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Review

The "homeostasis hormone" and its CRF_1 receptor. From structure to function

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INTRODUCTION

The maintenance of homeostasis is a basic prerequisite for life, ensuring the stability of our body in response to external or internal stimuli (stressful stimuli). The maintenance of homeostasis requires alterations in the function of the endocrine system, as well as several other adaptive responses, involving changes in the behavior and the function of the central nervous system (CNS), immune, cardiovascular and other systems. A distorted regulation of the adaptive responses to various stressful stimuli may affect our physiologic functions, thus rendering us vulnerable to various disorders, such as depression and anxiety.

Corticotropin releasing hormone (CRH), or corticotropin releasing factor (CRF), a hypothalamic hormone, plays a key role in the maintenance of homeostasis.[1] CRF is secreted by the paraventricular nucleus of the hypothalamus in response to stress and is transported via the portal vein to the anterior lobe of the pituitary gland where it causes the release of corticotropin (ACTH). Subsequently, the ACTH is transported by the blood to the adrenals, where it stimulates the release of glucocorticoids (Figure 1).[1,2] In addition to the regulation of the hypothalamic-pituitary-adrenal axis (HPA), CRF plays an important role in stress as well as in many physiological and pathophysiological processes by being involved in the control of the CNS[3-9] as well as the cardiovascular, gastrointestinal, behavioral, immune and reproductive systems.[10-26]

Key words: CRF-peptides, CRF-receptors, Non-peptide antagonists, Physiological/pathophysiological role, Structure

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PEPTIDE AND NON-PEPTIDE CRF ANALOGS

The CRF family

CRF, which was first isolated from ovine hypothalamus (oCRF), belongs to a family of structurally related, highly homologous peptides (CRF-peptides) from several species, such as rat (h/rCRF), human (h/rCRF), goat, cow, pig and xenopus CRF.[1,27-33] In addition to CRF-peptides, the CRF family includes peptides from different species such as sauvagine, urotensin and urocortins, which are closely related to CRF (CRF-like peptides). Sauvagine (SVG) and urotensin (URO) have been characterized from the frog *Phyllomedusa sauvagei* and the sucker fish *Catostomus commersoni,* respectively, whereas urocortin (Ucn), urocortin II (UcnII) and urocortin III (UcnII) have been charac-

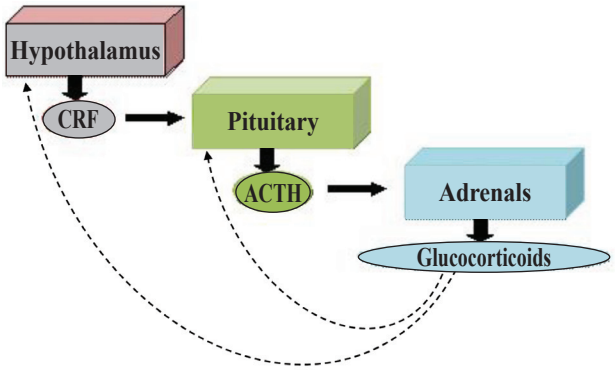


Figure 1. Schematic illustration of the hypothalamic-pituitary-adrenal axis (HPA). CRF is secreted by the paraventricular nucleus of the hypothalamus and is transported through the portal veins to the anterior lobe of the pituitary gland where it binds to CRF receptors (CRF-R) and causes the release of corticotropin (ACTH). Subsequently, the ACTH is transported through the blood to the adrenals, where it stimulates the release of glucocorticoids. Glucocorticoids exert a negative feedback control on both the hypothalamus and anterior pituitary (dashed arrows). In addition to the pituitary, CRF receptors (CRF-R) are also located in the hypothalamus and adrenals.

terized in mammals.[34-37] In addition to natural ligands, numerous peptide and non-peptide CRF analogs have been synthesized and are described below.

Structure and function of CRF family peptides

CRF, SVG and URO are peptides containing an amino-terminal (1-7 residues) and a carboxyl-terminal (36-41 residues) region connected to each other via an internal segment (8-32) of 25 amino acids.[38] This internal segment has a high chance of adopting an alpha-helical structure, which is the preferred conformation of CRF and its related peptides in hydrophobic or amphiphilic environments, whereas it is destabilized in aqueous environments.[38,39] The ability of CRF family peptides to adopt an alpha-helical conformation in hydrophobic or amphiphilic environments led to the hypothesis that their binding to specific CRF receptors located in the amphiphilic environment of the cell membrane alters the conformation of peptides to a biologically active alpha-helical form.[38-40] Indeed, replacement of several residues of oCRF with alpha-helical preferred ones resulted in an increase of the biological potency of the peptide.[41] Similarly, Beyermann et al (2000) have suggested that the alpha helicity of an internal region connecting the amino-terminal (1-21 residues) and carboxyl-terminal (33-40 residues) regions of CRF and UCN is critical for peptide function.[42] Peptide analogs with internal regions constructed with highly flexible linkers such as those composed by ε-aminocaproic acid residues had lower potencies than UCN and CRF.[42] In contrast, analogs with linkers rich in alanines (alpha helical promoting amino acids) were equipotent to CRF.[42,43] Similarly, connection of the amino-terminal and the carboxyl-terminal regions of UCN with a linker consisting exclusively of the negatively charged Glu and the positively charged Lys, which were arranged in such way that helix stabilization could occur by salt bridge formation between side chains at positions i and i4, resulted in an analog equipotent to UCN.[42]

Like the alpha-helical structure, the amino-terminal (first 21 residues) and carboxyl-terminal (last 8 residues) regions of CRF family peptides also play an important role in biological activity. Peptides having only one of these regions were biologically unimportant.[42,44] Furthermore, alanine substitution for several residues in the carboxyl-terminal region of CRF, removal of the last two amino acids of this region or replacement of the amidated carboxyl-terminal end of CRF with a free acid resulted in a significant to a complete loss of peptide biopotency.[1,45] Similar to the carboxyl-terminal region, modifications of the amino-terminal region of CRF family peptides seriously affected their biological activities. In particular, removal of the first 8 amino-terminal residues of oCRF resulted in the biologically inactive analog, oCRF (9-41), whereas alanine substitution for almost all of the amino acids in this region resulted in a significant to a complete loss of CRF potency to stimulate ACTH release.[41,45]

Removal of the first 8 amino-terminal residues from oCRF, thus creating the oCRF (9-41), abolished its ability to stimulate ACTH release, without largely affecting the binding capacity of peptide, as measured by its ability to antagonize the effects of CRF.[41] Additional modifications of oCRF (9-41) by replacing some of its residues with alpha helical preferred ones created the first CRF antagonist, alpha-helical-CRF (9-41).[41] Similarly, removal of the first 11 amino-terminal residues from r/hCRF, and modifications of the truncated peptide (r/hCRF (12-41)) created the antagonist cyclo(30-33)[D-Phe^{12}, Nle^{21,28}, Glu^{30}, Lys^{33}] h/rCRF (12-41).[46] This antagonist, termed astressin,

had negligible intrinsic activity and it was 100 times more potent than the alpha-helical CRF (9-41).[46] More recently, new CRF antagonists and agonists have been developed, which are described below.

Non-peptide CRF antagonists

Several non-peptide CRF antagonists were developed as new leads in drug discovery to treat various stress-related disorders like depression, anxiety and addictive disorders.[1] Most non-peptide CRF antagonists are substituted five-membered rings or bicyclic and tricyclic rings and are discussed in detail below.

