normalizes sexual behavior in male rats exposed to alarm pheromone (Kobayashi et al., 2011). Additionally, exposure to alarm pheromone increases the number of cells in the PVN double labeled for CRF and c-fos (Kobayashi et al., 2013). These findings suggest that alarm pheromone induces release of CRF from the PVN to modify sexual, and other behaviors, however, this was found in animals that were also tested for sexual behavior prior to tissue collection, so it should be interpreted cautiously. These studies clearly demonstrate behavioral responses to pheromones, a specifically social phenomenon, are mediated by the CRF system, in a sex-specific manner.

MALE AGGRESSION

Intermale aggression is often examined using the resident-intruder paradigm, in which an unfamiliar, non-aggressive intruder is placed in the home cage of the subject (Miczek, 1979). In these studies, the subjects are housed in isolation for an extended period of time prior to testing. Studies have implicated the CRF system in intermale aggression, although the exact mechanisms are unclear. Administration of CRF or its R2-acting paralogue sauvagine (i.c.v.) decreases aggression and sociability, while increasing defensive behaviors in mice (Mele et al., 1987). When tested in a novel arena, rather than home cage, CRF increased aggressive behavior in rats when administered into the amygdala (but not the highest dose), but not the lateral ventricle (Elkabir et al., 1990). Interestingly, i.c.v. administration of CRF decreased investigative behaviors, but at the highest dose in the amygdala promoted investigation (Elkabir et al., 1990). Male mice lacking Ucn2 show reduced aggression (Breu et al., 2012).

Contrasting effects were found in rats tested for stress-induced fighting. Stress-induced fighting consists of exposing a pair of rats to intermittent and inescapable foot shocks, with defensive aggression developing with repeated testing. Central administration of CRF facilitates aggressive behavior, and the antagonist α-helical CRF reduces stress-induced fighting (Tazi et al., 1987). The differences in effects observed in resident-intruder aggression and stress-induced fighting highlight the importance of dosage, site of administration, and behavioral conditions when comparing studies. This is further demonstrated in two studies of R2 KO mice. Using the resident-intruder test, Gammie et al. (2005) found no effect of R2 KO on aggression. However, when placed in a novel environment, R2 KOs show elevated levels of aggression (Coste et al., 2006).

Oral administration of the R1 antagonist SSR122343A reduces aggressive behavior in both hamsters and rhesus macaques

sociality were independently evolved, suggesting that convergent evolution of CRF receptor distribution is important for social behavior.

The CRF system also plays a role in pair bonding, as administration of CRF directly into the ventricles or nucleus accumbens facilitates partner preference formation in male prairie voles, even at doses that are thought to be too low to increase anxiety (DeVries et al., 2002; Lim et al., 2007). These effects are blocked by co-administration of the either a R1 or R2 antagonist, therefore both receptor subtypes are necessary for pair bond formation (Lim et al., 2007). These findings are particularly interesting as many studies have implicated the CRF system in social memory (described in the next section of the review).

Given that stress inhibits sexual behavior, it is not surprising that the CRF system has also been implicated in sexual behavior. Female white-crowned sparrows (*Zonotrichia leucophrys*) show stress-induced suppression of reproductive behavior during the breeding season. Exogenous CRF (i.c.v.) also inhibits reproductive behavior in this species (Maney and Wingfield, 1998). It is likely that these effects of CRF on behavior are centrally mediated, as this study used a dose of CRF (25 ng) that does not activate the HPA axis, and birds were estradiol implanted to maintain elevated sex steroid levels regardless of CRF treatment. A similar inhibition of sex behaviors is seen in both male and female mice that transgenically overexpress CRF in the brain (Heinrichs et al., 1997). These effects are not affected by adrenalectomy, further supporting a role for central CRF R1-acting peptides (CRF and/or Ucn1) in mediating sexual behavior (Heinrichs et al., 1997).

When injected directly into the arcuate-ventromedial area of the hypothalamus, MPOA, or periaqueductal gray of female rats, CRF inhibits lordosis and increases aggressive rejection of male attempts to sniff and mount (Sirinathsinghji et al., 1983; Sirinathsinghji, 1985, 1986). Similarly, i.c.v. CRF suppresses male copulatory behavior in rats (Sirinathsinghji, 1987). Female Syrian hamsters also show reduced lordosis when either Ucn1 or CRF is administered in the lateral ventricles, and this inhibitory effect of CRF is blocked by co-administration of the CRF receptor antagonist astressin, which suggests that these effects are receptor mediated (Jones et al., 2002). Although the role of each receptor subtype in modulating sexual behavior has not been explored, there is some evidence in support of the R1 receptor in primates. Oral administration of the R1 antagonist antalarmin increased masturbation in rhesus macaques (Habib et al., 2000) and potentiates separation-induced sexual behavior in marmosets (French et al., 2007). Clearly, the inhibitory effects of CRF administration on sexual behavior are seen across many species. However, i.c.v. CRF actually promotes sexual activity in female musk shrews (Schiml and Rissman, 2000), again highlighting the importance of species differences in interactions of the CRF system and social behaviors.

SOCIAL MEMORY

Heinrichs (2003) demonstrated bi-directional effects of pharmacological manipulation of the CRF system on social memory. In these experiments, adult female rats were given social recognition tests following i.c.v. injections of either the general CRF receptor antagonist D-Phe CRF (12–41) or the CRFBP ligand inhibitor r/h CRF (6–33). In this social recognition test, rats are briefly exposed to a novel juvenile conspecific. After a short delay, rats are re-introduced to the same juvenile. A reduction in time spent investigating the juvenile on the second exposure compared to the first is indicative of a social memory for that individual. Each the CRF receptor antagonist and CRFBP inhibitor dose-dependently impaired social memory when the delay between exposures was 30 min (Heinrichs, 2003). Paradoxically, after a 120 min delay, which normally is too long to maintain social recognition in this task, r/h CRF-treated animals displayed social recognition at both doses tested. Importantly, none of these manipulations affected exploration of the juvenile on first exposure, and therefore did not affect anxiety or SI, but only later recognition.

Social investigation and recognition are increased in mice centrally over-expressing CRF (CRF-OE; Kasahara et al., 2011). Mice were habituated to the same juvenile over four serial sessions. On the first habituation, the CRF-OE mice engaged in more social investigation of the juvenile. This contrasts with the effects of pharmacological treatment with CRF to reduce SI. Over the next three sessions, CRF-OE animals showed the same investigation and habituation as wild-types. Ten minutes following the last habituation session, each mouse was exposed to the familiar and a novel juvenile conspecific. Both groups selectively investigated the novel juvenile, indicative of normal short-term memory. However, when tested again 24 h later, only the CRF-OE mice retained social memory (Kasahara et al., 2011).

Both of these studies implicate the CRF system in social recognition, although it remains unclear if this is a general effect on memory, or if it is specific to social stimuli. Evidence from Ucn3 KO mice has identified a disassociation between social and objection recognition (Deussing et al., 2010). Mice lacking Ucn3 demonstrated slower extinction of social memory, but no difference in a novel object recognition task, social odor discrimination, or the SI test. These KOs also demonstrate similar elevated plus maze, forced swim test, acoustic startle, and prepulse inhibition behavior to their wild-type counterparts. In agreement with these findings, mice lacking the CRF R2 receptor also show improved social memory, but Ucn2 KO's have unaltered social memory. This study clearly demonstrates a specific role of Ucn3 on social memory via R2 receptors (Deussing et al., 2010).

CONCLUSIONS AND FUTURE DIRECTIONS

As reviewed here, the CRF system is both highly sensitive to the social environment and is implicated in the regulation of a broad array of social behaviors. This interaction between social environment and the CRF system is dynamic. It is developmentally sensitive, species-specific, and often sex-specific (Lee et al., 2008; Deussing et al., 2010). The specificity of contribution of CRF R1 vs. R2 receptors to particular social behaviors is in agreement with the general tendency of involvement of various components of the CRF system in different bodily functions across evolution. Perhaps this specificity is a reflection of differential need to control different behavioral states of the organism across behaviorally-different taxa (for example, parental state in

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monogamous vs. promiscuous species, aggression in territorial vs. communal species, etc).

While it is undeniable that many of the social functions of the CRF system are likely indirect due to stress or anxiety, there are studies indicating differential sensitivity or specificity for social factors and the CRF system. Perhaps the most compelling evidence of this comes from comparative research on the CRF system in closely related species with divergent social behaviors (Lim et al., 2005; Goodson et al., 2006). Further characterization of how the CRF system is involved in species-specific social behavior is a major frontier in understanding the evolution and neurobiology of sociality.

With the rapid progress of the social neuroscience field and in understanding the complex structure of the CRF system, the next challenge is in figuring out the exact contribution of individual components of this system to specific facets of social behaviors. There is substantial evidence that R2 receptors are more responsive to several types of social behaviors. Yet, the original papers frequently only discuss CRF as the ligand potentially mediating this effect. Often this is done without performing parallel studies on the primary ligands of the R2 receptor: the urocortins. Sometimes, to confirm involvement of CRF in a behavior regulated by a CRF antagonist, CRF immunoreactivity or mRNA levels are examined, and changes are taken as an evidence for CRF's involvement. However, expression of CRF and Ucns are co-dependent (for example, CRF KOs have higher levels of Ucn1, and CRF overexpressors have lower levels of Ucn1). Therefore, in the absence of Ucn studies, regulation of CRF is only a suggestive evidence for its involvement over involvement of Ucns (Weninger et al., 2000; Kozicz et al., 2004). While there is direct evidence for the Ucns role in some social behaviors, such as parental behavior and social memory, the Ucns have been largely neglected in many areas areas, including social stress and SI. Providing a complete characterization of the neurocircuity that includes all elements of the CRF system will be invaluable in our understanding of the neurobiology of social behavior and relationships.

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Corticotropin-releasing factor-related peptides, serotonergic systems, and emotional behavior

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Corticotropin-releasing factor (CRF) is a 41-amino acid neuropeptide that is involved in stress-related physiology and behavior, including control of the hypothalamic-pituitary-adrenal (HPA) axis. Members of the CRF family of neuropeptides, including urocortin 1 (UCN 1), UCN 2, and UCN 3, bind to the G protein-coupled receptors, CRF type 1 (CRF_1) and CRF_2 receptors. In addition, CRF binding protein (CRFBP) binds both CRF and UCN 1 and can modulate their activities. There are multiple mechanisms through which CRF-related peptides may influence emotional behavior, one of which is through altering the activity of brainstem neuromodulatory systems, including serotonergic systems. CRF and CRF-related peptides act within the dorsal raphe nucleus (DR), the major source for serotonin (5-HT) in the brain, to alter the neuronal activity of specific subsets of serotonergic neurons and to influence stress-related behavior. CRF-containing axonal fibers innervate the DR in a topographically organized manner, which may contribute to the ability of CRF to alter the activity of specific subsets of serotonergic neurons. CRF and CRF-related peptides can either increase or decrease serotonergic neuronal firing rates and serotonin release, depending on their concentrations and on the specific CRF receptor subtype(s) involved. This review aims to describe the interactions between CRF-related peptides and serotonergic systems, the consequences for stress-related behavior, and implications for vulnerability to anxiety and affective disorders.

Keywords: anxiety, corticotropin-releasing factor, dorsal raphe nucleus, emotional behavior, serotonin

INTRODUCTION

Corticotropin-releasing factor (CRF) is a 41-amino acid neuropeptide that is involved in stress-related physiology and behavior, including control of the hypothalamic-pituitary-adrenal (HPA) axis (Vale et al., 1981, 1983). CRF has been implicated in the etiology and pathophysiology of stress-related disorders such as anxiety and affective disorders (Dunn and Berridge, 1990; Binder and Nemeroff, 2010). One of the ways in which CRF may play a role in the etiology and pathophysiology of anxiety and affective disorders is through modulation of brainstem neuromodulatory systems such as serotonergic systems. Serotonin (5-hydroxytryptamine; 5-HT) has long been implicated in control of emotional behavior as well as anxiety and affective disorders (Ressler and Nemeroff, 2000). Consequently, understanding the interactions between CRF, CRF-related neuropeptides, and serotonergic systems is likely to lead to advances in understanding the biological basis of anxiety and affective disorders. This review aims to describe the interactions among CRF, CRF-related neuropeptides, and serotonergic systems and the importance of these interactions in modulating emotional behaviors involved in anxiety and affective disorders.

CRF FAMILY OF PEPTIDES

The CRF family of neuropeptides includes CRF as well as the urocortins (UCN), UCN 1, UCN 2, and UCN 3, structurally related peptides that have been discovered more recently (Vaughan et al., 1995; Donaldson et al., 1996; Zhao et al., 1998; Lewis et al., 2001; Reyes et al., 2001; Lovejoy and Jahan, 2006; Fekete and Zorrilla, 2007). UCN 1 is a 40-amino acid peptide while both UCN 2 and 3 are 38-amino acid peptides. The UCN's, like CRF, have been implicated in stress-related physiology and behavior, including modulation of the HPA axis (Vaughan et al., 1995; Reul and Holsboer, 2002). There are two receptors that CRF and the UCN's bind to with high affinity, which are designated as CRF_1 (Perrin et al., 1993) and CRF_2 receptors (Lovenberg et al., 1995). They are both G protein-coupled receptors belonging to the B1 subfamily of G-coupled receptors and couple to both G_s and G_q (Perrin et al., 2006) with varying affinities for the neuropeptides in the CRF family. CRF itself has a greater affinity for CRF_1 receptors while UCN 1 binds with high affinity to both receptors and UCN 2 and UCN 3 both preferentially bind to CRF_2 receptors (Vaughan et al., 1995; Lewis et al., 2001; Reyes et al., 2001). Several splice variants for both receptor subtypes have also been reported and the structural and functional properties of these splice variants have been reviewed previously (Dautzenberg et al., 2001). Finally, the CRF binding protein (CRFBP) shows high affinity for both CRF and UCN 1 but has little affinity for UCN 2 or 3 (Lewis et al., 2001).

DISTRIBUTION OF CRF CONTAINING NEURONS IN NEURAL CIRCUITS CONTROLLING EMOTIONAL BEHAVIOR

Corticotropin-releasing factor-containing neurons are widely distributed throughout both the rat and mouse brains, with several areas differing in expression levels, based on patterns of

immunohistochemical staining in the two species (Wang et al., 2011). Given the wide distribution of CRF-containing neurons within the central nervous system, the idea that CRF works as a neuromodulator has received considerable attention in the past few decades. The main focus of this review is the role of CRF and CRF-related neuropeptides in stress-related emotional behavior, and therefore we focus on the distribution of these neuropeptides in neural circuits implicated in control of stress-related emotional behavior. A full consideration of the distribution of CRF and CRF-related neuropeptides can be found in previous reviews focusing on the chemical neuroanatomy (Swanson et al., 1983; Sakanaka et al., 1987; Kozicz, 2007).

A major source for CRF in the brain is the paraventricular nucleus of the hypothalamus (PVN) (Sakanaka et al., 1987). CRF synthesized in the PVN, via projections to the median eminence, plays a primary role in control of the HPA axis. However, several extrahypothalamic brain regions involved in control of emotional behavior have CRF-containing neurons. In particular, both the central nucleus of the amygdala (CE) and the bed nucleus of the stria terminalis (BNST) contain CRF-immunoreactive neurons with extensive projections to brainstem structures controlling emotional behavior (Gray, 1993; Wang et al., 2011). Other regions with CRF expressing neurons that are involved in control of emotional behavior include the hippocampus, subiculum, lateral septum, and periaqueductal gray (Sakanaka et al., 1987; Calandreau et al., 2007). The localization of CRF in brain regions involved in control of emotional behavior implicated CRF as an important neuromodulator, in addition to an important neurohormonal function (Gray, 1993).

DISTRIBUTION OF UCN 1, 2, AND 3 CONTAINING NEURONS

The UCN's are expressed in discrete regions within the brain. The non-preganglionic Edinger-Westphal nucleus has a large number of UCN 1 neurons (Kozicz et al., 1998). Additionally, the lateral superior olivary and supraoptic nuclei also have been shown to have mRNA and immunoreactivity for UCN 1 (Bittencourt et al., 1999; Lewis et al., 2001). UCN 2 is mainly localized in subcortical structures including the locus coeruleus (Reyes et al., 2001). UCN 3 is also localized to discrete areas of the brain including an area encircling the columns of the fornix in the rostral hypothalamus, the posterior portion of the BNST and an area dorsolateral to the caudal portion of the dorsomedial hypothalamic nucleus (Kuperman et al., 2010). Another grouping of UCN 3 neurons is located in the anterodorsal part of the medial amygdaloid nucleus (Lewis et al., 2001; Li et al., 2002).

DISTRIBUTION OF CRF RECEPTORS IN EMOTION-RELATED BRAIN REGIONS

The distribution of CRF_1 and CRF_2 receptors within rodent brain has been well-described with CRF_1 receptors being more widely distributed while CRF_2 receptors are more restricted to subcortical areas (Potter et al., 1994; Chalmers et al., 1995; Van Pett et al., 2000). The hippocampus contains both CRF receptors as does the periaqueductal gray (Van Pett et al., 2000). The amygdala expresses both receptor subtypes with low levels of only CRF 1 receptors in the CE (Van Pett et al., 2000). All portions of the BNST have been shown to have CRF_1 receptors while the posterior portion of the BNST also has CRF_2 receptors (Van Pett et al., 2000). Importantly for this review, the raphe nuclei including the DR and median raphe nucleus (MnR) both have CRF_1 and CRF_2 receptors with the DR having higher levels of CRF_2 and the MnR having about equal amounts of both receptors (Van Pett et al., 2000; Day et al., 2004).

THE FUNCTIONAL SUBSETS OF 5-HT NEURONS BASED ON FUNCTIONAL NEUROANATOMY AND AFFERENT AND EFFERENT CONNECTIONS

In order to discuss the possibility that CRF and CRF-related peptides control functional subsets of serotonergic neurons involved in control of emotional behavior, it is first useful to consider the evidence for a topographical and functional organization of the midbrain raphe complex. The midbrain raphe complex includes serotonergic systems located within the DR, the median raphe nucleus, caudal linear nucleus, pontomesencephalic reticular formation, supralemniscal cell group, and interpeduncular nucleus (Hale et al., 2012) Here we will focus on the organization of the DR. The DR is topographically organized and can be divided into subregions making up the rostral, dorsal, ventral, ventrolateral, interfascicular, and caudal portions. It is beyond the scope of this review to describe in detail the topography but we will, in brief, describe the major subdivisions here and refer the reader to previous reviews for a thorough review of the DR serotonergic system and its topography (Lowry, 2002; Lowry et al., 2005, 2008; Hale and Lowry, 2011; Hale et al., 2012).

THE ROSTRAL DR

The rostral portion of the DR, which is located from approximately −7.04 to −7.30 mm from bregma in the rat brain (Paxinos and Watson, 1998), receives projections from cingulate, orbital and infralimbic cortices, as well as a small number of projections from the CE, BNST, and substantia inominata and larger numbers from the paraventricular and other hypothalamic nuclei (Peyron et al., 1997). In turn, the rostral DR projects to the caudate putamen with collaterals to the substantia nigra, and also projects to the subthalamic nucleus and substantia inominata (Steinbusch, 1981; Imai et al., 1986; Canteras et al., 1990; Grove, 1998). Data show that 6 weeks of voluntary wheel running increases 5-HT_{1A} receptor mRNA in the rostral and mid-rostrocaudal DR as well as decreases 5-HT_{1B} receptor and 5-HT transporter mRNA (Greenwood et al., 2003, 2005). Voluntary wheel running is also associated with a protective effect against the behavioral deficits associated with uncontrollable tail shock such as exaggerated freezing in a shuttle box (Greenwood et al., 2003, 2005). These data show that the rostral DR is connected with emotion-related brain regions and that altered emotional behavior is associated with serotonergic changes in this brain region.

THE DORSAL PART OF THE DR

The DRD is located from approximately −7.30 to −8.30 mm from bregma in the rat brain (Paxinos and Watson, 1998). The DRD receives projections from areas associated with the control of emotional behaviors including the lateral and ventral orbitofrontal and infralimbic cortices, CE, BNST, and the dorsal,

dorsomedial, lateral, and posterior hypothalamic nuclei (Peyron et al., 1997). Further, the DRD projects to areas associated with control of emotional behaviors including the CE, BLA, BNST, nucleus accumbens (Acb), medial prefrontal cortex (mPFC), and dorsal hypothalamus (Van Bockstaele et al., 1993; Commons et al., 2003; Hale et al., 2008). In addition, the DRD sends a number of collateral projections to functionally related forebrain targets involved in emotional behavior. Anxiety related stimuli such as multiple classes of anxiogenic drugs including UCN 2, or anxiety producing situations including exposure to an open-field test arena, or social defeat, lead to increased activation of DRD serotonergic neurons as measured by c-Fos immunoreactivity (Abrams et al., 2005; Gardner et al., 2005; Bouwknecht et al., 2007; Hale et al., 2008, 2010; Paul et al., 2011). Lastly, chronic corticosterone in the drinking water, which increases anxiety-like behavior in a social interaction task, open field task, and elevated plus maze, increases tryptophan hydroxylase 2 (TPH) mRNA in the DRD (Donner et al., 2012b). The DRD is connected with emotion-related brain regions including the BNST (see **Figure 1**) and activation by anxiogenic stimuli show that it may be an important region involved in the control of emotion-related behavioral output.

