Corticotropin-Releasing Factor Receptor Antagonists: Potential Novel Therapies for Human Disease

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The concept that the hypothalamus plays a primary role in the regulation of the hypothalamus pituitary-adrenocortical (HPA) axis was first proposed more than a half a century ago by Sir Geoffrey Harris. Soon after, using extracts from the hypothalamus, two groups, Guillemin and Rosenberg [1] and Saffran and Schally [2], independently observed that a factor contained within these extracts could potently stimulate the release of **adrenocorticotropic hormone** (ACTH, corticotropin; Prod. No. A 0423) from anterior pituitary cells *in vitro*. This hypothalamic extract was named **corticotropin-releasing factor** (CRF; Prod. No. C 3042), although it was another 30 years before Vale and colleagues, working at the Salk Institute, purified and characterized material from sheep hypothalami and determined its structure to be a 41 amino acid peptide [3]. The *in vitro* and *in vivo* biological activity of this peptide fulfilled all the criteria to confirm that this peptide as the primary mediator of the HPA axis and responses to physical, emotional and environmental stress. In the decade that followed, many studies demonstrated the importance of this peptide in the central nervous system (CNS) where it produces a wide spectrum of autonomic, electrophysiological and behavioral effects consistent with its role as a neurotransmitter or neuromodulator.

Within the past ten years, the cloning of multiple CRF receptor subtypes and a specific CRF binding protein, together with the identification of other peptide family members (i.e. the urocortins), has precipitated a new era in CRF research. Moreover, these discoveries have expanded the notion that this system plays a more complex role in the CNS than was previously thought.

The CRF Receptor Family

CRF receptors belong to the superfamily of G protein-coupled receptors and fall within the recently described, and still growing family of "brain-gut" neuropeptide receptors. This family includes receptors for **calcitonin** (Prod. No. T 3535), **vasoactive intestinal peptide** (Prod. No. V 6130), **parathyroid hormone** (Prod. No. P 7036), **secretin** (Prod. No. S 7147), **pituitary adenylate cyclase-activating peptide** (Prod. No. A 1439), **glucagon** (Prod. No. G 1774) and **growth hormone-releasing factor** (Prod. No. G 8895). These receptors all share considerable sequence homology, and function through the coupling of a stimulatory guanine-nucleotide binding protein (G protein) and activation of **adenylyl cyclase** (Prod. No. A 0951) in response to their respective agonists. Almost a decade following the isolation and characterization of CRF itself, Vale and colleagues were the first to clone the CRF_1 receptor from a human ATCH-secreting adenoma using the technique of expression cloning [4]. The protein was characterized as containing 415 amino acid residues with seven putative transmembrane domains and five potential extracellular N-linked glycosylation sites. The human gene has since been localized on chromosome 17 at position 17q12-22 [5,6]. Several groups subsequently reported the cloning of cDNAs from other species, including rat and mouse [4,7-9]. All species of CRF_1 receptor mRNAs thus far identified encode proteins of 415 amino acid residues that are 98% homologous to one another. Moreover, the mRNA distribution for the CRF_1 receptor correlates well with the distribution of previously identified CRF binding sites in brain. While there have been examples in the literature describing splice variants of the CRF_1 receptor mRNA, these have not yet been demonstrated to produce physiologically functional proteins [10-12].

Following the cloning and characterization of the CRF_1 receptor, a second receptor subtype was identified and named the CRF_2 receptor [13]. This protein was cloned from a variety of species [13-15] and shown to exhibit approximately 71% homology to the CRF_1 receptor. Subsequently it has been shown to exist as three independent splice variants differing from each other only at the amino terminus. Indeed, the sequence beyond the wholly conserved glycine residue at position 41 (using the numbering of human CRF_1 receptor sequence) is identical for all three CRF_2 receptor isoforms. These isoforms have been given a number of different designations in the literature, but are now referred to as $CRF_{2(a)}$, $CRF_{2(b)}$ and $CRF_{2(c)}$ according to the IUPHAR receptor nomenclature committee [16]. Figure 1 shows a schematic representation of the two receptor subtypes and the three isoforms of the CRF_2 receptor. In the figure, the blue spheres represent the amino acid sequence of the CRF_1 receptor and those amino acids that are common between the CRF_1 and CRF_2 receptor subtypes. Those amino acid residues depicted in yellow represent the substitutions in the amino acid sequence that exist in the CRF_2 receptor.

About the Author

Dimitri Grigoriadis received his Ph.D. in 1987 from the University of Toronto, Ontario, Canada for work that focused on the pharmacological characterization of dopamine receptors and their role in diseases such as schizophrenia. Following post-doctoral research at the National Institute on Drug Abuse, where he began working on the biochemistry and localization of CRF receptors, he joined the Neuroscience group at The Du Pont Pharmaceutical Company. In 1993, he joined Neurocrine Biosciences Inc. in San Diego where he led a collaborative effort with Janssen Pharmaceuticals to identify non-peptide, orally active CRF receptor antagonists. This collaboration culminated in the selection of the first CRF_1 receptor antagonist to be tested in a the clinic for the treatment of major depression. He is currently Senior Director of Pharmacology and Lead Discovery at Neurocrine Biosciences Inc. where he leads a discovery team in collaboration with GlaxoSmithKline that aims to develop next generation CRF receptor antagonists.

