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Corticotropin Releasing Factor (CRF) Receptor Signaling in the Central Nervous System: New Molecular Targets

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Abstract

Corticotropin-releasing factor (CRF) and the related urocortin peptides mediate behavioral, cognitive, autonomic, neuroendocrine and immunologic responses to aversive stimuli by activating CRF_1 or CRF_2 receptors in the central nervous system and anterior pituitary. Markers of hyperactive central CRF systems, including CRF hypersecretion and abnormal hypothalamic-pituitary-adrenal axis functioning, have been identified in subpopulations of patients with anxiety, stress and depressive disorders. Because CRF receptors are rapidly desensitized in the presence of high agonist concentrations, CRF hypersecretion alone may be insufficient to account for the enhanced CRF neurotransmission observed in these patients. Concomitant dysregulation of mechanisms stringently controlling magnitude and duration of CRF receptor signaling also may contribute to this phenomenon. While it is well established that the CRF_1 receptor mediates many anxiety- and depression-like behaviors as well as HPA axis stress responses, CRF_2 receptor functions are not well understood at present. One hypothesis holds that CRF_1 receptor activation initiates fear and anxiety-like responses, while CRF_2 receptor activation re-establishes homeostasis by counteracting the aversive effects of CRF_1 receptor signaling. An alternative hypothesis posits that CRF_1 and CRF_2 receptors contribute to opposite defensive modes, with CRF_1 receptors mediating active defensive responses triggered by escapable stressors, and CRF_2 receptors mediating anxiety- and depression-like responses induced by inescapable, uncontrollable stressors. CRF_1 receptor antagonists are being developed as novel treatments for affective and stress disorders. If it is confirmed that the CRF_2 receptor contributes importantly to anxiety and depression, the development of small molecule CRF_2 receptor antagonists would be therapeutically useful.

Keywords

Corticotropin-releasing factor; CRF receptor signaling; anxiety

I. INTRODUCTION

The aim of this review is to describe recent advances in the molecular and behavioral biology of corticotropin releasing factor type 1 (CRF_1) and type 2 (CRF_2) receptors. We will review current knowledge regarding CRF receptor ligands, CRF receptor subtypes, signaling pathway

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diversity, and regulation of signaling by kinases and arrestins. We will also discuss the current controversy concerning functions subserved by central CRF_1 and CRF_2 receptors, and the potential relevance of abnormal CRF_1 and CRF_2 receptor signal transduction to the pathophysiology of human affective and stress disorders.

Although activation of stress systems is critical for survival in the context of internal or external threats to homeostasis, rapid counterregulation of these systems is equally important for re-establishing normal mood and functioning upon threat termination. Studies suggest that genetic abnormalities, exposure to stress early in life, or exposure to traumatic, unpredictable stress at any age can increase an individual's sensitivity to stress and reduce the "resilience" he or she display in coping with aversive events. "Resilience" is a function of both the threshold at which particular internal or external perturbations activate stress systems, and the rapidity and degree to which stress responses cease with termination of the aversive stimulus.

Corticotropin releasing factor (CRF) and the related peptides urocortin 1 (UCN1), urocortin 2 (UCN2) and urocortin 3 (UCN3) are important mediators of central and peripheral stress responses [1–5]. In the central nervous system, these peptides participate in the regulation of behavioral, cognitive, neuroendocrine, autonomic, and immunologic responses to aversive stimuli [1–5]. Following the isolation and sequencing of ovine CRF (oCRF) in 1981, Vale and colleagues at the Salk Institute considered naming their newly discovered neuropeptide "Amunine," a Greek word meaning "to ward off" or "to defend" [1]. The Vale group posited that CRF's ability to activate the hypothalamic-pituitary-adrenal (HPA) axis constituted an "acute defense of homeostasis" [1]. They also anticipated the discovery, which would subsequently be confirmed, that CRF exerts important actions in the brain beyond stimulation of pituitary-adrenocortical hormone secretion. Today, CRF and the related urocortin peptides are known to play diverse biological roles in stress physiology through their actions at one or both of the two CRF receptor subtypes that have been identified to date, CRF_1 and CRF_2. Although CRF_1 receptors are widely distributed throughout neocortical, limbic and brainstem regions of the central nervous system, central distribution of CRF_2 receptors is limited to discrete brain regions, including raphe nuclei, lateral septum (LS), cortical and medial amygdalar nuclei, and paraventricular (PVN) and ventromedial hypothalamic nuclei [2–5].

Clinical and pre-clinical studies suggest that abnormally persistent central CRF neurotransmission, presumably triggered by CRF hypersecretion and sensitized CRF receptor signal transduction, contributes importantly to the etiology of anxiety, stress and depressive illnesses. At present, small molecule antagonists of the CRF_1 receptor are being studied intensively in the hope of developing pharmacotherapeutic agents for the treatment of generalized anxiety disorder, panic disorder, posttraumatic stress disorder, and major depression [6–8]. The status of small molecule CRF_1 receptor antagonist development has been described in detail in a previous issue of this journal [8]. In addition to subserving central functions related to stress physiology, CRF receptors also exert peripheral actions relevant to cardiovascular, muscular, gastrointestinal, pancreatic, inflammatory, and neoplastic diseases. These functions are beyond the scope of this review.

II. CRF PEPTIDES

II.1. Corticotropin Releasing Factor

II.1.1. CRF Isolation and Sequencing—Biochemical and molecular studies have established that CRF exists as a 41-amino acid neuropeptide in mammals [1–5]. Two non-mammalian CRF-like peptides, sauvagine and urotensin I, have been isolated from frog and fish, respectively [9,10]. Human and ovine CRF are more closely related to the 41-amino acid peptide urotensin I (~55% homology) than to the 40-amino acid peptide sauvagine (45–48% homology) [2–5]. Because urotensin I and sauvagine were originally presumed to be the fish

and amphibian homologues of mammalian CRF, they were expected to share CRF's strong ACTH secretagogue action. Sauvagine, however, failed to stimulate ACTH release from frog pituitaries [11]. This discrepancy indicated that more than one CRF-like peptide exists in amphibian species. The subsequent isolation of additional CRF-like peptides from teleost fish (*Catostomus commersoni*) [12] and frog (*Xenopus laevis*) [13] proved this hypothesis. Mammalian, fish, and amphibian CRF peptides show a remarkable degree of sequence homology [3,14]. Among mammalian CRF peptides, human, rat, mouse and pig CRF homologues are identical. Only two amino acid residues differ between dog and fish CRF homologues, while the amphibian form of CRF diverges by only three amino acids from human CRF (hCRF). Ovine and bovine CRF appear to have been subjected to less evolutionary pressure than other mammalian forms of CRF since they share significantly less sequence homology with human and rodent CRF than with fish and amphibian CRF. In sheep, ovine CRF is a less potent stimulator of corticotrope ACTH secretion than vasopressin [15,16].

The biological function of CRF and CRF-like peptides is determined by the amidated end of the peptide's C-terminus [2]. The CRF neuropeptide's C-terminus binds to the extracellular binding pocket of the CRF receptor, while its N-terminus contacts other sites on the receptor to initiate cellular signaling [2,17,18]. A synthetic form of CRF possessing a free acid group at the end of the C-terminus displays three orders of magnitude lower potency compared to a synthetic counterpart with an amidated C-terminus [1,2].

II.1.2. CRF Gene—Although CRF mRNAs from fish [19], amphibians [13], rats and mice [20,21], sheep [22,23], pigs [24], and humans [25] differ in length, the genomic and peptidic organization of CRF is highly conserved across these species. In all phyla analyzed to date, the CRF gene contains two exons separated by one ~600–800 base-pair intron [13,19–25], with exon 2 encoding the entire translated region of the CRF precursor. The CRF pro-peptide is formed by removing a 24-aminoacid signal peptide from prepro-CRF. Cleavage of the pro-peptide at dibasic amino acids (lysine or arginine residues) generates the mature 41-amino acid CRF peptide [2,13,19,23,25]. The C-terminal Gly-Lys serves as an amidation motif with the glycine residue providing the template for the C-terminal amide [2]. In contrast to other neuropeptide precursors, no additional bioactive peptide seems to be encoded by prepro-CRF [3].

II.1.3. Regulation of CRF Expression—The promoter and 5′-upstream region of the CRF gene contains glucocorticoid (GRE) and cAMP response elements (CRE), and POU transcription factor binding sites [23,26,27]. These mechanisms permit exogenous stimuli to regulate CRF gene transcription. Importantly, stress provokes adaptational changes resulting in upregulation or downregulation of CRF expression, depending on the brain region. In rodents, CRF mRNA expression in the hypothalamic PVN and central nucleus of the amygdala (CeA) significantly increases in response to acute or chronic stress [28–31]. Similarly, central CRF administration generates a large increase in CRF mRNA expression in the PVN [32]. Acute stress-induced increases in CRF heteronuclear RNA and CREB phosphorylation in PVN neurons follow a similar temporal pattern [33]. Furthermore, the ability of a protein kinase A (PKA) activator to increase PVN CRF mRNA levels is additional evidence that CREB mediates stress-induced increases in CRF gene expression [34]. Investigators recently reported that acute restraint stress triggered an initial, rapid increase in CRF primary transcripts in the PVN in rats [31]. As stress continued, however, CRF mRNA expression decreased and inducible cyclic AMP early repressor (ICER) mRNA expression increased [31]. The latter finding suggests that under persistently stressful conditions ICER prevents the development of unrestrained CRF mRNA expression by inhibiting cyclic AMP-dependent CRF gene transcription [31]. Failure of this regulatory mechanism could result in central CRF hypersecretion, a putative contributor to the pathophysiology of mood and anxiety disorders. Other research has shown that acute stress increases CRF mRNA levels in Barrington's nucleus and the dorsolateral bed nucleus

of the stria terminalis (BNST), but decreases CRF mRNA expression in the olfactory bulbs [35]. Glucocorticoids reduce the number of CRF transcripts in the PVN during negative feedback regulation of the HPA axis, but upregulate CRF mRNA expression in the CeA and the BNST [23,36]. A recent study in rainbow trout showed that stress markedly increased hypothalamic and forebrain CRF mRNA expression as well as cortisol secretion, confirming the view that mechanisms regulating central CRF mRNA expression are highly conserved across species [37].

11.1.4. CRF Distribution in the Central Nervous System—CRF-expressing neurons are widely distributed throughout the mammalian central nervous system [2,3,38]. Particularly high levels of CRF expression have been observed in the following brain regions: PVN and lateral area of the hypothalamus; olfactory bulbs; neocortex; limbic neurocircuits, including amygdalar nuclei and BNST; hippocampus; and brainstem regions such as the nucleus of the solitary tract and locus coeruleus (LC) [38]. The dorsal raphe nucleus (DRN) is also innervated by CRF-expressing neurons whose axon terminals primarily contact GABA- rather than 5-HT-containing dendrites [39]. CRF has a greater affinity for the CRF_1 than for the CRF_2 receptor [2–4]. Since CRF_2 mRNA expression is significantly more abundant than CRF_1 receptor mRNA expression within the DRN, investigators have proposed that low concentrations of CRF selectively activate CRF_1 receptors in this brain region, while high concentrations activate both receptor subtypes. [39–43].

II.2. Urocortin 1

II.2.1. Urocortin 1 Cloning and Sequencing—In 1995, Vale and colleagues cloned a 40-amino acid mammalian urotensin I-like peptide from a rat Edinger-Westphal nucleus cDNA library. They named the new peptide “urocortin” because it resembled both urotensin I (63% homology) and CRF (45% homology) [44]. When additional urocortin-like peptides were discovered in 2001, the first urocortin to be cloned was re-named “urocortin 1” (UCN1) [4]. In contrast to mammalian CRF, mammalian UCN1, which has been isolated and sequenced from human, rat, mouse and sheep tissues, is highly conserved across species [44–47]. Although rat, mouse, and sheep UCN1 peptides are identical, they differ from their human homologue by two amino acids. Since the mammalian forms of UCN1 discovered to date all exhibit a high degree of sequence conservation, the functions of this peptide may be similar or identical across mammalian species. The mature UCN1 peptide is generated by cleavage of 42 amino acids in the C-terminus followed by amidation to eliminate the C-terminal dipeptide Gly-Lys [45].

II.2.2. Urocortin 1 Gene—The UCN1 gene comprises two exons and one intron, with exon 2 encoding the entire 122–124 amino acid urocortin 1 precursor peptide [44–46,48]. Structurally, the UCN1 and CRF genes are similar.

II.2.3. Regulation of Urocortin 1 Expression—Since the promoter region of the UCN1 gene contains cyclic AMP-responsive elements, increases in cyclic AMP levels stimulate UCN1 mRNA expression [46]. UCN1 mRNA expression increases in the Edinger-Westphal (EW) nucleus of acutely and chronically stressed rats [49,50]. Interestingly, UCN1 mRNA also upregulates in the EW nucleus in mice with a targeted deletion of either the CRF_2 receptor or the CRF peptide gene [5,49].

II.2.4. Urocortin 1 Distribution in Central Nervous System—In the rat central nervous system, UCN1 is highly expressed in the following brain regions: EW nucleus, hypothalamic nuclei, small populations of forebrain neurons, lateral septum (LS), DRN, substantia nigra, and motor nuclei of the brainstem [44,47,51]. The UCN1 projection from the EW nucleus to the intermediate LS is the most prominent UCN1 input to the forebrain [42,52]. UCN1 neurons

projecting from the EW nucleus to the DRN and other brainstem nuclei represent an important hindbrain pathway [42,52]. UCN1 is also expressed in the rodent pituitary [53]. Since the distribution of UCN1 expression does not correspond closely to the pattern of CRF_2 receptor expression in the rat brain, UCN1 may be an endogenous ligand for the CRF_1 receptor [2, 42]. In primates, as in rats, the highest levels of UCN1 immunoreactivity and mRNA expression have been detected in the EW nucleus, which suggests that the functions subserved by this ligand are highly conserved across mammalian species [54,55]. In humans, UCN1 expression has been detected in neocortex, hypothalamus, septal nuclei, cerebellum, and anterior pituitary somatotroph cells [54,56].

II.3. Urocortin 2 and 3: Novel CRF_2 Receptor-Selective Ligands

II.3.1. Cloning and Sequencing of Urocortin 2 and 3—In 2001, the Vale group at the Salk Institute cloned and sequenced from mouse and human cDNA libraries two UCN1-like peptides, which they named “urocortin 2” (UCN2) and “urocortin 3” (UCN3) [57,58]. Almost simultaneously, a group headed by Aaron Hsueh at Stanford University cloned and sequenced from human cDNA libraries a 40-amino acid UCN3-like peptide, which they named “stresscopin,” and a 43-amino acid UCN2-like peptide, which they called “stresscopin-related peptide” [59]. The name “stresscopin” was chosen to suggest the possibility that the newly discovered peptides promote “coping” responses to stress. The predicted sequences of these UCN1-like peptides indicate that the stresscopin N-terminus is two amino acids longer than the human UCN3 N-terminus, while the stresscopin-related peptide N-terminus is five amino acids longer than the human UCN2 N-terminus. UCN2 and stresscopin-related peptide possess similar pharmacological properties, as do UCN3 and stresscopin [60]. The names “urocortin 2” and “urocortin 3” have become accepted nomenclature for these peptides for two reasons: (a) both peptides are highly homologous to UCN1; and (b) their behavioral and physiological actions extend beyond the facilitation of stress “coping” responses [4]. The recent cloning of two urocortins orthologous to UCN1 and UCN3 from brain of the South African clawed frog *Xenopus laevis* demonstrates that urocortin peptides appeared before the evolutionary divergence of actinopterygian and sarcopterygian fishes and subsequent emergence of modern bony fishes and tetrapods [61]. The appearance of urocortins early in vertebrate evolution and their subsequent conservation underscores the importance of these peptides and their receptors for homeostasis and survival. To date, native peptides corresponding to UCN2 and UCN3 have not been biochemically isolated from human tissues. In contrast to mouse UCN2, human UCN2 lacks the classical consensus site for C-terminal amidation (Gly-Lys/Arg). Hence, it has not yet been demonstrated *in vivo* in humans that UCN2 is a biologically active peptide. Although human UCN3 possesses a good canonical C-terminal processing site, the action of this peptide at the CRF_2 receptor is less potent by one order of magnitude than that of its mouse homologue [57].