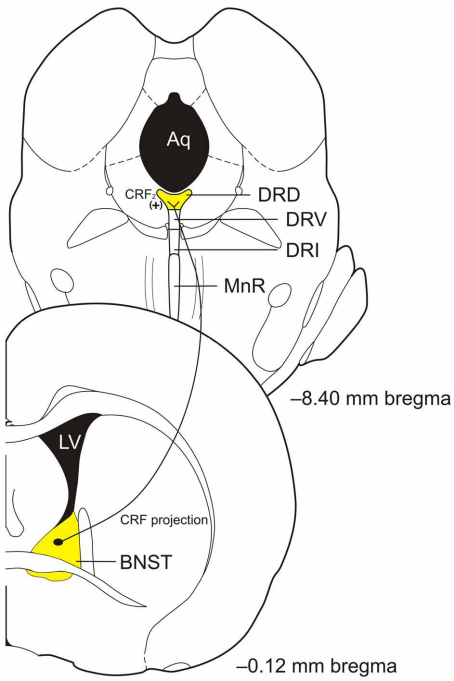

FIGURE 1 | Diagram of proposed bed nucleus of the stria terminalis (BNST) corticotropin-releasing factor (CRF) projections to the caudal portion of the dorsal part of the dorsal raphe nucleus (DRD) to activate CRF_2 receptors to modulate serotonin (5-hydroxytryptamine; 5-HT) output in control of anxiety-like behaviors. Abbreviations: Aq, aqueduct; CRF, corticotropin-releasing factor; DRD, dorsal part of the dorsal raphe nucleus; DRI, interfascicular part of the dorsal raphe nucleus; DRV, ventral part of the dorsal raphe nucleus; LV, lateral ventricle; MnR, median raphe nucleus; (+), excitation. Coronal section templates reproduced from Paxinos and Watson (1998), The Rat Brain in Stereotaxic Coordinates, 4th Edition. San Diego: Academic Press: 1998 with permission from Elsevier.

THE VENTRAL PART OF THE DR

The DRV is located from approximately −7.30 to −8.30 mm from bregma in the rat brain (Paxinos and Watson, 1998). The DRV receives projections from the cingulate and lateral orbital cortices, CE, and dorsomedial hypothalamic nucleus, with less dense projections from other amygdaloid nuclei and cortex (Peyron et al., 1997). The DRV in turn projects to sensorimotor, ventrolateral orbital, frontal, motor, and visual cortices and the caudate putamen (Steinbusch et al., 1980; Steinbusch, 1981; Waterhouse et al., 1986; Kazakov et al., 1993). Because of the projections to frontal, visual, and motor cortex, it is likely that this region of the DR is involved in directed behaviors that may or may not have an emotional content. Further research of DRV serotonergic neurons is needed for a better understanding of their functional properties.

THE VENTROLATERAL PART OF THE DR AND VENTROLATERAL PERIAQUEDUCTAL GRAY

The DRVL/VLPAG is located lateral to the DRD and occurs approximately from −7.64 to −8.54 mm from bregma in the rat brain (Paxinos and Watson, 1998). The DRVL/VLPAG receives projections from a number of brain regions involved in autonomic and emotional control. These include projections from the amygdala, with heavy innervation by the CE and moderate innervation by the dorsolateral medial amygdala with additional projections from the ventromedial prefrontal cortex, hypothalamus, and the retina (Hurley et al., 1991; Shen and Semba, 1994; Lee et al., 2003, 2007). The DRVL/VLPAG gives rise to projections involved in visual function including the superior colliculus and lateral geniculate nucleus (O'Hearn and Molliver, 1984; Waterhouse et al., 1993). Further, the DRVL/VLPAG also projects to the hypothalamus, medulla, PAG, and subcortical somatosensory regions, and data also suggest that DRVL/VLPAG serotonergic neurons control, via multisynaptic connections, presympathomotor neurons in the spinal cord (Beitz, 1982; Stezhka and Lovick, 1997; Ljubic-Thibal et al., 1999; Underwood et al., 1999; Kirifides et al., 2001; Bago et al., 2002; Kerman et al., 2006). The DRVL/VLPAG connections suggest that this region is important in control over panic-like and fight-or-flight behaviors. Consistent with this hypothesis, data show that panic-inducing stimuli such as hypercapnia or sodium lactate activate DRVL/VLPAG serotonergic neurons, but not in rats that are made panic prone, suggesting that the DRVL/VLPAG may inhibit panic in normal rats (Johnson et al., 2005, 2008).

Recent data suggest that the DRVL/VLPAG may be an important component in the interdependence of fear- and panic-like responses. Data show that when a rat is fear conditioned and experiencing CE-mediated fear it is less likely to exhibit panic-like behaviors when given dorsal PAG electrical stimulation (Magierek et al., 2003). Clinical evidence supporting the hypothesis that fear may inhibit panic-like responses comes from a recent study in which 3 people with selective lesions of the amygdala were unable to experience normal fear but experienced panic when given CO_2 inhalation (Feinstein et al., 2013). We propose that the CE, when activated, may serve to selectively inhibit panic through connections with DRVL/VLPAG serotonergic neurons (see **Figure 2** for the proposed circuit). People with bilateral amygdala lesions are unable to inhibit CO_2-induced panic responses while healthy

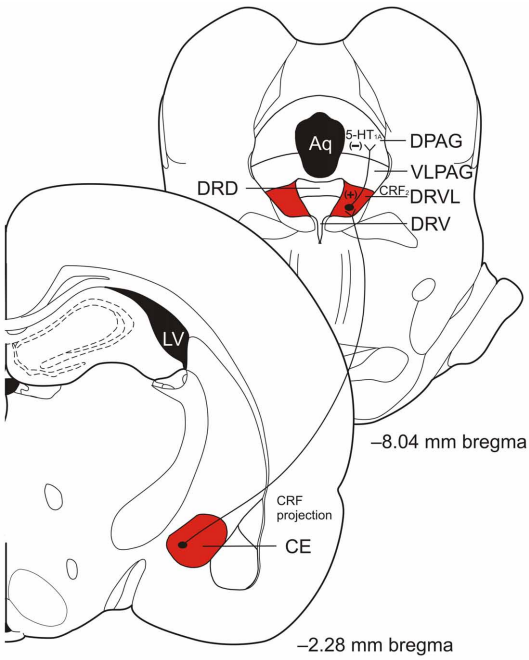

FIGURE 2 | Diagram of proposed central nucleus of the amygdala (CE) corticotropin-releasing factor (CRF) projections to the ventrolateral part of the dorsal raphe nucleus (DRVL) and DRVL serotonergic projections to the dorsal periaqueductal gray (DPAG) involved in panic inhibition during fear expression, such as freezing behavior. Excitatory projections from the CE excite serotonergic neurons in the DRVL that in turn release serotonin (5-hydroxytryptamine; 5-HT) in the DPAG to act on inhibitory 5-HT_{1A} receptors to inhibit panic. Abbreviations: Aq, aqueduct; CE, central nucleus of the amygdala; CRF, corticotropin- releasing factor; DPAG, dorsal periaqueductal gray; DRD, dorsal part of the dorsal raphe nucleus; DRV, ventral part of the dorsal raphe nucleus; DRVL, ventrolateral part of the dorsal raphe nucleus; LV, lateral ventricle; VLPAG, ventrolateral periaqueductal gray; (+), excitation; (−), inhibition. Coronal section templates reproduced from Paxinos and Watson (1998), The Rat Brain in Stereotaxic Coordinates, 4th Edition. San Diego: Academic Press: 1998 with permission from Elsevier.

controls generally do not experience panic when given CO_2 (Goetz et al., 2001). The data thus suggest an important role of serotonergic neurons in the DRVL/VLPAG for control of emotional behaviors including fear and panic.

THE CAUDAL PORTION OF THE DR

That most caudal subdivision of the DR (DRC) is located from approximately −8.30 to −9.30 mm bregma in the rat brain (Paxinos and Watson, 1998). The DRC receives a number of projections including from the mPFC, the preoptic area, arcuate nucleus, and perifornical and lateral hypothalamic areas, lateral habenula, and substantia nigra with more sparse afferents from both the CE and BNST (Lee et al., 2003). In turn, the DRC projects to brain regions involved in control of emotional behavior including the LC, amygdala, paraventricular nucleus of the thalamus and ventral hippocampus (Imai et al., 1986; Krout et al., 2002). Furthermore, the DRC has been shown to be involved in the behavioral deficits seen with inescapable stress and is activated following social defeat, administration of anxiogenic drugs, and administration of ligands including CRF and UCN 2 suggesting it, along with other regions of the DR, plays in an important role in the control of emotional behavior (Hammack et al., 2002, 2003a; Abrams et al., 2005; Gardner et al., 2005).

THE INTERFASCICULAR PART OF THE DR

The interfascicular part of the DR (DRI) is located approximately between −8.18 and −8.80 mm from bregma in the rat brain (Paxinos and Watson, 1998). Although not well-researched, studies have shown projections from the LC, median preoptic area, and the lateral parabrachial nucleus to the DRI (Saper and Loewy, 1980; Holstege, 1995; Lee et al., 2003; Kim et al., 2004). The DRI has, however, been shown to project to a number of regions. The DRI has projections to both dorsal and ventral hippocampus, medial septum, entorhinal, dorsolateral prefrontal, medial orbital, and anterior cingulate cortex and mediodorsal thalamus (Azmitia and Segal, 1978; Kohler and Steinbusch, 1982; Kohler et al., 1982; Porrino and Goldman-Rakic, 1982; Krout et al., 2002). The DRI, along with the DRVL/VLPAG, has been shown to be activated by a number of peripheral sensory stimuli, including peripheral injection of heat-killed *Mycobacterium vaccae* or lipopolysaccharide (LPS), and exposure to warm and cold temperature (Hollis et al., 2006; Hale et al., 2011; Kelly et al., 2011). Activation of DRI serotonergic neurons is associated with antidepressant-like behavioral responses (Lowry et al., 2007). This then suggests, consistent with its pattern of efferents that this area of the DR may also be important in controlling certain emotional behaviors.

DISTRIBUTION OF 5-HT RECEPTORS IN EMOTION RELATED BRAIN REGIONS

There are at least 14 different 5-HT receptors that have been identified, all of which have been thoroughly reviewed previously (Hoyer et al., 1994, 2002; Barnes and Sharp, 1999; Smythies, 2005; Hannon and Hoyer, 2008). The receptors are divided into 7 families (1–7), and all, except the 5-HT_3 receptors, are G protein-coupled metabotrobic receptors whereas the 5-HT_3 receptor is a ligand-gated ion channel (Barnes and Sharp, 1999). Specifically, the 5-HT_1 and 5-HT_5 receptors are $G_{i/o}$ coupled, 5-HT_2 receptors are $G_{q/11}$ coupled, and lastly, 5-HT_4, 5-HT_6, and 5-HT_7 receptors are G_s coupled (Hannon and Hoyer, 2008). As well, 5-HT receptors are located both pre and post-synaptically, can be inhibitory or excitatory, and can be located on both γ-aminobutyric acid (GABA)ergic and glutamatergic neurons leading to a highly intricate and complex system within the brain (Rainnie, 1999; Guo and Rainnie, 2010).

Serotonin receptors are located in the amygdala and the BNST, which are thought to be important regions for fear and anxiety-behaviors. In addition, serotonin receptors have been implicated in playing a role in emotion-related behaviors in the hippocampus and the mPFC. Although all 5-HT receptors have been identified within the amygdala, particular attention has been paid to the 5-HT_{1A} and 5-HT_{2C} receptors (Park and Williams, 2012; Asan et al., 2013). Specifically, the 5-HT_{1A} receptors in the CE have been shown to be involved in the reduction of anxiety-like behaviors while 5-HT_{2C} receptors are associated with an increase in anxiety-like behaviors (Li et al., 2012). The hippocampus plays

an important role in conditioned fear and 5-HT receptors appear to play an integral role (Eriksson et al., 2012). In the hippocampus, 5-HT_{1A} receptor activation appears to inhibit emotion-related behavior associated with fear conditioning (Stiedl et al., 2000). However, 5-HT_7 receptor activation appears to enhance emotion-related behavior, especially when the 5-HT_{1A} receptors are blocked (Eriksson et al., 2012), while a lack of 5-HT_7 receptors impairs fear learning (Roberts et al., 2004). Further, evidence suggests a role for activating 5-$HT_{2A/C}$ receptors in the hippocampus in increasing GABA release (Shen and Andrade, 1998) while hippocampal 5-HT_{2C} activation has been associated with an increase in anxiety-like behaviors (Alves et al., 2004). Additionally, 5-HT_{1A}, 5-HT_{1B}, 5-HT_{2A}, 5-HT_{2C}, and 5-HT_7 receptors have been implicated in playing a role in emotional behaviors in the BNST, with the 5-HT_1 receptor activation linked to reduced anxiety and the others linked to increased anxiety (Levita et al., 2004; Guo et al., 2009; Hammack et al., 2009; Guo and Rainnie, 2010). Lastly, the mPFC has been shown to be an important area involved in emotion-related behaviors and partially controlled by 5-HT (Amat et al., 2005). While 5-HT_{2C} receptors are located in this region, they have yet to be implicated in emotion-related behaviors outside of drug seeking (Pentkowski et al., 2010). However, 5-HT_{1A} receptors in the mPFC appear to play an important role in regulating 5-HT release from the DR such that stressful environments produce an increase in 5-HT release, which in turn appears to enact a negative feedback loop that turns off 5-HT release in the mPFC through glutamatergic and GABAergic neurons (Altieri et al., 2012). While both inhibitory and excitatory receptors are located in these brain regions, it is likely they work in concert depending on the context of the environment. For example, anxiety and fear are restrained unless the situation dictates the appropriateness of these emotions or in terms of psychopathology, these systems are no longer in concert to restrain anxiety and fear in inappropriate situations (Hammack et al., 2009).

DISTRIBUTION OF CRF AND CRF RECEPTORS WITHIN THE DORSAL RAPHE NUCLEUS

As discussed briefly above, one mechanism through which CRF and the UCN's can influence emotional behavior is through actions on brainstem neuromodulatory systems such as serotonergic systems. The DR, along with the MnR, is the major source for 5-HT in the brain (Steinbusch, 1981). Although the DR is a main source for 5-HT, it also contains neurons that express other neurotransmitters and neuropeptides, including CRF. Corticotropin-releasing factor-immunoreactive neurons have been observed in the DR in colchicine-treated rats (Commons et al., 2003). Corticotropin-releasing factor-positive neurons were predominately found in the dorsomedial subregion of the mid-rostrocaudal DRD with smaller numbers of positive cells in the ventrolateral part of the DRVL/VLPAG. Importantly, these CRF-immunoreative cells were mostly dual labeled for TPH indicating that these were also serotonergic neurons. In addition, CRF-positive cells were largely absent from the ventromedial and most caudal portions of the DRD and DRVL/VLPAG while CRF-positive fibers were seen to traverse the lateral edge of the rostral DRV. It was also demonstrated through anterograde tracing that the dorsomedial neurons had dense projections to the CE, a region involved in the control of emotion such as fear and anxiety (Gray, 1993; Davis, 1997; Commons et al., 2003; Phelps and LeDoux, 2005). Moreover, CRF application in the mid-rostrocaudal DRD increases 5-HT in the CE and freezing behaviors (Forster et al., 2006). Because the CE is involved in emotion and has direct CRF connections with specific regions of the DR, it is situated to control serotonergic systems and modulate emotion-related behavioral output.

The dorsomedial neurons of the mid-rostrocaudal DR have dense CRF projections to the BNST while the BNST also has reciprocal connections with the DRD and DRC (Van Bockstaele et al., 1993; Petit et al., 1995; Peyron et al., 1997; Dong et al., 2001; Commons et al., 2003; Dong and Swanson, 2004). The BNST has been shown to be an important region controlling emotional behaviors such as alterations in acoustic startle behavior (Davis et al., 2010). CRF innervation of the DRD/DRC by BNST CRF positive neurons has not been directly shown, however, control of emotional-like behavior by CRF projections from the BNST to the caudal DRD has been suggested as BNST lesions block escape deficiencies produced after inescapable shock while administration of CRF into the DRC mimics the effects of inescapable shock on inhibiting escape behaviors (Hammack et al., 2002, 2004). Moreover, overexpression (OE) of CRF in the BNST induces a decrease in CRF_2 receptor mRNA specifically within the DRD suggesting that the BNST has direct CRF projections to this brain region (Sink et al., 2013). Given the role of the BNST in control of emotional behaviors and its potential connections with subregions of the DR, it is an important structure that most likely contributes to control of emotional behavioral output through CRF-5-HT interactions.

The distribution of CRF receptors within the DR is also topographically organized and both CRF_1 and CRF_2 receptors are colocalized with serotonergic neurons as well as non-serotonergic neurons (Day et al., 2004; Waselus et al., 2009). CRF_1 receptor mRNA density is considerably lower than CRF_2 receptor mRNA in the DR (Van Pett et al., 2000). CRF_1 receptors, using immunohistochemistry and electron microscopy, are present on the plasma membrane of dendrites in the DR as well as within the cytoplasm in roughly equal distribution (Waselus et al., 2009). CRF_1 receptors are located in the dorsal portion of the DRVL/VLPAG and have been shown to be colocalized there with GABA in 36% of neurons triple labeled for c-Fos, GABA, and CRF_1 receptors after forced swim stress (Roche et al., 2003; Day et al., 2004). These data suggest that CRF receptor activation can serve to modulate serotonergic activity both directly and indirectly.

CRF_2 receptors are topographically organized in the DR and are expressed in both serotonergic and non-serotonergic neurons. CRF_2 receptors have been demonstrated using immunohistochemistry and electron microscopy in the DR on both axon terminals and in dendrites with a predominant level within the cytoplasm (Waselus et al., 2009). The CRF_2 receptor is apparent in low numbers in the rostral portion of the DR but increases in greater number in more caudal sections of the DR as shown with *in situ* hybridization histochemistry and immunoreactivity (Day et al., 2004; Lukkes et al., 2011). In addition, the effects of CRF administration in the caudal portions of the DR is only blocked by

CRF_2 but not CRF_1 receptor antagonists (Hammack et al., 2002, 2003a; Staub et al., 2005, 2006). Further, CRF_2 receptors also show a topographically organized colocalization with 5-HT neurons and GABA neurons in the DR (Day et al., 2004). Specifically, CRF_2 receptor mRNA in the rostral and mid-rostrocaudal levels of the DR is almost exclusively colocalized with 5-HT neurons while in more caudal regions, about half of the CRF_2 receptor mRNA is colocalized in GABAergic neurons (Day et al., 2004).

CRF and UCN 1 fibers are topographically organized in the DR but much less is known about UCN 2 and UCN 3 fibers. Corticotropin-releasing factor-containing axons are more dense in the medial ventral portion of the rostral DR and then more dense in the dorsolateral DR in more caudal regions with less density in the medial ventral portion (Valentino et al., 2001). This topographical organization suggests that CRF is able to modulate 5-HT in a complex and dynamic manner to influence emotional behavior given that 5-HT neurons are also topographically organized (Hale and Lowry, 2011; Hale et al., 2012). UCN 1 fibers have also been described in the DR including within DRD, DRV, and DRVL subregions suggesting the possibility that UCN 1 also influences serotonergic systems and emotional behavior through CRF_2 (Vaughan et al., 1995). The physiological involvement of UCN 2 and 3 in the modulation of the DR and 5-HT is little known as the DR shows very sparse or no innervation by either UCN 2 or 3 (Reyes et al., 2001; Li et al., 2002). Nevertheless, selective activation of CRF_2 receptors in the DR does modulate and mediate emotional behaviors (described below), suggesting that further work looking into the role of the UCN's in the DR is necessary to elucidate their physiological role.

MECHANISMS OF CRF/5-HT INTERACTIONS

CRF AND UCNs EFFECTS ON DR NEURONAL FIRING

CRF has been shown to alter DR neuronal firing rates *in vivo* and *in vitro*. Studies looking at the response of DR neurons *in vivo* have shown a bimodal response to CRF within the medial rostral portion of the DR in that low doses given either intracerebroventricularly (i.c.v) or directly into the DR inhibit neuronal firing while higher doses increase firing (Kirby et al., 2000; Price et al., 2002) consistent with other data that show a similar pattern of effects on 5-HT release in the lateral striatum and the lateral septum (Price et al., 1998; Price and Lucki, 2001). The response of 5-HT neurons in the DR to CRF has been shown to be topographically organized *in vitro* with more serotonergic neurons responding in the ventral portion of the DR compared to the dorsomedial aspect of the DR (Lowry et al., 2000). Although the majority of neurons in this region respond with an increase in firing rates, many were non-responsive and several showed a decreased firing rate. Stress has been shown to alter the response of serotonergic neuron firing rates after CRF application in that more neurons respond with increased firing rates and have a greater rate of firing compared to control animals, suggesting that CRF plays an important role after stress to modulate the serotonergic system (Lowry et al., 2000). Further studies have shown that CRF_2 receptors move from being predominately within the cytoplasm and then move to the plasma membrane after stress (Waselus et al., 2009) although the mechanism for increased responsiveness and increased firing rates in serotonergic cells to CRF after stress is not known. These data thus show that the interaction between CRF and 5-HT on neuronal activity is modulated by the region of the DR, the amount of CRF released, and prior experience.

Further substantiating the evidence that the members of the CRF family of peptides can have multiple influences on 5-HT, CRF_2 receptors seem to play a dual role in the DR on neuronal firing while CRF_1 receptor activation seems to be inhibitory (Kirby et al., 2000). Small amounts (0.1 − 10 ng) of UCN 2 injected into the mid-rostrocaudal DR inhibit neuronal firing in 5-HT neurons whereas higher amounts (30 ng) of UCN 2 increase firing in 5-HT neurons and this effect is blocked by selective CRF_2 receptor antagonists (Pernar et al., 2004). Furthermore, this high dose of UCN 2 also inhibited more non-5-HT neurons than the low dose which could indicate inhibition of GABA neurons through CRF_2 receptors, which would disinhibit 5-HT neurons and result in increased serotonergic firing. CRF_1 receptors also appear to be involved in the inhibition of neuronal firing after CRF administration in the DR as this effect can be blocked with a specific CRF_1 receptor antagonist (Kirby et al., 2000).