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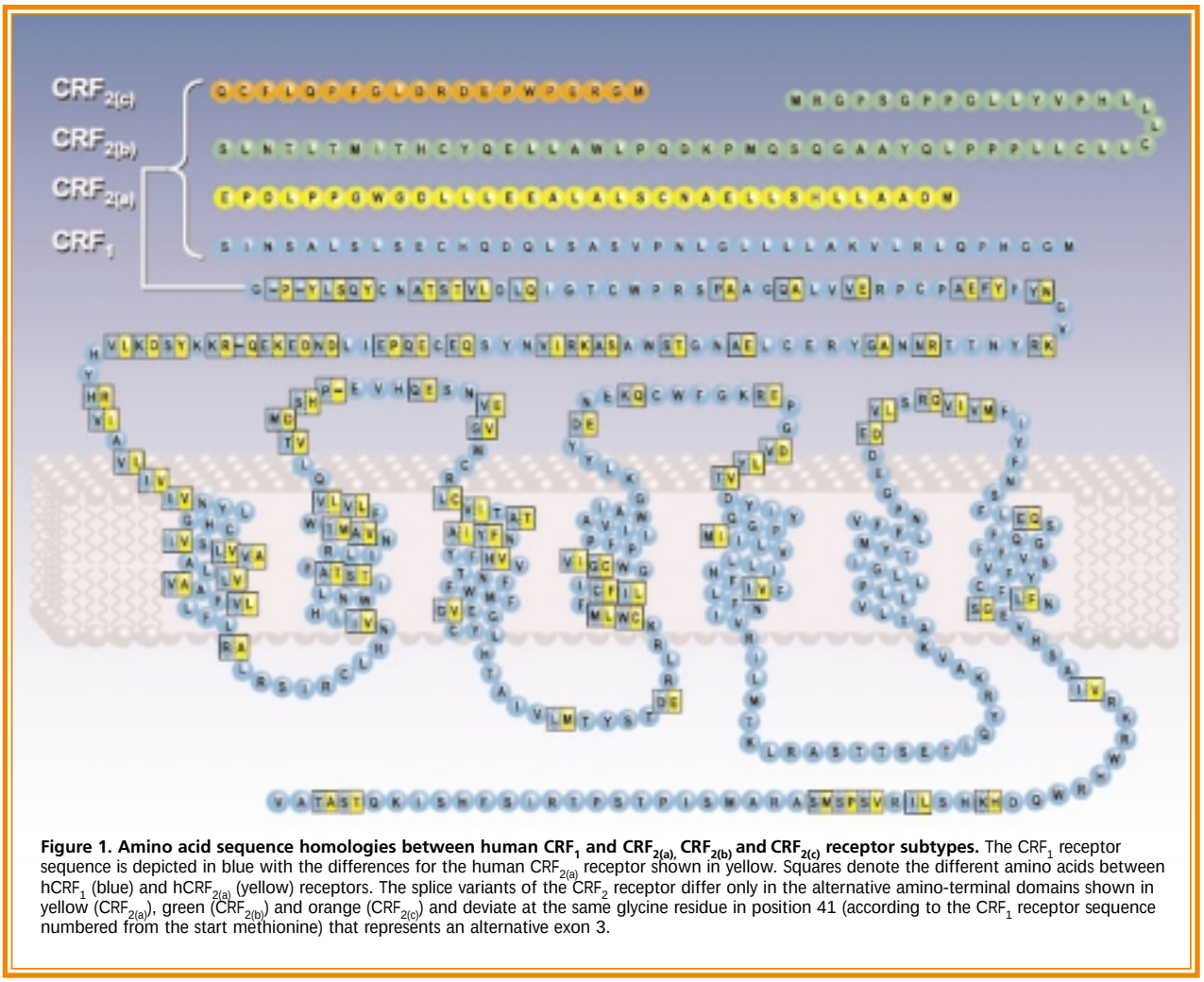

Figure 1. Amino acid sequence homologies between human CRF_1 and $CRF_{2(a)}$, $CRF_{2(b)}$ and $CRF_{2(c)}$ receptor subtypes. The CRF_1 receptor sequence is depicted in blue with the differences for the human $CRF_{2(a)}$ receptor shown in yellow. Squares denote the different amino acids between $hCRF_1$ (blue) and $hCRF_{2(a)}$ (yellow) receptors. The splice variants of the CRF_2 receptor differ only in the alternative amino-terminal domains shown in yellow ($CRF_{2(a)}$), green ($CRF_{2(b)}$) and orange ($CRF_{2(c)}$) and deviate at the same glycine residue in position 41 (according to the CRF_1 receptor sequence numbered from the start methionine) that represents an alternative exon 3.