II.3.2. Regulation of Urocortin 2 and 3 Expression—UCN2 mRNA expression has been reported to increase in the hypothalamus of mice injected with dexamethasone due to the presence of a GRE in the promoter of the UCN2 gene [5]. Although acute stress upregulated UCN3 mRNA expression in the hypothalamic PVN, it failed to alter expression of this peptide in basomedial and cortical amygdalar nuclei [62]. In mice, constitutive knockout of the CRF_2 receptor gene has been reported to increase UCN3 mRNA expression in the lateral perifornical region, UCN1 mRNA expression in the EW nucleus, and CRF mRNA expression in the CeA [5].

II.3.3. Distribution of Urocortin 2 and 3 in Central Nervous System—The data concerning UCN2 and UCN3 distribution cited below pertains to the rat central nervous system since a detailed study of expression of these two CRF_2 receptor agonists in the human brain has not been completed. The patterns of UCN2 and UCN3 mRNA expression in rat brain are

discrete, in contrast to the broad pattern of distribution of CRF mRNA expression [52,57–59]. The small number of brain regions expressing UCN2 mRNA include paraventricular, supraoptic, and arcuate nuclei of the hypothalamus; LC; and brainstem motor nuclei [58]. UCN3 projections innervating the LS, amygdala, BNST, and hypothalamus constitute an important forebrain pathway [52,57]. The UCN3 projections innervating the intermediate and ventral regions of the LS most likely arise from the medial hypothalamus [52]. A high level of UCN3 mRNA expression has been measured in the LS, which also contains a large number of $CRF_{2(a)}$ receptor-expressing neurons [40–43,52]. The LS and DRN are the only brain regions in which CRF_2 receptor expression is significantly more abundant than CRF_1 receptor expression [40–43]. Although the DRN expresses a high level of $CRF_{2(a)}$ receptor mRNA, UCN3 mRNA expression in this brain region is low [52]. An abundance of UCN3-immunoreactive fibers and CRF_2 receptor mRNA expression has been identified in the posterior region of the BNST and the posterior cortical and medial nuclei of the amygdala [52].

III. CRF RECEPTOR MOLECULAR AND STRUCTURAL BIOLOGY

III.1. Molecular Cloning and Genomic Organization of CRF Receptor Genes

III.1.1. Cloning of CRF_1 and CRF_2 Receptors—Two major CRF receptor subtypes, CRF_1 and CRF_2, have been cloned during the past decade [2–4]. Both CRF receptors belong to the class B1 group of the G protein-coupled receptor (GPCR) superfamily. The CRF_1 receptor, a 415–446 amino acid polypeptide, has been cloned from vertebrate species across a phylogenetic spectrum [16,63–72]. Similarly, complementary DNAs for the CRF_2 receptor have been isolated from many species as evolutionarily distant as man and pufferfish [68–83]. Recently, a third CRF receptor subtype termed "CRF_3", which is highly homologous to the CRF_1 receptor, was cloned from catfish [80]. The CRF_3 receptor was not found, however, in other fish species [70,71]. The CRF_3 receptor appears to be unique to the catfish species for the following reasons: (a) CRF_3 receptor expression is restricted to the catfish pituitary, a tissue normally expressing CRF_1 receptors in other species; (b) only small pharmacological differences exist between catfish CRF_1 and CRF_3 receptors; and (c) a CRF_3-like receptor does not appear to exist in other fish or vertebrate species.

III.1.2. Sequences, Structures, and Homologies of CRF Receptor Subtypes—The species homologues of either the CRF_1 or the CRF_2 receptor are ≥80% identical with each other; a >70% identity is found between CRF_1 and CRF_2 receptors from all species [83]. Both CRF receptors share ~30% sequence homology with other class B GPCRs. The first 24 amino acids of human and rat CRF_1 receptors and the first 26 amino acids of the $CRF_{2(b)}$ receptor splice variant form signal peptide motifs [83–87]. The highest degree of sequence conservation between CRF_1 and CRF_2 receptors is found in the intracellular loops (IC_1–IC_4; ~90% identity) and the seven putative transmembrane helices (TM1-7; ~85% identity), while the extacellular domains (EC_1–EC_4) are conserved to a lesser extent (~60%) (Fig. 1). Importantly, the IC_3 loop, the putative coupling site to the stimulatory G protein, is 100% conserved in all CRF receptors discovered to date [2–4]. Several protein kinase phosphorylation consensus sites that may be involved in the regulation of CRF receptor function are located in the IC_1–IC_4 domains of CRF_1 and CRF_2 receptors (Fig. 1) [2–4].

Four N-glycosylation sites have been identified in the EC_1 domain and eight conserved cysteine residues have been mapped to the EC_1–EC_3 loops of both CRF receptors [83]. Glycosylation is important for cell surface expression of CRF receptors. Six out of the eight cysteines are crucial for the proper folding of CRF receptors [84]. Recently, an elegant nuclear magnetic resonance analysis established that a short consensus repeat (SCR) or "sushi" domain is present in the first extracellular domain of the mouse $CRF_{2(b)}$ receptor [18]. The SCR module is formed by the following components: (a) two antiparallel β-sheet regions with a polypeptide fold

stabilized by three disulfide bonds (Cys^{45}-Cys^{70}; Cys^{60}-Cys^{103}; and Cys^{84}-Cys^{118}); (b) a central hydrophobic core consisting of a salt bridge (Asp^{65}-Arg^{101}) sandwiched between aromatic rings of Trp^{71} and Trp^{109} [18]. The three disulfide bonds, Asp^{65}, Arg^{101}, Trp^{71} and Trp^{109} are highly conserved in both CRF receptors and the B1 GPCR family [18]. The SCR domain may govern important protein-protein interactions that mediate CRF receptor signaling and regulation.

III.1.3. CRF Receptor Splice Variants—A large number of putative splice variants of the CRF_1 receptor have been identified [16,63,65,88,89]. In general, these "splice variants" only occur in human tissues and are deficient in binding and/or signaling activity [4,14]. Due to their lack of physiological function, they do not meet the International Union of Pharmacology Committee on Receptor Nomenclature and Drug Classification (NC-IUPHAR) criteria for a receptor splice variant [4]. In contrast to the CRF_1 receptor, three functional splice variants [74,79] and two truncation variants [90,91] have been identified for the mammalian CRF_2 receptor. The functional splice variants have a common C-terminal region [92], whereas the extreme N-termini, which are encoded by one or two exons [93,94], differ substantially from one another. The 406–414 amino acid $CRF_{(2a)}$ receptor variant is the only variant found in non-mammalian species [68,70,80,82], while the 430–438 aminoacid $CRF_{(2b)}$ receptor and the $CRF_{(2a)}$ receptor are both expressed in mammals [73–76,78,95]. Expression of a 397-amino acid $CRF_{(2c)}$ receptor has only been detected in limbic regions of the human central nervous system [79]. Since only minimal pharmacological differences have been reported for $CRF_{2(a–c)}$ receptors [79,81,95], splicing most likely determines tissue distribution and cellular expression of the CRF_2 receptor. In non-primate mammals, neurons express $CRF_{2(a)}$ mRNA [40,81,96], while non-neuronal (e.g. choroids plexus) and peripheral cells express $CRF_{2(b)}$ receptor mRNA [73–76,81,91,96]. In humans and non-human primates, however, expression of $CRF_{2(a)}$ and $CRF_{2(b)}$ mRNA can overlap. For example, in the central nervous system, neuronal and non-neuronal cells both express $CRF_{2(a)}$ and $CRF_{2(b)}$ receptor mRNA [79,95, 97].

III.1.4. CRF Receptor Genes—The human CRF_1 and CRF_2 receptor genes have been mapped to chromosomes 17q21 and 7p14, respectively [98,99]. Both genes contain multiple exons and introns, which is typical for class B GPCRs [100]. The human CRF_1 receptor gene is encoded by 14 exons spanning ~20 kb of genomic DNA [101]. Thirteen out of these 14 exons encode the functional human CRF_1 receptor protein. Exon 6 encodes the unusual 29 amino-acid sequence of one of the non-functional CRF_1 receptor splice variants that is restricted to humans [66]. The rat CRF_1 receptor gene contains only 13 exons that are separated by 12 introns [102]. The human CRF_2 receptor gene (~50 kb) is larger than the human CRF_1 receptor gene and contains 15 exons [81,94]. The first four encode the three different 5′-ends of the splice variants; exons 5–15 then encode the common parts of the CRF_2 receptor.

III.2. Regulation of CRF Receptor Expression

Recent studies have provided insight into the regulation of CRF receptor genes. An ATG sequence is present in the upstream open reading frame (ORF) of the CRF_1 receptor promoter [103]. When the ATG sequence was mutated to ATA and this construct was transfected into cells, the following effects were observed: (a) the level of CRF_1 receptor mRNA was not altered; (b) CRF_1 receptor protein expression and binding upregulated; and (c) CRF-stimulated cyclic AMP accumulation increased [103]. Consequently, the upstream AUG motif in the 5′-UTR of CRF_1 receptor mRNA inhibits its translation. In another study, CRF_1 receptor promoter activity and mRNA expression upregulated in human teratocarcinoma NT2 and pregnant myometrial cells during prolonged stimulation with CRF [104]. Inhibition of PKA abolished CRF-induced enhancement of CRF_1 receptor transcription [104]. Pretreatment with CREB antisense or the PKA inhibitor H89 inhibited CRF-induced reduction in CRF_1 receptor mRNA

levels in rat anterior pituitary cells in agreement with PKA regulation of CRF_1 receptor transcription [105]. Furthermore, prolonged exposure of neuronal-derived CATH.a cells to CRF decreased the rate of CRF_1 receptor transcription *via* a PKA mechanism without altering mRNA stability [106]. Insertion of a PKA inhibitor into the nucleus of CATH.a cells failed to change CRF_1 receptor transcription measured by a reporter gene during CRF exposure [107]. Protein kinase C (PKC) activation decreased steady-state CRF_1 receptor mRNA levels in CATH.a cells [108], while PKC inhibition blocked the ability of CRF to stimulate CRF_1 receptor transcription in several cell lines [104]. Therefore, the CRF_1 receptor promoter is regulated by both adenylyl cyclase-PKA and phospholipase C-PKC signaling pathways.

Cell culture studies have shown that a rapid, large decline in steady-state levels of CRF_1 receptor mRNA occurs in rat anterior pituitary corticotropes during prolonged exposure to CRF [105,109–111]. However, CRF_1 receptor mRNA expression increased in mouse AtT-20 pituitary tumor and human corticotropic adenoma cells exposed to CRF [106,111,112]. Incubating AtT-20 cells with CRF or forskolin increased the rate of CRF_1 receptor transcription [106], whereas exposing rat anterior pituitary or mouse Cath.a cells to CRF decreased CRF_1 receptor mRNA *via* a PKA-dependent mechanism [105,107]. *In vivo*, CRF_1 receptor mRNA remains at normal levels or increases in the rat anterior pituitary during stress [113,114]. CRF_1 receptor mRNA expression and binding sites were upregulated in the PVN of rats exposed to stress [114]. In contrast, glucocorticoids appear to inhibit translation of CRF_1 receptor mRNA and/or increase degradation of CRF_1 receptor protein [114,115]. No decrease in CRF_1 receptor mRNA levels was observed in human retinoblastoma Y79, neuroblastoma IMR-32, and transfected HEK293 cells treated with CRF for up to 24 hours [116–118]. While CRF_1 receptor expression has been reported to increase in the rat frontal cortex and hippocampal CA3 region following central CRF administration, no changes in amygdalar CRF_1 or brain CRF_2 receptor expression were observed [119].

Human $CRF_{2(a)}$ receptor promoter activity has been reported to increase in transfected CHO-K1 cells during prolonged forskolin exposure [120]. Mutation of the cyclic AMP response element (CRE) cis-regulatory site prevented forskolin from stimulating $CRF_{2(a)}$ receptor promoter activity [120]. Consistent with the presence of a GRE, $CRF_{2(a)}$ receptor promoter activity decreased in response to corticosteroid treatment [120,121]. $CRF_{2(a)}$ receptor mRNA levels decreased in the ventromedial hypothalamus of rodents exposed to stress or glucocorticoid treatment [121,122].

III.3. CRF Receptor Distribution

In rodents, CRF_1 receptor mRNA expression is found throughout the CNS, while CRF_2 receptor mRNA expression is concentrated in discrete brain regions such as the DRN and LS (Table 1). *In situ* hybridization studies of rat brain show that CRF_1 receptor mRNA expression is abundant in the following CNS regions where CRF_2 receptor expression is very low or absent: neocortex; hypothalamic dorsomedial nucleus; basolateral amygdaloid nucleus (BLA); medial septum; and dopaminergic neurons of the substantia nigra, ventral tegmental area, and brainstem [2,3,40–43,96,122–124]. The high level of CRF_1 receptor immunoreactivity observed in cholinergic neurons of the rat basal forebrain and brainstem suggests that CRF modulates cholinergic neurotransmission in these brain regions [125]. In both rat and mouse, CRF_2 receptor expression is prominent in the following brain regions: ventromedial hypothalamic nucleus; LS; cortical and medial amygdalar nuclei; DRN; and nucleus of the solitary tract [40–43,96]. While CRF_1 mRNA expression is low in the DRN, CRF_2 receptor mRNA expression is high, occurring in serotonergic neurons at the middle level of the nucleus and in GABAergic neurons at the caudal level (Table 1) [40–43,125].

Although CRF_2 receptors are certainly localized postsynaptically, recent evidence suggests that they are also expressed presynaptically on vagal [126] and cerebellar [127] afferent

terminals. In the hypothalamic PVN, co-expression of mRNAs for the $CRF_{2(a)}$ receptor and CRF suggests that $CRF_{2(a)}$ receptor signaling may regulate PVN CRF expression [40–43]. In the rat, both CRF receptor subtypes are expressed in the hypothalamic PVN, olfactory bulb, hippocampus, entorhinal cortex, BNST, and periaqueductal grey (PAG) (Table 1) [40–43,96, 123]. In contrast, rat anterior pituitary corticotrophs express very high levels of CRF_1 receptor mRNA, but no CRF_2 receptor mRNA, while gonadotrophs express low levels of CRF_2 receptor mRNA, but no CRF_1 receptor mRNA (Table 1) [128].

In rats, injecting either CRF or UCN1 into the lateral ventricle increases Fos expression in the LC and CeA [41]. While a few investigators have detected low levels of CRF_1 receptor binding and immunoreactivity in rodent LC noradrenergic neurons [123], most have not observed CRF_1 receptor mRNA expression in this brain region [40–43,114]. The rat LC expresses a high level of UCN2 mRNA, but no measurable CRF_2 receptor mRNA [52]. In the rhesus monkey, however, investigators have detected CRF_1 receptor mRNA and binding in the LC, and mRNAs for both CRF receptor subtypes in the CeA (Table 1) [97]. Although CRF_1 and CRF_2 receptor mRNA expression has not been found in the rat CeA using *in situ* hybridization, a recent electron microscopic immunocytochemistry study detected CRF_1 receptors in membranes and intracellular vesicles of CeA neuronal dendrites in the vicinity of CRF-expressing axons [129]. It has been posited that the CRF_1 receptors detected in intracellular vesicles are held in cytosolic reserve and translocate to the cell membrane in response to stress.