CRF AND UCNs EFFECTS ON SEROTONERGIC NEURONS AS MEASURED BY c-FOS

The members of the CRF family of neuropeptides induce topographically organized neuronal activation as measured by c-Fos. CRF, when infused i.c.v., produces a topographically organized neuronal activation within the DR in medial prefrontal cortex-projecting neurons with a higher percent of activation in caudal portions of the DR as measured by c-Fos (Meloni et al., 2008). Specifically, i.c.v. infusion of CRF (1 μg), induces c-Fos positive neurons in the entire DR, with higher numbers of positive cells seen in more caudal regions compared to more rostral regions (Meloni et al., 2008). UCN 1 (1–10 μg) given i.c.v. has also been shown to increase c-Fos in the DR although topographical activation has not been described (Bittencourt and Sawchenko, 2000). Additional studies show topographically organized activation of serotonergic and non-serotonergic DR neurons by i.c.v. infusion of 2 μg of UCN 2 (Staub et al., 2005, 2006; Hale et al., 2010). Specifically, c-Fos was seen in the mid-rostrocaudal DRD and the DRC and this activation was blocked by a specific CRF_2 receptor antagonist. Consistent with these data, direct infusion of UCN 2 (100 ng) into the DR also produces increased c-Fos within the DR but the effect is more widespread throughout the DR and again this is blocked using a specific CRF_2 receptor antagonist (Amat et al., 2004). Moreover, UCN 2 activation of DR neurons includes activation of ventricle/periventricular-projecting serotonergic neurons as well as non-ventricle/periventricular-projecting serotonergic neurons, suggesting that CRF_2 ligands could play an important physiological role in behavioral consequences of CRF_2 receptor activation although further examination is needed (Amat et al., 2004; Hale et al., 2010). Taken together, these data show that both CRF_1 and CRF_2 receptor agonists activate DR neurons in a topographically organized fashion such that the mid-rostrocaudal and caudal portions of the DR appear to be preferentially activated, suggesting that these regions are important in CRF-5HT interactions.

CRF RECEPTOR ACTIVATION ALTERS SEROTONERGIC NEUROTRANSMISSION AS MEASURED BY MICRODIALYSIS

CRF receptor activation following i.c.v. administration of CRF or CRF-related neuropeptides induces changes in extracellular 5-HT concentrations in specific brain regions involved in control of emotional behavior, including the hippocampus, as measured by microdialysis (Linthorst et al., 2002; Kagamiishi et al., 2003; De Groote et al., 2005). However, chronic i.c.v. CRF infusion (1 μg/1 μL/h for 7 days) produces no basal difference in 5-HT levels in the hippocampus but does blunt the elevation in 5-HT after LPS injection, suggesting chronic elevation of CRF blunts stress-induced release of 5-HT in projection regions of the DR (Linthorst et al., 1997). While CRF receptor activation following i.c.v. administration of CRF or CRF-related neuropeptides can increase 5-HT release, it can also decrease release. I.c.v. CRF produces a decrease in 5-HT release in both the lateral septum and lateral striatum at a low dose (0.3 μg) while this manipulation either does not change 5-HT concentrations or increases concentrations at a higher dose (3.0 μg) (Price et al., 1998, 2002; Price and Lucki, 2001). Both 5-HT_{1A} and $_{2A}$ receptor activation in the lateral septum has been associated with increased anxiety-like behavior, which would correspond with increased release of 5-HT in this region (Cheeta et al., 2000; de Paula et al., 2012). The decrease in 5-HT release in the lateral septum may allow for more proactive behaviors such as exploration to occur when placed in a mildly stressful situation (low dose of CRF) while a very stressful (high CRF dose) situation could induce more reactive behaviors like freezing. Because CRF receptors are located in other regions besides the DR, these effects of i.c.v CRF receptor agonists likely result from activating circuits that are connected with the DR as well as directly activating receptors within the DR.

Intra-DR CRF receptor activation induces changes in 5-HT concentrations in specific emotion-related brain regions, similar to effects seen with i.c.v. administration of CRF receptor agonists. CRF (0.5 μg) injected into the medial portion of the DR, including both the dorsal and ventral aspects, increases 5-HT release in the prefrontal cortex after a 60 min delay (Forster et al., 2006). Activation of the DR by 0.5 μg CRF injection also produces an immediate increase in 5-HT in the CE (Forster et al., 2006; Lukkes et al., 2008; Scholl et al., 2010). Further, CRF_2 receptor activation in the caudal DR produces an increase in 5-HT in the BLA and increases c-Fos in serotonergic neurons within the rostral, mid-rostrocaudal, and caudal DR (Amat et al., 2004). Serotonin concentrations in the Acb are also modified by CRF receptor activation in the DR. Low doses of CRF (0.1 ug) injected into the DR, including both the DRD and DRV aspects of the mid-rostrocaudal DR, reduces 5-HT concentrations whereas higher doses (0.5 μg) increases 5-HT in the Acb (Lukkes et al., 2008). Importantly, a CRF_1 receptor antagonist blocked the effect at the low dose while a CRF_2 receptor antagonist blocked the effect of the high dose, showing that CRF is activating CRF_1, perhaps on GABA interneurons, to inhibit 5-HT release while at higher concentrations is also activating CRF_2 receptors, resulting in increased release of 5-HT in the Acb. These data show that direct activation of CRF receptors in the DR modulate 5-HT release in emotion-related brain regions.

The changes in 5-HT release in the BLA, CE, and Acb are associated with varying behavioral outputs related to emotion. Activation of 5-HT_{2C} receptors in the BLA increases fear-like behaviors (Campbell and Merchant, 2003; Greenwood et al., 2012) which corresponds with the increased release of 5-HT seen in this brain region after CRF_2 activation in the DR (Amat et al., 2004). Additionally, 5-HT receptor activation in the CE has also been implicated in increased anxiety and fear-like behaviors as increased 5-HT in the CE leads to increased freezing behavior (Forster et al., 2006), presumably through activation of the excitatory 5-$HT_{2A/C}$ receptors located in the CE (Asan et al., 2013). Further, foot shock has been shown to also increase 5-HT release in the Acb in association with freezing behavior, indicating a role for fear-like behavioral responses in relation to increased 5-HT release in the Acb (Fulford and Marsden, 1997, 2007). It is not clear which 5-HT receptors in the Acb would be playing a role in fear-like behaviors. However, both 5-HT_{2A} and5-HT_{2C} receptors in this region have been shown to be involved in drug reward behavior, which can be modulated by stress (Erb and Stewart, 1999; Zayara et al., 2011). It is likely that increasing and decreasing 5-HT levels in various emotion-related brain regions involves a complex interplay of 5-HT receptors given that their binding can both inhibit and excite neuronal activation and 5-HT receptors have been shown on GABA neurons and glutamate neurons (Rainnie, 1999; Guo and Rainnie, 2010; Asan et al., 2013).

CRF, UCNs, AND 5-HT INTERACTIONS CONTROLLING EMOTIONAL BEHAVIOR

Numerous studies have demonstrated the involvement of members of the CRF family of peptides and their respective receptors and 5-HT in emotional behaviors in rodents. These include studies involving administration of specific CRF receptor agonists and antagonists with both i.c.v. and intra-DR applications as discussed above. Additionally, development of numerous mutant mice with genetic knock out (KO) or OE of one or more of these peptides or receptors have helped further our knowledge about the important role CRF plays in emotional behaviors (described below). Here, we will focus on behaviors that have been shown to involve the interactions between CRF, the UCNs, and 5-HT.

A number of studies have implicated CRF/5-HT interactions in control of emotional behavior. Administration of CRF through i.c.v increases the acoustic startle response and the CRF-induced startle is correlated with activation of c-Fos within the DR (Meloni et al., 2008). Partially, the c-Fos positive neurons were also positive for 5-HT and projected to the mPFC, a region that is important in the effects of controllability on behavioral consequences of stress (Rozeske et al., 2011; Patel et al., 2012). Behavioral consequences of uncontrollable stress are mediated by CRF receptors in the DR (Hammack et al., 2002, 2003a,b). In particular, it has been shown that CRF_2 receptors in the DRC are responsible for the behavioral consequences, observed 24 h later, of inescapable shock and that activation of CRF_2 receptors in the DRC can mimic the effects of uncontrollable shock on behavior (Hammack et al., 2002). Further, activation of CRF_1 receptors by low doses of CRF acts to inhibit the DR and can block the behavioral consequences of uncontrollable stress (Hammack et al., 2003a). Stressors such as foot shock and

restraint induce neuronal activation, as measured by c-Fos, in anxiety-related regions including the DR, amygdala, and BNST while at the same time also increasing mRNA for CRF in the BNST and CE, suggesting that anxiety- or fear-inducing stimuli alter CRF function while activating neurons in the DR (Funk et al., 2006). Importantly, this implicates a complex system that is responsive to the effects of an acute stressor, which can lead to alterations in emotional behaviors such as increased freezing behaviors (Hammack et al., 2004). These data thus suggest a crucial role of CRF receptors within the DR in control of stress-related behaviors and suggest that CRF/5-HT interactions are important in the behavioral consequences of uncontrollable stress.

Chronic activation of the CRF system is associated with changes in emotional behavior. Specifically, OE of CRF within the BNST does not produce basal changes in anxiety yet when induced prior to fear conditioning, it interferes with learning, while induction after fear conditioning but before fear testing produces an exaggerated fear response (Sink et al., 2013). Importantly, OE of CRF in the BNST results in changes in binding density for CRF_1 in the BNST and CRF_2 in the dorsal and caudal portions of the DR. This suggests that alterations of CRF expression in specific areas connected to the DR can lead to both physiological and emotional changes, depending on time points, and can disrupt fear learning or enhance fear expression, possibly through alterations in 5-HT function through decreased CRF_2 receptors in the dorsal and caudal DR as these receptors have been shown to be involved in fear related behaviors (Hammack et al., 2003a). Repeated administration of UCN 1 into the BLA has also been shown to induce serotonergic changes in the DR, including an increase in *tph2* mRNA in specifically the DRVL that was correlated with increases in anxiety-like behavior (Donner et al., 2012a). Further, maternal separation and later social defeat also serve to increase *tph2* mRNA in the DRVL/VLPAG and produce a more passive-like coping behavior, and this increase in *tph2* mRNA could serve as a common factor related to altered emotionality brought on by multiple environmental stressors (Gardner et al., 2005, 2009). These studies suggest that the interaction between CRF receptor activation and 5-HT can modify emotional behavior while environmental experience can serve to alter 5-HT systems in a manner that is dependent on CRF receptor activation.

Interactions between CRF and serotonergic systems have also been implicated in control of active vs. passive behavioral coping responses during forced swim stress. The swim stress-induced reduction in 5-HT concentration in the lateral septum has been shown to be dependent on CRF receptor activation as an i.c.v. $CRF_{1,2}$ receptor antagonist blocks this effect on 5-HT (Price et al., 2002). This effect may be specific to swim stress, however, as 5-HT concentrations increase in the lateral septum in mice in the presence of predator odor (Beekman et al., 2005). Further, the increase in 5-HT seen in the hippocampus during forced swim can also be blocked using a non-specific CRF antagonist given i.c.v. (Linthorst et al., 2002; Kagamiishi et al., 2003; De Groote et al., 2005). These studies suggest a role for CRF receptor activation in control of serotonergic systems by diverse stress-related stimuli.

CRF, UCN 1, 2, AND 3, AND CRF RECEPTOR TRANSGENIC ANIMALS, SEROTONERGIC SYSTEMS, AND EMOTIONAL BEHAVIOR

One line of research taken to investigate the roles of CRF, UCNs, and CRF receptors in control of serotonergic systems and emotional behavior is to use transgenic animals. Genes can be removed or added in to influence development from fertilization or can be conditionally changed after birth to avoid developmental alterations associated with transgenic manipulations that may lead to unintended consequences (Smith et al., 1998; Timpl et al., 1998). It is important to keep in mind as well-that many of these studies will produce essentially the overall sum effect of adding or removing peptides or receptors from the entire brain and peripheral systems on behavior and that further work with more selective changes will be informative as to their roles within specific brain regions.

Both CRF_1 and CRF_2 receptor KO mice have been developed and used to investigate the role of the receptors in control of emotional behaviors. The CRF_1 receptor KO mice display a decrease in anxiety-like behaviors while CRF_2 receptor KO mice tend to display an increase in anxiety and depression-like behaviors, although not in all cases or in all measures (Smith et al., 1998; Timpl et al., 1998; Bale et al., 2000; Coste et al., 2000, 2006). In $CRF_{1,2}$ receptor double KO mice, only males show an increase in anxiety-like behavior while the females tend to show normal or decreased anxiety, which suggests that there is an interaction between sex and genotype on anxiety-like behavioral output in these animals (Bale et al., 2002). The rearing behavior of heterozygous and homozygous CRF_2 receptor KO dams seems supports an increase in anxiety-like behavior in their male offspring regardless of the males' genotype, implying that both environmental and genetic factors play a role in anxiety-like behaviors and that sex is an important factor (Bale et al., 2002). Further evidence suggests that CRF_1 receptors in the limbic system specifically are important in controlling anxiety-like behaviors (Muller et al., 2003). Conditional KO of CRF_1 receptors in the limbic system has no effect on the HPA axis, but results in reduced anxiety-like behavior and increased active coping in depression models (Muller et al., 2003). CRF_1 receptors in the limbic system therefore seem to play a critical role in initiating an anxiety response.

Mouse models have also been developed to investigate the effects of OE or deletion of CRF. Chronic OE of CRF results in a downregulation of UCN 1 in the Edinger-Westphal nucleus (Kozicz et al., 2004) while CRF KO results in an upregulation of UCN 1 in the Edinger-Westphal nucleus, suggesting that CRF may control the level of UCN 1 expression or that changes in CRF can be compensated for by UCN 1 (Weninger et al., 2000). Moreover, CRF OE mice also show a change in CRF_1 and CRF_2 receptor mRNA expression throughout the brain (Korosi et al., 2006). In particular, OE of CRF induces a down-regulation of CRF_1 receptors while at the same time it induces an upregulation of CRF_2 receptors in the brain while the overall distribution of receptors remains the same showing that receptor mRNA expression is dependent on the level of CRF expression in these animals. CRF OE mice also show an increase in anxiety-like behavior, perhaps mediated in part by overactivation of CRF_1 receptors or by the increase in CRF_2 receptors in the DR (Korosi et al., 2006) and

changes in responsiveness to alterations in 5-HT release in regions connected with the DR (Stenzel-Poore et al., 1994; Heinrichs et al., 1997; van Gaalen et al., 2002). These mouse models demonstrate that CRF KO or OE alters the expression of other members of the CRF family of neuropeptides and their receptors, implying that an overall increase or decrease of CRF or the UCNs can contribute to changes in receptor expression and alter behavioral output.

Chronic OE of UCN 3 is associated with changes in the serotonergic system and altered emotion-like behaviors. Chronic OE of UCN 3 produces a change in post-stress 5-HT and 5-HIAA concentrations in the caudal and dorsal DR and lateral septum as well as a basal change in 5-HT_{1A} receptor mRNA in both the DR and amygdala (Neufeld-Cohen et al., 2012). UCN 3 OE also alters basal anxiety-related behavior compared to wild type animals (Neufeld-Cohen et al., 2012), which may implicate desensitized CRF_2 receptors resulting in the increased anxiety, similar to the CRF_2 receptor KO mice. In particular, UCN 3 OE mice have increased anxiety-like behavior in both the elevated plus-maze and the light-dark box and an increase in immobility in the tail suspension test suggesting that chronic CRF_2 receptor activation results in a basal increase in emotionality. Consistent with these data, conditional OE of UCN 3 in the rostral portion of the perifornical area, where UCN 3 is normally expressed, also increases anxiety-like behavior (Kuperman et al., 2010). Interestingly, post-stress anxiety-like behavior in UCN 3 OE mice is either not increased or is in fact reduced suggesting that chronic activation of CRF_2 receptors creates a chronic anxiety-like state but resistance to further stress-induced anxiety (Neufeld-Cohen et al., 2012).

A number of studies have also investigated the role of UCN 1, 2, and 3 in emotional behaviors using transgenic mice. UCN 1 KO mice have been shown to have normal anxiety-like behavior although a decrease in the acoustic startle response is seen in males (Wang et al., 2002) and a lower startle response has been associated with decreased anxiety in other measures of anxiety (Salam et al., 2009). These animals are described as having normal hearing although another line of UCN 1 KO mice appeared to have disruption of hearing and an increase in anxiety-like behavior (Vetter et al., 2002) so a reduced startle response maybe due do a disruption in the startle neuronal pathway. Both UCN 2 and 3 KO animals do not show any disruption in anxiety-like behaviors, although reduced aggressiveness is seen in male UCN 2 KO's and reduced depression-like behaviors were noted in female UCN 2 KO mice (Chen et al., 2006; Deussing et al., 2010; Breu et al., 2012). However, given that both CRF and UCN's can activate CRF_2 receptors, it is not surprising that there is not a noticeable change in anxiety or consistent decrease in depression-like behaviors in the UCN 2 or 3 KO mice. A double KO of UCN 1 and 2, however, results in a anxiolytic-like phenotype (Neufeld-Cohen et al., 2010a) while UCN triple KO mice show normal basal anxiety levels but increased anxiety-like behavior 24 h after a stressor (Neufeld-Cohen et al., 2010b). Notably, the UCN triple KO mice show some basal and 24 h post-stress differences in serotonergic activities in the amygdala, subiculum and medial and later septum, which is consistent with the hypothesis that the increase in anxiety-like behavior is mediated in part by changes in serotonin and implicates a role for CRF_2 receptor activation in controlling 5-HT and its potential role during the stress recovery period.

NON-HUMAN PRIMATE DATA ON CRF/5-HT INTERACTIONS AND EMOTIONAL BEHAVIOR

Non-human primate data gives further insight into the interaction between CRF, serotonergic systems, and emotional behavior. Recent data suggest that, in a particular subset of cynomolgus macaques deemed to be more stress-sensitive than their cohorts because of interrupted menstrual cycles, chronic treatment with a serotonin selective reuptake inhibitor (SSRI) produces significant changes in CRF receptors after 15-weeks of administration but not in less-stress sensitive animals (Senashova et al., 2012). Specifically, after chronic treatment with an SSRI, the stress sensitive monkeys had an increased number of CRF_2 receptor mRNA positive cells as found through digoxigenin-*in situ hybridization* staining, compared to less stress-sensitive monkeys. Similar studies have shown that the stress-sensitive monkeys have an increased cortisol response to serotonin release induced by fenfluramine while showing a blunted prolactin release (Bethea et al., 2005a) and basal increased cortisol release during the day (Herod et al., 2011a,b), suggesting further disruption in normal serotonin systems. Interestingly, these monkeys, along with the altered CRF receptors in response to an SSRI, also have lowered CRF fiber density in the DR with an increased number of UCN 1 cell bodies after SSRI treatment (Weissheimer et al., 2010). This suggests that altered levels of CRF and UCN 1 contribute to the stress sensitivity witnessed in the animals and that altered 5-HT systems contribute to these differences, perhaps through increased CRF or decreased UCN signaling to the DR. In fact, stress-sensitive cynomolgus macaques, compared to less stress sensitive cynomolgus macaques, have decreased serotonin transporter and tryptophan hydroxylase 2 (TPH) mRNA and, in one study, had lower 5-HT_{1A} receptor mRNA in the DR, all suggesting that alterations in these 5-HT systems contribute along with the altered CRF/UCN system to the stress-sensitive phenotype of these monkeys (Bethea et al., 2005b; Lima et al., 2009).

CLINICAL DATA ON CRF/5-HT INTERACTIONS AND EMOTIONAL BEHAVIOR

There is a wealth of knowledge gained from studies showing that SSRI's can be a useful way to treat anxiety and depression (Goldstein and Goodnick, 1998) although a meta-analysis of clinical data also show that this may be dependent on the severity of the disorder and less useful in mild to moderate depression (Fournier et al., 2010) although see (Gibbons et al., 2012). There has also been interest in modulating CRF in patients with affective disorders to help alleviate their symptoms (Künzel et al., 2003). Corticotropin-releasing factor-positive terminals are apposed to serotonergic neurons in the human brain suggesting that they play a role in modulating 5-HT as seen in animal models (Ruggiero et al., 1999). Importantly, elevated CRF concentrations have been described in the cerebral spinal fluid in suicide victims, suggesting a dysregulation of the central CRF system (Arató et al., 1989). Although the data have not been completely consistent in findings of elevated CRF concentrations, with reports of no difference or even decreased CRF in CSF, it does appear that there

can be abnormal levels of CRF in CSF in people with depression and the discrepancies may represent subgroups of depression or perhaps how long they have been in the episode of depression (Mitchell, 1998). One study of interest concerning the interplay between CRF and 5-HT showed elevated levels of CRF in CSF which subsequently normalized after treatment with the SSRI fluoxetine, suggesting that the abnormality in 5-HT function in depression could be related to the elevation of CRF (De Bellis et al., 1993).

Depression has also been associated with alterations in 5-HT function in specific regions of the DR in humans. Data collected from postmortem human brain tissue show an increase in TPH immunoreactivity (Underwood et al., 1999; Boldrini et al., 2005) and mRNA (Bach-Mizrachi et al., 2006, 2008) in the DR of depressed suicides, with a more pronounced increase in the DRC. Data also show an increase of TPH immunoreactivity specifically in the DRD of depressed alcoholic suicides (Bonkale et al., 2006) with a trend for an increase in *tph2* mRNA expression in the same region in non-alcoholic depressed suicides (Bach-Mizrachi et al., 2006). These data are in contrast to those described in the stress-sensitive monkeys in which it had been found that there is decreased *tph2* mRNA compared to less stress sensitive monkeys, which may indicate that there are multiple pathways leading to emotion-related disorders (Bethea et al., 2011). In male, but not female, suicide victims, *UCN* mRNA is significantly elevated in the Edinger-Westphal nucleus (Kozicz et al., 2008) further implicating a disruption in central CRF-related peptides related to depression. Other data show that CRF is upregulated as well in the DR and MnR in depressed suicides providing direct evidence for changes in CRF in brain regions that are the major sources for 5-HT in humans, which could be one reason for the altered TPH seen in other studies, perhaps through increased activation of serotonergic neurons by CRF (Austin et al., 2003).