The three CRF_2 receptor isoforms, differing only in their N-terminal domains are shown in yellow, green and orange for the $CRF_{2(a)}$, $CRF_{2(b)}$ and $CRF_{2(c)}$ receptors, respectively. The $CRF_{2(b)}$ receptor, which has been cloned from rat, mouse and human, contains 431 amino acid residues and differs from the 411 amino acid $CRF_{2(a)}$ isoform in that the first 34 amino acid residues in the amino-terminal extracellular domain are replaced by a unique sequence that is 54 amino acid residues in length [13,17]. The $CRF_{2(c)}$ receptor has most recently been identified only in human brain [18]. This splice variant uses a different 5'-alternative exon for its amino terminus and replaces the first 34 amino acid sequence of the $CRF_{2(a)}$ receptor with a unique 20 amino acid sequence (see Figure 1). The chromosomal mapping of the human CRF_2 gene has also been determined and has been localized to chromosome 7 p21-p15 [19].

Pharmacological Characteristics of CRF Receptor Subtypes

The cloning of the CRF receptor subtypes and isoforms, coupled with their expression in heterologous mammalian cell systems, has greatly enhanced our ability to study this receptor family and develop the pharmacological tools required to aid the discovery of potentially novel therapeutics. Early studies of the CRF system utilized the radiolabeled agonists [^{125}I]-rat/human CRF and [^{125}I]-ovine CRF to elucidate the binding characteristics and anatomical distribution of CRF receptors in brain and a variety of tissues [for reviews see 20-23]. However, prior to the recent elucidation of the CRF_2 subfamily of receptors, all of the *in vitro* and *in vivo* characteristics had been ascribed to a single receptor subtype. Fortunately, this body of data remains unaffected by the discovery of a second family member by virtue of the fact that [^{125}I]-r/hCRF and [^{125}I]-oCRF have lower affinity for the CRF_2 receptor subtype (10-100 nM), making them essentially selective *in vitro* tools for the CRF_1 receptor. It was not until the synthesis of a radiolabeled form of the peptide **sauvagine** (from frog skin, *Phyllomedusa sauvagei;* Prod. No. **S 3884**) [24], which possesses high affinity for both CRF_1 and CRF_2 receptor subtypes, that it became possible to visualize and characterize both receptors in cell lines and native tissues [25].

CRF receptor subtypes exhibit a clear and distinct pharmacological profile with respect to the endogenous and related CRF peptides. For the CRF_1 receptor, the pharmacological rank orders of potency in cell lines stably transfected with either human or rat CRF_1 receptors were

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found to be identical to the established profile for the CRF receptor present in rat frontal cortex. The rank order of potency is as follows: **urocortin** (Prod. No. U 4127) ~ **urotensin I** (Prod. No. U 7253) ~ sauvagine > **ovine CRF** (oCRF; Prod. No. C 3167) ~ rat/human CRF (r/h CRF) ~ **bovine CRF** (Prod. No. C 2671) > d-Phe r/hCRF(12-41) > **α-helical CRF(9-41)** (Prod. No. C-246) >> r/hCRF(1-41)OH, VIP and **arginine vasopressin** (AVP; Prod. No. V 9879). In contrast to other neuropeptide systems, these data demonstrated that although there are subtle differences between the rat and human forms of the CRF_1 receptor in terms of amino acid sequence, these differences are not significant enough to alter the ability of the above peptides to bind to or interact with this receptor subtype.

For the CRF_2 receptor, expression of all three isoforms in mammalian cell lines gave rise to a different pharmacological profile than that determined for CRF_1. Thus, CRF_2 receptors labeled with [^{125}I]-sauvagine exhibited the following rank order profile: urocortin ~ sauvagine ~ urotensin I > **astressin** (Prod. No. A 4933) > r/hCRF > d-Phe CRF(12-41) ~ α-helical CRF(9-41) > oCRF >> r/hCRF(1-41)OH, VIP and AVP with only very subtle differences in the absolute affinities for these peptides across the three CRF_2 receptor isoforms, suggesting a possible contribution of the different amino-terminal domains (refer to Figure 1). Without exception, it is clear that the mammalian CRF peptides oCRF and r/hCRF possess weaker affinity for the CRF_2 receptor subtype, while urocortin possesses equal affinity for both the CRF_1 and CRF_2 receptor subtypes. In addition, it is important to mention that all peptides that interact with any of the CRF receptors must be amidated at the carboxyl-terminus. Deamidation of any of these peptides results in a significant, if not complete, loss of affinity for these receptors. The sequence homology and affinities of agonist and antagonist peptides at CRF_1 and $CRF_{2(a)}$ receptors are shown in Figure 2.