In contrast to rodents, CRF_2 receptor mRNA expression is found throughout the central nervous system of the tree shrew and rhesus monkey [95,97]. While CRF_1 and CRF_2 receptor mRNA expression has been detected in many regions of the primate neocortex, expression of both receptors is especially prevalent in the prefrontal and cingulate cortices, which interconnect with limbic brain regions [97,130]. CRF_2 receptor mRNA expression has been detected in the CeA and anterior pituitary of the rhesus monkey [97]. In human anterior pituitary, both CRF_1 and CRF_2 receptors are expressed in corticotroph cells [64,111,131]. Co-expression of CRF_1 and CRF_2 receptors in the neocortex and pituitary of human and non-human primates suggests that CRF receptor subtypes may regulate these regions in a coordinated manner.

III.4. CRF Receptor Ligands

III.4.1. Peptide Agonists for CRF Receptors—The affinity of ovine CRF (oCRF) for the CRF_1 receptor is 40- to 200-fold greater than for the CRF_2 receptor. Similarly, the affinity of human/rat CRF (h/rCRF) for the CRF_1 receptor is 4–20 fold greater than for the CRF_2 receptor (Table 2) [2,3,14,132,133]. Although UCN2 and UCN3 are highly selective CRF_2 receptor ligands, the affinity of the $CRF_{2(a)}$ receptor for UCN2 is 3- to 13-fold greater than for UCN3 (Table 2) [57–59]. Two artificial CRF receptor agonists have been developed, α-helical CRF and cortagine. Alpha-helical CRF is a more potent CRF_1 receptor ligand than oCRF [134,135]. Compared to oCRF, cortagine a chimeric peptide derived from oCRF, h/rCRF and sauvagine exhibits a slightly greater affinity for the CRF_1 than for the CRF_2 receptor [136].

III.4.2. Peptide Antagonists for CRF Receptors—Investigation of the structure-activity-relationship (SAR) of peptidic CRF receptor antagonists has been a major endeavor within the field of CRF research. Deleting eight amino acids from the CRF peptide's N-terminus produced the first CRF receptor antagonist, α-helCRF$_{(9–41)}$, which binds non-selectively to both CRF receptor subtypes [137]. Initially, investigators assumed that the CRF peptide's C-terminus determined receptor binding affinity, while its N-terminus governed agonist potency. They posited that successful antagonist design depended upon separating the mutant peptide's N- and C-termini using artificial α-helical spacers. Recent findings suggest, however, that antagonists generated by separating the CRF peptide's N- and C- termini using artificial α-helical spacers of variable length still possess significant agonist potency [138].

The relative orientation of the CRF peptide's N and C termini rather than their distance from one another appears to be the essential determinant of agonist potency. The middle part of the receptor may contribute only in a minor way to biological activity.

SAR studies attempted to identify specific residues governing the effect of a CRF ligand's secondary structure on biological activity [138–141]. The discovery that a ligand's structural constraints contribute to its affinity for a receptor led to the development of two new CRF receptor antagonists: (a) [D-Phe^{12},Nle^{21,38} C$^{\alpha}$MeLeu^{37}]h/rCRF$_{(12–41)}$ (D-Phe CRF) [142]; and (b) {cyclo(30–33)-[D-Phe^{12}, Nle^{21,38}, Glu^{30}, Lys^{33} h/rCRF$_{(12–41)}$} (astressin) [143]. Compared to α-helCRF$_{(9–41)}$, D-PheCRF and astressin exhibit less intrinsic agonist potency and are 10- to 100-fold more effective in inhibiting ACTH release. Astressin was the first antagonist to be synthesized with a lactam bridge between residues 30 and 33, a region essential for the antagonist's high affinity binding. D-PheCRF, the linear analogue of astressin, is 300-fold less potent than its cyclic counterpart. Several astressin-like antagonists with longer duration of action than astressin share the common feature of a lactam bridge between residues 30–33 [144,145]. Neither antagonist, however, is selective for either the CRF$_1$ or CRF$_2$ receptor [14,133].

A milestone in the CRF receptor field was reached in 1998 when a CRF$_2$ receptor-selective peptide antagonist called anti-sauvagine-30 ([D-Phe^{11},His^{12}]Svg$_{(11–40)}$, antisauvagine) was synthesized by truncating sauvagine's N-terminus [146]. Anti-sauvagine-30 potently inhibits agonist and antagonist binding to both CRF$_{2(a)}$ and CRF$_{2(b)}$ receptors [14,81,146]. Radio-iodinated anti-sauvagine-30 is a superior ligand for selectively labeling CRF$_2$ receptors [147]. Amino acid exchanges and conformational constraints have been incorporated into the anti-sauvagine-30 sequence to develop other CRF$_2$ receptor antagonists. Interestingly, elongating anti-sauvagine-30 by one amino acid and incorporating Tyr^{11} produces a peptide antagonist ([Tyr^{11}His^{12}]Svg$_{(10–40)}$) that is significantly more selective for the CRF$_2$ receptor than anti-sauvagine-30 [147,148]. Since [D-Tyr^{11}His^{12}]Svg$_{(10–40)}$ is sixfold less selective than [Tyr^{11}His^{12}]Svg$_{(10–40)}$, CRF$_2$ receptor antagonist selectivity appears to be sensitive to orientation of the Tyr^{11}-residue [148]. Lengthening the anti-sauvagine-30 sequence by adding Ser^{10} produces a peptide that is more selective for the CRF$_2$ receptor than shorter analogues [148].

Recently, CRF$_2$ receptor antagonists have been developed by exchanging positions 13 and 39 against C$_{\alpha}$MeLeu residues in the N-terminus and introducing a cyclic constraint between positions 31 and 34. This antagonist, called astressin$_2$-B {cyclo(31–34)[D-Phe^{11},His^{12},Nle^{17},C$_{\alpha}$MeLeu^{13,39}, Glu^{31}, Lys^{34}]Ac-Svg$_{(8–40)}$}, has a longer duration of action than anti-sauvagine-30 [149]. Lactamization of [D-Phe^{11}, His^{12},Nle^{17},Glu^{29},Lys^{32}] Svg$_{(10–40)}$ between position 29–32 produces an antagonist that binds with high potency to both CRF receptors. Thus, the ability of {(cyclo29–32)[Trp^{11}, His^{12},Nle^{17},Glu^{29},Lys^{32}] Svg$_{(10–40)}$} to inhibit CRF$_1$ and CRF$_2$ receptor-mediated cyclic AMP accumulation is ~30-times greater than that of astressin [149]. Because astressin is not completely soluble under physiological pH conditions [150], it exhibits high *in vitro* but moderate to low *in vivo* potency. The high solubility of anti-sauvagine analogues makes them advantageous for use in behavioral experiments.

III.4.3. Small Molecule Receptor Antagonists for CRF—Since 1996, a large number of small molecule CRF$_1$ receptor antagonists have been developed. Most small molecule antagonists for the CRF$_1$ receptor consist of a central scaffold of monocyclic, bicyclic or tricyclic structure that is coupled to a pyridine or pyrollo- or pyrazolo-pyrimidine and an amine [6–8]. Small molecule antagonists bind to amino acids within TM3 and TM5 of the CRF$_1$ receptor in an allosteric manner [6]. Surprisingly, no small molecule CRF$_2$ receptor antagonists have been synthesized.

III.5. Structural Determinants of CRF Receptor Ligand Selectivity and Conformation

CRF_1 and CRF_2 receptors display distinctly different preferences for the various CRF and urocortin ligands. All species homologues of CRF, UCN1, urotensin I and sauvagine bind to and activate CRF_1 receptors with high affinity in the nanomolar to subnanomolar range (Table 2) [14,63–68,81,133]. The *Xenopus laevis* CRF_1 receptor displays a very restricted ligand selective profile which discriminates it from its mammalian counterpart (Table 2) [14,67,81, 151,152]. In contrast to the CRF_1 receptor, all CRF_2 receptor splice variants exhibit distinct ligand selectivity profiles. UCN1, urotensin I and sauvagine bind to CRF_2 receptors with high affinity whereas h/rCRF and oCRF bind with significantly lower affinities to this receptor subtype [57–59,67,73–77,80,95]. UCN2 and UCN3 exclusively bind to CRF_2 receptors (Table 2) [57–59,81]. The selectivity profiles of agonists observed in recombinant heterologous cell systems, however, may not resemble those found in cell lines endogenously expressing CRF receptors. For example, a recent study revealed that oCRF more potently activated $CRF_{2(b)}$ receptors endogenously expressed in a native cell line compared to those artificially expressed in a recombinant cell system [153].

The ligand binding preferences of CRF_1 and CRF_2 receptors have been studied in detail by generating receptor chimera or mutants. It is now well established that CRF agonists mainly bind to the N-terminal EC_1 domain and portions of the EC_2 and EC_3 domains of both receptor subtypes [85–87,132,151–157]. Furthermore, a chimera composed of the CRF_1 receptor's EC_1 domain and the closely related parathyroid hormone receptor (without its EC_1 domain) provided data in support of the hypothesis that the CRF_1 receptor's EC_1 domain plays a major role in peptide ligand binding, but not receptor activation [154]. When a tethered receptor was created by substituting the 16 amino acids in the N-terminus of the CRF peptide for the EC_1 domain of the CRF_1 receptor, the resulting chimera exhibited a high level of constitutive activity [158]. This finding suggests that CRF's N-terminus is presented to the body of the CRF_1 receptor during agonist-induced activation. Because small molecule antagonists bind directly to the CRF_1 receptor's TM3 and TM5 and thereby block its activation [6,159], small molecule but not peptide antagonists can interfere with signaling by the tethered CRF_1 receptor [6]. Peptide ligands and small molecule antagonists preferentially bind to different conformational states of the CRF_1 receptor and do not compete to occupy the same binding site. These studies suggest the applicability of a two-domain model of the CRF_1 receptor-ligand interaction [17,159,160]. According to this model, a peptide ligand's C-terminus binds to the EC_1 domain of the receptor while its N-terminus binds to an extracellular juxtamembrane domain (J-domain), influencing the conformation of the intracellular Gα protein binding site [17,159,160]. Recently, an alternative model was proposed according to which the last eleven amino acids of the UCN1 N-terminus interact with a CRF_1 receptor J-domain [161]. Small molecule antagonists, on the other hand, bind almost exclusively to a J-domain of the receptor, which produces an allosteric interaction that partially inhibits peptide ligand binding [17, 159,160]. These studies provide evidence that small molecules are allosteric inhibitors rather than competitive CRF_1 receptor antagonists [159].

IV. CRF RECEPTOR SIGNAL TRANSDUCTION

IV.1. Gs-Coupling and the Adenylyl Cyclase-Protein Kinase A Pathway

A large number of studies have investigated the signal transduction profiles of CRF_1 and CRF_2 receptors. The transmembrane and intracellular domains of both CRF receptor subtypes are highly homologous. Thus, it is not surprising that both receptor subtypes couple to the same Gα proteins and signal through similar second messengers. There is consensus among investigators that the dominant mode of CRF_1 receptor signaling in endogenous and recombinant cell lines is activation of the adenylyl cyclase-protein kinase A pathway (Figs. 2,3) [2,3,63,68,83,117,162–164]. The binding of CRF or UCN1 to the membrane-bound

CRF_1 receptor changes the receptor's conformation from an inactive to an active signaling state, thereby increasing its affinity for G_S, the stimulatory heterotrimeric GTP binding protein. The coupling of the α subunit of the G_S protein to the third intracellular loop of the agonist-activated receptor stimulates adenylyl cyclase activity which, in turn, generates production of the second messenger cyclic AMP. The coupling of $G_S\alpha$ to the CRF_1 receptor's third intracellular loop produces a ~1300-fold increase in the receptor's affinity for the CRF peptide [17]. G_S-mediated CRF_1 receptor signaling generates cyclic AMP-dependent activation of protein kinase A and subsequent downstream events such as CREB phosphorylation (Fig. 2) [2,3,63,83,162–169].

Similar to the CRF_1 receptor, the binding of selective (UCN2, UCN3) and non-selective (UCN1, CRF) agonists to extracellular domains of the CRF_2 receptor changes the conformation of the receptor on the cell membrane to an active state characterized by high affinity for the G_S binding protein. $G_S\alpha$ couples to the third intracellular loop of the agonist-activated CRF_2 receptor, stimulating adenylyl cyclase activity and initiating cyclic AMP-dependent signaling *via* the protein kinase A pathway (Figs. 2,3) [2,3,73–76,81]. The ability of the PKA inhibitor H89 to block sauvagine-induced pCREB formation in transfected CHO cells demonstrates that phosphorylation of CREB during $CRF_{2(a)}$ receptor signaling is mediated by the cyclic AMP-PKA pathway [169]. Although a majority of studies indicate that CRF receptors couple to $G_S\alpha$ and activate the protein kinase A pathway, additional second messengers have been implicated in CRF receptor signal transduction.

IV.2. Gq-Coupling and the Phospholipase C-Protein Kinase C Pathway

Endogenously and recombinantly expressed CRF_1 receptors activate the phospholipase C-protein kinase C (PKC) pathway, possibly by coupling to $G_q\alpha$ (Fig. 2). For example, stimulating rat Leydig cells with CRF induced rapid translocation of PKC from cytosol to cell membrane [170]. CRF also has been shown to stimulate calcium signaling in rat neonatal astrocytes [171]. Incubating CRF_1 receptor-expressing epidermal and dermal cell lines with CRF, UCN1, or sauvagine increased intracellular calcium mobilization and inositol 1,4,5-triphosphate (IP_3) formation as well as cyclic AMP accumulation [172–175]. Activating CRF_1 receptors endogenously expressed in human endometrial cells increased formation of both IP_3 and cyclic AMP [176,177]. Activating CRF_1 receptors endogenously expressed in feto-placental cells, however, generated only IP_3 signals [176,177]. CRF-stimulated formation of both cyclic AMP and IP_3 has been observed in transfected HEK293 and COS-7 cells recombinantly expressing the human CRF_1 receptor [89,178–181]. oCRF-, UCN1-, or sauvagine-induced activation of CRF_1 receptors stably expressed in HEK293 cells stimulated intracellular calcium mobilization (Fig. 3) and IP_3 formation [180,181]. In contrast, intracellular calcium signaling did not occur in CRF_1 receptor-expressing SK-N-MC neuroblastoma cells stimulated with these three agonists (Fig. 3) [180]. Inhibiting phospholipase C by pretreating CRF_1 receptor-expressing HEK293 cells with U-73,122 completely blocked sauvagine-stimulated calcium signaling [180]. Neither the PKA inhibitor Rp-cAMPS nor the calcium-release channel inhibitor ryanodine altered agonist-stimulated calcium mobilization in these cells [180].

Incubating $CRF_{2(b)}$ receptors endogenously expressed in human epidermoid A431 cells with sauvagine increased intracellular calcium levels, stimulated IP_3 formation, and triggered translocation of PKC-α and -β from cytosol to cell membrane [182,183]. In addition, sauvagine mobilized intracellular calcium stores in $CRF_{2(a)}$ receptor-expressing CHO cells [169]. Incubating $CRF_{2(a)}$ receptors stably expressed in HEK293 cells with UCN1, UCN2, UCN3, oCRF, or sauvagine mobilized intracellular calcium (Fig. 3) and increased IP_3 formation [180]. Calcium mobilization did not increase in SK-N-MC neuroblastoma cells stably expressing $CRF_{2(a)}$ receptors [180]. Pretreating $CRF_{2(a)}$ receptor-expressing HEK293 cells with the phospholipase C inhibitor U-73,122 completely inhibited sauvagine- and UCN3-

stimulated calcium signaling [180]. Neither the PKA inhibitor Rp-cAMPS nor the calcium-release channel inhibitor ryanodine altered sauvagine- or UCN3-stimulated calcium mobilization in these cells [180]. Since CRF_1 and $CRF_{2(a)}$ receptors endogenously expressed in human neuroblastoma SK-N-MC cells did not increase calcium mobilization and IP_3 formation, CRF receptor signaling *via* the phospholipase C pathway appears to depend on hitherto unidentified characteristics of the cellular background [180].