CONCLUSIONS

Corticotropin releasing factor and the UCNs interact with serotonergic systems in a topographically organized manner and, depending on the receptor and the connectivity with limbic brain regions and concentrations of peptide, can lead to alterations in gene expression, changes in serotonergic output, and increased or decreased emotional behaviors. Focus on the relationship between the members of the CRF family of peptides and serotonergic systems should take into consideration the complex topographical organization of serotonergic systems. Increased understanding of these relationships in specific brain regions could lead to novel therapeutic strategies to more directly modulate emotional outcomes with fewer side effects relative to current treatments for anxiety and affective disorders.

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Central CRF neurons are not created equal: phenotypic differences in CRF-containing neurons of the rat paraventricular hypothalamus and the bed nucleus of the stria terminalis

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Corticotrophin-releasing factor (CRF) plays a key role in initiating many of the endocrine, autonomic, and behavioral responses to stress. CRF-containing neurons of the paraventricular nucleus of the hypothalamus (PVN) are classically involved in regulating endocrine function through activation of the stress axis. However, CRF is also thought to play a critical role in mediating anxiety-like responses to environmental stressors, and dysfunction of the CRF system in extra-hypothalamic brain regions, like the bed nucleus of stria terminalis (BNST), has been linked to the etiology of many psychiatric disorders including anxiety and depression. Thus, although CRF neurons of the PVN and BNST share a common neuropeptide phenotype, they may represent two functionally diverse neuronal populations. Here, we employed dual-immunofluorescence, single-cell RT-PCR, and electrophysiological techniques to further examine this question and report that CRF neurons of the PVN and BNST are fundamentally different such that PVN CRF neurons are glutamatergic, whereas BNST CRF neurons are GABAergic. Moreover, these two neuronal populations can be further distinguished based on their electrophysiological properties, their co-expression of peptide neurotransmitters such as oxytocin and arginine-vasopressin, and their cognate receptors. Our results suggest that CRF neurons in the PVN and the BNST would not only differ in their response to local neurotransmitter release, but also in their action on downstream target structures.

Keywords: CRF, BNST, PVN, Oxytocin, VGLUT2, GAD67, vasopressin

INTRODUCTION

Corticotrophin releasing factor (CRF) is a neuropeptide that is essential for coordinating the adaptive response of an organism to stressful situations (Vale et al., 1981). CRF is produced predominantly by neurons in the parvocellular division of the paraventricular nucleus of the hypothalamus (PVN) (Swanson et al., 1983; Herman et al., 2003). However, CRF is also produced by neurons in extra-hypothalamic limbic structures like the extended amygdala, which includes the bed nucleus of the stria terminalis (BNST) and the central nucleus of the amygdala (CeA), as well as in hindbrain structures like the locus coeruleus and dorsal Raphé nuclei (Cummings et al., 1983; Curtis and Valentino, 1994; Snyder et al., 2012). CRF that is synthesized and released by PVN neurons plays a major role in regulating activity of the hypothalamic-pituitary-adrenal (HPA) axis and triggers the classic endocrine stress response (Vale et al., 1981; Rivier and Vale, 1983), whereas outside the HPA axis CRF acts not as a hormone, but as a modulator of synaptic transmission at pre- and postsynaptic sites within specific central neuronal circuits (Lowry and Moore, 2006; Orozco-Cabal et al., 2006). In the BNST, the highest concentration of CRF neurons are found in the oval and fusiform nuclei (Cummings et al., 1983; Morin et al., 1999), which is also rich in CRF fibers and terminals, many of which originate from the CeA (Cummings et al., 1983; Sakanaka et al., 1986; Morin et al., 1999). Growing evidence suggests that CRF neurons of the anterolateral cell group of the BNST, $BNST_{ALG}$, play a major role in the affective response to stressors (Lee and Davis, 1997; Liang et al., 2001; Nijsen et al., 2001; Ciccocioppo et al., 2003; Sahuque et al., 2006; Dabrowska et al., 2013), and that dysfunction of this CRF system contributes to the etiology of several psychiatric disorders, including depression (Crestani et al., 2010), anxiety-disorders (Walker et al., 2009), as well as addiction (Koob, 2010).

However, despite sharing a common neuropeptide phenotype, central CRF neurons should not be regarded as a homogeneous cell population. For example, the Raphé nuclei contain a population of CRF neurons that co-localize serotonin (5-HT) (Valentino et al., 2010), whereas locus coeruleus CRF neurons co-localize noradrenalin (NA) (Valentino et al., 1983, 2010). Hence, target structures that receive input from CRF neurons in these two midbrain nuclei would be predicted to have a markedly different response to afferent input. Consistent with this premise, evidence from *in situ* hybridization and immunohistochemical studies suggest that PVN CRF neurons could have a glutamatergic

phenotype (Ziegler et al., 2002; Lin et al., 2003; Hrabovszky et al., 2005; Hrabovszky and Liposits, 2008), whereas BNST CRF neurons could be GABAergic (Sun and Cassell, 1993; Bowers et al., 1998; Day et al., 1999; Pompolo et al., 2002). This raises the intriguing possibility that either CRF neurons in these two stress-reactive structures have a phenotype that is independent of the surrounding structure or that they co-express two functionally opposing neurotransmitters. Support for the latter premise comes from the observation that stress responsive neurons in the BNST, but not the PVN, are primarily GABAergic (Herman et al., 1996; Day et al., 1999; Bali et al., 2005; Janitzky et al., 2009). However, no study to date has systematically examined the neurochemical phenotype of CRF neurons in these two regions at the single cell level.

As noted above, the PVN and BNST also contain subpopulations of neurons that express a wide variety of neuropeptides including, but not limited to, oxytocin (OT), arginine-vasopressin (AVP), neurotensin (NT), and enkephalin (ENK) (Sawchenko et al., 1984a,b; Lightman, 1993), some of which are co-expressed in the same cell populations. For example, although AVP is primarily produced by magnocellular PVN neurons, under stress conditions parvocellular CRF neurons in the PVN also co-synthesize AVP (Sawchenko et al., 1984a; Arima et al., 2001), suggesting that the neurochemical phenotype of these neurons can be dynamically regulated in response to environmental demands. Interestingly, CRF and OT are also reported to be co-expressed in a subset of PVN neurons (Sawchenko et al., 1984b), and our recent single-cell RT-PCR study confirmed that some magnocellular OT neurons in the PVN also co-express mRNA transcripts for CRF (Dabrowska et al., 2011). Together these data suggest that stress responsive CRF neurons may have a phenotype that is both region-specific and dynamically regulated in response to environmental stimuli.

Finally, we recently reported that a reciprocal relationship exists between CRF neurons of the $BNST_{ALG}$ and OT neurons of the PVN (Dabrowska et al., 2011). Hence, magnocellular OT neurons in the PVN express high levels of type 2 CRF receptors (CRFR2), whereas $BNST_{ALG}$ CRF neurons express high levels of OT receptor mRNA (OTR), suggesting that OT neurons of the PVN might directly regulate the excitability of the CRF neurons in the $BNST_{ALG}$ and *vice versa*. However, OT is also released locally in the PVN (Neumann, 2007), raising the possibility that CRF neurons in the PVN may also be regulated by local OT release. Indeed, we have shown that magnocellular CRF neurons in the PVN express OTR mRNA (Dabrowska et al., 2011), however, nothing is known about OTR expression in the parvocellular CRF cell population. Furthermore, although multiple neuronal subtypes have been characterized in the rat PVN (Tasker and Dudek, 1991) and BNST (Hammack et al., 2007; Hazra et al., 2011) based on their electrophysiological properties, little is known about the properties of CRF expressing neurons due to the lack of a cell-type specific marker for these neurons. To address these knowledge gaps, we have used a combination of dual-immunofluorescence and whole-cell patch clamp recording in conjunction with single-cell RT-PCR to probe rat CRF neurons in both regions for their expression of GABAergic (glutamic acid decarboxylase, GAD67) and glutamatergic (vesicular glutamate transporter, VGLUT) markers, as well as for other neuropeptides (OT, AVP) and their cognate receptors (OTR, V1AR, V1BR).

METHODS

ANIMAL SUBJECTS

All experiments were performed on brain tissue obtained from adult (60 days old), male, Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA). Animals were housed 4 animals per cage and were maintained on a 12:12-h light–dark cycle with *ad libitum* access to food and water. For experiments that required stereotaxic surgery and colchicine injections, rats were anaesthetized with an IP injection of a mixture of dexdormitor (0.16 mg/kg; Pfizer Animal Health, New York, NY, USA) and ketamine hydrochloride (48 mg/kg; Butler-Schein Animal Health, Dublin, OH, USA). All procedures used were approved by the Institutional Animal Care and Use Committees (IACUC) of Emory University, and were in compliance with National Institutes of Health (NIH) guidelines for the care and use of laboratory animals.

TISSUE PROCESSING FOR IMMUNOFLUORESCENCE

To facilitate immunohistochemical analysis, 6 rats received a 2 μl intracerebroventricular (icv) colchicine (Sigma Aldrich, St. Louis, MO, USA, 60 μg/μl) infusion 48 h prior to transcardial perfusion to maximize CRF peptide content in neuronal cell bodies, as described previously (Dabrowska et al., 2011). Subsequently, all rats were transcardially perfused with 4% paraformaldehyde and subsequent fixation procedure was performed as described elsewhere (Dabrowska and Rainnie, 2010).

DUAL-IMMUNOFLUORESCENCE EXPERIMENTS

The specificity of the antibodies used in this study has been confirmed and described previously (Martin et al., 2010; Dabrowska et al., 2011). Dual-immunofluorescence experiments were performed using the following primary antibodies: rabbit polyclonal anti-CRF antibody (1:250, ab11133, Abcam, Cambridge, MA), mouse monoclonal anti-GAD67 (1:500, MAB5406, Chemicon-Millipore, Billerica, MA), mouse monoclonal anti-VGLUT2 antibody (1:1000, clone N29/29, 75-067, UC Davis/NIH NeuroMab Facility, Davis, CA, USA, Antibodies Incorporated) and using protocols that have been described previously (Dabrowska et al., 2011).

To examine the co-localization of CRF with GAD67 and VGLUT2, we performed dual-immunofluorescence experiments on free-floating serial sections of the rat $BNST_{ALG}$ and PVN as described before (Dabrowska et al., 2011). Here, representative sections were taken from Bregma +0.22 to −0.4 mm for the $BNST_{ALG}$, and from −1.6 to −2.12 mm for PVN. The sections were rinsed 3× (10 min each) in phosphate buffer saline (PBS), permeabilized with 0.5% Triton-X 100 in PBS, and incubated for 48 h at 4°C with the following primary antibodies pairs diluted in 0.5% Triton-X/PBS solution: CRF/GAD67 and CRF/VGLUT2. Sections were rinsed 3× (10 min each) in PBS and then incubated at room temperature for 2 h with Alexa-Fluor secondary antibodies specific for the primary antibody host: namely Alexa-Fluor 488 goat anti-mouse IgG and Alexa-Fluor 568 goat anti-rabbit

IgG (1:500, Invitrogen, Carlsbad, CA, USA). Following incubation with secondary antibodies, sections were rinsed 3× in PBS and 1× in 0.05 M phosphate buffer (PB), mounted on gelatin-coated glass slides and coverslipped using mounting medium consisting of 12% mowiol (Sigma Aldrich, St. Louis, MO, USA) and 30% glycerol. Confocal spinning disk laser microscopy was used to analyze dual-immunofluorescence patterns and to obtain high-resolution photomicrographs using an Orca R2 cooled CCD camera (Hamamatsu, Bridgewater, NJ, USA) mounted on a Leica DM5500B microscope (Leica Mircosystems, Bannockburn, IL) equipped with a CSU10B Spinning Disk (Yokagawa Electronic Corporation, Tokyo, Japan). Analysis and semi-quantitative analysis of dual-labeled neurons in the PVN and the $BNST_{ALG}$ was performed with Simple PCI 6.6 software (Hamamatsu Corporation, Sewickley, PA, USA). For each pair of primary antibodies, immunoreactive neurons from representative sections containing either the $BNST_{ALG}$, or the PVN, (from three animals, thirty sections total) were counted and analyzed for dual-labeling. Co-localization was determined by capturing confocal Z-stack images and counting the number of neurons co-localizing the two markers (VGLUT2/CRF or GAD67/CRF) in all focal plans. Cell counts were then expressed as a percentage of the total number of neurons expressing CRF.

SINGLE-CELL RT-PCR

Single cell RT-PCR (scRT-PCR) protocols and the procedures used to determine mRNA transcript expression in single cells of the PVN and $BNST_{ALG}$ have been described in detail elsewhere (Dabrowska et al., 2011; Hazra et al., 2011). Briefly, following recording of the electrophysiological properties of neurons in each area, the cytoplasm was aspirated from recorded neurons into the patch recording pipette and then expelled into a microcentrifuge tube containing a reverse transcription (RT) cocktail (Applied Biosystems, Foster City, California). The RT product was then amplified in triplicate and screened for 18S rRNA expression. Only those cells positive for 18S rRNA were subjected to amplification with primers. Oligonucleotide primers used in the current study have been described elsewhere (Dabrowska et al., 2011; Hazra et al., 2011), with the exception of V1AR and V1BR, as well as VGLUT1 and VGLUT3. The primers used for V1 receptors were as follows: 5′-CGACACAGCAAGGGTGACAAGG-3′ and 5′-AGGAAGCCA G CAACGCCG-3′ (accession number NM_053019.2, 265 bp) for V1AR, and 5′-AGCATCAGTACCATCTCCAGG-3′ and 5′-TGGTCTCCATAGTGGCTTCC-3′ (NM_017205.2, 463 bp) for V1BR. The primers used for VGLUT were as follows: 5′-ACCC ATCGGAGGCCAGATCG-3′ and 5′-GCCACTCCTCCCGC GT CTTGTGC-3′ (NM_053859, 416 bp) for VGLUT1 and 5′-GGAA TCATTGACC AAGATGAGTTAGCTGA-3′and 5′-TTT AGGT GTTTCTGAGAAGT CTCCTTCGG-3′ (AY117026, 200 bp) for VGLUT3.

CONTROLS FOR THE scRT-PCR

PCR conditions were optimized using total RNA isolated from rat $BNST_{ALG}$ so that a PCR product could be detected from (250 pg − 1 ng) of total RNA without contamination caused by non-specific amplification. For each PCR amplification, sterile water was used instead of cDNA as a control for contaminating artifacts. A second negative control was performed in which the RT enzyme was excluded from the initial reaction mixture for each amplification reaction. All of the control tests gave negative results throughout the study. In addition, all of the primers used in this study were intron-spanning to exclude amplification of genomic DNA. Moreover, the cell nucleus was never harvested while isolating mRNA from single cells further reducing the possibility of contamination by genomic DNA.

IN VITRO WHOLE CELL PATCH-CLAMP RECORDING OF VISUALLY IDENTIFIED NEURONS IN THE PVN AND THE $BNST_{ALG}$

In vitro patch-clamp recordings were performed as previously described (Guo and Rainnie, 2010; Hazra et al., 2011). In brief, rat were anesthetized using isoflurane and the brains rapidly removed and placed into ice-cold kynurenic-based artificial cerebrospinal fluid ($ACSF_{KA}$), which contained (in mM): NaCl (130), KCl (3.5), KH_2PO_4 (1.1), $MgCl_2$ (6.0), $CaCl_2$ (1.0), $NaHCO_3$ (30), glucose (10), and kynurenic acid (2). Subsequently, 350 μm slices containing the $BNST_{ALG}$ or PVN were obtained using a Leica VTS-1000 vibrating microtome (Leica Microsystems, Bannockburn, IL). $ACSF_{KA}$ was used to minimize any potential excitotoxicity associated with glutamate release during tissue slicing. Immediately after slicing, slices were hemisected, trimmed, and placed in a holding chamber containing oxygenated $ACSF_{KA}$ at room temperature for 1 h. Slices were then transferred to oxygenated regular ACSF containing (in mM): NaCl (130), NaHCO3 (30), KCl (3.50), KH2PO4 (1.10), MgCl2 (1.30), CaCl2 (2.50), and glucose (10). Slices were kept in the regular ACSF at room temperature for at least 30 min before recording. For recording, individual slices were transferred to a Warner Series 20 recording chamber (0.5 ml volume) mounted on the fixed stage of a Leica DM-LFS microscope (Leica Microsystems). The slices were maintained fully submerged and continuously perfused with ACSF heated to 32°C, and gassed with a 95–5% oxygen/carbon dioxide mixture. Neurons were visually identified using differential interference contrast (DIC) optics, infrared (IR) illumination, and an IR sensitive CCD camera (Orca ER, Hamamatsu, Tokyo Japan).

Standard whole-cell recordings in current- and voltage-clamp mode were obtained from $BNST_{ALG}$ and PVN neurons using a MultiClamp 700B amplifier (Molecular Devices, Sunnyvale, CA), with a Digidata 1320A A-D interface, and pClamp 10 software (Molecular Devices). Current-clamp signals were filtered at 5 kHz and digitized at 10–20 KHz. Patch pipettes were fabricated from borosilicate glass (resistance 4–7 MΩ) and filled with a recording solution of the following composition (in mM): 130 K-gluconate, 2 KCl, 10 HEPES, 3 $MgCl_2$, and 5 phosphocreatine, 2 K-ATP, 0.2 NaGTP.

For all experiments, whole-cell patch-clamp configuration was established only when the seal resistance was >1.5 GΩ. Neurons were excluded from analysis if they showed a resting membrane potential (Vm) more positive than −50 mV and/or had an action potential that did not overshoot +10 mV. Series resistance (Rs) was bridge balanced and neurons with Rs > 25 MΩ were not included in analysis. Standardized protocols were used to determine the membrane properties of PVN and BNST neurons as previously described (Hammack et al., 2007). Briefly,

the voltage response of neurons was determined using transient (750 ms) outward and inward current steps of 10–50 pA based on membrane input resistance (Rm) of neurons, with the maximum hyperpolarizing voltage deflection restricted to ∼ −90 mV. Action potential properties were determined using an inward ramp of current (250 ms) sufficient to drive a single action potential. All electrophysiological data were analyzed with a custom made MATLAB 2009a script (Mathwork, Natick, MA). In a subpopulation of PVN neurons, 0.3% biocytin was included in the patch recording solution for morphological reconstructions as previously described (Hazra et al., 2011). PVNpc and PVNmc cells were distinguished from one another based on their relative medial / lateral location in the PVN, as well as by the apparent size of their soma (PVNpc = 10.7 ± 0.5 μm, $n = 18$; PVNmc = 18.8 ± 0.8 μm, $n = 18$).

STATISTICAL ANALYSIS

Statistical analyses were carried out using Prism 4 (GraphPad, La Jolla, CA). Tests for significant effect of cell type were performed using one way analysis of variance (ANOVA). To perform pairwise comparisons following significant main effects in ANOVA, Bonferroni multiple comparisons test were used. A *t*-test was used when only two cell types (PVNpc and PVNmc) were being compared. For all tests, significance was defined at $\alpha = 0.05$.

RESULTS

DUAL-IMMUNOFLUORESCENCE

GAD67 and VGLUT2 expression in the PVN

To determine the predominant amino-acid neurotransmitter phenotype of CRF neurons in the PVN and $BNST_{ALG}$, we performed dual-immunofluorescence for CRF together with specific markers for glutamatergic and GABAergic neurons. We chose GAD67 as the marker of GABAergic neurons because previous studies had shown a high levels of GAD67 mRNA expression in the BNST (Pompolo et al., 2002). Similarly, although three different isoforms of VGLUT are found in the CNS (Ziegler et al., 2002; Herzog et al., 2004), VGLUT2 is the predominant glutamate transporter found in the PVN (Hrabovszky et al., 2005). Hence, we conducted dual-immunofluorescence studies to examine the relative degree of co-expression of CRF with GAD67 or VGLUT2 in the PVN and the $BNST_{ALG}$.

As expected, CRF-immunoreactivity in the PVN was mainly localized in a subpopulation of parvocellular neurons in the medial parvocellular division and, to a lesser extent, in a subpopulation of magnocellular neurons (**Figures 1A–C**). In contrast, GAD67-immunoreactivity showed high somatodendritic expression in the perinuclear division of the PVN and low expression in either the parvocellular or magnocellular subdivisions (**Figure 1B'**). Dual-immunofluorescence experiments demonstrated that CRF- and GAD67-immunoreactive neurons exist mainly in adjacent, but mutually exclusive, neuronal subpopulations in the hypothalamus, and only 13% (16/124) of CRF neurons in the PVN co-localize GAD67 (**Figure 1B"**). Conversely, VGLUT2-immunoreactivity showed high somatodendritic labeling throughout the PVN (**Figure 1C'**) and our dual-immunofluorescence experiments revealed that the great majority (82%, 67/82) of CRF-positive neurons in the PVN also co-localized VGLUT2 (**Figure 1C"**). However, numerous VGLUT2 positive neurons were also observed in the PVN that did not co-localize CRF.

GAD67 and VGLUT2 expression in the $BNST_{ALG}$

In agreement with previous studies, CRF-immunoreactive neurons were concentrated in the oval and fusiform nuclei of the BNST, which also showed a dense network of CRF-positive fibers (**Figures 2A–C**). Consistent with previous *in situ* studies (Day et al., 1999; Pompolo et al., 2002), a high level of GAD67 somatodendritic immunoreactivity was observed throughout the $BNST_{ALG}$ (**Figure 2B'**), and dual-immunofluorescence experiments further revealed that 95% (61/64) of CRF-positive neurons in the oval nucleus co-localized GAD67 (**Figure 2B"**). However, the majority of GAD67-positive neurons in the $BNST_{ALG}$ do not co-localize CRF. Unlike GAD67, VGLUT2 immunoreactivity in the oval nucleus of the $BNST_{ALG}$ was low, and restricted mainly to the neuropil where it presented as more punctuate-like labeling rather than the somatodendritic labeling of GAD67 (**Figure 2C'**). Furthermore, unlike GAD67, a somatic co-expression of VGLUT2 in CRF-positive neurons of the $BNST_{ALG}$ was never observed (**Figure 2C"**).