The exclusive link to a G protein is characteristic of receptor systems that couple to adenylyl cyclase as a second messenger. These transducing proteins link the ligand binding event at the receptor to the activation of an intracellular pathway, in this case the conversion of **adenosine 5′ triphosphate** (ATP; Prod. No. A 2383) to **adenosine 3′-5′-cyclic monophosphate** (cAMP; Prod. No. A 9501). *In vitro*, guanine nucleotides have been shown to decrease the specific binding of agonists for a large number of seven transmembrane neurohormone or neurotransmitter receptors coupled to G proteins, presumably by uncoupling the G protein from the receptor and changing its conformation such that its affinity for its agonist is reduced. When transfected in stable mammalian cell lines, both CRF receptor subtypes were shown to be sensitive to guanine nucleotides and stimulated cAMP production with a similar rank order profile as was observed for the inhibition of binding studies. These data confirmed that human or rat CRF_1 or CRF_2 receptors heterologously expressed in mammalian cell lines functioned identically to those previously characterized in native tissues [25,26]. Furthermore, CRF- or sauvagine-stimulated cAMP production could be competitively inhibited by the peptide antagonists d-Phe CRF(12-41) and α-helical CRF(9-41) in both CRF_1 and CRF_2 expressing cell lines, again demonstrating that the expressed CRF receptors in these cell lines were indeed functional. The peptide antagonists d-Phe CRF(12-41) and α-helical CRF(9-41) inhibited the stimulated cAMP response with approximately the same IC_{50} value in both receptor subtype preparations. These results clearly indicate not only that subtype-specific and subtype-selective agonists can be identified for these two receptor subtypes, but also that mixed antagonists could bring to light subtle functional differences between the two receptor systems.

Figure 2. Peptide sequence and homology of agonists and antagonists with affinities at CRF_1 and $CRF_{2(a)}$ receptors.
(Numbers in brackets refer to references on pages 9 and 10 that detail the assays and tissues in which the various Ki values were determined.)

Peptide	Amino Acid Sequence	CRF_1 Ki (nM)	$CRF_{2(a)}$ Ki (nM)	Prod. No.
Human/Rat CRF	SEEPPISLDLTFHLLREVLEMARAEQLAQQAHSNRKLMEII-NH_2	1.5 [11]	42 [11]	C 3042
Ovine/Caprine CRF	SQEPPISLDLTFHLLREVLEMTKADQLAQQAHSNRKLLDIA-NH_2	1.1 [11]	230 [11]	C 3167
Frog Sauvagine	-ZGPPISIDLSLELLRKMIEIEKQEKEKQQAANNRLLLDTI-NH_2	1.6 [51]	5.2 [51]	S 3884
Urotensin I	NDDPPISIDLTFHLLRNMIEMARIENEREQAGLNRKYLDEV-NH_2	0.4 [12]	1.8 [12]	U 7253
Human Urocortin 1	-DNPSLSIDLTFHLLRTLLELARTQSQRERAEQNRIIFDSV-NH_2	0.4 [11]	0.3 [11]	U 4127
Human Urocortin 2	---IVLSLDVPIGLLQILLEQARARAAREQATTNARILARV-NH_2	>100 [92]	1.7 [92]	---
Human Urocortin 3	-TKPTLSLDVPTNIMNLLFNIAKAKNLRAQAAANAHLMAQI-NH_2	>100 [92]	22 [92]	U 1008
Rat/Mouse Urocortin 1	-DDPPLSIDLTFHLLRTLLELARTQSQRERAEQNRIIFDSV-NH_2	0.3 [93]	0.6 [93]	U 6631
Mouse Urocortin 2	---VILSLDVPIGLLRILLEQARYKAARNQAATNAQILAHV-NH_2	>100 [93]	0.7 [93]	U 9507
α-Helical CRF(9-41)	--------DLTFHLLREMLEMAKAEQEAEQAALNRLLLEEA-NH_2	19 [94]	1.1 [94]	C 2917
dPhe^{12}, Nle^{21,38} rCRF(12-41)	----------fHLLREVLExARAEQLAQQAHSNRKLxEII-NH_2	19 [94]	4.4 [94]	---
Antisauvagine-30	----------fHLLRKXIEIEKQEKEKQQAANNRLLLDTI-NH_2	400 [94]	1.1 [94]	A 4727
Astressin	----------fHLLREVLEXARAEQLAQEAHKNRKLxEII-NH_2	0.7 [94]	0.6 [94]	A 4933
Astressin$_2$B	--------DLSfHBLRKXIEIEKQEKEKQQAENNRLLLDBI-NH_2	>500 [94]	1.3 [94]	A 5227