IV.3. ERK-Mitogen-Activated Protein (MAP) Kinase Pathway

CRF receptor signaling *via* the extracellular signal-regulated kinase (ERK)-MAP kinase cascade has been observed in neuronal, cardiac, and myometrial cells, as well as in recombinant expression systems (Fig. 2). Sauvagine and UCN1 (but not h/rCRF) activated the MAP kinase pathway in recombinant CHO and HEK293 cells overexpressing CRF_1 receptors [169,179]. Since the PKA inhibitor H89 blocked CREB (but not ERK1/2) phosphorylation in transfected CHO cells [169], CRF_1 receptor-mediated MAP kinase signaling does not appear to occur *via* the PKA pathway in this cell line. In contrast, both PKA and MAP kinase inhibitors blocked CRF_1 receptor-induced neurite growth in CATH.a cells [184]. Similarly, PKA (but not PKC) induced phosphorylation of p44/42-MAP kinase (ERK1/2) in AtT20 cells [185,186]. Exposing CRF_1 receptor-expressing human pregnant myometrial or HEK293 cells to UCN1 stimulated MAP kinase phosphorylation, possibly *via* activation of the phospholipase C pathway [179]. These studies suggest that elements in the cellular background determine whether CRF_1 receptor signaling activates the MAP kinase pathway in a particular cell system.

The role of MAP kinase in CRF_2 receptor signal transduction has also been investigated. Sauvagine was found to stimulate phosphorylation of p44/42-MAP kinase, but not p38-MAP or JNK-stress-activated protein kinases in CHO cells expressing $CRF_{2(a)}$ receptors [169]. UCN1 (but not h/rCRF) induced phosphorylation of ERK1/2 in HEK293 or CHO cells stably transfected with $CRF_{2(b)}$ receptors [179,185]. Pretreating $CRF_{2(a)}$ receptor-expressing CHO cells with PD98059, a MAP kinase MEK1/2 inhibitor, abolished sauvagine-induced phosphorylation of p44/42-MAP kinase [169]. Pretreating the same cells with the PI-3 kinase inhibitor LY294002 or the phosphoinositide-specific phospholipase C inhibitor U73122 partially attenuated sauvagine-stimulated p44/42-MAP kinase phosphorylation [169]. These findings suggest that $CRF_{2(a)}$ receptor-mediated activation of the MAP kinase pathway is calcium- and PI-3 kinase-dependent. Evidence that MEK1/2, PI-3 kinase and phospholipase C mediate $CRF_{2(b)}$ receptor-stimulated MAP kinase phosphorylation was obtained by pretreating $CRF_{2(b)}$ receptor-expressing CHO cells with the inhibitors PD98059, LY2420 02, or U73122, respectively. All three inhibitors completely suppressed UCN1-induced phosphorylation of ERK1/2 [185].

Studies indicate that UCN1 also stimulates phosphorylation of p44/42-MAP kinase in A7r5 cells and mouse neonatal cardiomyocytes endogenously expressing $CRF_{2(b)}$ receptors, possibly *via* phospholipase C, MEK1, and PI-3 kinase mechanisms [185,187]. Pretreating cardiomyocytes with manumycin A (a Ras inhibitor) or R1K1 (a Raf-1 kinase inhibitor) abolished UCN2- and UCN3-stimulated phosphorylation of ERK1/2 [187]. These data suggest that CRF_2 receptor signaling in cardiomyocytes stimulates PI-3 kinase, which, in turn, activates the Ras-Raf1 kinase pathway, inducing MEK1/2-mediated phosphorylation of p42/p44-MAP kinase. Since the PKA inhibitor H89 failed to block sauvagine-induced MAP kinase phosphorylation in transfected CHO cells [169], PKA does not appear to be involved in CRF_2 receptor-mediated activation of the MAP kinase pathway. Pretreating cardiomyocytes with the adenylyl cyclase inhibitor SQ-22,536 showed that UCN2- and UCN3-stimulated phosphorylation of ERK1/2 does not depend upon cyclic AMP generation [187]. Functional coupling of CRF_1 and CRF_2 receptors to multiple Gα proteins has been reported to occur in a yeast overexpression system [188]. The hypothesis that CRF receptors have a propensity to

couple to G proteins other than G_S is unproven and requires further investigation. A recent study has found that $CRF_{2(b)}$ receptor signaling *via* the ERK1/2 pathway depends upon the activation of residues in the receptor's first intracellular loop (especially Ser^{163} and Arg^{237}) and Tyr^{237} in the second intracellular loop [185]. A short consensus repeat (SCR) in the three-dimensional structure of the $CRF_{2(b)}$ receptor [18] may provide a site for transactivation of the EGF receptor, which would stimulate ERK1/2 phosphorylation.

The MAP kinase signaling pathway regulates synaptic plasticity by stabilizing dendritic spine structures and inducing scaffolding proteins to form localized domains of receptor signaling molecules [189]. Translocation of phosphorylated ERK1/2 to the cell nucleus activates transcription factors controlling gene expression [189,190]. The binding of βarrestin-2 to phosphorylated, membrane-bound GPCRs stimulates formation of a protein scaffold to recruit and activate ERK1/2 [190], which, in turn, regulates intracellular processes, including cell motility, chemotaxis, and apoptosis [190]. CRF receptors may activate the MAP kinase cascade *via* β-arrestin2 recruitment.

IV.4. Akt/Protein Kinase B-PI-3 Kinase Pathway

CRF receptors can also signal *via* the Akt/protein kinase B (PKB) pathway. Activation of CRF_1 receptors in LY294 002-treated rat neonatal cerebellar granule neurons by CRF, UCN1, sauvagine, or urotensin I resulted in phosphorylation of Ser^9 on GSK-3β [191]. While forskolin mimicked the effect of agonist-induced CRF_1 receptor activation on GSK-3β phosphorylation, inhibition of PKA by Rp-8-Br-cAMPS or H89 blocked it [192]. Thus, PKA phosphorylates GSK-3β in this experimental setting. The ERK-MAP kinase pathway did not play a role in CRF_1 receptor-mediated phosphorylation of GSK-3β [193]. UCN1, stresscopin-related peptide, and stresscopin have been shown to stimulate phosphorylation of Akt *via* a PI-3 kinase mechanism in $CRF_{2(b)}$ receptor-expressing cardiomyocytes [192,193]. Transfecting cardiomyocytes with a MEK1/2, Akt, or PI-3 kinase dominant negative mutants abolished the anti-apoptotic and cardioprotective effects of UCN1-, stresscopin- and stresscopin-related peptide-induced $CRF_{2(b)}$ receptor signaling [192,193].

IV.5. NOS-Guanylyl Cyclase Pathway

Agonist-activated CRF_1 receptors have been reported to signal *via* the nitric oxide synthase (NOS)-guanylyl cyclase (GC) pathway in cultured human myometrial cells [194]. Antalarmin (but not antisauvagine-30) blocked CRF-stimulated NOS expression and cyclic GMP accumulation [194]. The failure of UCN2 or UCN3 to upregulate NOS expression and GC activity in myometrial cells provides additional evidence that the CRF_1 (but not the CRF_2) receptor mediates NOS-GC pathway activation in this cell system [194]. Incubating CRF_2 receptor-expressing human umbilical vein endothelial (HUVEC) cells with CRF significantly upregulated protein levels of inducible nitric oxide synthase (iNOS) [195]. This effect of CRF was attenuated by pretreating cells with antisauvagine-30 [195]. Although CRF_2 receptors are known to activate the NOS-GC pathway in HUVEC cells, NOS expression was upregulated by activating CRF_1 (but not CRF_2) receptors in murine endothelioma H5V cells [194,195]. Thus, elements of the cellular background appear to determine which CRF receptor subtype can activate the NOS-guanylyl cyclase pathway in a particular cell system.

IV.6. Caspase Pro-Apoptotic Pathway

Exposing human retinoblastoma Y79 cells to the cytotoxic agent camptothecin (CT) upregulated caspase 3-like activity, while exposure to CRF suppressed this effect [196]. Inhibiting protein kinase A with H89 eliminated CRF-induced suppression of CT-stimulated caspase 3 upregulation [196].

IV.7. NF-κB, Nur1/Nur77, and Other Transcription Factor Pathways

In AtT20 cells, CRF_1 receptor signaling *via* the cyclic AMP-PKA pathway suppressed nuclear factor-kappaB (NF-κB) DNA-binding and transcriptional activity [197,198], processes that are necessary for POMC gene transcription [198]. CRF_1 receptor signaling also suppressed NF-κB transcriptional activity in human keratinocytes [199,200]. NGFI-B, Nur1, and Nur77 are involved in CRF_1 receptor-mediated regulation of POMC gene transcription in AtT20 cells [186,201–204]. Glucocorticoid negative feedback inhibited CRF_1 receptor-mediated Nur77-induced POMC gene transcription [204]. Further, PKA-dependent activation of the MAP kinase cascade during CRF_1 receptor signaling resulted in ERK2-induced phosphorylation of Nur77 and POMC gene transcription in AtT20 cells [186]. In other research, the sonic hedgehog protein (Shh) has been found to potentiate CRF-mediated POMC gene transcription in AtT20 cells [205]. In HaCaT keratinocytes, CRF-induced CRF_1 receptor activation increased calcium influx and the binding activity of AP-1 transcription factors regulating cell proliferation and differentiation [206].

IV.8. Role of CRF Receptor Signaling Pathways in Stress and Anxiety

The cyclic AMP-PKA pathway has been shown to regulate stress and anxiety responses. Mice with a targeted deletion of the adenylyl cyclase type 8 gene exhibited reduced anxiety-like behavior and failed to develop HPA axis super-sensitivity during chronic stress [207]. Mice with mutations of the PKA RIIβ subunit and CREB also exhibited abnormal anxiety responses [208–210]. Further, activation of CREB in amygdalar neurons produced anxiety-like behavior, suggesting that CREB-mediated mechanisms regulate anxiogenesis in the amygdala [210]. Overexpressing a constitutively active Gsα subunit in forebrain of transgenic mice disrupted prepulse inhibition (PPI) of the startle reflex [211]. Thus, anxiety-like responses may be initiated and regulated by Gs-coupled CRF receptor signaling *via* the cyclic AMP-PKA pathway.

In BALB/c mice, context-dependent fear conditioning was increased by acute stress or an ICV or intrahippocampal injection of CRF and blocked by pretreatment with a PKC inhibitor [212,213]. In contrast, central CRF administration failed to enhance conditioned fear in C57BL/6N mice [2131]. It remains to be determined if preferential coupling of hippocampal CRF receptors to Gq in BALB/c mice and to Gs in C57BL/6 mice explains these findings [213]. Another study has shown that CRF_2 receptors preferentially activate protein kinase C in LS nerve terminals during cocaine withdrawal [214]. Further investigation should be undertaken to determine if molecular mechanisms shift CRF_1 and/or CRF_2 receptor G protein-coupling and signaling cascades from one mode to another, as such shifts may be important for regulating defensive responses.

A pattern of strong ERK1/2 activation was observed in hippocampal CA1 and CA3 pyramidal cells and basolateral amygdalar neurons of mice given an intracerebroventricular (ICV) injection of CRF [215]. CRF-induced phosphorylation of ERK1/2 was absent, however, in low-anxiety mice with a conditional knockout of forebrain and limbic CRF_1 receptors [216]. Hence, CRF_1 receptor signaling *via* the MAP kinase pathway may play a role in anxiogenesis. Other work suggests that CRF_2 receptor-mediated phosphorylation of ERK1/2 in hippocampal neurons contributes to the potentiation of fear conditioning by acute stress [215].

V. CRF RECEPTOR REGULATION

V.1. Regulation of CRF_1 Receptor Signaling in Anterior Pituitary Cells

CRF-stimulated cyclic AMP accumulation and ACTH release decreased in a time-dependent manner in mouse AtT-20 pituitary tumor cells and primary cultures of rat anterior pituitary corticotropes exposed to CRF [163]. In rat anterior pituitary corticotropes, homologous

CRF_1 receptor desensitization was accompanied by a large reduction in steady-state levels of CRF_1 receptor mRNA expression [109]. In contrast, CRF_1 receptor mRNA increased in AtT-20 cells incubated with CRF [106]. *In vivo*, stress, CRF infusion, chronic glucocorticoid treatment, and adrenalectomy all downregulate anterior pituitary CRF_1 receptors [83,103,113,114,217–223]. While low levels of CRF binding have been measured in anterior pituitary membranes prepared from chronically stressed rats, anterior pituitary CRF_1 receptor mRNA expression has been observed to decrease during the first two hours of stress and then recover to normal levels as stress continues [113,114,219–221]. Chronic stress- or adrenalectomy-induced downregulation of CRF_1 receptors in anterior pituitary corticotropes is accompanied by desensitization of CRF-stimulated cyclic AMP accumulation and ACTH release [217,219]. Western blot experiments have provided evidence that adrenalectomy-induced CRF hypersecretion desensitizes and internalizes anterior pituitary CRF_1 receptors without reducing CRF_1 receptor synthesis [103,114]. A recent study has demonstrated that long-term adrenalectomy increases cytosolic levels of the 5′-leader sequence RNA binding protein that inhibits CRF_1 receptor translation [224]. Glucocorticoids have been reported to decrease CRF_1 receptor mRNA levels and downregulate CRF receptors in cultured rat anterior pituitary cells [109]. Glucocorticoids appear to influence post-transcriptional regulation of CRF_1 receptor function by inhibiting translation of CRF_1 receptor mRNA expression and/or increasing CRF_1 receptor protein degradation [113,114,223]. *In vivo*, glucocorticoid treatment generates a large, sustained reduction in CRF binding measured in anterior pituitary membranes, but only a transient decrease in anterior pituitary CRF_1 receptor mRNA expression [114].

V.2. Regulation of Brain CRF Receptor Signaling

In vivo, repeated central administration of CRF for several days downregulated CRF_1 receptors in the rat amygdala [222]. CRF_1 receptor mRNA levels increased in the rat frontal cortex and hippocampal CA3 following central CRF administration [119]. CRF receptors were also downregulated in primary cultures of fetal rat extrahypothalamic cells incubated with CRF for one to three days [225]. CRF_1 receptor mRNA expression increased in the hypothalamic PVN during acute stress and over the first 18 hours of adrenalectomy [114]. Chronic stress, glucocorticoid treatment, or adrenalectomy failed, however, to downregulate CRF receptors in brain regions outside the hypothalamus [218–221,226].

V.3. General Characteristics of CRF_1 Receptor Desensitization in Cell Lines

Exposing human retinoblastoma Y79, neuroblastoma IMR-32, or primary cultures of human pregnant myometrial cells to CRF, or exposing transfected fibroblast Ltk- or HEK293 cells to CRF or UCN1, markedly decreased cyclic AMP accumulation and rapidly desensitized and internalized membrane CRF_1 receptors without changing steady-state CRF_1 receptor mRNA levels [116–118,227–229]. Prominent desensitization of CRF_1 receptor signaling *via* both adenylyl cyclase and phospholipase C pathways was observed in transfected HEK293 cells following 24-h exposure to a high concentration of sauvagine [181]. Although CRF_1 receptors in Y79 and IMR-32 cells recovered slowly following desensitization, requiring 24 h for cyclic AMP responsiveness to CRF to be fully restored [117,118], desensitized CRF_1 receptors in primary anterior pituitary cells or transfected HEK293 cells were fully resensitized within 1–2 h after agonist removal [229,230]. Cellular differences in GRK- and/or arrestin-mediated mechanisms regulating CRF_1 receptor desensitization and internalization may determine the pace of receptor recovery.