Having determined that CRF neurons of the oval nucleus of the $BNST_{ALG}$ and PVN could be differentiated based on their amino acid neurotransmitter phenotype, we were interested to see if they could be further differentiated based on their relative expression of other neuropeptides and their cognate receptors. Technical constraints limit the number of epitopes that can be labeled by antibodies in a single neuron and, hence, we next used a combination of whole-cell patch-clamp recording and single-cell RT-PCR to screen physiologically identified neurons in the parvocellular and magnocellular divisions of the PVN (PVNpc and PVNmc, respectively), and $BNST_{ALG}$ for their expression of mRNA transcripts for multiple peptide neurotransmitters and their receptors.

SINGLE-CELL RT-PCR

Having visually identified putative PVNmc and PVNpc neurons, and $BNST_{ALG}$ neurons, we first recorded their basic electrophysiological properties (see below) and then extracted cytosolic mRNA (see Methods). **Figures 3A–C** shows representative photomicrographs of putative magnocellular and parvocellular neurons in the PVN. In a subpopulation of neurons biocytin was included in the recording pipette to allow subsequent morphological reconstruction of the PVN neurons. Typical examples of a putative PVNmc (right) and PVNpc neuron (left) in the medial dorsal PVN are shown in **Figure 3D**. The combined results of our scRT-PCR analysis of mRNA transcripts obtain from neurons in the two divisions of the PVN and $BNST_{ALG}$ are summarized below and in **Tables 1, 3**. The physiological properties of $BNST_{ALG}$ and PVN neurons that express CRF transcripts are described briefly in the following section, and summarized in **Table 2**.

Expression of mRNA transcripts in PVN neurons

Single-cell RT-PCR analysis was performed on mRNA isolated from 43 PVN neurons, of which 21 were putative PVNpc

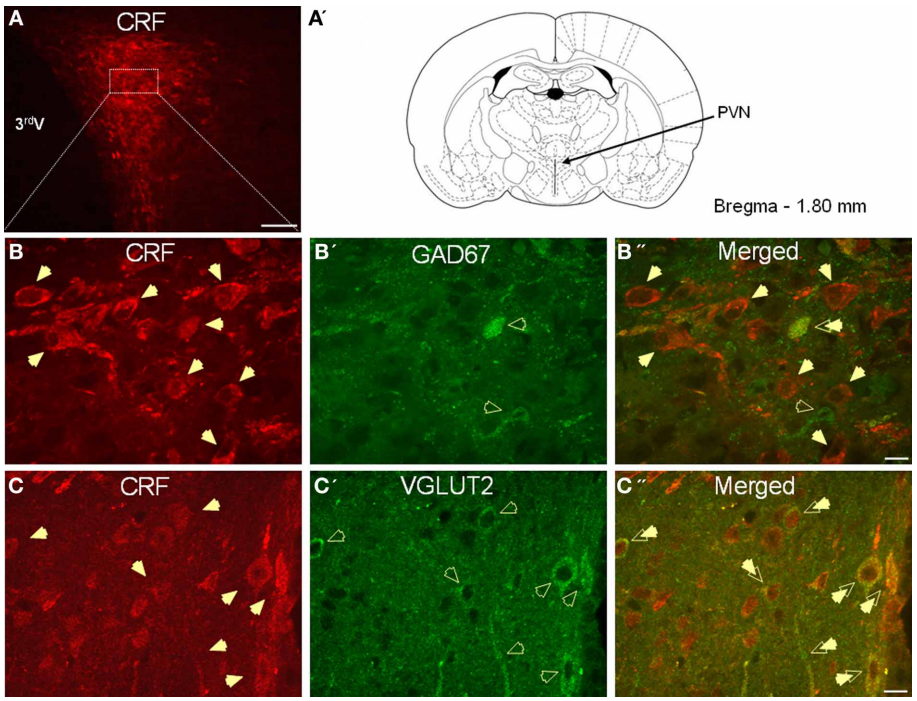

FIGURE 1 | (A) Photomicrograph showing high somatodendritic immunoreactivity of CRF in the parvocellular neurons of the PVN (magnification 10×, scale bar 10 μ, 3rd V- third ventricle). **(B–B″):** Photomicrographs showing non-overlapping somatodendritic immunoreactivity of CRF (**B,** red, closed arrows) and GAD67 (**B′,** green, open arrows) in the PVN (**B″,** merged). Occasionally, CRF-positive cells demonstrate immunolabeling for GAD67 as indicated by double arrow. **(C–C″)** In contrast, CRF-immunoreactive neurons (**B,** red) are highly co-localized with VGLUT2-positive neurons (**B′,** green) in the parvocellular PVN (merged arrows, magnification 63×, scale bar 10 μm).

neurons, and 22 were putative PVNmc neurons. Previously, we have briefly described the genetic phenotype of a sample population of magnocellular PVN neurons (Dabrowska et al., 2011). Here, we extend these studies to include a more detailed examination of the phenotype of PVNpc and PVNmc neurons.

To verify the results from our immunohistochemical studies, all neurons were initially screened for the presence of mRNA transcripts for GAD67, as well as VGLUT1, VGLUT2, and VGLUT3. As expected, 86% (37/43 neurons) of PVN neurons screened in this study expressed mRNA transcripts for VGLUT2, and none expressed GAD67 transcripts, further confirming that PVN neurons have a predominantly glutamatergic phenotype. Moreover, consistent with results from previous *in situ* hybridization studies (Ziegler et al., 2002; Herzog et al., 2004; Singru et al., 2012), none of the PVN neurons screened expressed mRNA transcripts for either VGLUT1 or VGLUT3 (data not shown), suggesting that VGLUT2 is a unique identifier for glutamatergic neurons of the PVN. Having established that PVN neurons were predominantly glutamatergic we next examined the relative extent of co-expression of CRF mRNA transcripts with VGLUT2 transcripts. In 21 PVNpc neurons screened, 62% (13/21) expressed mRNA transcripts for CRF. Consistent with our immunohistochemical data, 92% (12/13) of PVNpc neurons that expressed CRF mRNA transcripts also expressed VGLUT2, and none expressed GAD67 transcripts.

Next, we examined the relative extent of co-expression of CRF transcripts in PVNpc neurons with transcripts for OT, AVP, and ENK, together with the cognate receptors for OT and AVP. As illustrated in **Table 1**, 77% of parvocellular CRF-positive neurons (10/13) were seen to co-express mRNA transcripts for OT and V1BR, 54% for CRFR2 (7/13), and only 8% expressed transcripts for ENK (1/13). None of the parvocellular CRF neurons examined in this study expressed transcripts for CRFR1, AVP, OTR, or V1AR mRNA. In contrast, 75% of non-CRF parvocellular neurons expressed ENK and OTR (6/8), 62% expressed VGLUT2 and CRFR2 (5/8), 37% expressed transcripts for AVP (3/8), and only 25% expressed transcripts for OT and V1BR (2/8).

We have previously briefly described the expression phenotype of a sample population of magnocellular CRF-positive neurons (Dabrowska et al., 2011). Here we extend these observations and compare their expression phenotype with that of parvocellular CRF neurons. Out of 22 PVNmc neurons screened in this study, only four expressed the mRNA for CRF. Like parvocellular CRF neurons, 75% of magnocellular CRF neurons (3/4) expressed mRNA transcripts for OT, CRFR2, and V1BR, 50% expressed transcripts for VGLUT2 (2/4), and 25% expressed transcripts for AVP and ENK (1/4). Interestingly, whereas no parvocellular CRF neurons expressed transcripts for OTR, 50% of magnocellular CRF neurons showed OTR expression. Finally, V1BR transcripts were preferentially expressed

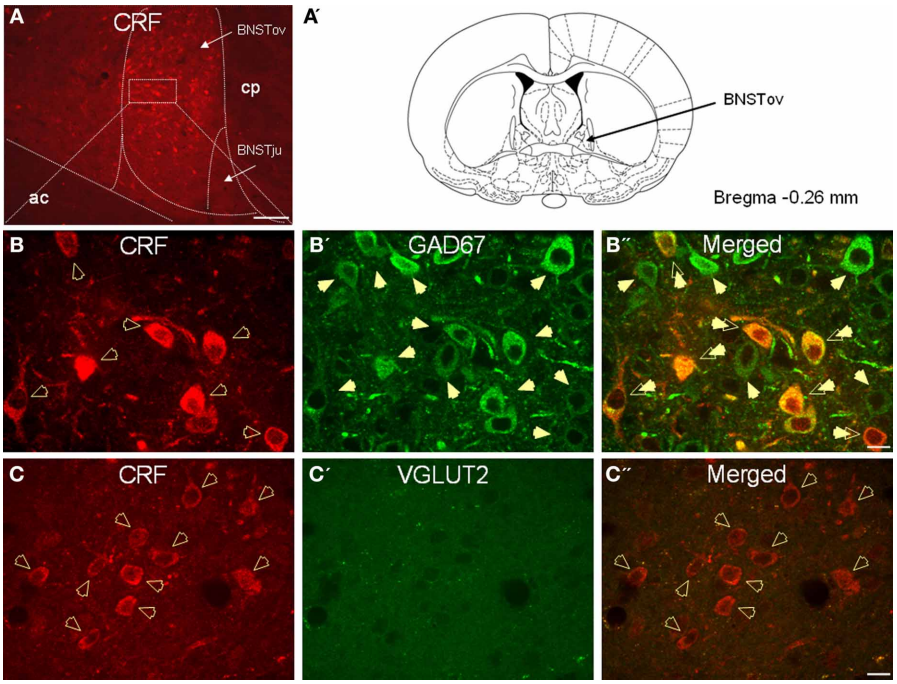

FIGURE 2 | (A) Photomicrograph showing high somatodendritic immunoreactivity of CRF in the $BNST_{ALG}$ (magnification 10×, scale bar 100 μ, ac, anterior commissure; cp, caudate putamen; BNSTov, oval nucleus of the BNST; BNSTju, juxtacapsular nucleus of the BNST). **(B–B")**: Photomicrographs showing high-level co-localization of CRF (**B,** red) and GAD67 (**B′,** green) in the $BNST_{ALG}$ (**B",** merged, double arrows). Virtually all CRF-immunoreactive neurons co-express GAD67, but numerous GAD67-positive neurons do not co-localize CRF in the $BNST_{ALG}$. **(C–C")** In contrast, CRF-immunoreactive neurons (**B,** red, open arrows) do not co-localize VGLUT2 (**C′,** green) in the $BNST_{ALG}$ (magnification 63×, scale bar 10 μm).

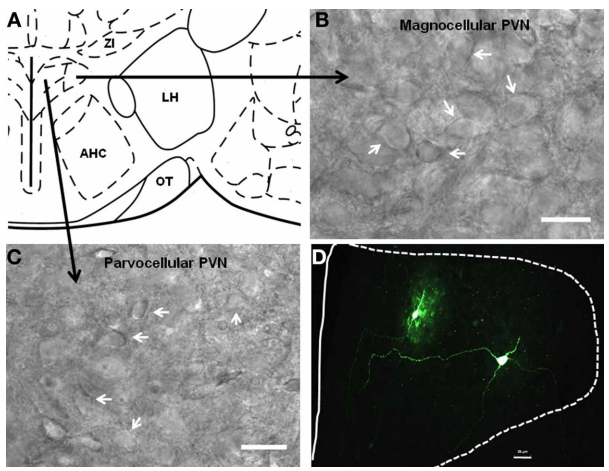

FIGURE 3 | Localization of parvocellular and magnocellular neurons in the PVN. (A) A schematic diagram showing the recording sites from the PVN neurons. **(B,C)** Representative images showing the cytoarchitecture of magnocellular and parvocellular neurons under DIC illumination. **(D)** Immunofluoroscence image showing two anatomically reconstructed PVN neurons, one parvocellular and one magnocellular. Scale bar 25 μm.

by CRF neurons and were never observed to co-express with AVP mRNA transcripts in either PVNpc or PVNmc, suggesting that V1BR does not serve as an autoreceptor in AVP neurons.

Expression of mRNA transcripts in $BNST_{ALG}$ neurons

Previously, we have shown that three physiologically and genetically distinct subclasses of neurons are present in the $BNST_{ALG}$, Type I–III (Hammack et al., 2007; Hazra et al., 2011). Type III $BNST_{ALG}$ neurons are putative CRF neurons, since the majority (95%) of these neurons express mRNA transcripts for CRF, and display a unique ion channel expression pattern that correlates with their electrophysiological features (Hazra et al., 2011). To determine if CRF neurons of the $BNST_{ALG}$ share a phenotype with parvocellular CRF neurons of the PVN we screened Type I–III BNST neurons for the presence of GAD67 and VGLUT1-3 transcripts. Significantly, all $BNST_{ALG}$ neurons tested, irrespective of cell type, expressed GAD67 mRNA transcripts, but none of these neurons expressed transcripts for VGLUT1-3. These findings confirmed our immunohistochemical observations that CRF neurons in the $BNST_{ALG}$ have a predominantly GABAergic phenotype.

Having established that CRF neurons of the PVN and $BNST_{ALG}$ could be differentiated based on their amino-acid phenotype, we next examined whether additional differences could be found in their peptidergic phenotype. The results of this study are summarized in **Table 1**. Significantly, unlike parvocellular CRF neurons that showed 83% co-expression with OT mRNA transcripts, none of the Type III CRF neurons tested co-expressed OT. Conversely, whereas 95% of Type III neurons expressed transcripts for OTR (18/19), no parvocellular CRF neurons expressed OTR transcripts. Moreover, although 54%

Table 1 | Transcriptomic analysis of CRF, non-CRF PVN cells, and Type I–III $BNST_{ALG}$ neurons.

Neurons	CRF	CRFR1	CRFR2	OT	OTR	AVP	V1AR	V1BR	ENK	VGLUT2	GAD67
PVNpc (21)											
CRF 13	13	0	7	10	0	0	0	10	1	12	0
Non-CRF 8	0	0	5	2	6	3	0	3	6	5	0
PVNmc (22)											
CRF 4	4	0	3	3	2	1	0	3	1	2	0
Non-CRF 18	0	1	15	17	3	2	0	4	10	18	0
Type I $BNST_{ALG}$ (20)											
CRF 3	3	0	0	0	0	0	1	0	1	0	3
Non-CRF 17	0	4	0	0	0	0	16	1	1	0	17
Type II $BNST_{ALG}$ (20)											
CRF 7	7	1	0	2	2	0	0	4	1	0	7
Non-CRF 13	0	1	1	1	6	0	0	7	9	0	13
Type III $BNST_{ALG}$ (20)											
CRF 19	19	0	0	0	18	0	0	2	4	0	19
Non-CRF 1	0	1	1	0	1	0	0	1	1	0	1

Table 2 | Distinct membrane properties of CRF neurons in the PVN and $BNST_{ALG}$.

CRF neurons	RMP (mV)	1st ISI (ms)	Rin (mΩ)	Spike					
				Amplitude (mV)	Half width (ms)	Threshold (mV)	Rise time (ms)	Decay time (ms)	fAHP (mV)
PVNpc (12)	-56.3 ± 0.94	24.1 ± 3.2	1180 ± 102	70.1 ± 2.4	1.003 ± 0.072	-33.1 ± 0.89	0.36 ± 0.022	0.98 ± 0.094	-14.5 ± 0.9
PVNmc (4)	-55.2 ± 0.63	19.0 ± 3.3	373 ± 114**	73.3 ± 4.5	1.01 ± 0.042	-33.2 ± 1.12	0.37 ± 0.024	0.89 ± 0.030	-19.2 ± 2.6*
Type III BNST (19)	-66.4 ± 0.97**,##	26.6 ± 4.5	226 ± 17**	76.9 ± 1.4	1.04 ± 0.024	-39.0 ± 0.75*,#	0.44 ± 0.016	1.12 ± 0.045	-4.9 ± 0.5**,##

*,** $p < 0.05$ and 0.01 respectively, vs. PVNpc; #,## $p < 0.05$ and 0.01 respectively, vs. PVNmc. ISI, inter-spike-interval; PVNpc, parvocellular neurons of the PVN; PVNmc, magnocellular neurons of the PVN.

of parvocellular CRF neurons expressed CRFR2 transcripts, no expression was observed in Type III CRF neurons and, finally only 10% of Type III CRF neurons expressed V1BR transcripts (2/19), whereas 77% of the parvocellular CRF neurons did. Hence, CRF neurons in the $BNST_{ALG}$ and the PVNpc would be expected to respond in distinctly different ways to local release of peptide neurotransmitters.

Examination of mRNA transcript expression for the different peptides and their receptors in Type I and Type II $BNST_{ALG}$ neurons revealed that like CRF neurons, Type II $BNST_{ALG}$ neurons never express V1AR transcripts, whereas 94% of non-CRF Type I BNST neurons do. Interestingly, 36% of those Type II neurons that co-expressed V1BR also expressed transcripts for CRF, similar to Type III neurons.

ELECTROPHYSIOLOGICAL PROPERTIES OF PUTATIVE CRF NEURONS IN THE PVN AND $BNST_{ALG}$

Having determined that CRF neurons of the PVN and $BNST_{ALG}$ have significantly different neurochemical phenotypes, we then examined whether the electrophysiological properties of these neurons was also different. The typical voltage response of CRF neurons in the PVNpc, PVNmc, and $BNST_{ALG}$ to transient (750 ms) depolarizing and hyperpolarizing current injection is illustrated in **Figure 4**. As can be seen in **Figures 4A,B** (left upper traces), CRF neurons from both PVN regions displayed a similar voltage response to transient current injection. Hence, all neurons recorded from each region showed a moderate level of spike-frequency adaptation in response to 750 ms depolarizing current injections (PVNpc $n = 12$, PVNmc $n = 4$). In contrast, $BNST_{ALG}$ CRF neurons exhibited a mixed firing pattern in response to depolarizing current injection. Like CRF neurons in the PVN, 11/19 $BNST_{ALG}$ CRF neurons showed spike-frequency adaptation in the first 175 ± 20 ms, and then settled down to a regular firing pattern for the remainder of the pulse. The remaining 8/19 neurons showed no spike-frequency adaptation and fired regularly throughout the current injection protocol (**Figure 4C**). In response to hyperpolarizing current injection, voltage transients of neurons in each region showed a marked time-independent anomalous rectification. However, on closer examination one-way ANOVA tests found significant differences in resting membrane potential [RMP, $F_{(2,\ 32)} = 31.7$, $p < 0.001$], input resistance [Rin, $F_{(2,\ 32)} = 76$, $p < 0.001$], spike threshold [$F_{(2,\ 32)} = 13.7$, $p < 0.001$], and fast afterhyperpolarization [fAHP, $F_{(2,\ 32)} = 31.7$, $p < 0.001$], as summarized in **Table 2**. *Post-hoc* Bonferroni tests revealed that parvocellular CRF neurons had a significantly higher mean Rin than both the magnocellular

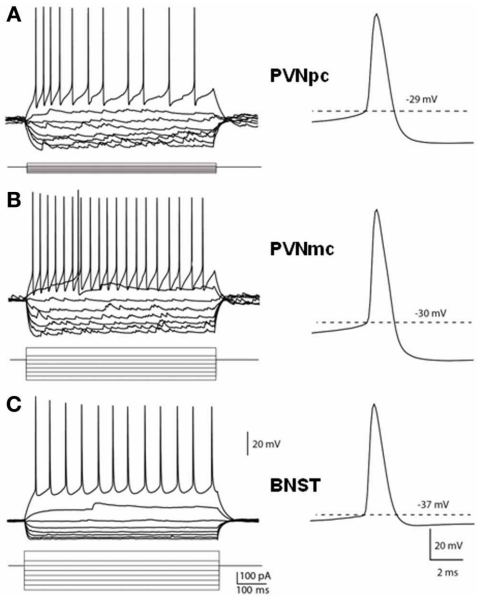

FIGURE 4 | Distinct membrane properties of putative CRF neurons in the PVN and type III CRF neurons in the $BNST_{ALG}$. Representative traces demonstrate the membrane responses of these neurons to membrane current injections and typical spikes. **(A)** PVNpc neurons have extremely high input resistance and high threshold for spike firing. When hyperpolarized, these neurons exhibited an inward rectification current. No outward rectification was observed during depolarization current injections. Right trace shows a typical spike that has high firing threshold and a prominent fAHP. **(B)** PVNmc neurons have smaller input resistance than that of PNVpc neurons and also have high threshold for spike firing. Single spike trace also shows this neuron has a high spike threshold and prominent fAHP. **(C)** In comparison to the PVN CRF neurons, CRF neurons in the BNST have lower input resistances, lower spike threshold and smaller fAHP. PVNmc, PVNpc represent magnocellular and parvocellular neurons of PVN respectively.

Table 3 | Comparison of mRNA transcript expression of I_T current channel subunits in PVN and $BNST_{ALG}$ CRF neurons.

CRF neurons	Cav3.1	Cav3.2	Cav3.3
PVNpc (13)	10	6	0
PVNmc (4)	0	3	0
Type III $BNST_{ALG}$ (19)	0	0	16

CRF neurons, and BNST CRF neurons. On the other hand, both the parvo- and magnocelluar CRF neurons in the PVN exhibited a more pronounced fast after-hyperpolarizing potential (fAHP) following each spike, as well as a more depolarized resting membrane potential compared to that of $BNST_{ALG}$ CRF neurons. Additionally, CRF neurons in the $BNST_{ALG}$ had a significantly more hyperpolarized threshold for action potential than both PVNpc and PVNmc neurons. The basic membrane properties of putative CRF neurons in the PVN and $BNST_{ALG}$ are summarized in **Table 2**.