Anatomical Distribution of CRF Receptor Subtypes

The anatomical distribution of CRF receptor subtypes and isoforms has been well documented in a variety of species using both receptor autoradiography and *in situ* hybridization studies. Using these techniques, CRF binding sites and CRF receptor mRNA have been localized in anatomically and physiologically relevant areas. For example, the highest density of CRF_1 receptors exists in the pituitary gland, where CRF_1 expression is detectable in both anterior and intermediate lobes with particularly high expression in clusters observed within the anterior lobe. This correlates well with the action of CRF on corticotropes of the anterior pituitary associated with the release of ACTH [27]. In addition, high levels of CRF_1 receptors have been localized in the cerebral cortical areas, amygdala and hippocampus of rodent brain and also to hypothalamus and amygdala in non-human primates [27,28]. While there is a general correlation between the distribution of [^{125}I]-oCRF or [^{125}I]-sauvagine binding sites and CRF_1 or CRF_2 mRNA in brain with the functional actions of CRF, there are several brain areas where apparent discrepancies exist. For example, CRF elicits potent electrophysiological and behavioral effects in the locus coeruleus, albeit the distribution of binding sites and CRF_1 or CRF_2 mRNA is not noteworthy in this brainstem nucleus. Physiologically therefore, it is tempting to speculate that other CRF receptor subtypes, as yet unidentified, may be present in those regions where CRF seems to exert profound actions, but where no appreciable labeling of the receptor is observed. Within the periphery, CRF_1 receptors have been localized to the adrenal glands and gastrointestinal tract as well as to the immune and reproductive system where levels of CRF_1 mRNA have been localized to the testis and ovary [9,29,30].

In contrast to CRF_1 receptors, CRF_2 receptors show a quite different and more varied distribution within the CNS and across species. Accordingly, species differences in the localization of receptor subtypes between rodent, non-human primate and human tissues have made it somewhat difficult to extrapolate data obtained from behavioral studies in rodent models to human effects. Within the rat brain, $CRF_{2(a)}$ receptor expression is generally confined to sub-cortical structures including the lateral septal region, the bed nucleus of the stria terminalis, the amygdalia and the olfactory bulb [27]. The lateral septum, by virtue of its widespread reciprocal connections throughout the brain, is implicated in a variety of physiological processes. These range from higher cognitive functions, such as learning and memory, to autonomic regulation, including food and water intake. In addition, the septum plays a central role in classical limbic circuitry and is thus important in a variety of emotional conditions, including fear and aggression. Indeed, it has recently been demonstrated that acute antagonism of CRF_2 receptors in the lateral septum produces a reduction in stress-induced behavior such that these receptors may represent a potential target for the development of novel anxiolytics [31].

Corticotropin-Releasing Factor Products Available from Sigma-RBI

Agonists/Ligands

C 2671	**Corticotropin-Releasing Factor, bovine**
C 3042	**Corticotropin-Releasing Factor, human, rat**
C 3167	**Corticotropin-Releasing Factor, sheep**
C 0961	**Corticotropin-Releasing Factor fragment 6-33**
S 3884	**Sauvagine**
U 4127	**Urocortin human**
U 6631	**Urocortin, rat**
U 9507	**Urocortin II, mouse**
U 1008	**Urocortin III, human**
U 0883	**Urocortin III, mouse**

Antagonists

A 8727	**Antalarmin**
A 4727	**Antisauvagine-30**
A 4933	**Astressin**
A 5227	**Astressin$_2$B**
C 2917	**Corticotropin-Releasing Factor antagonist**
C-246	**α-helical Corticotropin-Releasing Factor (9-41)**
N 3911	**NBI 27914**

The $CRF_{2(b)}$ isoform is localized primarily on non-neuronal elements, such as the choroid plexus of the ventricular system and cerebral arterioles. In the periphery, $CRF_{2(b)}$ mRNA is expressed at high levels in both cardiac and skeletal muscle with lower levels evident in both lung and intestine [27,32]. The $CRF_{2(c)}$ isoform has yet to be identified in rodents. However, RT-PCR analysis of human brain mRNA demonstrated expression in septum, amygdala, hippocampus and frontal cortex [18]. A full characterization of the $CRF_{2(c)}$ subtype and the role it may play in physiology or pathophysiology remains to be determined.

Peptide Ligands for CRF Receptors

Once the peptide structure of ovine CRF was elucidated, it was soon discovered that its amino acid sequence was closely related to the two non-mammalian peptides sauvagine and urotensin I. Initial functional assays determined that these peptides were potent releasers of ACTH from cultured rat pituitary cells, identifying them as high affinity agonists [33]. During that same period, several other hypothalamic CRF-related peptides were described and characterized from a variety of species including rat, human, goat, cow, pig, suckerfish and *Xenopus* [34-40]. All of these peptides demonstrated high affinity for the CRF_1 receptor, and were thus defined as the endogenous ligands for the CRF_1 receptor within their respective species. Interestingly, however, the non-mammalian peptides sauvagine and urotensin I also demonstrated high affinity for the mammalian CRF_2 receptor subtype. The fact that authentic CRF was also present in non-mammalian species [reviewed in 41] prompted many laboratories to investigate the existence of other CRF-like molecules in mammalian species. In 1995, the first unique peptide related to CRF was discovered in rat. Termed urocortin, it was found to posseses high affinity for the CRF_2

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receptor and shown to be localized in areas corresponding to the distribution of the CRF_2 receptor [42]. The human homolog of rat urocortin was subsequently identified and cloned from a human brain library and defined as the endogenous ligand for the CRF_2 receptor [43]. Among the subfamily of hypothalamic CRF peptides, the absolute amino acid sequence homology is > 80%. Homology of rat/human CRF to other family members, such as urotensin I, the urocortins and sauvagine, is much less ranging from 35–63% (see Figure 2).