V.4. G Protein-Coupled Receptor Kinases and CRF_1 Receptor Desensitization

Evidence suggests that GRK3 plays an important role in the homologous desensitization of CRF_1 receptors (Fig. 2). In Y79 cells, uptake of a GRK3 antisense oligonucleotide or

transfection of a GRK3 antisense cDNA construct decreased GRK3 expression by ~55% and inhibited homologous CRF_1 receptor desensitization by ~65% [227]. In addition, a large increase in GRK3 expression was observed in Y79 cells during the emergence of CRF-induced CRF_1 receptor desensitization [228]. A recent study reported that GRK2 protein levels increased in rat anterior pituitary and mouse AtT-20 pituitary tumor cells following prolonged exposure to CRF [231]. Further, transfecting AtT-20 cells with the GRK2 dominant negative mutant GRK2-K220R attenuated homologous CRF_1 receptor desensitization [231]. Although this study suggests that GRK2 plays a role in CRF_1 receptor desensitization, overexpressing GRK2 in AtT-20 cells did not increase CRF-induced desensitization of CRF_1 receptors [231]. HA-epitope-tagged CRF_1 receptors recombinantly expressed in COS-7 cells were rapidly phosphorylated following exposure to a saturating concentration of CRF, possibly *via* the action of GRK2 or GRK3 (Fig. 2) [232]. Additional evidence for GRK3-mediated regulation of CRF_1 receptor signaling includes recent data showing rapid translocation of GRK3 from cytosol to cell membrane in transfected HEK293 cells acutely stimulated with CRF [229]. In addition, CRF_1 receptors co-immunoprecipitated with GRK3 and GRK6 in membranes of HEK293 cells exposed to CRF [229]. Pretreating permeabilized CRF_1 receptor-expressing HEK293 cells with antibodies targeting GRK3 or GRK6 significantly inhibited homologous desensitization of CRF_1 receptors [229]. Substituting an alanine for Thr^{399} in the CRF_1 receptor C-terminus decreased by ~40% both phosphorylation and homologous desensitization. These data suggest that Thr^{399} may be a site for GRK3- or GRK6-catalyzed phosphorylation of the CRF_1 receptor [229].

V.5. βArrestins and CRF_1 Receptor Regulation

Recruitment of GRK3 by the CRF_1 receptor depends on Gβγ and βarrestin activity [190,233]. Arrestins promote rapid internalization of GRK-phosphorylated CRF_1 receptors by sterically hindering Gα coupling (Fig. 2) [190,233]. Overexpressing the arrestin dominant negative mutant βarrestin (319–418) in HEK293 cells co-transfected with a CRF_1 receptor cDNA inhibited homologous desensitization by 60% [229]. A recent confocal microscopy study revealed that CRF-induced activation of CRF_1 receptors triggered translocation of βarrestin1 to the cell membrane [234]. Interestingly, β-arrestin1 did not internalize along with the CRF_1 receptor into clathrin-coated pits [234]. Downregulation of CRF_1 receptors following a 24-h exposure to CRF depended upon receptor internalization [235]. The non-selective peptidergic antagonist astressin internalized and downregulated CRF_1 receptors, but at a slower pace than agonist-dependent internalization and downregulation [235]. Astressin-induced internalization of CRF_1 receptors did not depend upon agonist-induced phosphorylation of the receptor and arrestin recruitment, but did require interaction of the antagonist's N-terminus with the CRF_1 receptor's juxtamembrane domain [235].

V.6. Dysregulation of CRF Receptor Signaling in Cushing's Disease

Cushing's disease is characterized by ACTH-dependent hypercortisolemia resulting from abnormally enhanced CRF_1 receptor signaling in corticotrope adenoma cells. The level of CRF_1 receptor expression in adenoma cells is two-fold greater than in normal corticotropes [236,237]. Although CRF_1 receptor mRNA expression decreases in normal anterior pituitary corticotropes exposed to CRF, it increases in human pituitary corticotrope adenoma cells and mouse AtT-20 pituitary corticotrope tumor cells incubated with CRF [106,111,112]. Remarkably, CRF_1 receptors fail to desensitize and downregulate in Cushing's pituitary cells exposed to CRF for 4 hours to 10 days [238]. Instead, CRF-stimulated ACTH secretion increases [238]. In addition, agonist-stimulated CRF_1 receptors do not internalize but instead remain at the membrane in human corticotrope adenoma cells [239]. Hypothetically, Cushing's disease could develop as a result of inherent or induced malfunctioning of G-protein receptor kinase- and/or arrestin-mediated signaling pathways regulating homologous desensitization and internalization of CRF_1 receptors (Fig. 2).

V.7. Protein Kinase C and CRF_1 Receptor Desensitization

In retinoblastoma cells, direct activation of PKC desensitized CRF_1 receptors, an effect that was blocked by administering the PKC inhibitor bisindolylmaleimide I (BIM) or by downregulating PKC isoforms *via* a 48-h exposure to the phorbol ester PMA [240]. In transfected COS-7 cells, CRF_1 receptor phosphorylation was 2.3-fold higher than basal levels following a 5-min exposure to PMA [240]. Pretreating COS-7 cells with BIM abolished PMA-induced CRF_1 receptor phosphorylation [240]. These findings suggest that CRF_1 receptor signaling is regulated by both homologous and heterologous desensitization mechanisms (Fig. 2). Since CRF_1 receptors have been shown to activate the phospholipase C-PKC pathway [180], protein kinase C may phosphorylate and homologously desensitize CRF_1 receptors in certain cellular settings.

VI. CRF RECEPTOR SIGNALING AND BEHAVIORAL REGULATION

VI.1. Introduction

This section will focus on studies of specific CRF_1 and CRF_2 receptor functions related to animal models of mood and anxiety disorders, as larger reviews of the effects of CRF receptor activation in models of feeding and addiction can be found elsewhere [241]. Current studies of CRF-induced behavioral responses and CRF receptor distribution have focused on the hypothesis that the constellation of developmental, behavioral and physiological effects of endogenous CRF ligands may be attributed to separate functions of each of the CRF receptors [2–7,241,242].

VI.2. CRF_1 Receptor Regulation of HPA Axis and Behavioral Responses to Stress

The CRF_1 receptor is thought to be the subtype through which CRF primarily initiates its hypothalamic-pituitary-adrenal (HPA) responses to stress [2–7,114,241–243]. In rodents, CRF_1 receptor antisense has been reported to reduce avoidance behaviors in approach/avoidance conflict tests (i.e., paradigms measuring the behavioral result of two competing drives-exploration of novelty and avoidance of potential threat). Avoidance behavior is interpreted as an “anxiety-like” behavior in these paradigms. Antisense inhibition of CRF_1 receptor function has produced varied findings due to the length of treatment, magnitude and location of antisense-induced knockdown, and differences in comparison groups [244–247]. In contrast, pharmacological blockade or genetic knockout (KO) of CRF_1 receptors has been reported to attenuate many rodent defensive behaviors, including freezing [248,249], avoidance [241,250–253], startle responses [254,255], and defensive attack [256]. A study in primates reported that the CRF_1 receptor-specific antagonist antalarmin reduced both anxiety-like behaviors and HPA axis responses to social stress [257]. CRF_1 receptors may also be involved in stress-induced changes in heart rate variability, which can occur in anxiety disorders [258]. The effectiveness of CRF_1 receptor antagonists in reducing anxiety-like behavior appears to depend on an animal’s baseline anxiety-like behavior. For example, CRF_1 receptor antagonism is more effective in reducing anxiety-like behavior in rodents with high “trait” anxiety-like behavior or those previously exposed to stress [7,241,242,256,259]. These data suggest that CRF_1 receptor antagonist pharmacotherapy may be especially effective in the treatment of affectively ill patients experiencing considerable stress - particularly individuals who are either genetically predisposed to develop anxiety disorders or have been exposed to high levels of stress that “sensitize” subsequent stress responses.

Site-specific, selective antagonism of CRF_1 receptors in the central nucleus of the amygdala, but not the lateral septum, significantly increased the total duration and latency to begin freezing following shock administration [249]. Recently, a mouse was molecularly engineered to have a conditional deletion of the CRF_1 receptor gene in forebrain, hippocampal, and amygdalar neurons, but normal expression of anterior pituitary CRF_1 receptors [243]. Although

this mutant mouse exhibited robust and prolonged ACTH and corticosterone secretion in response to acute stress, its anxiety-like behavioral responses in two avoidance tests were markedly reduced [243]. These findings suggest that CRF_1 receptors in the forebrain, hippocampus and/or amygdala directly mediate the expression of certain anxiety-like behaviors independent of HPA axis functioning. Thus, compelling evidence indicates CRF_1 receptor signaling is sufficient, and in some cases necessary, to initiate many anxiety-like defensive responses, and CRF_1 receptor antagonists may be efficacious for treating anxiety and stress disorders [5–8,241,242,260].

VI.3. CRF_2 Receptor Regulation of HPA Axis and Behavioral Responses to Stress

Unfortunately, studies of the role of CRF_2 receptors in anxiety and stress responses are much less conclusive. Global manipulations of CRF_2 receptor signaling have produced conflicting results on anxiety and stress responding in rodents. Mice with a constitutive CRF_2 receptor gene deletion exhibit either increased [261,262] or normal [260] basal and stress-induced defensive behaviors as measured by approach/avoidance conflict tests (i.e. elevated plus maze and open field). The duration of the ACTH response to stress is significantly reduced in CRF_2 receptor KO mice and even more abbreviated in mice with double CRF_1/CRF_2 receptor gene deletions [263,264]. The latter finding prompts the hypothesis that CRF_2 receptors may be important for the maintenance of neuroendocrine responses to stress [260]. Conflicting results produced by CRF_2 receptor gene deletion have been attributed to differences in genetic background across the three CRF_2 receptor KO lines [265]. In addition, increased expression of CRF mRNA in the central nucleus of the amygdala, UCN1 mRNA in the Edinger-Westphal nucleus, and UCN3 mRNA in the lateral perifornical area are developmental compensations that may also account for increased anxiety- and depression-like phenotypes exhibited by certain CRF_2 receptor KO mice [5,261]. Interestingly, blockade of CRF_1 receptors normalizes the depression-like phenotype observed in CRF_2 receptor KO mice subjected to swim stress [266], while CRF_1 receptor antagonists attenuate depression-like behavior in wild-type mice and rats [267,268]. Hence, increased CRF_1 receptor signaling in CRF_2 receptor KO mice may mediate the depression-like phenotype these mice exhibit. Two studies of CRF_2 receptor antisense administration reported no effect on avoidance behaviors [244,246]. It is important to note, however, that the level of antisense-induced CRF_2 receptor reduction was very small or unspecified in these studies. In contrast, site-specific administration of a CRF_2 receptor antisense oligonucleotide, which reduced CRF_2 receptor expression ~80% in the lateral septum, significantly attenuated contextual fear-conditioned freezing [269]. Selective activation of CRF_2 receptors in the lateral septum has been shown to induce anxiety-like behaviors such as avoidance, freezing and reduced exploration [249,269,270]. Global pharmacological blockade of CRF_2 receptors has resulted in both increases [262] and decreases [271–273] in anxiety-like behaviors in approach/avoidance tests. In contrast to non-selective CRF receptor agonism, selective agonism of CRF_2 receptors with UCN2 or UCN3 suppresses locomotor activity in the elevated plus maze [274] or in a habituated environment following CRF challenge [275], but increases time spent in the open arms of the elevated plus maze [274], an anxiolytic response. These findings suggest that CRF_2 receptor activation alone can reduce certain anxiety-like behaviors and may oppose some CRF_1 receptor-mediated anxiety-like effects. Behavioral outcomes produced by CRF_2 receptor manipulations may depend greatly on the cell type or neuroanatomical substrate impacted by a particular experimental procedure.

VI.4. CRF_1 and CRF_2 Receptors and Behavioral Responses to Uncontrollable and Chronic Stressors

Studies performed in rodents show that exposure to uncontrollable, inescapable shock (IS) sensitizes the response of the dorsal raphe nucleus (DRN) serotonergic system to subsequent stressors for 24–72 hours and induces or potentiates, for the same time period, a set of fear-

and anxiety-like responses collectively termed “learned helplessness” or “behavioral depression.” These responses include enhanced fear conditioning; deficits in shuttle box escape testing; reduced food and water intake; reduced swimming when the animal is placed in water; reduced aggression and social dominance; and reduced social interaction [276]. Rats exposed to the same amount of escapable shock (ES) fail to develop these behavioral changes [276]. Importantly, effects of IS are measured in a context different from the one in which IS was delivered. Hence, uncontrollability of the stressor rather than contextual fear conditioning accounts for learned helplessness effects [276]. The DRN is one of the few brain regions in which CRF_2 receptors are significantly more abundant than CRF_1 receptors [40–43]. Moreover, it receives extensive CRF projections, possesses many CRF-immunoreactive neurons, and is a major target of a UCN1 projection from the EW nucleus [38–43,51,125, 277]. While IS excites DRN serotonergic neurons, increasing serotonin efflux in limbic and forebrain projection regions, escapable shock is without effect [278,279]. Low concentrations of CRF inhibit and high concentrations excite the DRN serotonergic system [276,280,281]. CRF is thought to produce opposite effects on serotonergic neuronal activity *via* differential activation of CRF_1 and CRF_2 receptors within the DRN. The inhibitory effect of a low concentration of CRF can be reversed by administering a CRF_1 receptor-selective antagonist [280]. In contrast, the excitatory effect of a high concentration of CRF appears to be mediated by the CRF_2 receptor, as intra-DRN injection of the CRF_2 receptor-selective agonist UCN2 or a dose of CRF sufficient to activate CRF_2 receptors has been reported to produce behavioral changes associated with learned helplessness in the absence of IS [282–284]. Further, intra-DRN injection of the selective CRF_2 receptor antagonist ASV30 before IS dose-dependently blocked the acquisition of learned helplessness, while intra-DRN administration of the selective CRF_1 receptor antagonist NBI-27914 was without effect [283]. Finally, intra-DRN injection of a low dose of CRF blocked the learned helplessness effects produced by intra-DRN administration of UCN2 [284].

While two studies have reported that systemic administration of selective CRF_1 receptor antagonists blocked IS-induced escape deficits [285,286], a third study has shown that the same antagonists were without effect [287]. These discrepant findings can be explained by the use of different protocols to measure escape behavior. Investigators who found that selective CRF_1 receptor antagonism reduced escape deficits measured escape behavior in the same context in which IS was delivered, while those who reported that selective CRF_1 receptor antagonists failed to reduce escape deficits measured escape behavior in a different context [276]. Data indicating that learned helplessness is mediated by DRN CRF_2 receptors conflict with reports that mice with constitutive deletion of the CRF_2 receptor gene exhibit increased immobility in response to forced swim stress [266]. This discrepancy may arise from compensatory changes produced by constitutive CRF_2 receptor gene deletion, including increased CRF expression in the central nucleus of the amygdala (CeA) and potential disinhibition of CRF_1 receptor signaling in the basolateral amygdala (BLA) due to the absence of CRF_2 receptors in the lateral septum [5,266].

Investigators studying the behavioral effects of forced swim stress have proposed that CRF_1 receptor-mediated inhibition of the DRN serotonergic system facilitates short-duration, active defensive responses elicited by stressors that can be rapidly terminated, while CRF_2 receptor-mediated excitation of this system promotes long-duration, passive defensive responses characteristic of learned helplessness [281]. Similarly, researchers studying the learned helplessness effects of IS have proposed that behavioral changes induced by IS can be characterized as a reduction in fight/flight defensive behaviors accompanied by sensitization of fear/anxiety-related behaviors [276]. Both groups posit that CRF_1 and CRF_2 receptors regulate the DRN serotonergic system in an opponent manner [276,281]. This hypothesis receives support from studies showing that CRF_1 receptor-mediated inhibition of serotonergic terminal release is engaged during an initial exposure to forced swimming when rats

predominantly exhibit swimming and climbing responses, but is absent during a subsequent exposure to forced swimming when active responses wane and immobility increases [281, 288–291]. Further, injecting a low dose of CRF into the DRN, which presumably activates CRF_1 but not CRF_2 receptors, decreases immobility in the forced swim test [281,291]. In contrast, activation of CRF_2 receptors in the DRN is necessary for the loss of active escape behavior induced by IS [282–284]. A recent study reports that the ventral medial prefrontal cortex (mPFCv) blocks the development of learned helplessness when a stressor is controllable by inhibiting the DRN serotonergic system [292].