Previous studies in the PVN had reported that expression of the low-threshold calcium current, I_T, was a unique identifier of parvocellular PVN neurons, which was markedly attenuated, or absent, in PVNmc neurons (Tasker and Dudek, 1991; Luther and Tasker, 2000), and that expression of this current may underlie differences in the firing patterns observed in these two regions. Similarly, we have shown that the firing pattern of $BNST_{ALG}$ neurons is dependent on and interplay between I_T and the transient outward potassium current, I_A (Hammack et al., 2007). Hence, activation of I_T may play a significant role in regulating the firing activity in CRF neurons in both the PVN and the $BNST_{ALG}$. As illustrated in **Figure 4**, CRF neurons in both the PVNpc and PVNmc failed to exhibit any of the rebound burst firing properties that were previously reported for type II neurons in the PVNpc (Luther and Tasker, 2000). However, burst firing was observed in a small population of non-CRF PVNpc neurons (data not shown). Alternatively, we reasoned that I_T activation may influence the firing activity of PVNpc CRF neurons by regulating the inter-spike interval (ISI) between the first two spikes of a train of action potentials. Indeed, when looking at all of the PVNpc and PVNmc neurons, including those that do not express CRF, PVNpc neurons displayed a tendency toward a shorter 1st ISI in comparison to PVNmc neurons (26.8 ± 4.7 vs. 42.5 ± 11.2 ms; $p > 0.05$), which may be indicative of more I_T activation. However, no significant difference was observed for the 1st ISI in CRF PVNpc and PVNmc neurons (24.1 ± 3.2 vs. 19.0 ± 3.3 ms, respectively), suggesting that CRF neurons in these two regions may share common properties that are distinct from non-CRF neurons. Although CRF neurons of the PVN and $BNST_{ALG}$ share many basic electrophysiological properties; **Table 2** illustrates that $BNST_{ALG}$ CRF neurons can be differentiated from their PVN counterparts in some of their basic membrane properties, which could significantly influence their response to excitatory afferent inputs. These data are consistent with our dual-immunofluorescence results, further suggesting that CRF neurons in the PVN and BNST represent distinct neuronal populations.

EXPRESSION OF I_T CHANNEL SUBUNITS IN CRF NEURONS OF THE PVN AND $BNST_{ALG}$

To determine if the differences observed in the 1st ISI may be dependent on differential expression of I_T channel subunits, we next examined the relative expression of mRNA transcripts for the I_T channel subunits $Cav_{3.1}$, $Cav_{3.2}$, and $Cav_{3.3}$ in CRF neurons of the PVN and $BNST_{ALG}$. As illustrated in **Table 3**, 54% (7/13) of CRF-positive neurons in the PVNpc expressed mRNA transcripts for the $Cav_{3.1}$ subunit, whereas only 38% (5/13) expressed transcripts for the $Cav_{3.2}$ subunit. In contrast, the only I_T channel subunit transcript expressed by PVNmc CRF neurons was the $Cav_{3.2}$ subunit. Hence, 75% (3/4) of PVNmc CRF neurons co-expressed $Cav_{3.2}$ subunit transcripts. Significantly, the majority of non-CRF PVNmc neurons (15/22) failed to show mRNA transcript expression for any of the I_T channel subunits (data not shown). In contrast to CRF neurons in the PVN, 85% of Type III CRF neurons in the $BNST_{ALG}$ expressed mRNA transcripts for the Cav3.3 subunit, but did not express transcripts for either Cav3.1 or Cav3.2 subunits.

DISCUSSION

In this study we have demonstrated that CRF neurons in the PVNpc and in the $BNST_{ALG}$ display unique and regionally distinct expression patterns not only for the major amino acid neurotransmitters glutamate and GABA, but also for other neuropeptides and their receptors that are known to play a major role in the behavioral response to stress. Hence, our dual-immunofluorescence and scRT-PCR experiments revealed that PVNpc CRF neurons are predominantly glutamatergic, have the potential to synthesize and release oxytocin (OT), are unlikely to respond to local OT release, but could respond to local AVP and CRF release through activation of V1B and CRF2 receptors, respectively. Notably, PVNmc CRF neurons have a similar profile to that of PVNpc neurons. Conversely, Type III CRF neurons in the $BNST_{ALG}$ are predominantly GABAergic, do not have the ability to release OT or AVP, but could respond to local OT and AVP release via activation of OT and V1B receptors. Moreover, CRF neurons in the $BNST_{ALG}$ do not express CRF2 receptors and, hence, local CRF release would be predicted to differentially modulate the activity of CRF neurons in these two regions. Finally, our physiological data further suggests that excitatory afferents would be more likely to drive PVNpc CRF neurons to fire action potentials than they would CRF neurons of the $BNST_{ALG}$, even though the action potential threshold is lower in $BNST_{ALG}$ neurons, due to PVNpc neurons having a more depolarized resting membrane potential and higher input resistance.

Vesicular glutamate transporters are the main regulators of glutamate uptake into synaptic vesicles prior to its release from axon terminals (Fujiyama et al., 2001). To date three isoforms have been identified, VGLUT1-3, each of which show differential expression in the CNS (Ziegler et al., 2002; Herzog et al., 2004). Here we report that CRF neurons of the PVNpc and PVNmc exclusively express mRNA transcripts for VGLUT2, but not VGLUT1 or VGLUT3. The data is consistent with previous *in situ* hybridization and immunocytochemical studies showing that VGLUT2 is the predominant isoform expressed in the PVN (Ziegler et al., 2002), and that hypophysiotropic parvocellular CRF neurons of the PVN express VGLUT2 (Lin et al., 2003; Hrabovszky et al., 2005). Similarly, VGLUT2 has been reported to be the predominant glutamate transporter expressed by almost all hypothalamic neuroendocrine neurons, including CRF, thyrotropin releasing hormone (TRH; Herman et al., 2002; Hrabovszky and Liposits, 2008), OT, and AVP expressing neurons (Takamori et al., 2000; Herzog et al., 2001; Dabrowska et al., 2011), suggesting that glutamate may be the principal neurotransmitter released by these neurons under basal firing conditions. Unlike fast amino-acid synaptic transmission, release of peptide neurotransmitters is slow, requiring high frequency firing to elicit release, and is thought to modulate subsequent fast amino-acid transmission (Kits and Mansvelder, 2000; Greengard, 2001). Hence, transmission in efferent pathways from PVN CRF neurons may switch from solely glutamate, to glutamate and CRF during times of high PVN activation. Significantly, CRF has been shown to facilitate glutamatergic transmission in multiple brain regions (Liu et al., 2004; Rainnie et al., 2004; Hahn et al., 2009) but see (Gallagher et al., 2008), suggesting that concurrent activation of postsynaptic glutamate and CRF receptors may enhance HPA axis activity in response to chronic or intense stress stimuli.

Consistent with previous immunohistochemical studies (Cullinan et al., 2008) our scRT-PCR data confirm that neurons in the PVNmc (Dabrowska et al., 2011) and PVNpc do not express GAD67 mRNA transcripts. However, we have observed GAD67-immunoreactive neurons at high levels in the perinuclear region adjacent to the PVN, which might be consistent with the presence of local inhibitory interneurons reported in earlier *in situ* hybridization studies of GAD67 expression in the PVN (Cole and Sawchenko, 2002). Indeed, CRF neurons of the medial PVNpc were shown to be targets of GABAergic inputs originating from neurons in the perinuclear zone (Roland and Sawchenko, 1993; Boudaba et al., 1996; Miklos and Kovacs, 2002), as well as from extrinsic sources such as the BNST (Herman and Cullinan, 1997).

The BNST is thought to be a major relay site for limbic input into the PVN (Cullinan et al., 1993; Dong et al., 2001; Zhu et al., 2001; Crane et al., 2003) and, notably, the PVNpc receives the heaviest BNST projection from the oval and fusiform nuclei, which contain the CRF neurons (Dong et al., 2001; Dong and Swanson, 2006). Lesion studies have shown that the anterior BNST mediates HPA axis activation, while the posterior BNST is involved in HPA axis inhibition (Choi et al., 2007) and therefore it has been suggested that CRF neurons of the anterior BNST might co-express glutamate (Silverman et al., 1989). However, we have shown that CRF neurons of the $BNST_{ALG}$ do not express mRNA transcripts for any of the three VGLUT isoforms and, instead, express transcripts and protein for GAD67. Our results are consistent with previous *in situ* hybridization studies showing that the BNST is predominantly a GABAergic structure (Sun and Cassell, 1993; Bowers et al., 1998; Day et al., 1999; Bali et al., 2005). At first glance these results would seem to contradict the results of the lesion studies showing activation of the HPA axis by the anterior BNST. However, Choi and colleagues proposed that it was the anterior BNST, ventral to the commissure, in the region of the fusiform nucleus that was responsible for activation of the PVN, and not the $BNST_{ALG}$ (Choi et al., 2007). The oval nucleus of the $BNST_{ALG}$ projects heavily to the fusiform nucleus (Dong et al., 2001) and, hence, inhibition of fusiform neurons may functionally disinhibit the PVN.

More recently, it has been proposed that neurons in the region of the fusiform nucleus are glutamatergic (Georges and Aston-Jones, 2002; Jennings et al., 2013). However, there is some question about the validity this observation as the results from several *in situ* hybridization studies, including those reported in the Allen Brain Atlas, suggest that only a few putative VGLUT2-3 mRNA expressing neurons are present in this region (Herzog et al., 2001; Kudo et al., 2012). Nevertheless, if CRF neurons of the $BNST_{ALG}$ are involved in the acute response to stressors via an indirect pathway then inhibition of putative glutamatergic neurons in the ventral BNST would paradoxically decrease activation of the PVN. An alternative mechanism might be that activation of GABA/CRF neurons in the $BNST_{ALG}$ could indirectly activate the HPA axis by dis-inhibiting GABAergic interneurons of the perinuclear zone. However, no study to date has reported direct projections from the $BNST_{ALG}$ to this region.

Notably, Day and colleagues have suggested that systemic stressors, such as infection, do not activate CRF neurons in the $BNST_{ALG}$, but instead activate ENK neurons (Day et al., 1999) as measured by alterations in c-*fos* expression. However, Cullinan and colleagues (Cullinan et al., 1995) have reported that psychological stressors, such as restraint stress, cause a dramatic increase in the expression of another immediate early gene, Zif/268, but caused only low level c-*fos* expression. Hence, activation of CRF neurons on the $BNST_{ALG}$ may be critically dependent on the nature of the stressor, and detection of their activation is dependent on which immediate early gene is screened.

Interestingly, the majority of PVN CRF neurons also co-expressed mRNA transcripts for OT, and some of the magnocellular neurons also co-expressed transcripts for the OTR. OT is classically viewed as an anxiolytic neurotransmitter (Mccarthy et al., 1996; Ebner et al., 2005; Lee et al., 2009) and, hence, the potential for OT expression in parvocellular CRF neurons is intriguing and raises the possibility that these neurons could switch their neurochemical phenotype depending on environmental demands. Dynamic regulation of neuronal phenotype is not without precedence in the CNS. Hence, during lactation tuberoinfundibular dopamine neurons begin to express ENK, which prevents dopamine from inhibiting prolactin secretion (Merchenthaler, 1993). Dynamic changes in the expression of both CRF1 and CRF2 receptors were also reported in hypothalamic magnocellular neurons expressing AVP and OT in response to hyperosmotic stress (Arima and Aguilera, 2000). It is possible that prolonged periods of stress may dynamically regulate the neurochemical phenotype of parvocellular CRF neurons in a similar manner.

Moreover, our data showing co-expression of OT and OTR transcripts is consistent with previous autoradiography studies showing OTR expression in OT neurons (Adan et al., 1995), suggesting that the OTR in this cell population might serve as an autoreceptor to regulate OT release (Freund-Mercier and Richard, 1984). Interestingly, we reported previously that the majority of CRF neurons in the $BNST_{ALG}$ also expressed mRNA transcripts for the OTR (Dabrowska et al., 2011), suggesting that OT could regulate the activity of CRF neurons in the $BNST_{ALG}$ and therefore directly modulate affective behavior. In the current study we show that the majority of non-CRF neurons in the PVN have the potential to produce OTR, suggesting that local OT release could differentially modulate the excitability of the parvocellular neurons, and therefore impact the activity of the HPA axis as it was suggested before (Neumann et al., 2000). However, mRNA transcript expression does not necessarily translate into expression of the mature peptide, since we have previously demonstrated relatively sparse co-localization of CRF- and OT-neurons in the PVN (Dabrowska et al., 2011).

It is noteworthy that we saw no co-expression of CRF with AVP in either the PVN or $BNST_{ALG}$, particularly considering that CRF and AVP have previously been reported to co-localize in both the PVNpc and PVNmc (Ma et al., 1997; Arima et al., 2001). Sawchenko and colleagues have reported that acute stress can cause a delayed induction of AVP mRNA transcripts in PVNpc CRF neurons (Kovacs and Sawchenko, 1993; Sawchenko et al., 1993). It is possible that in our unstressed animals the expression level of AVP transcripts is just below our detection threshold. In addition, is has been suggested that two populations of CRF neurons exist in the PVNpc, one that co-express AVP and one that does not (Whitnall and Gainer, 1988). It is possible that with our relatively small sample size we have unintentionally biased our recordings for scRT-PCR toward the former population.

We have also shown that Type III CRF neurons in the $BNST_{ALG}$ and PVNpc CRF neurons both express mRNA transcripts for V1BR, but not V1AR, whereas non-CRF Type I neurons of the $BNST_{ALG}$ express V1AR but not V1BR. These findings are consistent with previous studies showing a high level of expression of the V1B receptor in the PVN, where it is known to play an key role in modulating activity of the HPA axis (Nair and Young, 2006); for review see (Roper et al., 2011). Significantly, V1B receptor knockout mice have been shown to have a blunted ACTH response to both acute and repeated stress, however, this response is critically dependent on the context of the stressor (Lolait et al., 2007). Our results showing V1BR transcript expression in $BNST_{ALG}$, as well as PVN, CRF neurons would suggest that the effect of the receptor knockout would depend on whether or not a particular stressor activated one or both of these pathways. Consistent with this hypothesis recent studies with the non-peptide V1B receptor antagonist, suggest that extra-hypothalamic V1B receptors may play a critical role in the regulation of affect (Salome et al., 2006). We have preliminary scRT-PCR evidence suggesting that those Type I $BNST_{ALG}$ neurons that express V1AR co-express GABA and either somatostatin or enkephalin (Hazra and Rainnie, unpublished observations). Hence, local AVP release might differentially regulate sub-populations of $BNST_{ALG}$ neurons and modulate different behavioral outputs. Consistent with this notion, previous studies have suggested a complementary or cooperative effect of AVP receptor activation on behavioral output (Ring, 2005; Veenema and Neumann, 2008). Central V1B receptors were shown to mediate anxiety (Ishizuka et al., 2010), while V1A receptors in the BNST were positively correlated with maternal aggression (Bosch et al., 2010; Caughey et al., 2011). The BNST is known to mediate both anxiety-like and maternal aggression behavior, thus our results suggest that in the BNST, Type III CRF neurons and Type I neurons might mediate distinct anxiety- and aggression-related behavioral outcomes, respectively.

Finally, using *in vitro* patch-clamp techniques, we have demonstrated that CRF neurons of the PVN and $BNST_{ALG}$ display similar, and yet distinct basic membrane properties. Previously, we have identified three electrophysiologically and genetically distinct cell types (Type I–III) in the $BNST_{ALG}$ (Hammack et al., 2007; Hazra et al., 2011), and have further identified Type III neurons as corresponding to the CRF containing cell population (Martin et al., 2010; Dabrowska et al., 2011). Previous studies in the PVN have also identified three neuronal subtypes (Type I–III) based on their electrophysiological properties (Hoffman et al., 1991; Tasker and Dudek, 1991; Luther and Tasker, 2000), and type I and II neurons were subsequently identified as being magnocellular and parvocellular neurons, respectively (Luther and Tasker, 2000). Significantly, the parvocellular CRF neurons recorded in our study have many electrophysiological properties similar to those previously reported for the neurosecretory subpopulation of type II parvocellular PVN neurons (Tasker and Dudek, 1991);

in that PVNpc CRF neurons displayed moderate spike-frequency adaptation of the action potential firing pattern from rest, and had a pronounced post-spike fast AHP. However, no rebound low-threshold burst firing activity was observed in PVNpc CRF neurons following transient hyperpolarizing current injection. Two distinct cell populations have been reported in the PVNpc (Hermes et al., 1996), one of which shows robust rebound low-threshold burst firing activity (type III) and one that does not (type II). Consistent with this observation we observed a subpopulation of non-CRF PVNpc neurons that did show low-threshold burst firing activity. Moreover, CRF PVNpc neurons showed a marked time-independent anomalous rectification of the voltage transient in response to hyperpolarizing current injection similar to that previously reported in type II and III PVNpc neurons.

Like the PVNpc CRF neurons, the PVNmc CRF neurons in this study also exhibited a marked time-independent anomalous rectification. However, PVNmc neurons have previously been reported to lack this time-independent inward rectification (Tasker and Dudek, 1991). Consistent with the previous studies, non-CRF PVNmc cells in the current study did not exhibit the inward rectification, indicating that the CRF PVNmc cells exhibit electrophysiological properties more similar to that of PVNpc neurons than other PVNmc neurons. Another distinguishing property of PVNmc, or Type I neurons, is a large A-type K^+ current (I_A; Luther and Tasker, 2000). Although we did not measure I_A directly in this study, we did observe an ∼200 ms delay in the onset to the first action potential in 3/4 CRF PVNmc cells compared to PVNpc neurons in response to low intensity current injection (**Figure 4B**). However, due to the small sample size of PVNmc neurons ($n = 4$) it is impossible to draw any definitive conclusions about the factors mediating this response.

Intriguingly, CRF neurons of the PVN and $BNST_{ALG}$ had many overlapping physiological properties such as a regular firing pattern, and a strong inward-rectification of the voltage response to hyperpolarizing current injection. However, consistent with differences observed in our immunohistochemical and transcriptomic analysis, Type III CRF neurons and PVNpc CRF neurons were significantly different in many other aspects of their membrane properties. Hence, PVNpc CRF neurons had a much higher input resistance (1180 MΩ) compared to the CRF neurons in the $BNST_{ALG}$ (226 MΩ), which suggests that the same excitatory input would cause a greater depolarization in PVNpc CRF neurons. Although CRF neurons in the $BNST_{ALG}$ had a more hyperpolarized threshold for action potential (−39 mV) than PVNpc CRF neurons (−33 mV), PVNpc CRF neurons also showed a more depolarized resting membrane potential (−56 mV) compared to $BNST_{ALG}$ CRF neurons (−66 mV), which together with the higher input resistance suggests that excitatory synaptic input would be more likely to drive PVNpc CRF neurons to action potential threshold than those in the $BNST_{ALG}$. Furthermore, previous studies have shown that PVNpc neurons exhibit a strong I_T current that contributes to a low-threshold spiking pattern and only a little I_A (Tasker and Dudek, 1991; Luther and Tasker, 2000). Although we did not observe burst firing in the current study, we have confirmed that the parvocellular PVN neurons, which expressed CRF, also co-expressed $Cav_{3.1}$ and $Cav_{3.2}$ subunits mediating the I_T currents. Conversely, the Type III putative CRF neurons in the BNST do not exhibit low-threshold firing indicative of an I_T current (Hammack et al., 2007), but do show significant I_A currents (Rainnie et al., 2013). However, in the current study we have shown that the great majority of Type III, putative CRF neurons of the $BNST_{ALG}$ co-expressed $Cav_{3.3}$ subunit. The lack of low-threshold firing in these neurons, despite the probable presence of voltage-gated calcium channels, is most likely due to the opposing I_A current found in these cells.

PVNpc CRF neurons are responsible for a rapid activation of the HPA axis to initiate the endocrine response to stress stimuli (Rivier and Vale, 1983), while the CRF neurons in the $BNST_{ALG}$ are thought to modulate the affective component of the stress response, which is most likely context-dependent and slower (Walker et al., 2009) but see Sterrenburg et al. (2012). Thus, the functional separation of these two neuronal populations might be partially explained by their distinct membrane properties and concomitant ion channel expression pattern.

In conclusion, we reported that although CRF neurons in the PVN and the $BNST_{ALG}$ share the same neuropeptide phenotype, they represent distinct neuronal populations. Hypothalamic CRF neurons are glutamatergic (excitatory), whereas the CRF neurons in the $BNST_{ALG}$ are GABAergic (inhibitory). Functional separation of these two groups of CRF neurons can be further distinguished by their distinct membrane properties, such that hypothalamic CRF neurons are more likely to be rapidly activated in response to stress to initiate HPA axis activation, while CRF neurons in the $BNST_{ALG}$ display membrane properties which suggest that greater stimulus is needed to reach action potential threshold in these neurons. Consistent with this observation, CRF neurons in the BNST are thought to modulate the affective component of the stress response, and previous studies have suggested that in contrast to the PVN neurons, CRF neurons in the BNST may only be activated in response to chronic, but not acute stress (Kim et al., 2006). Furthermore, we have shown that OT and AVP as well as their cognate receptors co-exist in intimate relationships with CRF neurons in both PVN and the $BNST_{ALG}$. However, OT is more likely to regulate the excitability of CRF neurons in the $BNST_{ALG}$ than in the PVN, therefore OT-CRF neurotransmission seems to be more likely involved in the affective component of the stress response than in the classic endocrine response to stress. In contrast, V1BR were widely expressed in both PVN and the BNST CRF neurons, which suggests that AVP may be involved in both HPA axis activation as well as in the affective component of the stress response, which is mediated by CRF neurons in the $BNST_{ALG}$.