To date, there are no known endogenous antagonists for the CRF receptor system. However, various truncations of agonist peptides, with deletions of the first eight to eleven amino-terminal amino acid residues, have resulted in potent peptide antagonists (see Figure 2). The first such peptide to be synthesized was α-helical CRF(9-41) [44]. This peptide was shown to potently reduce CRF-mediated responses when administered either peripherally or centrally. More potent truncated peptides were identified and synthesized, with d-Phe CRF(12-41) [45] being approximately three times more potent than astressin [46], a structurally constrained derivative of d-Phe CRF(12-41), which was found to be approximately 30 times more potent than α-helical CRF(9-41). These antagonists greatly enhanced the understanding of the physiology of the CRF system and the important role this neurohormone plays in the mediation of the stress response. These first generation antagonists exhibited equal affinities for both the CRF_1 and CRF_2 receptor subtypes and did not discriminate between any of the CRF_2 receptor isoforms.

The first examples of CRF_2 selective peptide antagonists were designed by amino-terminal truncations and cyclic modifications of the agonist sauvagine. **Antisauvagine-30** (Prod. No. A 4727) [47] and cyclized **astressin$_2$B** (Prod. No. A 5227) possess approximately 400 to 500-fold selectivity for the CRF_2 receptor over the CRF_1 receptor. As a result, these peptides have been used as tools to dissect out specific roles for the CRF_2 receptor subtype in a variety of *in vivo* studies.

Table 1. Summary of potential therapeutic indications for CRF receptor antagonists.

Potential Therapeutic Indication	Key Evidence for CRF Receptor Involvement	References
Depression	CRF is hypersecreted in patients with major depression and has been implicated in melancholic and atypical depression as well as in post-traumatic stress disorder (PTSD). CRF receptor antagonists have demonstrated clinical effects in major depression.	[68,95-97]
Anxiety	CRF is hypersecreted in patients with anxiety disorders. Antagonism of both CRF_1 and CRF_2 receptors has been shown to reduce anxiogenic behavior. CRF_1-deficient mice exhibit anti-anxiety behaviors. CRF_2-deficient mice exhibit heightened anxiety-like behaviors.	[31,98-101]
Neurodegeneration/ Stroke	Under pathologic conditions, CRF expression is elevated in areas of neurodegeneration. Selective CRF_1 receptor antagonists demonstrate anti-ischemic effects in rat models of permanent focal cerebral ischemia.	[102,103]
Infantile Seizures	Selective CRF_1 receptor antagonists reverse limbic seizures in neonatal rats.	[104]
Migraine	Stress activates intracranial mast cells and promotes degranulation. Etiology of cyclic vomiting syndrome and migraine in children has been associated with heightened HPA axis activity.	[105,106]
Substance Abuse	Patients suffering from alcoholism have elevated CRF levels in their cerebral spinal fluid (CSF). Alcohol withdrawal symptoms are decreased in CRF_1 receptor knockout mice. CRF seems to play a major role in the maintenance of cocaine self-administration, and CRF_1 receptor antagonists block stress-induced reinstatement of cocaine/heroin use.	[107-112]
Irritable Bowel Syndrome	Irritable bowel syndrome has been linked to psychiatric disorders. CRF_1 receptor antagonists block stress-induced hyperalgesia and colonic motor function.	[82,113]
Feeding Disorders	CRF and related peptide agonist are anorectic agents when administered centrally, an effect that may be mediated through CRF_2 receptors. Mixed CRF_1/CRF_2 receptor antagonists block the anorexia associated with conditions that activate the CRF system.	[114-117]
Inflammation/ Rheumatoid Arthritis	CRF mediates pro-inflammatory processes in a variety of cell types. CRF and related peptides have been localized to synovial fluid in rheumatoid arthritis.	[118-122]
Pre-Term Labor	CRF plays an important role in human pregnancy and the onset of labor. CRF levels are elevated and CRF_1 receptor concentrations are decreased in preeclampsia and premature labor. CRF_1 receptor antagonists delay parturition in sheep.	[123-127]
Atopic Dermatitis/ Psoriasis	Stress triggers mast cell degranulation in the skin through CRF, an effect that is mimicked by urocortin. Selective CRF_1 receptor antagonists block chronic contact dermatitis in rat.	[128-131]

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Non-Peptide Ligands for CRF Receptors