Evidence suggests that the BLA and bed nucleus of the stria terminalis (BNST) play important roles in anxiogenesis. CRF receptors appear to contribute to anxiety-like behaviors mediated by these brain regions both directly and indirectly. For example, injecting the selective CRF_2 receptor agonist UCN2 into the DRN has been reported to dose-dependently stimulate serotonin release from terminal fields in the BLA [293]. The action of serotonergic $5\text{-}HT_{2C}$ receptors within the BLA is thought to play a role in anxiogenesis [294–297]. In addition to exerting indirect effects on BLA functioning, CRF receptors within this brain region may contribute directly to long-term potentiation of anxiety-like responses triggered by chronic stress exposure. For example, intra-BLA injection of a subthreshold dose of UCN1 on five consecutive days (“priming”) has been shown to potentiate anxiety-like responses in the social interaction and elevated plus maze tests for more than a month [298]. Further, chronic stress has been reported to increase CRF_1 receptor immunoreactivity in the BLA and BNST, and CRF concentrations in the BNST [299,300]. Data collected in rats indicate that CRF receptors in the BNST mediate long-term potentiation of the startle reflex by CRF or the aversive stimulus bright light [301,302]. Moreover, lesioning the BNST has been reported to block potentiated fear conditioning and escape deficits observed 24 h after IS [303]. These data suggest that CRF receptors within the DRN, BLA and BNST are involved in experience-dependent changes in stress responding, particularly when the stressor is uncontrollable or chronic, as in learned helplessness and chronic stress paradigms.

VI.5. Interactions Between CRF Receptors and Neuro-transmitter Pathways

Opposing CRF_1 and CRF_2 receptor modulation of glutamate transmission in limbic regions and serotonergic transmission in raphe nuclei has been observed [281,304]. Activation of CRF_1 receptors in the amygdala or septum has been reported to decrease and increase, respectively, glutamate transmission *via* a post-synaptic mechanism [304]. CRF_2 receptor activation opposed CRF_1 receptor-mediated effects on glutamate transmission in both brain regions, perhaps *via* pre- and post-synaptic mechanisms [304]. Hence, across two limbic structures CRF_1 and CRF_2 receptors exerted differential effects on glutamate transmission, although in both cases the two CRF receptor subtypes functionally opposed one another. CRF_1 and CRF_2 receptors also appear to modulate the DRN serotonergic system in an opponent fashion. CRF receptors in the dorsal and median raphe acutely modulate serotonergic transmission and firing rates [276,280]. Current evidence regarding the differential roles of CRF_1 and CRF_2 receptors within the DRN suggests that CRF_1 receptors localized postsynaptically on $GABA_A$ receptor-expressing neurons and CRF_2 receptors localized presynaptically on serotonergic neurons inhibit 5-HT neuronal activity, while posts-ynaptic CRF_2 receptors localized on GABAergic or serotonergic neurons indirectly or directly stimulate 5-HT terminal release [276,281,288,306–308]. Investigators have hypothesized that uncontrollable stress activates the DRN serotonergic system by increasing CRF concentrations to a level sufficient to activate CRF_2 receptors within this brain structure [281,306–308]. Thus, final behavioral output could be significantly altered by subtle differences in the relative activation of CRF_1 and CRF_2 receptors at a given time point, as well as by relative activation of CRF receptors in reciprocal brain regions such as the amygdala and septum, or the raphe nuclei and their many neuroanatomical outputs.

VI.6. Models for Oppositional Functioning of CRF Receptors

A model with relative CRF_1 and CRF_2 receptor activation as a key component of final behavioral output could explain some of the conflicting pharmacological data in the literature if basal levels or stress-induced release of endogenous agonists differed across species, strain or stressor, resulting in different relative amounts of CRF_1 and CRF_2 receptor activation. Future pharmacological studies that systematically manipulate receptor activation levels may help elucidate the relative functions of CRF receptor subtypes in each behavior. Although CRF_1 and CRF_2 receptors may regulate particular defensive behaviors in an opponent fashion, both receptors play a role in the suppression of feeding behavior [241,259]. Hence, CRF_1 and CRF_2 receptors can exert either opposing or additive effects across distinct behavioral outputs.

VI.7. CRF Receptor Regulation of Startle Plasticity

We have demonstrated both opposing and additive effects of CRF_1 and CRF_2 receptors on defensive startle behaviors (Fig. 4) [254,309]. The startle response consists of a series of involuntary reflexes elicited by a sudden, intense auditory stimulus and is considered to be a defensive behavior evolved to protect the body from impact during attack [310,311]. It is a highly conserved behavior across mammalian species and the magnitude of the response is highly plastic. Fear-inducing stimuli or administration of CRF increase startle [254,301,312], while threat-reducing stimuli and sensory input in the case of prepulse inhibition (PPI) reduce startle [311–314]. Across species, presentation of a neutral, non-startling acoustic “prepulse” 30–300 ms before the startling stimulus reduces startle magnitude, presumably by requiring the organism to allocate attentional resources to process the prepulse and hence filter or “gate” the subsequent startling stimulus [311]. Acute stress or central CRF administration modulates startle responses, reducing startle threshold as well as reducing PPI of startle (Fig. 4) [309, 312,315–318]. CRF_1 receptor activation appears to be both necessary and sufficient for CRF-induced deficits in PPI and increases in startle behavior in mice (Fig. 4) [254,309]. CRF_2 receptor activation, however, appears to oppose the action of the CRF_1 receptor on PPI while enhancing CRF-induced increases in startle (Fig. 4) [309]. CRF’s ability to disrupt PPI may be mediated by Gs-coupled CRF_1 receptor signaling *via* the cyclic AMP-protein kinase A pathway [211]. These data support a possible role of CRF receptors in altering how sensory information impacts defensive and perhaps other types of behavior. PPI of startle is a measure of the ability of sensory information to modulate behavioral outcome, i.e. how well the forebrain can inhibit responding to incoming stimuli (the startle pulse) during active processing of preceding stimuli (the prepulse). Thus, CRF_1 receptor activation may block the ability of sensory information to modulate “hardwired” defensive behaviors like startle, protecting these behaviors from inhibition when they are most needed. Given the present data, this model would predict the following: (a) the CRF_1 receptor not only activates defensive behavior, but reduces its flexibility; and (b) the CRF_2 receptor increases flexibility of a given defensive behavior. The term “flexibility” is used here to refer to the modifiability of a behavior by sensory information. The idea that CRF_2 receptors increase the flexibility of CRF_1 receptor-mediated behaviors supports the hypothesis that CRF_2 receptor activation increases “stress coping” responses by modulating the initial effects of stress-induced CRF_1 receptor activation [5,260, 261,263]. Furthermore, a model of CRF_2 receptor action on behavioral flexibility, not just activation or inhibition of a particular behavior, supports the likelihood of subtle changes in available stimuli during testing having a large impact on behavioral outcome. Interestingly, anxiety disorder patients with PTSD or panic disorder who may have overactive, sensitized brain CRF neurotransmission exhibit startle and PPI abnormalities (i.e., exaggerated startle and reduced PPI) [see VII.3 and VII.4]. Abnormal CRF receptor signaling may play a role in these symptoms.

VI.8. CRF Receptor Regulation of Learning and Memory

Recent research suggests that CRF receptors enhance learning *via* two different mechanisms: (a) hippocampal CRF_1 receptor signaling *via* the PKC pathway [270], and (b) hippocampal CRF_2 receptor signaling *via* the MAP kinase pathway [215]. In contrast, activation of septal CRF_2 receptors interferes with the performance of a learned behavioral task that may depend upon activation of dopamine D_2 receptors for its expression [270,319]. It is important to note that these studies used fear conditioning as a model of cognition. Hence, it is not clear if these effects are specific to fear memory or cognition in general. CRF receptor signaling *via* phospholipase C-PKC and MAP kinase pathways may regulate anxiety-like defensive responses by increasing cognitive processing of environmental cues to identify potential threats while simultaneously suppressing on-going reward-seeking and active defensive behaviors.

VII. EVIDENCE FOR IMPAIRED FUNCTIONING OF CENTRAL CRF SYSTEMS AND THE HPA AXIS IN ANXIETY, STRESS AND DEPRESSIVE DISORDERS

VII.1. Trait Anxiety

Behaviorally inhibited children with anxious personality traits exhibit increased salivary cortisol levels compared to non-inhibited children [320]. These children are at increased risk for developing anxiety and depressive disorders in adulthood. Genetic abnormalities that increase the sensitivity of brain CRF systems may play a role in trait anxiety.

VII.2. Early Life Stress

Exposure to stress early in life produces persistent sensitization of CRF receptor-mediated stress responses that continued into adulthood [321–323]. Adult rats separated from their dams for 180 minutes daily on postnatal days 2–14 exhibited prolonged hypersecretion of ACTH and corticosterone in response to an acute psychological stressor [323]. Adult rats subjected to maternal separation as pups also manifested increased anxiety-like behavior and alterations in brain CRF systems, including increased CRF mRNA expression in the hypothalamic PVN, CeA, BNST, and LC; increased CRF_1 receptor mRNA expression and binding in the PVN; and high CSF levels of CRF [322,323]. High CSF CRF concentrations and sensitized behavioral responses to threatening stimuli have been observed in adult bonnet macaque monkeys exposed to unpredictable stress as infants [324].

In humans, exposure to stress early in life produces long-term sensitization of HPA axis responses and increases the risk for developing anxiety and depressive disorders in adulthood [325,326]. Preschool children of mothers currently exposed to major life stressors exhibited significantly increased salivary cortisol levels, but only if the mother had been subjected to a high level of stress during the child's first year of life [325]. Another study has shown that young children exposed to adverse experiences or psychological trauma developed increased cortisol secretion during the six months following stressor exposure [327]. Adult women subjected to abuse during childhood (with and without a current diagnosis of major depression) exhibited higher levels of ACTH secretion in response to an acute psychosocial stressor than did controls [328]. The increased ACTH response was more pronounced in abused women with current depression, and these women also showed greater stress-induced cortisol and heart rate responses than controls [328]. Abused women without current depression exhibited increased ACTH responses to a CRF injection, whereas abused women with depression showed blunted ACTH responses to the same challenge [328]. Together, these studies suggest that experiencing severe stress during childhood causes persistent increases in CRF expression in limbic and hypothalamic neurons, sensitized CRF receptor signal transduction, and HPA axis hyper-responsiveness. Thus, exposure to stress early in life shapes an individual's sensitivity

and responsiveness to stress, conferring increased vulnerability to anxiety, stress and depressive disorders in adulthood [329].

VII.3. Posttraumatic Stress Disorder

Studies of male combat veterans and elderly Holocaust survivors with chronic posttraumatic stress disorder (PTSD) have reported small but significant decreases in plasma cortisol levels and increased HPA axis sensitivity to low glucocorticoid negative feedback signals [330]. Studies of other PTSD patient populations, however, have documented persistent increases in HPA axis activity following trauma. For example, a recent study reported increased salivary cortisol levels in pediatric PTSD patients [327], while another study has shown that adult women with chronic PTSD had significantly greater CRF-induced ACTH and cortisol responses compared to controls [331]. Increased basal cortisol secretion among some groups of PTSD patients may reflect a genetic abnormality conferring increased risk for the development of PTSD as well as co-morbid affective disorders [332]. Abnormally high cerebrospinal fluid (CSF) levels of CRF have been documented in PTSD patients [333,334], and these levels are highest in patients with greater illness severity and psychosis [335]. In addition, a positive correlation between elevated CSF cortisol levels and increased CSF CRF levels was observed in a group of PTSD patients [336]. Similar to the enhancing and suppressant effects on startle responding and PPI, respectively, of central CRF administration observed in preclinical behavioral studies [see VI.7], some clinical studies have found enhanced startle responding in stressful experimental situations [337,338] and reduced PPI of startle [339,340)] in patients with PTSD. The exaggerated fear-potentiated startle responses exhibited by some PTSD patients [341,342] is reminiscent of the sensitized fear conditioning observed in rodents previously exposed to inescapable, uncontrollable shock (IS) [276]. These data suggest that PTSD patients acquire learned fear more readily and exhibit stronger fear responses than controls. Other investigators, however, have documented normal fear-potentiated startle in PTSD patients [343–345].

VII.4. Panic Disorder

Recent studies have identified genetic mutations in or near the CRF gene in a group of individuals exhibiting an anxious phenotype associated with increased risk for the development of panic disorder [346,347]. Investigators also have documented abnormal HPA axis regulation, including increased basal cortisol secretion, overnight hypercortisolemia, increased frequency of ultradian cortisol secretory episodes, and HPA axis hyperactivity following dexamethasone challenge in panic disorder patients [348–351]. Other studies have reported increased salivary cortisol levels during spontaneous panic attacks [349] and higher levels of stress-induced cortisol release in panic disorder patients compared to controls [352]. High salivary cortisol levels and persistent HPA axis overactivity in panic disorder patients predict a greater frequency of panic attacks and poor clinical outcome [349–351]. CSF CRF levels in panic disorder patients, however, did not differ from those of controls [353,354]. Panic disorder patients exhibit disrupted PPI [355,356], reduced startle habituation [355,357], and increased baseline startle [355,356,357].

VII.5. Major Depression

There is consensus among psychiatric researchers that individuals with recurrent major depression are hyper-reactive to stressful life events, which can precipitate onset and relapses, prolong episodes, and worsen the clinical course of the illness [7,242,358–362]. Patients with melancholia, the most severe form of major depression, manifest behavioral and neuroendocrine signs of exaggerated CRF neurotransmission, including severe anxiety and agitation, excessive activation of the HPA axis, and high CSF CRF levels [358–361]. Symptoms of HPA axis hyperactivity such as increased cortisol secretion, resistance to

dexamethasone suppression, and reduced ACTH responsiveness to a CRF challenge increase with the severity of the major depressive episode [358–361]. Investigators have found that the level of excessive cortisol secretion exhibited by 32% of first-degree relatives of patients with unipolar or bipolar depression in response to a dexamethasone-CRF challenge is similar to that of their psychiatrically ill kin [363]. Thus, HPA axis hyper-responsiveness may constitute a genetic marker for a subtype of depression.

Although it is not yet possible to investigate central CRF system functioning in depressed patients *in vivo*, recent efforts to synthesize CRF receptor ligands detectable by positron emission tomography may eventually enable researchers to visualize CRF receptor activity in the central nervous system [126,364]. The predominant hypothesis that CRF hypersecretion plays a leading role in the pathogenesis of major depression receives support from studies showing that severely depressed patients exhibit high CSF levels of CRF [358,361], and from postmortem studies of depressed suicide victims documenting very high CSF CRF levels [365]; increased CRF mRNA expression in certain regions of the frontal cortex [366]; and increased concentrations of CRF-immunoreactivity in the prefrontal and frontal cortex, locus coeruleus, and median and dorsal raphe nuclei [366–370]. Consistent with the finding that patients with melancholia manifest the highest incidence of HPA axis hyperactivity, studies of depressed suicide victims have revealed increased CRF mRNA expression and CRF-immunoreactivity in hypothalamic PVN neurons [371,372]. Although one postmortem study of depressed suicide victims reported downregulation of CRF_1 receptors in frontal cortex [373], two subsequent studies were unable to replicate this finding [366,374]. In addition, reduced CRF_1 receptor mRNA levels were found in the frontopolar cortex (but not in the prefrontal cortex) of depressed suicide victims [369]. Another study reported that CRF_1 receptor mRNA expression was the same in anterior pituitary of depressed suicide victims and controls [131]. Finally, a preliminary report noted that CRF_1 receptor binding was lower in the ventral basolateral amygdaloid nucleus of suicide victims compared to controls [375].