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Inhibition of corticotropin releasing factor expression in the central nucleus of the amygdala attenuates stress-induced behavioral and endocrine responses

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Corticotropin releasing factor (CRF) is a primary mediator of endocrine, autonomic and behavioral stress responses. Studies in both humans and animal models have implicated CRF in a wide-variety of psychiatric conditions including anxiety disorders such as post-traumatic stress disorder (PTSD), depression, sleep disorders and addiction among others. The central nucleus of the amygdala (CeA), a key limbic structure with one of the highest concentrations of CRF-producing cells outside of the hypothalamus, has been implicated in anxiety-like behavior and a number of stress-induced disorders. This study investigated the specific role of CRF in the CeA on both endocrine and behavioral responses to stress. We used RNA Interference (RNAi) techniques to locally and specifically knockdown CRF expression in CeA. Behavior was assessed using the elevated plus maze (EPM) and open field test (OF). Knocking down CRF expression in the CeA had no significant effect on measures of anxiety-like behavior in these tests. However, it did have an effect on grooming behavior, a CRF-induced behavior. Prior exposure to a stressor sensitized an amygdalar CRF effect on stress-induced HPA activation. In these stress-challenged animals silencing CRF in the CeA significantly attenuated corticosterone responses to a subsequent behavioral stressor. Thus, it appears that while CRF projecting from the CeA does not play a significant role in the expression stress-induced anxiety-like behaviors on the EPM and OF it does play a critical role in stress-induced HPA activation.

Keywords: CRF, RNA interference, CeA, corticosterone, stress, anxiety, knockdown

INTRODUCTION

Corticotropin releasing factor (CRF), also known as corticotropin releasing hormone or corticoliberin, is a 41 amino acid peptide (Vale et al., 1981) that plays an essential role in stress adaptation and coping. It regulates a wide range of both acute and chronic neuroendocrine, autonomic, and behavioral responses (Koob and Bloom, 1985; Dunn and Berridge, 1990; Heinrichs et al., 1995; Lehnert et al., 1998; Ronan and Summers, 2011). Dysregulation of CRF signaling may be a common molecular pathway for the myriad "stress-related" disorders. Measures of hyperactivity of the CRF system have been consistently implicated in the pathophysiology/etiology of a number of psychiatric conditions including anxiety disorders such as PTSD as well as depression and addiction (for reviews, see Arborelius et al., 1999; Bale and Vale, 2004; Ronan and Summers, 2011). Understanding the complexities of CRF regulated signaling and behaviors is essential to understanding these psychiatric disorders.

Many studies have revealed a role for CRF and CRF_1 receptors in a range of stress responses. In animal models CRF (icv) induces anxiety-like behavior and depressive symptoms, such as anhedonia, decreased appetite, reduced slow wave sleep, psychomotor alterations and reduced libido (Keck, 2006; Binder and Nemeroff, 2010). The anxiogenic character of endogenous CRF receptor ligands is verified by consistent anxiolytic effects of peptide and non-peptide CRF_1 antagonists (Zorrilla and Koob, 2004; Holsboer and Ising, 2008), and by reduced anxiety-like behavior in mice with a conditional knockout of limbic brain CRF_1 receptors (Muller et al., 2003; Nguyen et al., 2006). Thus, during stress CRF_1 receptor signaling produces anxious behavior (Coste et al., 2001; Risbrough et al., 2003, 2004, 2009; Bale and Vale, 2004; Heinrichs and Koob, 2004). Further results show decreased responsiveness to stressful stimuli in constitutive transgenic CRF_1 receptor knockout mice (Smith et al., 1998; Timpl et al., 1998; Contarino et al., 1999). Together these findings suggested that the CRF system would play an important role in depression and anxiety disorders.

The central nucleus of the amygdala (CeA) is a key component of an extended brain circuitry involved in stress responses and anxiety-like behaviors and is an important structure for CRF-mediated responses as it contains one of the highest concentrations of CRF immunoreactive neurons outside of the PVN (Cummings et al., 1983; Swanson et al., 1983; Cassell and Gray, 1989; Shimada et al., 1989). Numerous studies have shown that a wide variety of stressors and anxiogenic stimuli activate neurons of the CeA as measured by increased expression of the early immediate gene c-fos (Hayward et al., 1990; Campeau et al., 1991;

Honkaniemi et al., 1992; Funk et al., 2003; Asok et al., 2013) supporting the notion that stressors act, at least in part, through the CeA. A vast neuroimaging literature has linked hyperresponsivity of the amygdala to a range of anxiety and stress-induced disorders such as PTSD (Liberzon and Sripada, 2008; Hughes and Shin, 2011), addiction (Crunelle et al., 2012; Mihov and Hurlemann, 2012; Goldman et al., 2013) and depression (Bellani et al., 2011a,b). The CeA is the primary output of the amygdala. Fibers from dense pyramidal CRF neurons located primarily in the lateral part of the CeA project to other key regions including lateral hypothalamus, lateral BNST, mesencephalic reticular formation, locus ceruleus, raphé, ventral tegmental area, dorsal and ventral parabrachial nuclei, mesencephalic nucleus of the trigeminal nerve, core and shell ventromedial hypothalamus, ventral subiculum, corticomedial amygdala (Cummings et al., 1983; Sakanaka et al., 1986; Rodaros et al., 2007). Evidence suggests that CeA CRF neurons play a role in HPA axis activation. Few fibers from the CeA innervate the PVN directly (Sawchenko et al., 1993) but fibers do heavily innervate PVN projecting structures such as the BNST and very likely regulate the release of CRF from PVN to the pituitary where it is the primary secretagogue of ACTH causing HPA axis activation during stress (Choi et al., 2008).

Studies have suggested that CRF neurons in the CeA specifically mediate a range of behavioral and endocrine responses to stressors (Heinrichs et al., 1992; Heilig et al., 1994; Gray and Bingaman, 1996). Stressors, both physical and psychological, cause a rapid increase (within 1–3 h) in the expression of CRF mRNA (Mamalaki et al., 1992; Kalin et al., 1994; Hsu et al., 1998; Makino et al., 1999) and peptide (Makino et al., 1999). Administration of CRF (icv) elicits anxiety-like behaviors that are attenuated by infusion of CRF antagonists into the CeA (Heinrichs et al., 1992; Rassnick et al., 1993; Swiergiel et al., 1993). This suggests that CRF neurons in the CeA are involved in these behaviors since the majority of CRF in the CeA likely arises from local circuits (Merlo-Pich et al., 1995). Studies utilizing different animal models including rats, cats, and rabbits have shown that electrical stimulation of the CeA causes autonomic and anxiety-like responses mimicking those elicited by stress that are identical to responses caused by icv CRF administration (Hilton and Zbrozyna, 1963; Mogenson and Calaresu, 1973; Stock et al., 1978). Microdialysis studies have demonstrated local CRF release in the CeA during restraint stress (Merlo-Pich et al., 1995). Increased release of CRF in the CeA is also implicated as a potential mechanism underlying the anxiety associated with withdrawal in drug and alcohol abuse (Menzaghi et al., 1994; Merlo-Pich et al., 1995; Richter et al., 1995, 2000; Richter and Weiss, 1999). Lesions of the amygdala block fear-conditioned startle responses (Hitchcock and Davis, 1991). Bilateral lesions of the CeA in rhesus monkeys reduced fear-related behavior and freezing when exposed to an aversive stimulus and decreased levels of cerebral spinal fluid CRF and plasma ACTH in response to the stressor. This study showed that the CeA is involved in regulating hypothalamic CRF activity, peripheral endocrine responses and behavioral responses to fear-eliciting stimuli (Kalin et al., 2004). More recently, studies on adult mice showed that site specific manipulation of CeA CRF expression through lentiviral-based systems affected behavioral and endocrine systems (Regev et al., 2011, 2012; Flandreau et al., 2012) with sometimes contradictory results. In contrast to the wealth of evidence pointing to a role for CRF in the CeA in stress-induced anxiety-like behavior, long-term (4 month) viral-mediated overexpression of CRF in the CeA actually reduced anxiety-like behaviors (Regev et al., 2011) whereas long-term knockdown leads to decreases in anxiety-like behavior and cortisol responses to acute stressors (Regev et al., 2012).

Though a role of the CeA in stress and anxiety responses has been extensively characterized, the specific contribution of CRF neurons in the CeA to these responses has not. There are many other neurotransmitter systems in the CeA that could be playing a role including, among others, NPY and neurotensin. It has been estimated that up to 90% of neurotensin labeled cells in the CeA are also immunoreactive for CRF (Shimada et al., 1989). Also, most studies implicating the CeA in stress responsiveness have relied on stimulation or lesions; both of which could have non-specific effects. For example, fibers projecting from the basolateral amygdala to the BNST pass directly through the CeA. Some have speculated that electrolytic lesions of this pathway account for effects of what had been ascribed to CeA lesions (Davis et al., 2010).

To further clarify the role of CRF in the CeA on anxiety-like behavioral and corticosterone endocrine responses to stressors we silenced expression of CRF in the CeA of adult rats using RNA interference (RNAi). Our goal was to achieve a rapid localized knockdown of CRF to limit possible compensatory upregulation of other stress responsive systems that could mask the true role of amygdalar CRF in these responses. We hypothesized that unconditioned anxiety-like behaviors and corticosterone responses to stressors are mediated by CRF in the CeA and knocking down CRF expression would be sufficient to attenuate these responses.

MATERIALS AND METHODS

ANIMALS

Adult male Sprague-Dawley rats (Charles River; 225–250 g) were purchased from Charles River Breeders (CD-IGS Strain, Wilmington, MA). Rats were housed 2 to a cage (24′ × 10′), given free access to food and water and maintained on a 12:12 Light: dark schedule with lights on at 7:00 am. Rats were acclimated for at least 1 week before any procedures were carried out. All animal procedures were carried out under an approved IACUC protocol from the University of South Dakota in accordance with all applicable rules and regulations in an AALAC approved facility at the Sioux Falls VA Medical Center.

RNA INTERFERENCE

Short 21-mer double-stranded RNA oligonucleotides with TT overhangs (siRNA) were synthesized (IDT DNA, Coralville, IA) targeting the open reading frame of rat CRF mRNA (accession number NM_031019). Multiple candidate 21-mer targets of the CRF coding region of the CRF precursor gene were chosen using the web-based SDS Program (siRNA Design Software; http://i.cs.hku.hk/~sirna/software/sirna.php). This software tool makes use of multiple existing design tools to output a set of ranked candidate targets. These targets were further filtered based on RNA secondary structure using the online bioinformatics site

mfold Web Server (Zuker, 2003; http://mfold.rna.albany.edu/?q=mfold/RNA-Folding-Form). The selected target sequence has ∼50 G-C content and is located within one exon (mRNA nucleotides 697–716). A BLAST search confirmed uniqueness of sequence.

Duplex sequences

siCRF

```
   5'- GCUCACAGCAACAGGAAACTT -3'   Sense
       ||||||||||||||||||||
3'- TTCGAGUGUCGUUGUCCUUUG -5'      Antisense
```

siControl

```
     5'- CGAACAACAGAACCUACAGTT -3'   Sense
         ||||||||||||||||||||
  3'- TTGCUUGUUGUCUUGGAUGUC -5'     Antisense
```

Sense and antisense RNA strands were synthesized with 5′ tt overhangs (IDT DNA, Coralville, IA). The siRNA oligonucleotides were suspended in RNase-Free Duplex Buffer (IDT DNA, Coralville, IA). GeneSilencer siRNA transfection reagent (Gene Therapy Systems San Diego, CA) was mixed (1:1 ratio) with the diluted siRNA oligonucleotides for a final siRNA concentration of 3.3 μg/μl. The mixture was allowed to incubate at room temperature (RT) for 5 min before it was loaded into a sterilized RNase-free syringe.

SURGERY

Rats were anesthetized with isoflurane (2–2.5%; VetEquip Inhalation Anesthesia System, Pleasanton, CA). Scalps were shaved and rats were placed in a stereotactic apparatus (Kopf model #900, Tujunga, CA) with the incisor bar set to −3.3 to attain a flat skull position. Eye drops (LiquiTears™) were gently applied throughout the surgery to prevent irritation and drying. A Betadine scrub solution (provodone-iodine 7.5%) was applied to the surgical area three times with fresh, sterile cotton swabs; each time removed using sterile saline. A longitudinal incision was made in the scalp and sterile tissue clamps were applied to expose the skull. Fascia was scored and gently scraped with a scalpel. A dilute hydrogen peroxide solution was applied to assist with stopping any bleeding and it makes Bregma more visible. Stereotactic coordinates were determined for the CeA (AP −2.1; ML ± 4.25; DV −8.4; Paxinos and Watson, 1997) and holes drilled through the skull with a sterile 0.7 mm burr bit using a precision drill (Foredom) attached to a stereotactic arm, being careful to leave dura intact. With the aid of a surgical stereomicroscope, dura and any remaining bone disc were very carefully removed using a sterile 25-gauge needle. Sterilized 10 μl syringes were filled with either an siRNA duplex targeting CRF mRNA (siCRF) or scrambled sequence (siControl) diluted with transfection reagent as described above. Syringes were placed in a microinjector unit attached to the stereotactic frame (Model 5000/5001, David Kopf Instruments, Tujunga, CA) and the syringe tip zeroed at the brain surface. The syringe was then slowly (30–45 s) lowered to the correct depth. The injections were made with a slow, steady rate of injection (1.5 μl/15 min). This procedure was repeated on the contralateral side of the brain using the same RNAi duplex. For the dose response testing, the contralateral side of the brain received the scrambled RNAi duplex. Bone wax was applied to the drilled holes and the incision closed with staples (9 mm EZ Clips™). Triple antibiotic cream was applied to the wound and the rat was placed in a cage with an absorbable lining and a 125-Watt infrared heat lamp suspended over one end. Rats were closely monitored until fully ambulatory. Rats were given buprenorphine (0.01 mg/kg SQ) and returned to their housing unit where they were individually housed until experimental procedures.

DOSE RESPONSE AND TIME COURSE OF THE siRNA

A preliminary dose and time course experiment was run to determine the lowest effective dose that could be used for behavioral experiments. In order to evaluate the role of CRF in the CeA on endocrine and behavioral responses to stressors and minimize the potential for compensatory upregulation of other stress systems we wanted to find the most effective dose with the shortest latency to loss of peptide. Rats ($n = 2$/group) received unilateral stereotactic injections of three different concentrations of siRNA oligonucleotides were tested: 6.6 μg/μl, 3.3 μg/μl, 1.7 μg/μl. One side received siRNA oligonucleotides targeting CRF mRNA (siCRF) and the contralateral side received a scrambled sequence (siControl), into the CeA. Three different time points were tested: 24, 48, and 72 h post-injection. Rats were killed and brains removed for histological verification of both injection site and quantification of CRF knockdown.

VERIFICATION OF CRF KNOCKDOWN

Immunohistochemistry

Immunostaining for CRF on brain sections containing the CeA was performed. Rats were deeply anesthetized with isoflurane and perfused transcardially with 300 ml ice-cold saline (0.9%) with heparin (200 units/l) followed by 300 ml fresh 4% paraformaldehyde in 1X PBS using a gravity perfusion system. Brains were removed, post-fixed in 4% paraformaldehyde overnight (24 h) at 4°C then cryoprotected in 20% glycerol in 1X PBS before being immersed in OCT compound (Tissue-Tek, Sakura) frozen surrounded by powdered dry ice. Brains were sectioned at 35 μm on a cryostat microtome (chamber −25°C, specimen −21°C; Leica CM 1900, Leica Microsystems; Wetzlar, Germany) and stored at 4°C in 1X PBS with 0.01% NaAzide (Sigma, St. Louis, MO). Sections containing the CeA were chosen with reference to an atlas (Paxinos and Watson, 1997) washed twice for 5 min each in 1X PBS then placed in blocking buffer (3% Normal Goat Serum, 0.25% Triton X-100 in 1X PBS) for 60–90 min. Primary antibodies were diluted with blocking buffer (CRF 1:1000, rabbit anti-rat polyclonal cat# T-4037; Peninsula Labs, Belmont, CA; NeuN 1:500, mouse anti- rat monoclonal cat# MAB377; Millipore, Billerica, MA). Sections were incubated overnight (∼18 h, free floating) at RT with gentle agitation. The next day sections were rinsed 3 × 3 min in 1X PBS then incubated with fluorescently labeled secondary antibody (Cy2 or Cy3 Goat anti-rabbit or goat anti-mouse IgG; Jackson ImmunoResearch, West Grove, PA) diluted 1:200 in 1X PBS and incubated at RT in darkness with gentle agitation for 2–4 h. Sections were washed in 1X PBS three times for 3 min, mounted on poly-l-lysine coated and dried for

2 h. Slides were then washed in 1X PBS followed by an ethanol dehydration series (50, 75, 90, 100, 100% for 10 s each) and the xylene substitute Citrisolv (Fisher) 2 × 3 min before being coverslipped with DPX mountant (Fluka).

Microscopy

A Zeiss Axioskop microscope equipped with an Axiocam color camera was used to acquire digital images of CRF immunofluorescence in CeA using a 20x objective from 3–4 tissue sections from each animal. All images were acquired using identical parameters. Acquisition settings were established to prevent overexposure using native CRF immunoflourescence from homecage rats. Images from coded slides were analyzed using Adobe Photoshop 7.0 (Adobe, Mountain View, CA). Using a selection tool the CeA was manually selected and average luminosity (intensity) level ± SD of the pixels calculated using the histogram function. All values were normalized for differences in background fluorescence by selecting a standardized unlabeled area adjacent to the CeA in the same section. Corrected luminosity values were calculated by subtracting this background luminosity from luminosity in the CeA. These values were averaged for each animal.

BEHAVIORAL TESTING

Separate cohorts of rats for open field (OF) and elevated plus maze (EPM) testing were randomized into four groups (n = 9–12/group): (1) No Stress-siControl, (2) No Stress-siCRF, (3) Stress-siControl, and (4) Stress-siCRF. Separate cohorts were used to enable us to decipher the effects of prior stress on these behaviors. We were very cautious to control the amount of prior stress the animals had since that was one of our experimental conditions. Animals in the Stress groups were restrained 24 h prior to behavioral testing. Restraint stress was administered for 1 h in commercial acrylic restraint tube with extra ventilation holes. All testing was digitally recorded and analysis performed with Ethovison XP software (Noldus Information Technologies, Netherlands). Immediately following behavioral testing rats were killed and brains removed for histological verification of both injection site and knockdown of the CRF peptide in CeA.

Elevated plus maze

Rats were placed in the center of the EPM and behaviors recorded with a camera mounted on the ceiling for 5 min. Duration and frequency of open vs. closed arm entries along with total locomotion were quantified using Ethovision software. Other measures of exploratory and anxiety-like behaviors including head peaking (defined as breaking the plane of the entrance to open arm with their body remaining in the center), rearing and grooming were scored by two blinded observers.

Open field

Rats were placed in the center of the OF (oval field, 61.0 × 91.4 cm) and allowed to stay in the OF for 10 min. Frequency and duration of entries in the center zone along with rearing and locomotion were measured.

PLASMA CORTICOSTERONE

Blood samples (∼25–50 μl) were quickly collected (<30 s) from the tail vein of rats using heparinized capillary tubes after both restraint stress and behavioral testing as well as from home cage control rats. Blood was centrifuged, plasma collected and stored at −20°C until assay. Corticosterone concentrations were determined by ELISA (R&D Systems, Minneapolis, MN).

STATISTICS

Corrected luminosity values of siCRF and siControl rats were compared using an unpaired t-test. Data for corticosterone and behaviors as dependent variables were analyzed by two-way ANOVA (prior stress exposure X siRNA treatment) using SigmaStat 3.0 (Systat Inc.). Pairwise comparisons were performed using the Bonferroni *post-hoc* test. Significance level was set at $\alpha = 0.05$.

RESULTS

EFFECTIVENES OF siRNA KNOCKDOWN OF CRF

Decreased expression of CRF peptide with siRNA treatment was evident across a wide range of doses (6.6 μg/μl, 3.3 μg/μl, 1.7 μg/μl) and time points (24, 48, 72 h). Based on the results from this preliminary investigation we chose to use 3.3 μg/μl in these sets of experiments because it was the lowest consistently effective dose at 48 h. Immunohistochemical labeling of CRF and the neuron specific marker NeuN in the CeA shows an almost complete siRNA-induced silencing of CRF expression without any loss of cells (**Figure 1**). **Figure 2** shows a representative image of the mechanical lesion caused by the injector needle in the CeA. Corrected luminosity values of CRF immunofluorescence within the CeA of control (siControl) and siCRF injected rats (**Figure 3**; $p < 0.00001$).

PLASMA CORTICOSTERONE

As would be expected, home cage control rats had significantly lower concentrations of plasma corticosterone than rats that were either restrained [$F_{(6, 74)} = 12.82$; $p < 0.00$]) or after behavioral testing [OF—$F_{(4, 60)} = 6.20$; $p < 0.001$; Elevated Plus—$F_{(6, 74)} = 6.63$; $p < 0.001$] regardless of siRNA treatment (**Figures 4**, **5**). Prior stress had a significant main effect corticosterone levels [$F_{(1, 74)} = 14.91$; $p < 0.001$] but RNAi treatment alone did not [$F_{(1, 74)} = 0.90$; $p = 0.345$]. There was a significant interaction of RNAi x Prior Stress [$F_{(1, 74)} = 7.40$; $p = 0.008$]. Highest concentrations of corticosterone were found after EPM testing in control rats that had been restrained the day before. Knocking down CRF in the CeA blocked this heightened corticosterone response (**Figure 4**; $p < 0.05$).