CRF RECEPTORS

CRF and its analogs exert their actions by interacting with two types of plasma membrane receptors, type 1 (CRF_1) and type 2 (CRF_2), which belong to the secretin-like family B of G protein-coupled receptors (GPCRs).[47,48] In addition to CRF_1 and CRF_2, a third type of CRF receptor (CRF_3) has been characterized from the catfish and it is expressed in the pituitary gland, urophysis and brain.[49] Furthermore, CRF_1 and CRF_2 receptors have been shown to be expressed as several functional splice variants ($CRF_{1\alpha}$-CRF_{1m}, $CRF_{2\alpha}$, $CRF_{2\beta}$ and $CRF_{2\gamma}$) and were extensively reviewed by Hillhouse and Grammatopoulos.[50] Among the splice variants of CRF_1 receptor, $CRF_{1\alpha}$ (which will be mentioned in this manuscript as CRF_1 for simplicity) is the main functional variant that mediates the actions of CRF family peptides, whereas the other CRF_1 variants have impaired functional properties.[50] In contrast to CRF_1, the splice variants of CRF_2 did not significantly differ pharmacologically from each other.[51,52]

The CRF_1 and CRF_2 receptors bind the CRF family peptides with different affinities. In particular, h/rCRF and oCRF bind to CRF_1 with higher affinities than to CRF_2, with oCRF being the most CRF_1-selective peptide among the natural peptides of the CRF family. The affinity of oCRF for CRF_1 receptor is 180-fold higher than that for CRF_2. Similarly, the CRF non-peptide analogs (chemically described below) bind selectively to CRF_1 receptor and antagonize the actions of CRF. In marked contrast, UcnII and UcnIII are the CRF_2-selective natural peptides of the CRF family. On the other hand, UCN and SVG bind to CRF_1 and CRF_2 receptors with similar affinities. Similarly, the difference in the binding affinities of the synthetic analogs, astressin and alpha-helical CRF (9-41) for CRF_1 and CRF_2, is only 4-10-fold. Recently, a variety of modifications of CRF family peptides created CRF receptor subtype-selective ligands such as the CRF_2-selective antagonists, cyclo(31-34) [DPhe(11), His(12), C(alpha) MeLeu(13,39), Nle(17), Glu(31), Lys(34)] Ac SVG ((8-40)) (or astressin2-B), [DPhe11, His12]Svg(11-40), (or antiSVG-30), and [D-Phe11, His12, Nle17] Svg(11-40), (or K41498) and the CRF_1-selective agonists (cyclo(31-34)[DPhe12, Nle21,38, Glu31, Lys34]-Ac-hCRF(4-41) (or stressin1-A) and the chimeric peptide ([Glu(21), Ala(40)][Svg(1-12)]-[human/ratCRF(14-30)]-[Svg(30-40)]) (or cortagine).[53-57]

The binding of CRF family peptides to their receptors results in the activation of several heterotrimeric ($\alpha\beta\gamma$) G-proteins having different $G\alpha$ subunits, such as $G_{\alpha s}$, $G_{\alpha i}$, $G_{\alpha o}$, $G_{\alpha q}$, and $G_{\alpha z}$.[50] Activation of $G\alpha$ subunits by CRF receptors results in their dissociation from the $G\beta\gamma$ heterodimers and the subsequent regulation of several signaling pathways by the activated $G\alpha$ subunits and the $G\beta\gamma$ dimers as well. Thus, the CRF_1 receptor has been shown to activate through $G_{\alpha s}$ the adenylate cyclase, thus resulting in the accumulation of intracellular cAMP and the subsequent activation of protein kinase A (PKA) in various cell lines and tissues.[58-63] In CHO cells expressing the CRF_1 receptor, stimulation of the $G_{\alpha s}$-cAMP-PKA pathway has been shown to stimulate the MAPK kinase, MEK1, which in turn activates the extracellular signal regulated kinase 1/2 (ERK1/2).[63] In the locus coeruleus, however, the CRF_1-mediated stimulation of cAMP has been shown to activate ERK in a PKA-independent manner.[64] In addition to cAMP-dependent pathways, the activated CRF_1 is able to inhibit the proliferation of corticotropic tumour (AtT-20) cells via a cAMP independent pathway.[65] Furthermore, following peptide binding, CRF_1 and the CRF_2 could alter the intracellular Ca^{2+} signaling through $G_{\alpha q}$-mediated or $G_{\beta\gamma}$-mediated stimulation of phospholipace C (PLC).[63,66] Moreover, the CRF receptor-mediated activation of $G_{\beta\gamma}$-subunits has been shown to stimulate the phosphatidylinositol 3-kinase, which, through the production of PI(3, 4, 5) P3, activates the PLC, thus resulting in the mobilization of Ca^{2+}.[63] The CRF_1-mediated activation of PI3-K and PLC pathways, as well as mobilization of intracellular calcium stores, has been shown to activate ERK1/2.[63,67,68]

In addition to ERK1/2, CRF_1 and CRF_2 are able to activate another functionally important kinase, the p38 mitogen protein kinase (p38 MAPK).[67,69,70]

Even more interestingly, previous experiments have demonstrated that activation of CRF_1 receptor by CRF in several brain regions resulted in the activation of ERK1/2 in only a few of them which are related to external environmental information processing and behavioral aspects of stress, thus suggesting a specific involvement of this pathway in mediating behavioral adaptation to stress.[71] In addition to the brain, the regulation of ERK1/2 activity by CRF receptors has also been identified in other tissues, such as the myometrium.[72]

STRUCTURE AND FUNCTION OF CRF RECEPTORS

CRF receptors, like all GPCRs, consist of 7 alpha helical membrane-spanning segments (TMs), an extracellular amino-terminal domain (N-domain) and an intracellular carboxyl-terminal tail (C-domain). The TMs of CRF receptors are connected with each other with three extracellular loops (ELs) and three intracellular loops (ILs) (Figure 2).[47,73-76] Experimental findings from numerous studies suggest that the C-domain and the ILs of CRF receptors interact with various G-proteins, thus playing an important role in receptor-mediated signaling.[47,60,77-82] In contrast to ILs which interact with the G-proteins, the ELs and the N-domain of the CRF receptors may, it has been suggested, interact with the CRF family peptides. Substitution of the N-domain of CRF_1 with the corresponding one of GH-releasing hormone receptor abolished the binding of the radiolabelled astressin and UCN.[83] In contrast, the reverse chimeric receptor retained UCN and astressin binding, albeit with a reduced affinity, thus implying the participation of this region in peptide binding.[83] Similarly, a soluble form of the N-domain of CRF_1 as well as a chimera created by replacing the extracellular region of activin receptor (a single membrane-spanning segment receptor) with the N-domain of CRF_1 have been shown to bind to UCN and astressin with affinities that are lower than wild type receptor but still biologically considerable.[84-86]

Structure-function studies have established that the amino acids, important for ligand binding, in the

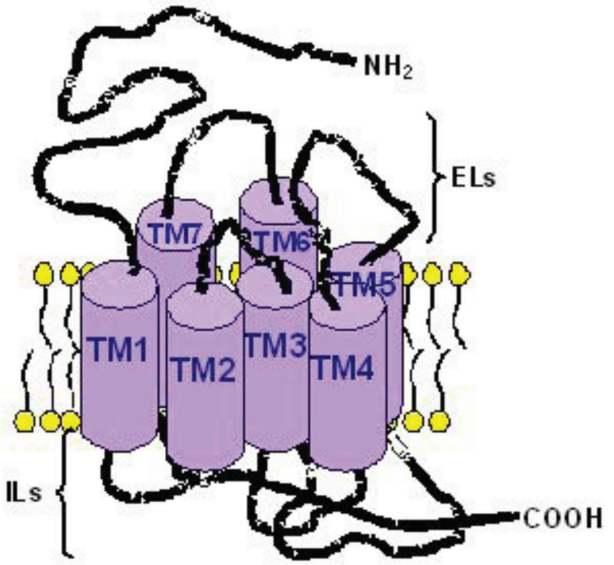


Figure 2. Schematic illustration of CRF_1 receptor. Hydropathy analysis of the cloned CRF receptors revealed the presence of seven hydrophobic regions (TM1-TM7) characteristic of the membrane-spanning segments of G-protein coupled receptors (GPCRs), which are depicted as cylinders and are connected to each other with three extracellular (ELs) and three intracellular loops (ILs).[47] These loops as well as the amino-terminal extracellular domain (N-domain) and the carboxy-terminal intracellular tail (C-domain) are depicted as lines.