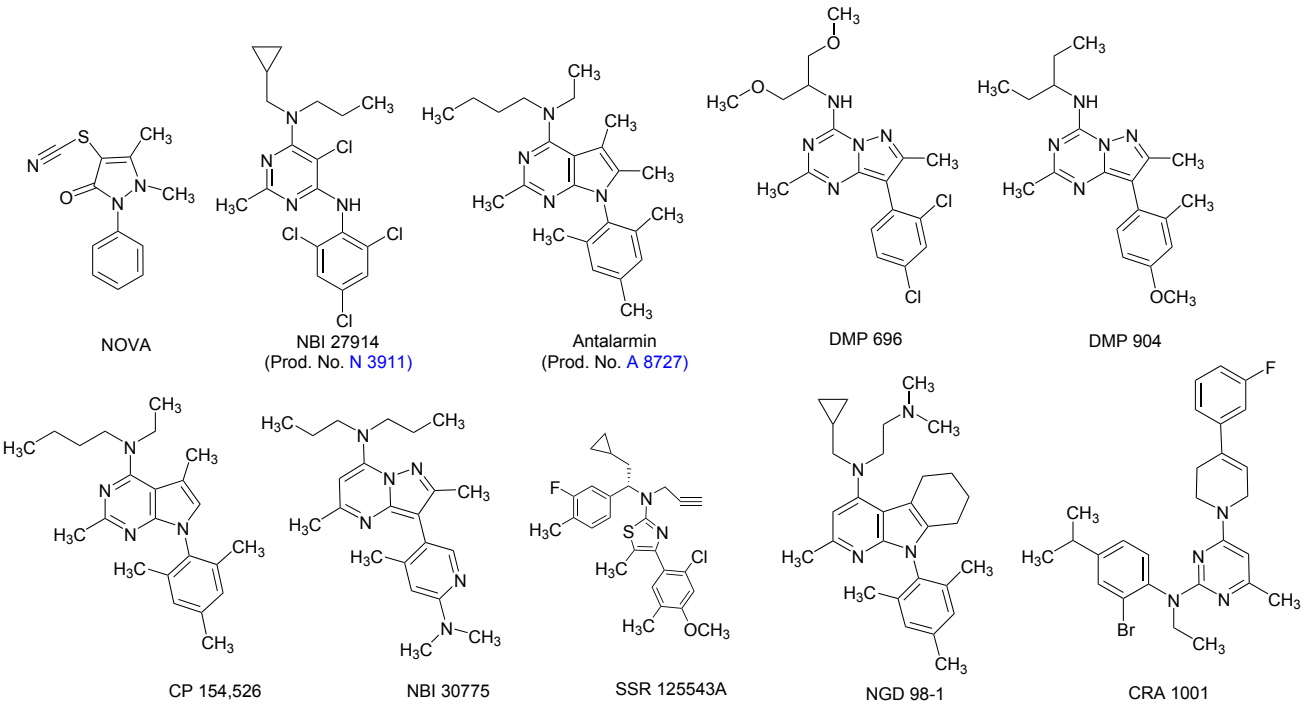
The search for small molecule non-peptide antagonists acting at CRF receptors was initiated well before there was any direct evidence for receptor subtypes or isoforms. The first reported antagonists were a series of oxopyrazoline thiocyanates patented by Nova Pharmaceutical Corporation in 1991 that displayed relatively weak activity (3-10 mM), but appeared to specifically inhibit the binding of [^{125}I]-oCRF to rat cortical membranes [48]. Since then, there have been well over 100 composition of matter patents covering a wide range of discrete chemical entities that claim to possess high affinity and selectivity for the CRF_1 receptor. Figure 3 details a few examples from the literature that are representative of some of the different chemical classes of compounds that display high affinity for the CRF_1 receptor. Interestingly, while all of these patents describe chemically distinct and unique molecules identified from very diverse starting points, they must all fit a common pharmacophore, indicating that the receptor has strict requirements for functional groups to allow a high affinity interaction [49-51]. Many of these molecules have proved crucial in elucidating the precise role of the CRF system in various *in vitro* and *in vivo* models of disease and have paved the way for the discovery of potential novel therapeutics for a variety of peripheral and CNS disorders. Multiple reviews exist that describe the evolution of these molecules from a structure-activity perspective [51-55].

The majority of compounds described to date are highly specific for the CRF_1 receptor subtype. The reason for this conundrum is not intuitively obvious. The expectation that the close homology of the two receptor proteins (refer to Figure 1) would yield some common contact points for small molecules has not been met. While it is true that certain small molecules (for example NBI 30775) display some activity at the CRF_2 receptor at low micromolar concentrations, exhaustive structure activity studies have not been able to greatly improve on potency. Despite significant interest from pharmaceutical companies in developing CRF receptor antagonists, few compounds have been reported that display high affinity or are selective for the CRF_2 receptor subtype. Structural studies using chimeras, point mutations, tethered domains and detailed pharmacological profiling using some of the radiolabeled small molecules, have begun to shed some light on the critical determinants of specificity between these two receptors [56-60]. For the CRF field, the discovery of receptor-selective molecules remains a major challenge.