BEHAVIORAL TESTING

Open field

Knockdown of CRF in the CeA had no effect on locomotion or measures of anxiety-like behavior in the OF. Total distance traveled was not different between treatment groups [**Figure 6**; $F_{(3,50)} = 0.94$; $p = 0.43$]. Measures of anxiety-like behavior included: frequency of entries into the center [**Figure 7**; $F_{(3,50)} =$

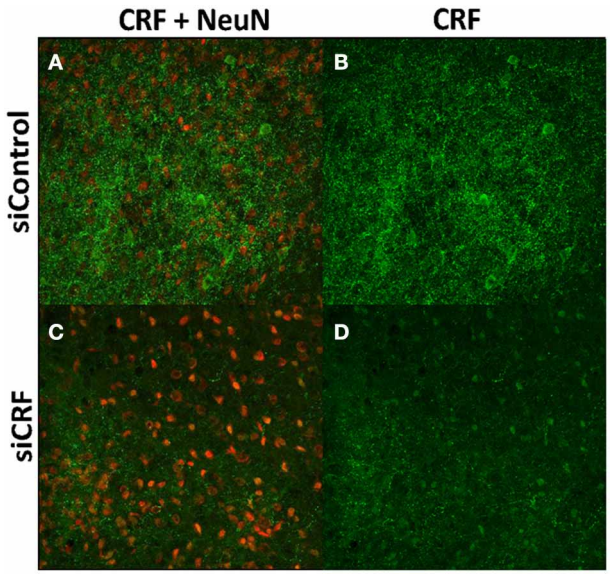

FIGURE 1 | Images (40X, Z-Scan) showing immunoreactivity for CRF (green) and NeuN (red) in the CeA of siControl injected (A,B) and siCRF injected CeA (C,D; 1.5 μl, 3.3 μg, 48 h post-injection). Immunoreactivity for CRF is robust in CeA of siControl injected while siCRF causes a substantial decrease in CRF signal with no effect on number of neurons.

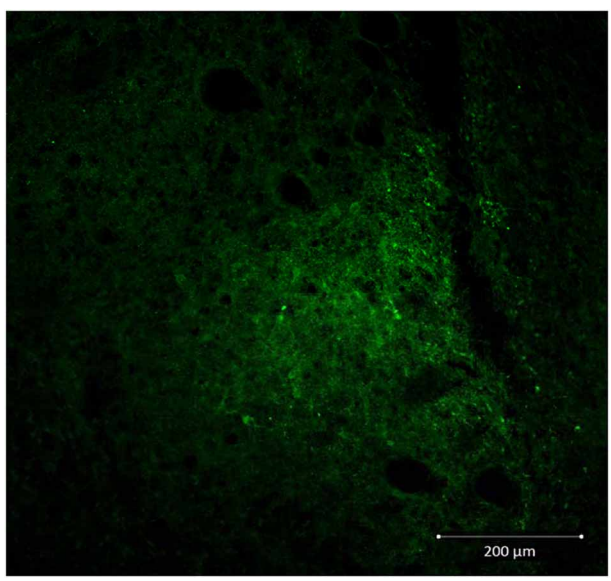

FIGURE 2 | Representative image (20X) showing injection needle tract in CeA of a siControl injected animal. CRF neurons are labeled in green.

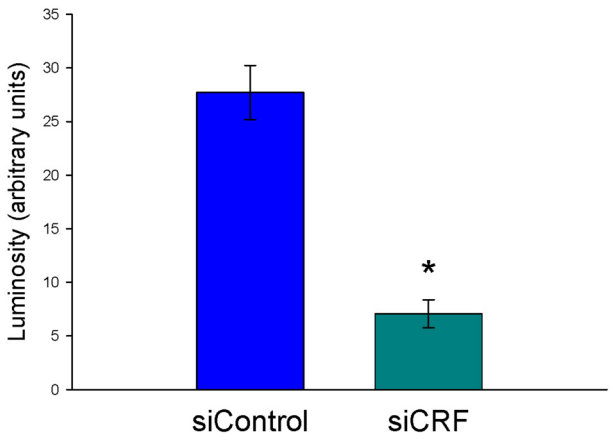

FIGURE 3 | Corrected luminosity (arbitrary units) of CRF immunofluorescence within the CeA of control (scrRNA) and siRNA injected rats. 1.5 μl injections (3.3 μg siRNA) of either scrambled siRNA control or siRNA targeting CRF mRNA (*$p = 0.00001$).

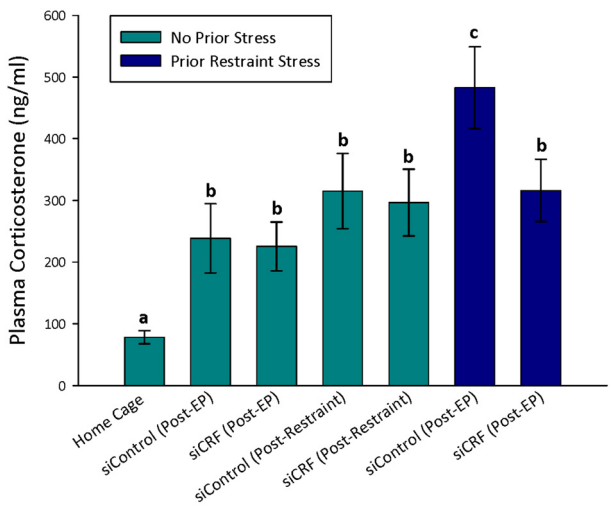

FIGURE 4 | Plasma corticosterone concentrations after restraint stress and EP testing in siContol and siCRF rats. Restraint stress and EPM induced a robust increase in corticosterone in both control and treatment rats ($p < 0.001$). The highest concentrations of plasma corticosterone were found after EPM testing in rats that had received restraint stress 24 h prior. This restraint stress sensitization of corticosterone response to a subsequent stressor was significantly attenuated by siRNA treatment ($p < 0.05$).

0.41; $p = 0.75$], duration of time spent in center [$F_{(3,50)} = 0.36$; $p = 0.78$] and rearing [$F_{(3,50)} = 2.17$; $p = 0.10$].

Elevated plus maze

Standard measures of anxiety-like behavior on the EPM (% time spent and % entries on open vs. closed arms) were not altered by siRNA treatment [$F_{(3,\ 41)} = 1.23$; $p = 0.311$ and $F_{(3,\ 41)} =$ 0.98; $p = 0.414$, respectively]. Interestingly, frequency of open entries was significantly increased by knocking down CRF expression in the CeA [$F_{(3,\ 41)} = 3.05$; $p < 0.05$]. This measure does not appear to be caused by increased locomotion as total distance traveled was not different significantly between groups [$F_{(3,\ 41)} = 1.65$; $p = 0.20$]. Time spent grooming was also significantly attenuated by siRNA treatment regardless of prior stress [$F_{(3,\ 41)} = 3.05$; $p < 0.05$]. Rats that received CRF siRNA (siCRF) groomed less than rats receiving siControl (**Figure 8**). Prior stress had no effect on this parameter (40.4 s ± 12.3 and

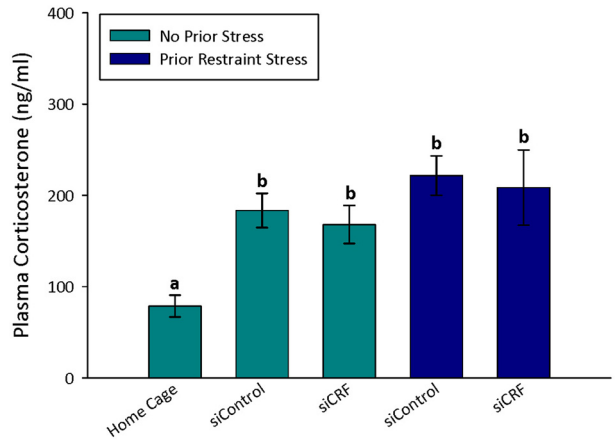

FIGURE 5 | Plasma corticosterone values after open field. Treatment with siCRF and prior restraint had no significant effect on the increase in plasma corticosterone induced by OF testing ($p < 0.001$).

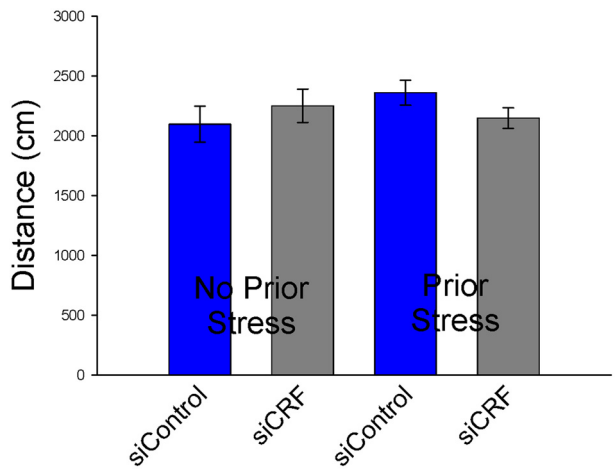

FIGURE 6 | Distance traveled (cm ± SEM) in Open Field was not significantly altered by siCRF treatment or restraint stress.

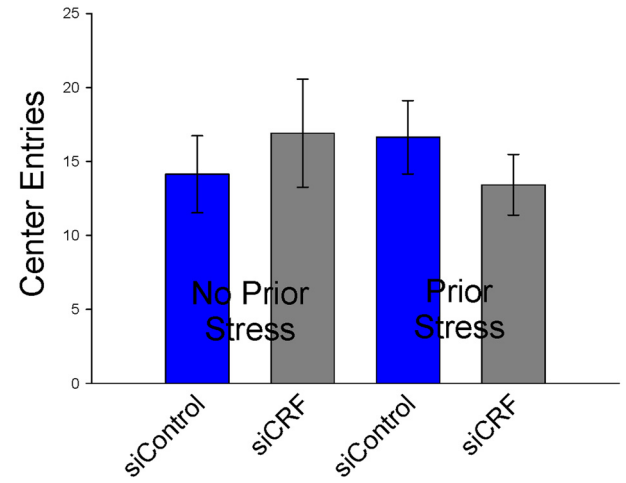

FIGURE 7 | Number of entries into center (± SEM), a measure of anxiolytic behavior, were not affected by siRNA treatment or restraint stress.

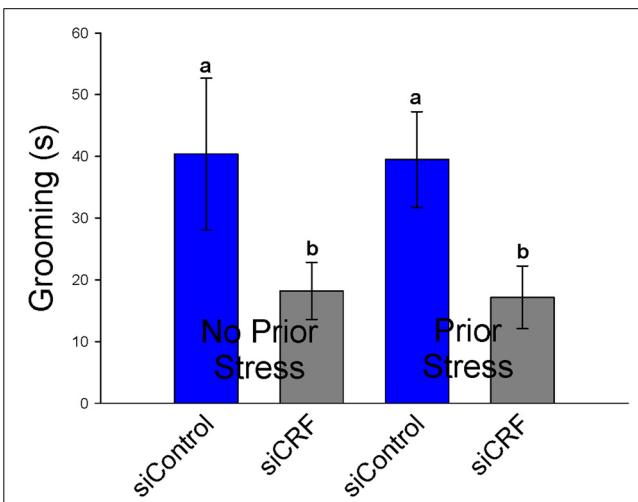

FIGURE 8 | Grooming Behavior on the EPM (s ± SEM) was significantly attenuated in rats with decreased CRF in CeA ($p < 0.05$). Prior restraint stress had no effect on this behavior.

39.5 ± 7.7). Rearing [$F_{(3, 41)} = 1.669$; $p = 0.19$] and number of head peeks [$F_{(3, 41)} = 0.512$; $p = 0.68$] were not affected by treatment.

DISCUSSION

We sought to determine if CRF in the CeA plays a role in stress-induced anxiety-like behaviors and HPA activation by using RNAi techniques to locally and rapidly (within 2 days) silence CRF expression in the CeA. This study demonstrates that while CRF in the CeA plays a role in the expression of certain CRF-related behaviors and HPA responses, knocking down amygdalar CRF expression is not sufficient to block the expression of anxiety-like behaviors on either the EPM or in the OF. Grooming, a CRF-inducible behavior, was attenuated in siCRF treated animals while measures of HPA activation (plasma corticosterone) were decreased by siCRF treatment animals with a prior stress exposure.

Prior exposure to a stressor sensitized an amygdalar CRF effect on stress-induced HPA activation. In these stress-challenged animals silencing CRF in the CeA significantly attenuated corticosterone responses to a behavioral stressor. Corticosterone release induced by restraint stress was not affected by treatment but was significantly attenuated in siCRF animals in response to behavioral anxiogenic stimuli 24 h after restraint. Thus, it appears that the role of CRF in the CeA in mediating stress-induced HPA activation is context specific and demonstrates plasticity in response to prior stress history. Activation of the CeA has been shown to activate HPA axis (Herman et al., 2005). One possibility is an indirect CRF activation of the BNST. CRF neurons in the paraventricular nucleus receive input from the CeA both directly and indirectly through the BNST among other regions (Gray

et al., 1989; Cullinan et al., 1993; Gray, 1993). However, direct input from the CeA is minor compared to that of the BNST. This anatomical evidence led to the proposal that the CeA influences the HPA axis via complex multisynaptic pathways that likely include the BNST (Herman et al., 1994, 2005; Prewitt and Herman, 1998). Interestingly, chronic overexpression of CRF in the CeA (10 weeks) leads to increased expression of both CRF and AVP in the PVN which correlated with increased measures of HPA hyperactivity (Flandreau et al., 2012).

Restraint stress has been demonstrated to cause activation and enhancement of CRF-mediated responses. It causes a rapid increase in both CRF mRNA and peptide in neurons of the CeA (Mamalaki et al., 1992; Kalin et al., 1994; Hsu et al., 1998; Makino et al., 1999) and directly stimulates CRF release in the CeA (Merlo-Pich et al., 1995). Prior restraint stress also causes a suppression of exploratory behavior in a number of paradigms that can be attenuated by CRF receptor antagonists (Berridge and Dunn, 1987, 1989). Restraint stress increases CRF-enhanced acoustic startle (Pelton et al., 1997) and markedly enhances CRF induced firing of populations of dorsal raphe serotonergic neurons (Lowry et al., 2000). This sensitization of CRF responses by prior stressors has also been demonstrated for CRF-mediated signaling specifically in the CeA. Administration of CRF (icv) alone has no effect on serotonin signaling in the CeA, however, following restraint (24 h prior) CRF significantly attenuated serotonin release as measured by microdialysis and hplc in freely moving rats (Ronan et al., 1999). In this experiment prior stress exposure unmasked a role for CeA CRF knockdown in stress-induced activation of the HPA and anxiety-like responses.

Another behavioral variable that is reliably increased by icv CRF administration is locomotion (Dunn and Berridge, 1990; Koob, 1999; Lowry et al., 1996). In our study, decreases in CeA CRF did not significantly alter locomotion either in the OF or on the EPM. In agreement with our findings two other studies found no effect of long-term viral mediated amygdalar CRF over- or underexpression on locomotion in homecage environments (Regev et al., 2011, 2012). Other studies, however, have suggested an inhibitory role of amygdalar CRF on locomotion. Rats treated with corticosterone early in development had decreased numbers of CRF-immunopositive neurons in the CeA and this correlated with increased locomotion (Roskoden et al., 2005). It is difficult to ascribe a central role of CRF to this altered locomotion since there are certainly many other systems that could contribute that would have been altered by early exposure to corticosterone. More recently, specific viral-mediated overexpression of CRF in the amygdala also led to decreased locomotion in the OF but not other tests leading the authors to postulate that amygdalar CRF does not cause a locomotor deficit but rather a "psychomotor retardation in a novel environment" (Flandreau et al., 2012). Together with our findings the evidence points to no significant role of amygdalar CRF on baseline locomotor activity.

Our results only partially support the literature suggesting a central role for CRF in the CeA with the expression of anxiety-like behavior and the hypothesized central role of CRF in stress-induced anxiety disorders (Davidson, 2002; Schwartz et al., 2003; Ronan and Summers, 2011). Inhibiting expression of CRF in the CeA had no effect on standard measures of anxiety-like behaviors on the EPM (% time or % entries on the open vs. closed arms) or in the OF (% time in center). In this experiment, grooming behavior on the EPM was significantly attenuated by loss of CRF in CeA regardless of whether or not animals had been previously exposed to restraint stress. Grooming behavior on the EPM is often used as a corollary measure of anxiety-like behavior (Bolanos et al., 2003; Estanislau, 2012; Tapia-Osorio et al., 2013), however, this is not universally accepted (Bolles, 1960; van Erp et al., 1994). Self-grooming is a behavioral response in rodents to stressful or anxiogenic stimuli (Spruijt et al., 1992). Central administration of CRF (icv) has long been known to cause increased grooming behavior (Dunn et al., 1987). This study highlights the important role of amygdalar CRF in this behavior. The significance, if any, of the finding that on the EPM, siCRF treatment rats had significantly more open entries than controls is unclear. When normalized to number of closed entries this significance is lost. Perhaps it is simply an expression of increased motor activity, though total locomotion did not differ. Or it could be indicative of an increase in exploratory behavior. Thus, knocking down CRF in the CeA alters some stress-induced behaviors it is not sufficient to completely attenuate anxiety-like responses.

Even though there is a wealth of evidence suggesting an anxiogenic role for CRF and the amygdala it is likely that CRF-mediated anxiety-like behaviors, like that hypothesized for endocrine responses, are mediated in part by other brain regions as well. Studies in rats, cats, and rabbits have shown that electrical stimulation of the CeA causes anxiety-like responses (Kaada et al., 1954; Hilton and Zbrozyna, 1963; Heinemann et al., 1973; Mogenson and Calaresu, 1973) and conversely, lesions of the CeA attenuate anxiety-like responses (Moller et al., 1997; Kalin et al., 2004). The primate amygdala plays a role in certain anxiety-like behaviors such as anxiety-related defensive responses, which may have a conditioned fear component (Kalin et al., 2004). The amygdala has been implicated in anxiety responses in a novel environment such as the EPM. However, amygdala lesions do not affect anxiogenic responses on the EPM except in rats that had received restraint stress (Moller et al., 1997).

It has been suggested that the CeA may be more specific to conditioned anxiety or fear, whereas the BNST may be more involved in unconditioned anxiogenic effects (Walker and Davis, 1997; Davis and Shi, 1999; Davis et al., 2010). The BNST and the CeA are anatomically, neurochemically, cytoarchitecturally, and embryologically related (Alheid and Heimer, 1988). The BNST is considered a site of convergence of information from brain regions associated with the control of emotional, cognitive, autonomic and behavioral responses related to stress. (Alheid and Heimer, 1988; Casada and Dafny, 1993a,b; Ciriello and Janssen, 1993; Cullinan et al., 1993). The lateral subdivision of the anterior BNST, containing high concentrations of CRF neurons is extensively connected with several brain areas that coordinate autonomic, neuroendocrine and behavioral responses to stress, such as a reciprocal connection with the CeA, parabrachial nucleus and PVN (De Olmos and Ingram, 1972; Moga et al., 1989; Moga and Saper, 1994; Dong et al., 2001; Dong and Swanson, 2004).

The involvement of CRF systems in both the BNST and CeA has been demonstrated for other stress-induced behaviors. CRF systems in both the CeA and BNST appear to be involved in stress-induced relapse to cocaine seeking behavior in rats. CRF microinfusions into the BNST but not the CeA mimic the effects of footshock on reinstatement (Erb et al., 2001). Also, infusions of the CRF_1 antagonist D-Phe CRF_{12-41} into the CeA are ineffective at blocking reinstatement but doses 10-fold lower in the BNST are capable of blocking the behavior. Lesions of the CeA lead to an attenuated reinstatement but do not block the behavior completely. Together these findings suggest a role for the CRF-containing pathway from the CeA to the BNST in stress-induced reinstatement but CRF release in the BNST appears to be the critical factor controlling this behavior. This release could be both from fibers originating in the CeA as well as from local circuits in the BNST. Another behavior mediated by CRF systems in both the CeA and BNST is fear-potentiated acoustic startle. The acoustic startle reflex is an unconditioned response to a loud noise. The startle reflex that is elicited by noise in a non-paired context can be increased in amplitude if the startle noise is given in the presence of a cue that has been previously paired with shock (Brown et al., 1951; Davis and Astrachan, 1978; Davis, 1990; Yeomans and Pollard, 1993). Electrolytic lesions of either the amygdala (Hitchcock and Davis, 1986) or the amygdalofugal pathways (Hitchcock and Davis, 1987) can block this fear potentiated startle but not the startle response itself. The direct pathway from the amygdala to the nucleus reticularis pontis caudalis was originally proposed as the critical pathway mediating fear-potentiated startle (Hitchcock and Davis, 1991). Further studies have demonstrated that this pathway is not direct. It is relayed by a synapse in the rostral midbrain (Yeomans and Pollard, 1993; Zhao and Davis, 2004). Injections of CRF (icv) can also enhance acoustic startle response in a non-paired context. This effect is mediated by the BNST. Lesions of the BNST but not CeA block CRF-mediated potentiation of acoustic startle. Also, CRF microinjected into BNST but not CeA mimics the effects of icv CRF and CRF antagonists in the BNST block CRF enhanced startle (Lee and Davis, 1997). Though the CeA is critical to the expression of fear-potentiated startle it is not critical to the expression of CRF enhanced startle. Much like the story with stress-induced relapse, it appears that the effects of CRF are mediated in the BNST, at least in part by a CRF pathway from the CeA. Thus, it is likely that activation of the BNST may be a critical output for behavioral responses.

In conclusion, CRF in the CeA certainly contributes to the expression of endocrine responses to stressors but has much less effect on behavioral responses. Silencing CRF expression in the amygdala attenuates a heightened HPA activation in response to a behavioral stressor in animals with prior stress exposure. Unconditioned stress-induced anxiety-like behavioral responses are attenuated by loss of CRF in the CeA in animals regardless of stress history. However, knocking down expression of CRF in the CeA is not sufficient to completely block expression of anxiety-like responses suggesting that amygdalar CRF is part of a wider response coordinated by this and other brain regions/neurotransmitter systems. This study adds further weight to the body of evidence linking CRF signaling to stress-induced anxiety disorders and helps to clarify the specific contribution of amygdalar CRF to stress responsiveness.

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