N-domain of CRF_1 are located between residues 43-50 and 76-84 of the receptor.[87,88] The precise interactions of the N-domain of CRF receptors with various peptides have been determined by crystallography and NMR studies using soluble forms of this receptor's segment. In particular, the crystal structure of the N-domain of the CRF_1 receptor complex with CRF revealed that the peptide adopts a relatively straight, continuous alpha-helix and docks into a hydrophobic surface composed of the β1-β2 hairpin loop, loop 2, Tyr99, Pro69 and Cys68-Cys102 disulfide of the receptor's N-domain.[89] This crystallographic study has also proposed that the CRF binds with its amino-terminal residues 1-25 pointing toward the ELs and TMs of the receptor, whereas the carboxyl-terminal amino acids such as Leu37, Met38 and the C-terminal amide group (of residue 41) interact with the N-domain of CRF_1.[89] Interestingly, NMR studies have revealed the important part played by the corresponding residues of astressin in peptide binding to a soluble form of $CRF_{2\beta}$, despite the fact that the mode of binding of

astressin has been proposed as being different than that of CRF.[89,90]

In contrast to several peptide residues that play a common role in the binding of different ligands, other peptide residues might be responsible for CRF_1 or CRF_2 selectivity. In particular, a crystallographic study on the N-domain of CRF_1 proposed that the selective binding of CRF to CRF_1 over UcnII and UcnIII may be due to interactions that place Arg35 of CRF between Glu39 of peptide and Glu104 of receptor.[89] The Arg35 of CRF is replaced with an Ala in UcnII and UcnIII, which is more compatible than Arg35 with the hydrophobic Pro of CRF_2 that corresponds to Glu104 of CRF_1, thus explaining the CRF_2-selectivity of UcnII and UcnIII.[89]

The N-domain of CRF_1 receptor also contains three disulfide bridges between Cys30 and Cys54, Cys44 and Cys87 and Cys68 and Cys102, which play a crucial role in maintaining the receptor in its functional conformation.[86,89,91] Reduction of these bonds with DTT or mutation of Cys which participate in these bonds to Ser or Ala significantly decreased CRF binding.[92] The disulfide arrangement in the N-domain of $CRF_{2\beta}$ is identical to that of the corresponding region of CRF_1, but different than that in the N-domain of $CRF_{2\alpha}$, which has four Cys linked to each other with disulfide bonds and one free Cys.[85,86,89,93]

In addition to the N-domain of CRF receptors, their extracellular loops as well as the extracellular portions of their TMs have been revealed as important for peptide binding. In particular, upon sauvagine binding to CRF_1, residues 17 and 16 were shown to be located in close proximity to residue 117 in the extracellular portion of the TM1 and residue 257 in the second extracellular loop (EL2) of the receptor, respectively.[94,95] The latter finding is in agreement with the results of an alanine mutagenesis study, which has suggested that Trp259 and Phe260 in the EL2 of CRF_1 receptor most likely interact with ligands, and specifically with the amino-terminal residues 8-10 of SVG and the corresponding ones of CRF.[96] Furthermore, this study has proposed that the interaction between the amino-terminal region of CRF family peptides and Trp259 and Phe260 of CRF_1 seems to be critical for receptor activation and the subsequent appearance of a biological effect.[96]

In addition to EL2, the first extracellular loop (EL1) of CRF_1 has been demonstrated as playing a part in peptide binding, given that the amino-terminally located residues 17 and 22 of UCN analogs have been shown in a recent study to be located in close proximity to residues Trp170-Glu179 in the EL1 of CRF_1.[97] These results are in agreement with the experimental findings of a previous structure-function study, which suggested that residues 174–178 of EL1 are implicated in peptide binding.[98,99] In addition to the binding sites, EL1 and EL2 contain two Cys, which are highly conserved among GPCRs and connect these regions of receptor with a disulfide bond that plays a very important role in receptor function.[92] Like EL1 and EL2, EL3 of CRF receptors also plays a role in peptide binding since it was demonstrated that Ala substitution for Tyr346, Phe347 and Asn348 in the EL3 of CRF_1 significantly reduced the binding affinity of CRF.[100]

The binding of CRF family peptides to CRF receptors has been proposed as being represented by a two-domain model, in which an initial interaction of the carboxyl-terminal region of peptides with the N-domain of receptor serves to dock the amino-terminal residues of peptides into a receptor's domain (J-domain) formed by the extracellular loops and the upper portions of TMs.[101,102]

THE MEMBRANE-SPANNING SEGMENTS

In contrast to the extracellular regions of CRF receptors which are important for the binding of the bulky peptides, the receptor TMs have been proposed as playing a role in the binding of small non-peptide CRF antagonists. Specifically, His199 and Met276 in the third (TM3) and the fifth (TM5) membrane-spanning segments of CRF_1 have been suggested as being involved in the binding of the non-peptide antagonist, NBI 27914 (structure shown under compound 16c), because mutation of these residues to the corresponding ones of CRF_2 significantly reduced NBI 27914 affinity for CRF_1.[98,103] Despite the fact that Met276 has been demonstrated as playing an indirect role in ligand binding, experimental findings from a structure-function study suggested that this residue is still very important for the interaction of CRF_1 with non-peptide antagonists, most likely by

positioning their heterocyclic core in the vicinity of Met276.[103] The involvement of CRF receptor TMs in ligand binding is further supported by the fact that the corresponding regions of family A GPCRs bind the non-peptide small molecules, such as catecholamines or acetylcholine.[104-107]

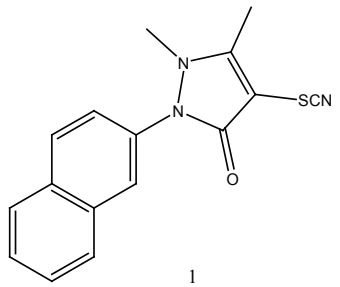
Although the TMs of CRF_1 have been proposed as playing a role in the binding of small non-peptide CRF analogs, the exact interactions have not yet been determined due to the lack of significant structural information about these regions of CRF receptors and all family B GPCRs as well, in contrast to the TMs of family A GPCRs, which have been structurally characterized in many crystallographic, biophysical and biochemical studies. In addition, the development of accurate molecular models of family B GPCRs, which would provide structural information concerning their TMs, is very difficult because these receptors display very little sequence similarity to those of family A receptors.[108-110] Only recently, a study using the substituted cysteine accessibility method (SCAM) provided structural information about the TMs of CRF_1 and suggested that, similarly to family A GPCRs, the TMs of this receptor form a water-accessible crevice, the binding-site crevice, which extends from the extracellular surface of CRF_1 into the plane of the membrane and that the contact sites of small non-peptide CRF analogs must be located on the surface of this crevice.[111] Specifically, this study has shown that the endogenous Cys211 (in TM3), Cys233 (in TM4) and Cys364 (in TM7) are located on the surface of the binding-site crevice of CRF_1.[111] Subsequently, Gkountelias et al. mapped the TM residues that form the surface of the binding-site crevice of CRF_1 receptor by applying SCAM and starting from the extracellular portion of TM3.[112] The results of this study have suggested that Thr192, Ala193, Tyr195 and Asn196 of TM3 are located on the water-accessible surface of the binding-site crevice of CRF_1 and that the pattern of accessibility is consistent with an alpha-helical conformation for this region of TM3.[112]

NON-PEPTIDE CRF_1-SELECTIVE ANTAGONISTS

Several non-peptide CRF antagonists were developed as new leads in drug discovery to treat various stress-related disorders like depression, anxiety and addictive disorders. Most non-peptide CRF antagonists discovered to date are substituted five-membered rings or bicyclic and tricyclic rings and bind selectively to CRF_1. The non-peptide CRF antagonists offer advantages over the peptide congeners in terms of stability, ease of preparation, ease of further modification to enhance the pharmacokinetic profile and better brain penetrability.