Several investigators have proposed that CRF hyper-secretion alone accounts for the symptoms of increased central CRF neurotransmission characteristic of patients with severe depression [242,358]. This hypothesis has not been rigorously tested and is inconsistent with current concepts concerning GPCR regulation [190]. Since agonist-activated CRF_1 receptors normally undergo rapid phosphorylation desensitization, and internalization in response to high agonist concentrations (Fig. 2) [116–118,163,227–232], it is unlikely that CRF hypersecretion alone is sufficient to account for the enhancement of central CRF neurotransmission seen in severe depression. Future research will likely show that abnormalities in the complex dynamic processes regulating CRF_1 and CRF_2 receptor phosphorylation, desensitization, internalization, and recycling, as well as shifts in CRF receptor-mediated intracellular signaling cascades, contribute importantly to the pathophysiology of depression. Hypothetically, CRF hypersecretion could produce exaggerated CRF receptor signaling if it were accompanied by a genetically- or environmentally-induced deficit in the functioning of one or more mechanisms regulating CRF receptor desensitization and internalization. Small molecule CRF_1 receptor antagonists are being developed as novel treatments for depression based on the concept that abnormally enhanced central CRF_1 receptor signaling contributes to the pathophysiology of major depression. In a preliminary clinical trial, the CRF_1 receptor antagonist NBI-30775 (R121919) decreased depression and anxiety scores in patients with major depression, presumably by blocking the activity of hyperactive CRF_1 receptors in brain regions mediating symptoms of depression [376].

VII.6. Bipolar Disorder

Abnormal HPA axis functioning has been detected in bipolar patients during both mania and depression, with the highest levels of cortisol hypersecretion and decreased HPA axis

sensitivity to glucocorticoid negative feedback signals occurring during mixed states and severe mania [377,378]. The magnitude of circulating cortisol levels is positively correlated with the severity of the mixed state, and with clinical symptoms of stage III mania such as dysphoria, high levels of anxiety, excessive fearfulness and paranoid ideation, hyperarousal, and psychomotor agitation [378]. Thus, hyperactive central CRF systems may play an important role in the induction of symptoms associated with mixed states and severe mania.

VII.7. Clinical CRF_2 Receptor Studies

To date, CSF levels of urocortins have not been measured in patients with anxiety, stress or depressive disorders, nor have any postmortem studies of depressed suicide victims assessed central mRNA expression levels for urocortins. One recent study has reported that CRF_2 receptor mRNA levels in several cortical regions did not differ in postmortem samples from controls and depressed suicide victims [369]. Another study indicated that the level of CRF_2 receptor mRNA was the same in the anterior pituitary of depressed suicide victims and controls [131]. A preliminary report did not detect any differences in CRF_2 receptor binding in the ventral basolateral amygdaloid nucleus of suicide victims and controls [375]. However, the role of UCN2, UCN3, and the CRF_2 receptor in anxiety disorders and major depression requires more intensive investigation. If future studies determine that the anxiogenic/depressogenic hypothesis of CRF_2 receptor function is correct, the development of a small molecule CRF_2 receptor antagonist may prove to be an important therapeutic breakthrough in the treatment of affective disorders. If the anxiolytic/antidepressant hypothesis of CRF_2 receptor function is proven, decreased central expression and release of UCN2 and UCN3, or deficient CRF_2 receptor signaling would be expected to contribute to the pathophysiology of anxiety, stress, and depressive disorders by impairing CRF_2 receptor-mediated counterregulation of central stress responses.

VIII. GENETICS AND STRESS HYPERSENSITIVITY

Recent evidence suggests that genetic abnormalities in brain CRF systems contribute to the pathophysiology of anxiety, stress and depressive disorders. A single nucleotide polymorphism (SNP) in the coding sequence for the CRF gene has been linked to trait anxiety in children with a familial history of panic disorder [347]. SNP analyses also have identified a GAG haplotype of the CRF_1 receptor in a subpopulation of patients with major depression whose high levels of anxiety predict a higher response rate to antidepres-sant treatment [379]. Further, quantitative trait loci (QTL) mapping of inbred BxD recombinant mice identified important elements for the genetic regulation of CRF and CRF_1 receptors on chromosome 12 [380]. Central CRF systems also may play an indirect role in the pathogenesis of anxiety, stress and depressive disorders *via* regulation of genetically altered components of the DRN serotonergic system. Infant Rhesus monkeys with a SNP in the promoter region of the serotonin transporter (5-HTT) gene that decreases 5-HTT expression exhibited greater emotional distress and HPA axis activation in response to early life stress than did infant monkeys without the SNP [381]. Reduced 5-HTT function likely increases the effect of CRF_2 receptor activation on serotonergic neurotransmission within the DRN and its projection sites, thereby potentiating the development of anxiety- and depression-like responses. In humans, the presence of a shortened allele for the 5-HTT gene, which is caused by a SNP in the gene's promoter region, is associated with high levels of innate trait anxiety [382]. Individuals exposed to stress early in life or in adulthood develop depression more readily if they have the short rather than the long allele for the 5-HTT gene [383].

IX. CONCLUDING REMARKS

During the past ten years, the discovery of exciting new technologies has enabled investigators to move the field of GPCR research forward at a rapid pace [190]. New molecular techniques

now make it possible to delete or overexpress receptors and their regulatory factors in model cell systems and in targeted brain regions; identify amino acid motifs regulating receptor interactions with agonists and intracellular regulatory factors using site-directed mutagenesis; detect changes in gene transcription using microarray screening; discover novel receptor-protein interactions using yeast two-hybrid screens; and track real-time interactions between receptors and other signaling molecules using confocal microscopy and bioluminescence resonance energy transfer (BRET). A recent confocal fluorescence microscopy study revealed that the agonist-activated CRF_1 receptor preferentially binds β-arrestin2 over β-arrestin1 [384]. Further, another study using site-directed mutagenesis and confocal microscopy showed that β-arrestin2 binding occurred at phosphorylation-dependent and phosphorylation-independent sites in the CRF_1 receptor's carboxyl terminus and intracellular loops, respectively [Oakley *et al*, submitted for publication] (see Fig. 2). Finally, a recent fluorescence resonance energy transfer (FRET) study found that CRF_1 receptors form dimers [385], a process that may contribute to the failure of β-arrestin2 to traffick with the receptor into endocytic vesicles [384,385]. These new research tools will make it possible to elucidate aspects of CRF receptor regulation and function that are not well understood at present, including (a) mechanisms that switch CRF receptor signaling from one mode of G protein signaling to another and the functional significance of such alterations; (b) intracellular mechanisms regulating the magnitude and duration of CRF receptor signaling; (c) brain region-specific behavioral effects of central CRF_1 and CRF_2 receptor activation; (d) the opponent manner in which CRF_1 and CRF_2 receptors modulate neuro-transmission in various brain regions; (e) CRF receptor-mediated mechanisms contributing to long-term sensitization of fear and anxiety-like responses and fear learning; and (f) the contribution of genetic variability in CRF systems functioning to the pathogenesis of affective and stress disorders. Finally, future investigations should attempt to clarify why non-peptidic CRF_1 receptor antagonists are most effective in reducing rodent anxiety-like behavior when they are administered to animals bred for high "trait" anxiety-like behavior or previously exposed to stress [7,241,242,256,259]. Interestingly, CRF_1 receptor antagonists do not appear to have an anti-depressant effect when rodents are tested in the traditional or modified versions of the forced swim test, or in the tail suspension test [386]. However, three different CRF_1 receptor antagonists have been shown to significantly reduce immobility in the forced swim test when administered chronically to Flinders Sensitive Line (FSL) rats, a strain that has been bred to express high levels of immobility in this test [386]. Similarly, CRF_1 receptor antagonists counteracted some of the effects of chronic mild stress when administered chronically [386]. Future studies should determine if the therapeutic effects of chronic CRF_1 receptor antagonist treatment in some animal models of depression result from adaptational changes in downstream signal transduction cascades. Together, these data suggest that CRF_1 receptor antagonist pharmacotherapy may be especially effective in treating individuals who are genetically predisposed to develop anxiety and depressive disorders or who have been exposed to high levels of stress that "sensitize" subsequent stress responses. Future investigations into the neurobiology of CRF receptor signaling will provide important insights into the complex cellular and molecular mechanisms underlying normal and pathological defense mechanisms, and their contribution to onset and relapse of anxiety, stress, and depressive disorders.

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ABBREVIATIONS

CRF
Corticotropin-releasing factor

CRF_1
CRF type 1 receptor

CRF_2
CRF type 2 receptor

UCN1-3
Urocortin type 1-3

URO
Urotensin I

SVG
Sauvagine

GPCR
G protein-coupled receptor

PKA
Protein kinase A

CREB
Cyclic AMP-response-element binding protein

pCREB
Phosphorylated CREB

PKC
Protein kinase C

MAP kinase
Mitogen-activated protein kinase

ERK
Extracellular signal-regulated kinase

MEK
Mitogen-activated protein kinase kinase

JNK
C-Jun kinase/stress-activated protein kinase

PI-3 kinase
Phosphatidylinositol 3-kinase

Akt/PKB
Acutely transforming retrovirus AKT8 in rodent T-cell lymphoma/protein kinase B

GSK-3β
Glycogen synthase kinase-3beta

GRE
Glucocorticoid response element

AP-1
: Activating protein-1 transcription factor

Nurr1
: Nur-related transcription factor 1

Nur77
: Nerve growth factor-inducible B (NGFIB) transcription factor

NF-κB
: Nuclear factor-kappa B

HEK cells
: Human embryonic kidney cells

CHO cells
: Chinese hamster ovary cells

CATH.a cells
: Catecholaminergic mouse brainstem tumor cells

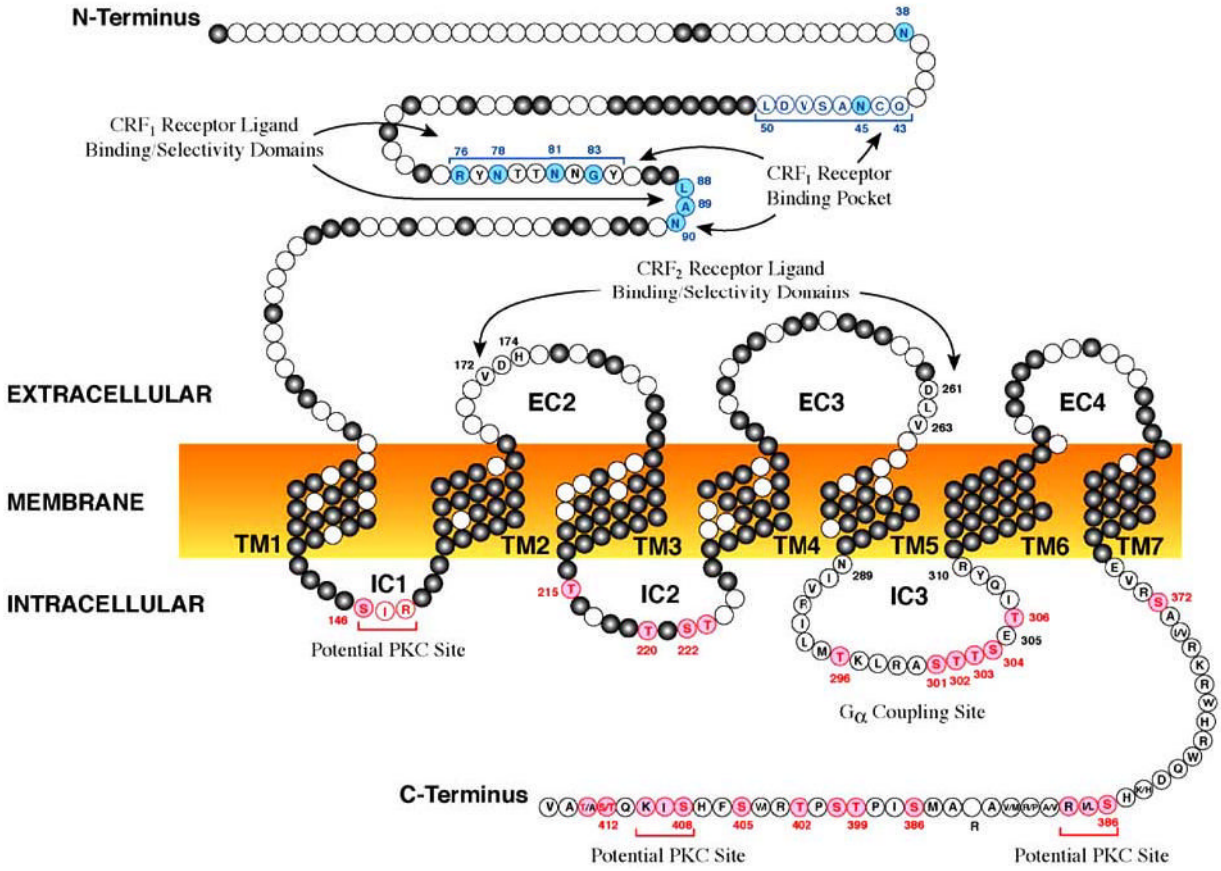

Fig 1. Diagram of the human CRF_1 and CRF_2 receptors

Depiction of the full-length, wild-type sequence indicates important extracellular amino acids comprising the binding pocket and ligand selectivity domain for both CRF receptors. Serines and threonines (red circles) located in CRF receptor intracellular loops and C terminus represent potential sites for phosphorylation by GRK and PKC isoforms.

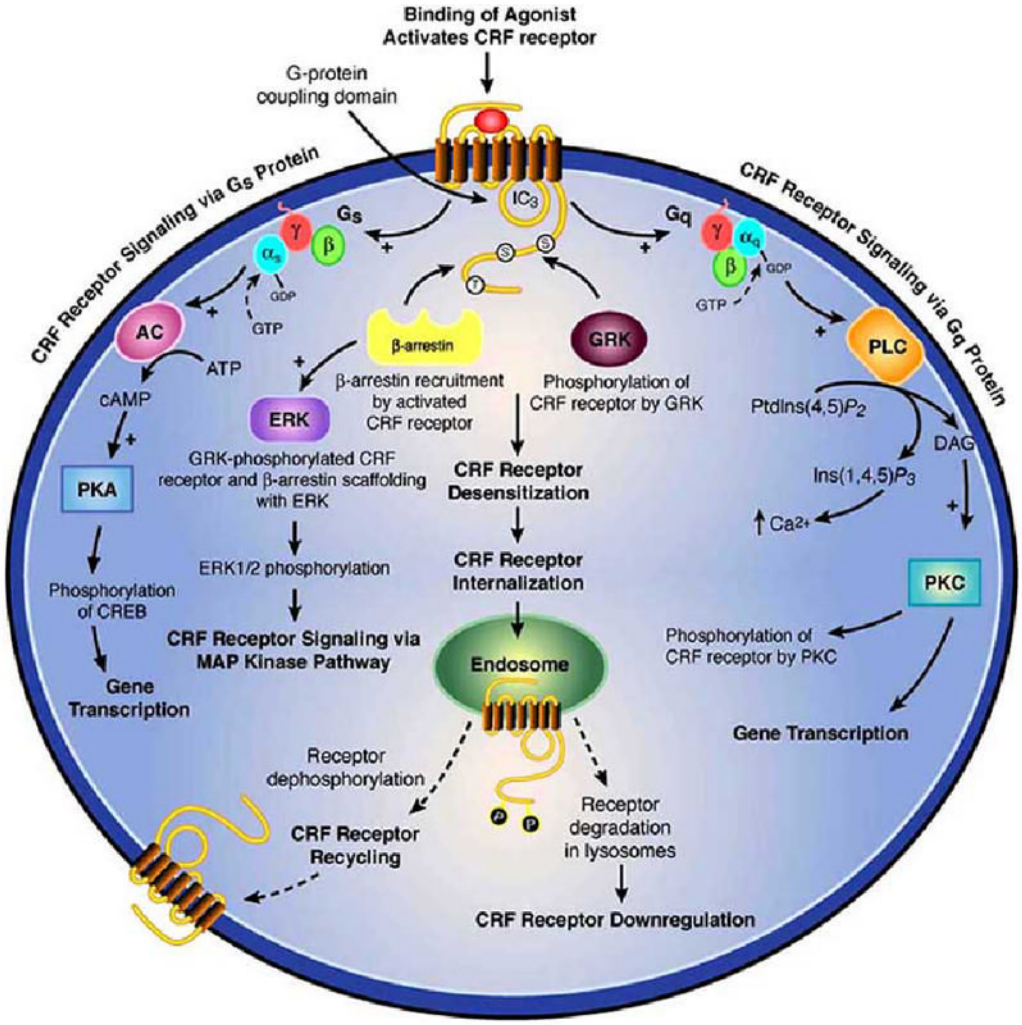

Fig 2. Major intracellular pathways for signal transduction by CRF_1 and CRF_2 receptors

Recent evidence indicates that CRF_1 receptors are regulated by GRK- and PKC-mediated phosphorylation and by interaction with β-arrestins [3,116–118,227–235].