CRF Receptor Antagonists and their Potential in Human Disease

Not unexpectedly, most of the biological studies involving CRF receptor antagonists have focused on the stress axis and those diseases or disorders that may be associated with stress. The predominant area of focus for the initial use of CRF_1 receptor antagonists has been in the treatment of major depression and anxiety disorders. While there are studies beginning to emerge supporting a role for the CRF_2 receptor in anxiety [31,61], a great deal of evidence has accumulated supporting the hypothesis that blockade of CRF_1 receptors will alleviate anxiety. A number of observations have suggested that the CRF system is dysregulated in major depression. Post mortem studies have suggested that the elevated levels of CRF in the cerebrospinal fluid of depressed patients, coupled with the decreased CRF receptor binding observed in the frontal cortex of suicide victims, is evidence that the CRF system is hyperactive in this condition [62-65]. Preclinical *in vivo* studies appear to support this hypothesis in that

Figure 3. Structures of non-peptide CRF_1 receptor antagonists.



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stress-induced alterations in behavior can mimic the effects of centrally administered CRF. Furthermore, transgenic studies have shown that mice overexpressing CRF are not only hypercortisolemic, but exhibit a heightened anxiety phenotype [66]. In contrast, CRF_1 receptor-deficient mice exhibit reduced ACTH and **corticosterone** (Prod. No. **C 2505**) levels, in addition to displaying a diminished ability to mount a stress response [67].

Despite the fact that some stress responses in humans can be mimicked in rodents, the predictability of the effects in animal studies for human disease is extremely weak. The hypothesis that CRF_1 receptor antagonists will prove efficacious in disease will need to be tested in well controlled clinical studies. While it is likely that several compounds have been through various phases of clinical trials, the only compound that has been reported to demonstrate clinical effects in patients with major depression is R121919 (NBI 30775). In a Phase IIA open label non-placebo controlled study, this pyrazolopyrimidine induced a significant reduction in HAM-D and HAM-A scores across the treatment period without affecting either basal HPA activity or significantly blunting an exogenously-administered CRF-induced ACTH response [68]. Although this particular compound displayed side effects that precluded further clinical development, this initial study significantly strengthened the hypothesis that CRF_1 receptor antagonists will prove efficacious in major depression. This study also demonstrated that it should be possible to separate the central efficacy of CRF_1 receptor blockade from the potential peripheral side effects of rendering the HPA axis unresponsive.

Apart from the potential neuropsychiatric indications for CRF_1 receptor antagonists, parallel studies performed since the discovery of CRF have concluded that CRF signaling pathways are very much involved in the various endocrine, immune, autonomic and visceral components of the stress response. For example, the influence of stress on the physiology of the gastrointestinal tract has long been recognized [69] and a great deal of evidence supports an important role for the CRF system in stress-related gut function [reviewed in 70,71]. Preclinical and clinical studies have shown that a variety of acute stressors inhibit gastric motility and emptying while also accelerating colonic motility and transit [72,73], and these effects can be reproduced by central administration of CRF [74-77]. Indeed, peptide agonists injected peripherally (both CRF and urocortins) can potently inhibit gastric motility and emptying while stimulating colonic motor function [78-81]. These effects can be effectively blocked by either mixed peptide or selective non-peptide CRF_1 receptor antagonists [82]. Recently, using newly characterized antibodies, the receptor proteins have been mapped in the upper and lower gastrointestinal tract and have shown some colocalization with urocortin, suggesting an autocrine or paracrine role for this system [83]. These data taken together suggest a therapeutic potential of CRF_1 receptor blockade in the treatment of stress-related gastrointestinal disorders.

As eluded to above, blockade of the CRF system seems to present a unique mechanism through which potentially novel therapeutics can be found. Table 1 provides a summary of a number of other indications that have been proposed to be mediated by the CRF system and the key supporting evidence. The references include both specific examples and reviews on each indication. The one factor underlying of all of these indications appears to be their link with the stress axis. On the one hand, it is tempting to speculate that compounds designed specifically to inhibit the CRF_1 receptor or the CRF_2 receptor subtype will have utility as potential therapeutics in a wide variety of stress disorders for which adequate treatment may not be currently available. On the other hand, with the ubiquitous nature of this system and its major role in the regulation of the stress response, the challenge will be to find agents that possess the desired efficacy yet lack significant side-effect liability.

Perspective

From the first descriptions of the stress axis to its hypothesized role in human disease, the characterization and elucidation of the receptors and ligands for the CRF system have led to the development of small, orally active non-peptide molecules that may possess therapeutic potential. These newly characterized tools have, in turn, led to the refinement and expansion of the initial hypotheses and have identified a tangible goal of producing selective molecules that will target specific CRF-mediated behavior and physiology. In preclinical studies, these peptide and non-peptide tools have been instrumental in providing a "proof-of-principle" that blockade of this system will ultimately provide some clinical benefit. Despite all of this work however, no compounds have progressed beyond Phase IIA clinical trials in any human disorder. Nonetheless, given the importance of this system in physiology and pathophysiology, there is little doubt that selective molecules for this family of receptors will soon become available and will yield exciting and novel therapeutic opportunities for the treatment of neuropsychiatric and stress-related diseases.

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