1. Substituted Pyrazolones:

Nova Pharmaceuticals reported the synthesis of 4-substituted thiopyrazolones and its disulfide congener.[113] These derivatives inhibited the binding of [^{125}I]Tyr-oCRF to rat cortical membranes as well as the CRF-mediated stimulation of adenylate cyclase. Among the reported compounds, compound **1** [Binding IC_{50}= 3.8μM & cyclase inhibition 3.6μM] and the disulfide derivatives **2** [Binding IC_{50}= 2.2μM & cyclase inhibition 1.1μM] and **3** [Binding IC_{50}= 3.3μM & cyclase inhibition 1.0μM] were potent.



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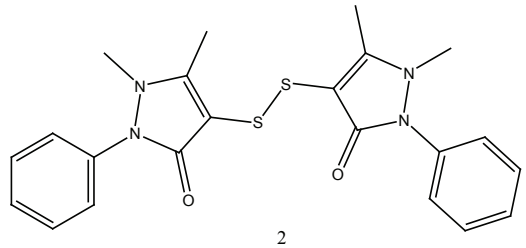

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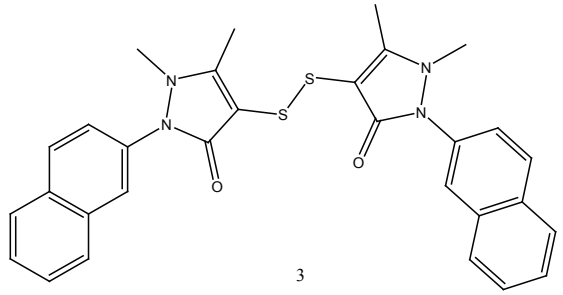

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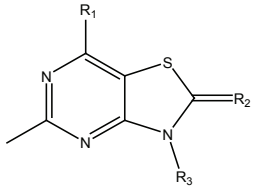
2. Thiazolo[4,5-d]-pyrimidines:

A research study performed by DuPont Pharmaceuticals reported various thiazolo[4,5-d]pyrimidines

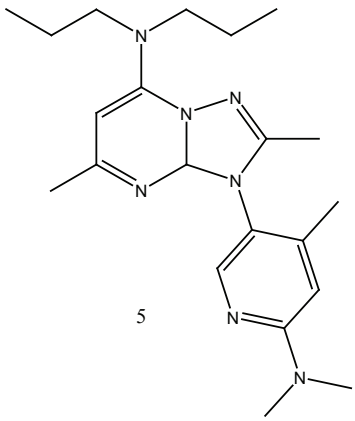
having the general structure **4**.[114] Substitution of noncyclic diamino groups in R_1 position was found to increase the binding affinity of CRF for the human CRF_1 receptor compared to the cyclic amino group (e.g. morpholine). The thiazolones (X = O) were also equipotent in binding to CRF_1 receptors compared to the precursor thiazolothiones (X = S).



4a, R_1= N(Et)$_2$	R_2= S	R_3= 2-Br^{-4}-isopropyphenyl	*Ki*= 4.1 nM
4b, R_1= N(Et)$_2$	R_2= O	R_3= 2-Br^{-4}-isopropyphenyl	*Ki*= 9.4 nM
4c, R_1= N(Pr)CH_2-cPr	R_2= S	R_3= 2-Br^{-4}-isopropyphenyl	*Ki*= 12.6 nM
4d, R_1= N(Pr)CH_2-cPr	R_2= O	R_3= 2-Br^{-4}-isopropyphenyl	*Ki*= 4.1 nM
4e, R_1= N($CH_2CH_2OCH_3$)$_2$	R_2= S	R_3= 2,4,6-trimethylphenyl	*Ki*= 8.6 nM
4e, R_1= N($CH_2CH_2OCH_3$)$_2$	R_2= O	R_3= 2,4,6-trimethylphenyl	*Ki*= 5.8 nM

3. Quinolines and Isoquinolines:

In search of novel CRF_1 antagonists with water solubility, Chen et al. prepared compound **5** with high pK_a (pK_a = 7.1).[115] However, clinical trials of this compound have been discontinued due to its hepatotoxicity.



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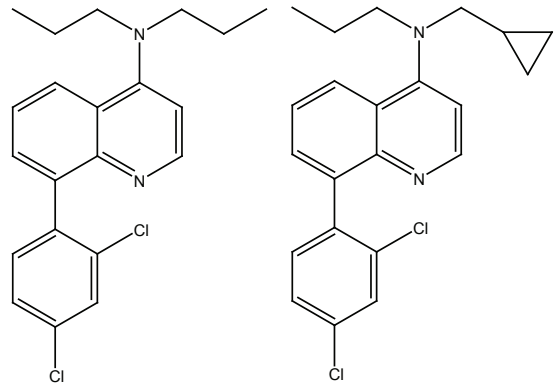
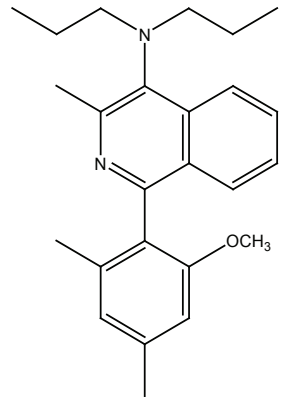
Furthermore, 4-substituted 8-aryl-2-methylquinolines with general structure **6** have recently been synthesized. Since the pK_a of the 4-amino quinolone was about 9.08, it has been speculated that these compounds should be largely charged at physiological pH (7.4) thus increasing water solubility. Presence of the dipropylamino and *N*-cyclopropane-methyl-*N*-propylamino group greatly enhanced activity compared to the smaller diethylamino group. Indeed, non-polar groups of the aromatic ring enhance activity.



6a, K_i = 0.9 nM

6b, K_i = 0.5 nM

By topological modification of previously reported high-affinity CRF antagonists, Yoon et al. prepared 1-aryl-4-aminoalkylisoquinolines and tested their affinities in competition binding studies in IMR-32 human neuroblastoma cells and using ^{125}I-Sauvagine as a radioligand. [116] Compounds with mono-substituted aryl groups were found to have lower affinity than those with di-substitution at both the *ortho* and *para* positions with at least one methoxy group at one *ortho* position. Compounds having a dipropylamino group at 4-position (-N-Pr_2) showed enhanced affinity, as seen in compound **7**.