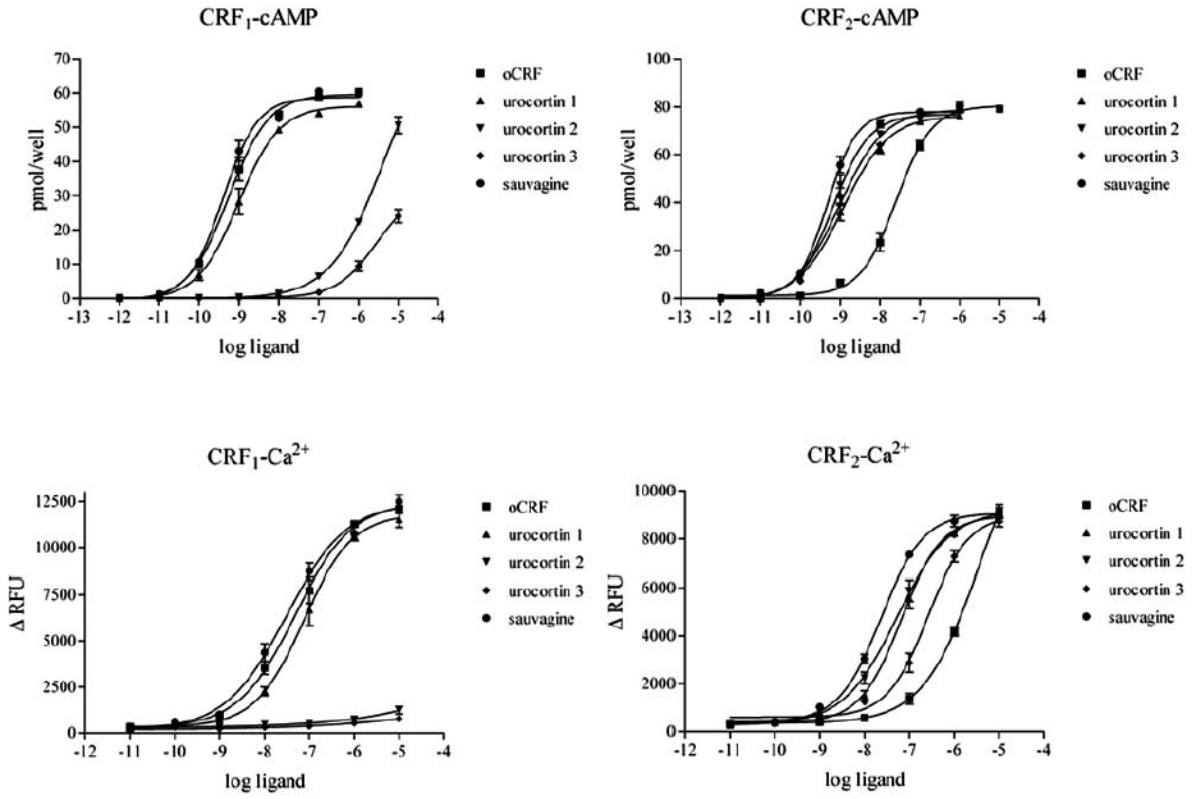

Fig 3.

Agonist-stimulated signaling of human CRF_1 and CRF_2 receptors *via* intracellular cyclic AMP accumulation and transient calcium mobilization. Concentration-response curves were generated for cyclic AMP accumulation (two upper panels) and calcium Ca^{2+} mobilization (two lower panels) stimulated by incubating HEK293 cells stably expressing human CRF_1 and $CRF_{2(a)}$ receptors with various agonists (0–10 μM) for 10 min at 37°C. The data points represent mean ± SEM for triplicate determinations of cyclic AMP (pmol/well) or Ca^{2+} mobilization (relative fluorescence units, RFU).

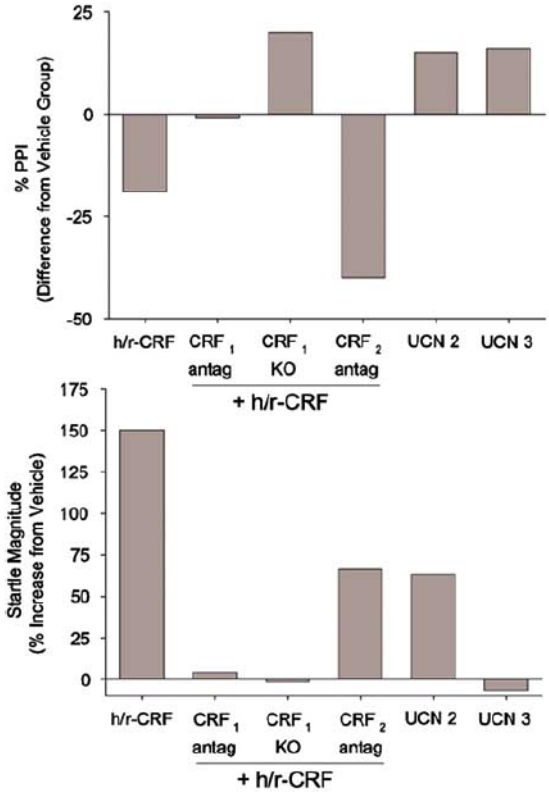

Fig 4. Comparison of effects on acoustic startle plasticity resulting from manipulations of CRF_1 and CRF_2 receptor signaling

Top Panel: CRF_1 and CRF_2 receptors exhibit opposing actions on prepulse inhibition (PPI) of startle. Bar graph data represent difference scores (%PPI-%PPI of respective vehicle group) after the following *in vivo* manipulations: (a) intracerebroventricular (ICV) injection of agonist alone, h/rCRF (0.2 nmol); (b) h/rCRF (0.2 nmol ICV) + CRF_1 receptor antagonist NBI-30775 (20 mg/kg intraperitoneal, IP); (c) h/rCRF (0.2 nmol ICV) in CRF_1 receptor knock-out mouse; (d) h/rCRF (0.2 nmol ICV) + CRF_2 receptor antagonist antisauvagine-30 (3 nmol ICV); (e) CRF_2 receptor-selective agonist, urocortin 2 (2 nmol ICV); and CRF_2 receptor-selective agonist, urocortin 3 (2.4 nmol ICV). *Bottom Panel:* CRF_1 and CRF_2 receptor signaling may exhibit an additive action on acoustic startle magnitude. Bar graph data represents percentage change from respective vehicle startle magnitude [(startle magnitude - startle magnitude of vehicle)/startle magnitude of vehicle) X 100]. The startle data summarized in this graph is described in our two recent publications [254,309].

Table 1

Distribution of CRF Ligands and CRF Receptors in Brain Regions Relevant to Stress, Anxiety and Depressive Disorders

Brain Region	CRF Ligand Localization	CRF Receptors	Adaptational and Pathophysiological Changes
Neocortex[a]	CRF neurons	Only CRF_1 expressed in rodent neocortex; both CRF receptors expressed in primate neocortex.	
Hypothalamus			
Paraventricular Nucleus (PVN)b	Abundant CRF neurons UCN 1 fibers UCN2 and UCN3 neurons	No PVN CRF receptor expression in basal state.	1. Stress ↑CRF, UCN2, UCN3 mRNA 2. Glucocorticoid administration ↑ UCN2 mRNA 3. Stress including maternal separation ↑ CRF1 mRNA & binding sites 4. Depressed patients committing suicide ↑ CRF- & AVP-expressing neurons ↑ CSF CRF levels
Medial Preoptic Nucleus	UCN 3 neurons	CRF_2	
Ventromedial Nucleus (VN)[c]	UCN3 terminals from medial amygdala Some UCN1 fibers	CRF_2	1. Stress including maternal deprivation ↓ $CRF_{2(a)}$ receptor mRNA 2. Glucocorticoid administration ↓ $CRF_{2(a)}$ receptor mRNA
Dorsomedial Nucleus	CRF neurons	$CRF_1 \gg CRF_2$	
Arcuate Nucleus	UCN1, UCN2 & UCN3 fibers CRF neurons	$CRF_1 > CRF_2$	
Hippocampus			
Entorhinal Area		$CRF_1 + CRF_2$	
CA1 & CA3, Dentate Gyrus[d]		$CRF_1 > CRF_2$	
Amygdala			
Central Nucleus (CeA),[ef]	Abundant CRF neurons; UCN1 fibers	CRF_1	1. Glucocorticoid administration or stress ↑ CRF mRNA 2. CRF_2 knockout mouse ↑ CRF mRNA
Medial Nucleus[h]	Abundant UCN3 neurons Some CRF fibres	$CRF_1 = CRF_2$	1. Stress ↑ UCN3 mRNA 2. Glucocorticoid adminstration ↑ UCN3 mRNA
Cortical Nuclei	UCN3 fibers; some UCN1 fibers	$CRF_2 > CRF_1$	
Septum			
Lateral Nucleus (LS)[i]	CRF neurons & fibers UCN1 fibers from the EW nucleus UCN3 fibers from medial hypothalamus	CRF_2	
Medial Nucleus (MS)		$CRF_1 \gg CRF_2$	
Bed Nucleus of the Stria Terminalis (BNST)[g]	CRF fibers from CeA UCN3 fibers from perfornical area	$CRF_1 = CRF_2$	1. Stress ↑ CRF mRNA
Nucleus Accumbens (NAC)	CRF neurons & fibers	CRF_1	
Edinger-Westphal Nucleus (EW)[k]	UCN1 neurons		1. Stress ↑ UCN1 mRNA
Locus Coeruleus (LC)	Abundant UCN2 neurons UCN1 fibers CRF fibers from CeA, BNST, PVN and PGi	CRF_1 & CRF_2 mRNA not detectable in rodent LC; primate & human LC express CRF_1 but not CRF_2 receptors.	1. Acute & chronic stress ↑ CRF mRNA & CRF-immunoreactive content 2. Maternal separation ↑ CRF_1 mRNA 3. Depressed patients committing suicide ↑ CRF-immunoreactive content
Dorsal Raphe Nucleus (DRN)[l]	UCN1 fibers from EW nucleus CRF fibers	$CRF_2 \gg CRF_1$	1. Depressed patients committing suicide ↑ CRF-immunoreactive content
Periacqueductal Gray (PAG)	UCN1 fibers from EW nucleus Some UCN 3 & CRF fibers	CRF_2	
Anterior Pituitary	None	CRF_1 (corticotrophs) CRF_2 (gonadotrophs)	1. Stress

Brain Region	CRF Ligand Localization	CRF Receptors	Adaptational and Pathophysiological Changes
			↓ CRF_1 receptors & ↑ CRF1 mRNA 2. Glucocorticoid administration ↓ CRF_1 receptors after transient ↑ CRF1 mRNA 3. CRF_1 receptor mRNA ↑↑ Cushing's Disease 4. Stress or glucocorticoid administration ↓ CRF_2 mRNA.

[a] CRF_1 receptor mRNA is widely expressed in rodent prefrontal, anterior cingulate, and frontoparietal cortex, with especially high levels in layer IV [40–43]. Both CRF_1 and CRF_2 receptors are expressed, however, in the primate neocortex, especially in prefrontal and cingulate cortices [97,130].

[b] ~2000 CRF cell bodies are present in the PVN which contains the highest number of CRF neurons in the central nervous system [38].

[c] The hypothalamic ventromedial nucleus possess the highest levels of CRF_2 receptors and UCN3 terminals within the hypothalamus [40–43,52].

[d] High expression of CRF_1 (but not CRF_2) receptors occurs in the primate dentate gyrus [97,130].

[e] Dopaminergic (DA) terminals have been localized to CRF-expressing neurons in CeA and the BNST [123]. The CeA expresses D_2 receptors while the BNST expresses D_1 receptors[123]. Therefore, mesocorticolimbic DA neurotransmission, which has been implicated in fear and anxiety, may regulate CRF neurons in the CeA and BNST.

[f] Recently, using electron microscopic immunocytochemistry, CRF_1 receptor protein was detected in membrane and intracellular organelles of rat CeA neurons [129]. CRF_1 receptor-immunoreactive dendrites were located in the vicinity of CRF-expressing axons. Because CeA neurons appeared to retain many CRF_1 receptors in a cytosolic reserve [129], CRF_1 receptors in the CeA may be subject to rapid internalization and downregulation. CRF_1 receptor mRNA and binding have been detected in the primate LC and mRNA for both CRF receptors in the primate CeA [97].

[g] CRF_2 receptor knockout mice develop large increases in CRF mRNA in the CeA, UCN1 mRNA in the EW, and UCN3 mRNA in the lateral perifornical area [5,261]. CRF_2 receptors may suppress release of CRF receptor ligands at brain synapses. CRF_2 receptor knockout mice exhibit anxiety-like behavior and stress hypersensitivity [5,261], possibly due to excessive CRF_1 receptor activation.

[h] Outside of the hypothalamus, the majority of UCN3 neurons are localized to the dorsal division of the medial amygdaloid nucleus [52]. High densities of UCN 3 terminals are foundin the LS, posterior BNST, and medial amygdala, which are brain structures with high CRF_2 receptor expression [40–43, 52].

[i] Perifornical UCN 3 neurons densely project to the LS, posterior BNST, and the VMH [52].

[j] A recent study has detected CRF_2 receptor mRNA expression in the VTA using RT-PCR [124].

[k] Very high concentrations of UCN 1 neurons in the EW nucleus project to the intermediate LS in the forebrain and to the dorsal raphe in hindbrain, which expressabundant CRF_2 receptors [40–44]. UCN 1 may be the major ligand for the hindbrain CRF_2 receptor system.

[l] The CRF_2 receptor appears to be the predominant subtype in the dorsal raphe [39–43]. Only very low CRF_1 receptor mRNA expression has been detected in the DRN [40–43].

Table 2

Affinities of Agonists Binding to Human and Amphibian CRF_1 and CRF_2 Receptors

Peptide	$hCRF_1$ (K_i)	$xCRF_1$ (K_i)	$hCRF_{2(a)}$ (K_i)	$hCRF_{2(b)}$ (K_i)	$xCRF_2$ (K_i)
Human CRF	1.5 nM	2.8 nM	42 nM	47 nM	130 nM
Ovine CRF	1.1 nM	34 nM	230 nM	320 nM	480 nM
Urocortin 1	0.3 nM	1.2 nM	0.4 nM	0.4 nM	0.8 nM
Urocortin 2	3,500 nM	2,000 nM	3.6 nM	4.5 nM	2.1 nM
Urocortin 3	>10,000 nM	8,800 nM	9.1 nM	12.6 nM	8.4 nM
Urotensin I	0.4 nM	4.9 nM	1.8 nM	5.7 nM	12 nM
Sauvagine	0.7 nM	62 nM	0.5 nM	2.1 nM	1.0 nM

Inhibitory binding constants (K_i) were measured in competitive binding experiments using HEK293cells stably expressing CRF_1 and CRF_2 receptors. ^{125}I-Tyr^0-human CRF was used as the radioligand for the Xenopus (x) CRF_1 receptor and ^{125}I-Tyr^0-SVG was used as the radioligand for human (h) CRF_1, $hCRF_{2(a)}$, $hCRF_{2(b)}$ and $xCRF_2$ receptors.