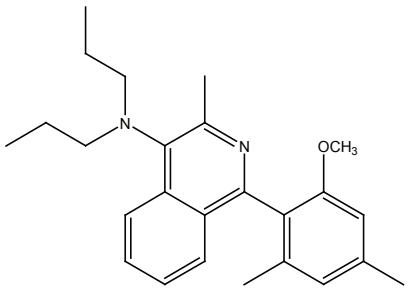


7, K_i = 6 nM

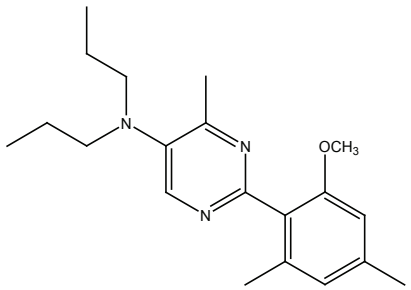
4. Pyrimidine derivatives:

High lipophilicity, poor water solubility and long half-life of many potent CRF_1 receptor antagonists make them unattractive for clinical development and hinder further development. Yoon et al. developed less lipophilic and more water soluble aryl pyrimidine derivatives in order to improve their pharmacokinetic profile.[117] Further introduction of a small alkoxy

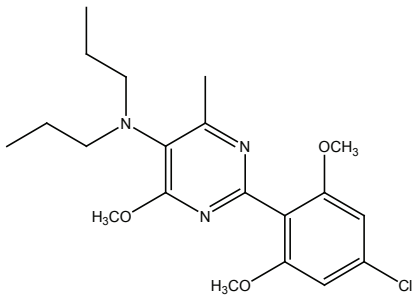
group at the available un-substituted positions in the pyrimidine ring enhanced the affinity. Further modifications of the pyrimidine 2-aryl group yielded 2-(2,4,6-tri-substituted) compounds, the most potent of which was the 2,4-dimethoxy-6-chloro derivative **8**.



K_i = 6 nM
cLogP = 7.4

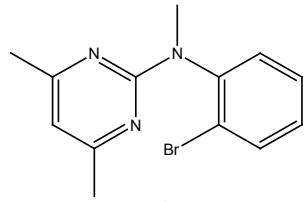


cLogP = 7.4

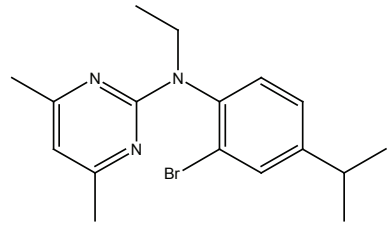


8, *K*i =2 nM
cLogP = 5.9

Screening of a library of compound by DuPont Pharmaceuticals revealed that compound **9** inhibited [^{125}I] Tyr-oCRH binding in rat frontal cortex homogenates.[118] Initial structure-activity relationship studies (SAR) of this lead compound resulted in the synthesis of compound **10,** which was subjected to further optimization. Replacement of the bromo group with methyl, trifluoromethyl or thiomethyl resulted in compounds having comparable affinities to **10**, while removal of any substituent at that position greatly reduced receptor binding affinity.



9



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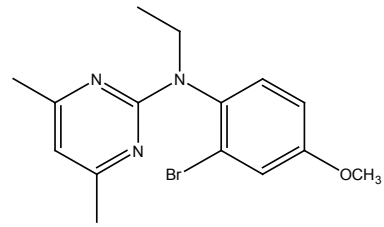
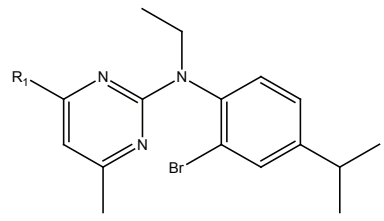


11 *K*i =35 nM

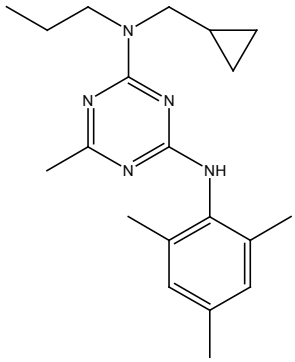
Substitution of the 4-position isopropyl group with larger groups (e.g. butyl, t-butyl) resulted in further decreased receptor binding than groups which have approximately the same size or smaller than isopropyl (e.g. methoxy) as in compound **11**.

In addition, it was noticed that substitution of the 4- or 6-positions of the pyrimidine ring with groups larger than methyl decreased receptor binding activity. Retaining the methyl at 6-position while having different selected substituents at 4-position (R_1) of the pyridine ring yielded many compounds **12a**, **12b**, **12c** and **12d** with enhanced activity.

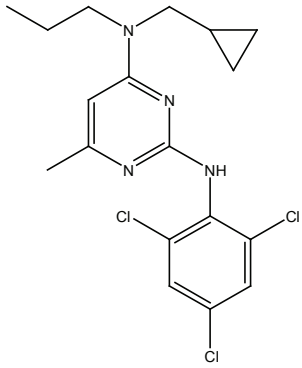


12a, R_1 = 3=pentyl K_i = 2 nM
12b, R_1 = $N(Pr)_2$ K_i = 9 nM
12c, R_1 = N(Pr)(*c*-C_3H_5) K_i = 5 nM
12d, R_1 = N(Pr)(CH_2-*c*-C_3H5) K_i = 9 nM

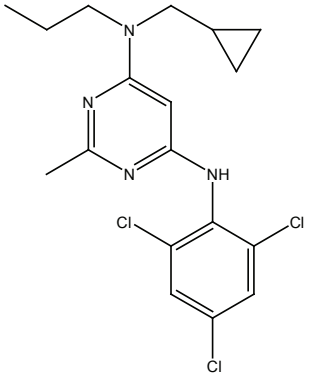
Removal of the 1- or 5-nitrogen in the triazene compound **13** [119] resulted in active pyrimdines **14** and **15** according to Chen et al.[120] However, removal of the 3-nitrogen of the triazine resulted in complete loss of activity.



13, K_i =57 nM

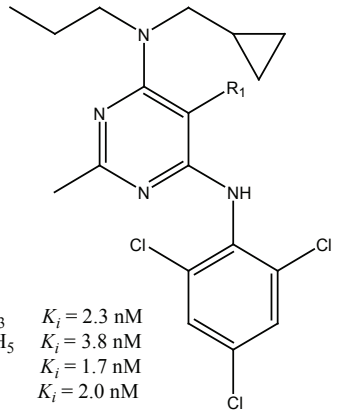


14, K_i = 70 nM



15, K_i = 30 nM

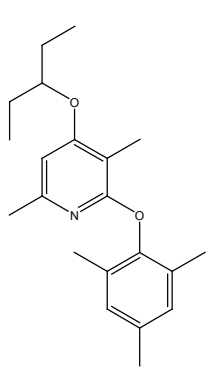
A pharmacophore model has been proposed where the aniline group was predicted to be orthogonal to and below the pyrimidine ring. Addition of small groups (e.g. methyl, ethyl, chloro and bromo) resulted in compounds **16a**, **16b**, **16c** (NBI 27914) and **16d** with nanomolar range receptor binding affinity.



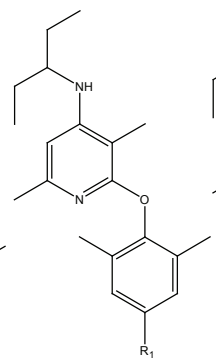
16a, R_1= CH_3 K_i = 2.3 nM
16b, R_1= C_2H_5 K_i = 3.8 nM
16c, R_1= Cl K_i = 1.7 nM
16d, R_1= Br K_i = 2.0 nM

5. Pyridine derivatives:

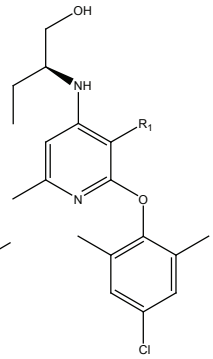
Several promising pyridines were developed at Pfizer. Compound **17** showed increased binding affinity but poor pharmacokinetic profile. To improve the pharmacokinetic properties, the oxygen atom in the alkoxy or aryloxy groups have been replaced by a nitrogen atom to increase basicity and thus water solubility.[121] Thus replacing the alkoxy group with alkylamino function resulted in compound **18a**, which had higher basicity and increased aqueous solubility in simulated gastrointestinal fluid. Further structural optimization has been achieved by replacing the methyl group at the para-position of the 2-phenoxy ring with chloro or bromo atoms, thus yielding the compounds **18b** and **18c**. Further structural modifications resulted in compound **19a** and **19b** with increased polarity and decreased binding.



17



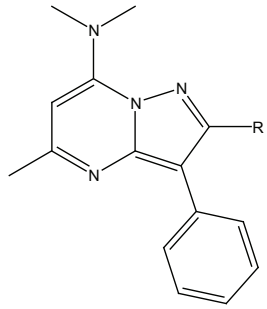
18a, R_1= CH_3 K_i = 5.1 nM
18b, R_1= Cl K_i = 5.1 nM
18c, R_1= Br K_i = 5.6 nM



19a, R_1= CH_3 K_i = 6.4 nM
19b, R_1= Cl K_i = 31 nM

6. Pyrazolo[1,2-b]pyrimidines:

Several pyrazolo[1,2-b]pyrimdines were prepared as potential CRF receptor antagonists. Compound **20** displayed promising activity.[122]



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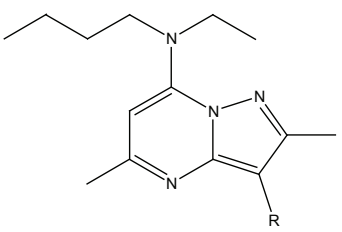
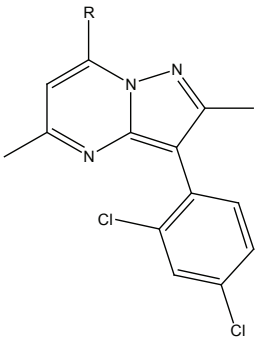
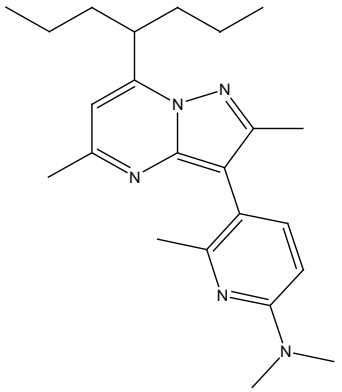


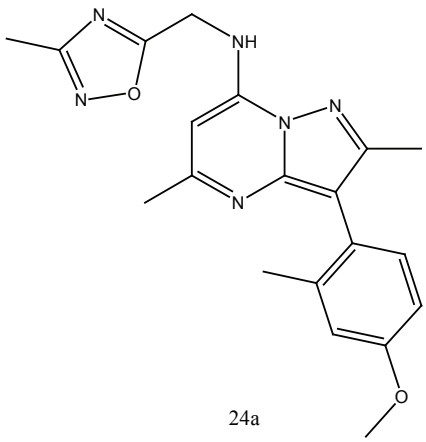
21a, R=phenyl K_i= 511 nM
21b, R=2-chlorophenyl K_i= 15 nM
21c, R=2,4-dichlorophenyl K_i= 5 nM
21d, R=2,4,6-trimethylphenyl K_i= 93 nM



22a, R=N(*n*-Pr)CH_2(*c*-Pr) K_i= 3.2 nM
22b, R=N(Et)($CH_2CH_2OCH_3$) K_i= 11 nM



23



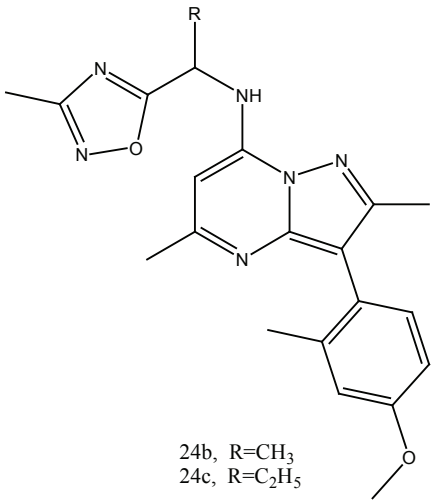
24a

SAR studies of this class demonstrated that replacement of the phenyl group (**21a**, K_i = 511 nM) with 2-Cl phenyl resulted in enhanced binding affinity (**21b,** K_i = 15 nM). Similarly, substitution with 2,4-dichlorophenyl created the most potent derivative of the series (**21c** with K_i = 5 nM). However, unlike most similar non-peptide CRF antagonists, the 2,4,6-trimethylphenyl derivative displayed 17-fold reduction of binding affinity (**21d**, K_i= 93 nM).

As expected, SAR studies also revealed that the presence of non-cyclic dialkyl amino groups with small groups had better activity than their cyclic counterparts (e.g. compounds **22a** and **22b**).

Compound **23** showed excellent potential in depression and anxiety tests; however, it caused reversible increase in hepatic enzymes, which hindered its further development.[123,124]

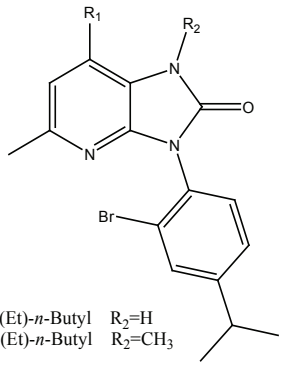
Attempts to lower the lipophilicity by substitution of N-alkyl side chain with heterocycles, while retaining the CRF antagonistic activity, were sought.[125] Several 1,2,4-oxadiazolyl derivatives were prepared, among them compound **24a** (pK_i=7.2±0.1) and **24b** (pK_i=7.6±0.1), which exhibited an improved metabolic stability. The best combination of metabolic stability and CRF binding affinity was observed in compound **24c** (pK_i=8.1±0.1).



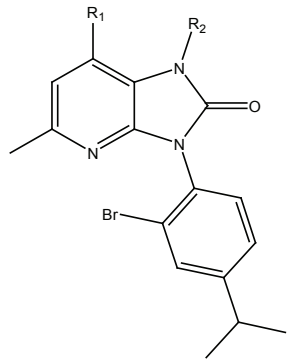
24b, R=CH_3
24c, R=C_2H_5

7. Purines:

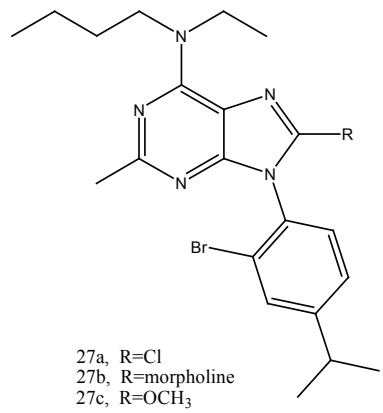
Many substituted purin-8-analogs have emerged as a new class of CRF antagonists.[126] Based on the previous SAR work, the 2-bromo-4-isopropylphenyl derivatives were selected as lead compounds. Compound **25a** had weak CRF binding affinity (K_i=890 nM), in contrast to its *N*-methyl derivative **25b**, which had a high binding affinity (K_i= 5 nM). Further modification with steric bulky groups resulted in decreased binding. The dialkylamine substituted derivatives had higher affinities than mono-substituted compounds (compounds **26a**, **26b**, **26c** and **26d**).



25a, R_1=N(Et)-*n*-Butyl R_2=H
25b, R_1=N(Et)-*n*-Butyl R_2=CH_3



26a, R_1=$N(Pr)_2$ R_2=CH_3 K_i= 7.6 nM
26b, R_1=N(Pr)CH_2-*c*-Pr R_2=CH_3 K_i= 5.7 nM
26c, R_1=N(H)(Et)CH_2OCH_3 R_2=CH_3 K_i= 42.8 nM
26d, R_1=N(H)$CH_2CH_2OCH_3$ R_2=H K_i= 1179 nM



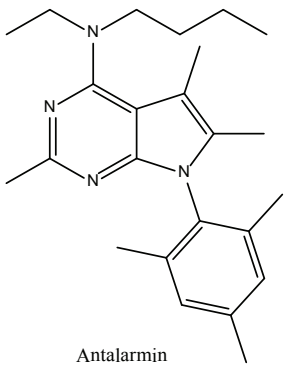
27a, R=Cl
27b, R=morpholine
27c, R=OCH_3

The replacement of the oxo group with a chloro function created a derivative with excellent binding affinity (**27a**, K_i=1.5 nM). Similarly, the replacement with small alkoxy groups (e.g. methoxy) resulted in the high affinity derivative **27c** (K_i=1.5 nM). In contrast, the binding affinity was dramatically reduced by the replacement of chloro function with morpholine (**27b**, K_i=345 nM). Larger-size groups resulted in loss of affinity.

NON-PEPTIDE CRF RECEPTOR ANTAGONISTS IN CLINICAL TRIALS

Several non-peptide CRF receptor antagonists, including antalarmin, are currently being studied in clinical trials. Antalarmin blocks the CRF_1 receptor and, consequently, reduces the release of ACTH in response to chronic stress.[127] Antalarmin reduces the behavioral response to stressful stimuli.[128] It should be mentioned that several newer non-peptide CRF antagonists are currently under development,[129] tar-

geting specific brain regions with the aim of ameliorating the health consequences of chronic stress and for use in the clinical management of anxiety and depression.[130-132]



Antalarmin

Promising results have also been observed using antalarmin as a potential treatment for CRF-induced hypertension. Central administration of CRF results in endocrinological, cardiovascular and behavioral effects that suggest stress or anxiety. Among these is a marked pressor response. Antalarmin was found to antagonize the pressor effect induced by central CRF.[133]

Similar promising results for antalarmin and other CRF_1 antagonists were also observed in the area of drug addiction disorders. Evaluation of antalarmin effects on cocaine dependence in cocaine-addicted monkeys showed a reduction of its use. Similarly, antalarmin tested on cocaine-addicted rats prevented dose escalation, suggesting that it might modulate the cocaine addictive effects over time. Antalarmin also displayed positive effects in reducing withdrawal symptoms from chronic opioid use and significantly reduced self-administration of ethanol in ethanol-addicted rodents. [134-137]

Antalarmin also showed anti-inflammatory effects and has been suggested as having potential uses in the treatment of inflammatory conditions such as arthritis[138] as well as stress-induced gastrointestinal ulcers[139] and irritable bowel syndrome.[140,141]

Chronic blockade of CRF_1 with systemic antalarmin significantly ameliorated rat adjuvant-induced arthritis, reducing the severity of inflammation in peripheral joints, this evidenced by clinical and histopathology results, and weight loss associated with disease onset. Antalarmin neither induced nor exacerbated arthritis expression in rats, despite suppression of levels of adjuvant-induced corticosterone, the major anti-inflammatory glucocorticoid in rats. Systemic blockade of CRF_1 appeared to predominantly block peripheral pro-inflammatory effects of immune CRF rather than the systemic glucocorticoid mediated antiinflammatory effects of hypothalamic CRF. Results indicate that chronic treatment with a CRF_1 antagonist attenuates progressive inflammation-induced degeneration of synovia, cartilage and bone in arthritic joints, suggesting that antalarmin may have therapeutic potential in treatment of human autoimmune and inflammatory disorders. [138]

Upon exposure to prolonged stress, rats develop gastric ulceration, enhanced colon motility with depletion of its mucin content and signs of physiological and behavioral arousal. When antidepressants (fluoxetine and bupropion), anxiolytics (diazepam and buspirone) or antalarmin were evaluated for their potential to modify these responses, fluoxetine, bupropion, diazepam and antalarmin all suppressed stress-induced gastric ulceration in male Sprague-Dawley rats exposed to four hours of plain immobilization. Antalarmin was found to produce the most pronounced anti-ulcer effect and additionally suppressed the stress-induced colonic hypermotility, mucin depletion, autonomic hyperarousal and struggling behavior. Non-peptide CRF_1 antagonists may therefore be of value as prophylactics against stress ulcer in the critically ill and as therapeutics for other related gastrointestinal disorders such as peptic ulcer disease and irritable bowel syndrome. [139]

The characterization of neuroendocrine-regulating CRF family peptides, in conjunction with the cloning and pharmacological characterization of the two major CRF receptor subtypes (CRF_1 and CRF_2) and the development of selective CRF receptor antagonists, provided new insights to explain the mechanisms of stress and the potential involvement of the CRF system in different pathophysiological conditions, including gastrointestinal disorders, mainly irritable bowel syndrome (IBS), and psychopathologies such as anxiety/depression. Compelling pre-clinical data demonstrated that central CRF administration mimics acute stress-induced colonic responses and enhances colorectal distension-induced visceral pain in rats through CRF_1 receptors. Similarly, peripheral CRF

reduced the pain threshold to colonic distension and increased colonic motility in both humans and rodents. These observations mimic the manifestations of IBS characterized by abdominal bloating and discomfort and altered bowel habits. CRF_1 pathways have been implicated in the development of anxiety/depression and these psychopathologies, together with stressful life events, have high co-morbidity with IBS and are considered significant components of the disease. CRF_1 receptors have been suggested as a target to treat IBS. Peripherally acting CRF_1 antagonists might directly improve IBS symptoms, as related to motility, secretion and immune response. On the other hand, central actions will be beneficial for the prevention of the psychopathologies that co-exist with IBS and as a way to modulate the central processing of stress- and visceral pain-related signals.[140,141]

SUMMARY

CRF plays a key role in the maintenance of homeostasis by regulating the hypothalamic-pituitary-adrenal axis, functioning as a neurotransmitter within the central nervous system and being involved in the control of the cardiovascular, gastrointestinal, behavioral, immune and reproductive systems. CRF exerts its actions by interacting with the CRF_1 and CRF_2 receptors, which belong to family B of G-protein coupled receptors. Considerable progress has been made in the determination of the structure and function of peptide and non-peptide CRF analogs, thus advancing to some extent the development of new compounds, including non-peptide CRF_1-selective antagonists. The non-peptide CRF analogs have significantly contributed to the determination of the role of CRF and its receptors in several physiological and pathophysiological conditions. Progress in elucidating the interactions of CRF ligands with their receptors will further advance the design and synthesis of new CRF analogs with potential clinical applications.

REFERENCES

1. Vale W, Spiess J, Rivier C, Rivier J, 1981 Characterization of a 41-residue ovine hypothalamic peptide that stimulates secretion of corticotropin and beta-endorphin. Science 213: 1394-1397.
2. Chrousos GP, 1995 The hypothalamic-pituitary-adrenal axis and immune-mediated inflammation. N Engl J Med 332: 1351-1362.
3. Owens MJ, Nemeroff CB, 1991 Physiology and pharmacology of corticotropin-releasing factor. Pharmacol Rev 43: 425-473.
4. Gold PW, Chrousos GP, 2002 Organization of the stress system and its dysregulation in melancholic and atypical depression: high vs low CRH/NE states. Mol Psychiatry 7: 254-275.
5. Keck ME, Holsboer F, 2001 Hyperactivity of CRH neuronal circuits as a target for therapeutic interventions in affective disorders. Peptides 22: 835-844.
6. Reul JM, Holsboer F, 2002 Corticotropin-releasing factor receptors 1 and 2 in anxiety and depression. Curr Opin Pharmacol 2: 23-33.
7. Dautzenberg FM, Kilpatrick GJ, Hauger RL, Moreau J, 2001 Molecular biology of the CRH receptors- in the mood. Peptides 22: 753-760.
8. Behan DP, Heinrichs SC, Troncoso JC, et al, 1995 Displacement of corticotropin releasing factor from its binding protein as a possible treatment for Alzheimer's disease. Nature 378: 284-287.
9. De Souza EB, Whitehouse PJ, Kuhar MJ, Price DL, Vale WW, 1986 Reciprocal changes in corticotropin-releasing factor (CRF)-like immunoreactivity and CRF receptors in cerebral cortex of Alzheimer's disease. Nature 319: 593-595.
10. Makrigiannakis A, Zoumakis E, Kalantaridou S, et al, 2001 Corticotropin-releasing hormone promotes blastocyst implantation and early maternal tolerance. Nat Immunol 2: 1018-1024.
11. Chrousos GP, Torpy DJ, Gold PW, 1998 Interactions between the hypothalamic-pituitary-adrenal axis and the female reproductive system: clinical implications. Ann Intern Med 129: 229-240.
12. Martinez V, Rivier J, Wang L, Tache Y, 1997 Central injection of a new corticotropin-releasing factor (CRF) antagonist, astressin, blocks CRF- and stress-related alterations of gastric and colonic motor function. J Pharmacol Exp Ther 280: 754-760.
13. Orth DN, 1992 Corticotropin-releasing hormone in humans. Endocr Rev 13: 164-191.
14. Parkes DG, Weisinger RS, May CN, 2001 Cardiovascular actions of CRH and urocortin: an update. Peptides 22: 821-827.
15. Venihaki M, Dikkes P, Carrigan A, Karalis KP, 2001 Corticotropin-releasing hormone regulates IL-6 expression during inflammation. J Clin Invest 108: 1159-1166.
16. Venihaki M, Majzoub JA, 1999 Animal models of CRH deficiency. Front Neuroendocrinol 20: 122-145.
17. Zoumakis E, Margioris AN, Makrigiannakis A, Stournaras C, Gravanis A, 1997 Human endometrium as a neuroendocrine tissue: expression, regulation and biological roles of endometrial corticotropin-releasing hormone (CRH) and opioid peptides. J Endocrinol Invest 20: 158-167.

18. Wang L, Martinez V, Rivier JE, Tache Y, 2001 Peripheral urocortin inhibits gastric emptying and food intake in mice: differential role of CRF receptor 2. Am J Physiol Regul Integr Comp Physiol 281: R1401-1410.
19. Martinez V, Wang L, Rivier JE, Vale W, Tache Y, 2002 Differential actions of peripheral corticotropin-releasing factor (CRF), urocortin II, and urocortin III on gastric emptying and colonic transit in mice: role of CRF receptor subtypes 1 and 2. J Pharmacol Exp Ther 301: 611-617.
20. Coste SC, Kesterson RA, Heldwein KA, et al, 2000 Abnormal adaptations to stress and impaired cardiovascular function in mice lacking corticotropin-releasing hormone receptor-2. Nat Genet 24: 403-409.
21. Coste SC, Quintos RF, Stenzel-Poore MP, 2002 Corticotropin-releasing hormone-related peptides and receptors: emergent regulators of cardiovascular adaptations to stress. Trends Cardiovasc Med 12: 176-182.
22. Karalis K, Sano H, Redwine J, et al, 1991 Autocrine or paracrine inflammatory actions of corticotropin-releasing hormone in vivo. Science 254: 421-423.
23. Agelaki S, Tsatsanis C, Gravanis A, Margioris AN, 2002 Corticotropin-releasing hormone augments proinflammatory cytokine production from macrophages in vitro and in lipopolysaccharide-induced endotoxin shock in mice. Infect Immun 70: 6068-6074.
24. Chatzaki E, Charalampopoulos I, Leontidis C, et al, 2003 Urocortin in human gastric mucosa: relationship to inflammatory activity. J Clin Endocrinol Metab 88: 478-483.
25. Koob GF, Bloom FE, 1985 Corticotropin-releasing factor and behavior. Fed Proc 44: 259-263.
26. Kempuraj D, Papadopoulou NG, Lytinas M, et al, 2004 Corticotropin-releasing hormone and its structurally related urocortin are synthesized and secreted by human mast cells. Endocrinology 145: 43-48.
27. Rivier J, Spiess J, Vale W, 1983 Characterization of rat hypothalamic corticotropin-releasing factor. Proc Natl Acad Sci U S A 80: 4851-4855.
28. Shibahara S, Morimoto Y, Furutani Y, et al, 1983 Isolation and sequence analysis of the human corticotropin-releasing factor precursor gene. Embo J 2: 775-779.
29. Ling N, Esch F, Bohlen P, Baird A, Guillemin R, 1984 Isolation and characterization of caprine corticotropin-releasing factor. Biochem Biophys Res Commun 122: 1218-1224.
30. Esch F, Ling N, Bohlen P, et al, 1984 Isolation and characterization of the bovine hypothalamic corticotropin-releasing factor. Biochem Biophys Res Commun 122: 899-905.
31. Patthy M, Horvath J, Mason-Garcia M, et al, 1985 Isolation and amino acid sequence of corticotropin-releasing factor from pig hypothalami. Proc Natl Acad Sci U S A 82: 8762-8766.
32. Okawara Y, Morley SD, Burzio LO, et al, 1988 Cloning and sequence analysis of cDNA for corticotropin-releasing factor precursor from the teleost fish Catostomus commersoni. Proc Natl Acad Sci U S A 85: 8439-8443.
33. Stenzel-Poore MP, Heldwein KA, Stenzel P, Lee S, Vale WW, 1992 Characterization of the genomic corticotropin-releasing factor (CRF) gene from Xenopus laevis: two members of the CRF family exist in amphibians. Mol Endocrinol 6: 1716-1724.
34. Montecucchi PC, Henschen A, 1981 Amino acid composition and sequence analysis of sauvagine, a new active peptide from the skin of Phyllomedusa sauvagei. Int J Pept Protein Res 18: 113-120.
35. Lederis K, Letter A, McMaster D, Moore G, Schlesinger D, 1982 Complete amino acid sequence of urotensin I, a hypotensive and corticotropin-releasing neuropeptide from Catostomus. Science 218: 162-165.
36. Reyes TM, Lewis K, Perrin MH, et al, 2001 Urocortin II: a member of the corticotropin-releasing factor (CRF) neuropeptide family that is selectively bound by type 2 CRF receptors. Proc Natl Acad Sci U S A 98: 2843-2848.
37. Lewis K, Li C, Perrin MH, et al, 2001 Identification of urocortin III, an additional member of the corticotropin-releasing factor (CRF) family with high affinity for the CRF2 receptor. Proc Natl Acad Sci U S A 98: 7570-7575.
38. Pallai PV, Mabilia M, Goodman M, Vale W, Rivier J, 1983 Structural homology of corticotropin-releasing factor, sauvagine, and urotensin I: circular dichroism and prediction studies. Proc Natl Acad Sci U S A 80: 6770-6774.
39. Dathe M, Fabian H, Gast K, et al, 1996 Conformational differences of ovine and human corticotropin releasing hormone. A CD, IR, NMR and dynamic light scattering study. Int J Pept Protein Res 47: 383-393.
40. Lau SH, Rivier J, Vale W, Kaiser ET, Kezdy FJ, 1983 Surface properties of an amphiphilic peptide hormone and of its analog: corticotropin-releasing factor and sauvagine. Proc Natl Acad Sci U S A 80: 7070-7074.
41. Rivier J, Rivier C, Vale W, 1984 Synthetic competitive antagonists of corticotropin-releasing factor: effect on ACTH secretion in the rat. Science 224: 889-891.
42. Beyermann M, Rothemund S, Heinrich N, et al, 2000 A role for a helical connector between two receptor binding sites of a long-chain peptide hormone. J Biol Chem 275: 5702-5709.
43. O'Neil KT, DeGrado WF, 1990 A thermodynamic scale for the helix-forming tendencies of the commonly occurring amino acids. Science 250: 646-651.
44. Ohta N, Mochizuki T, Hoshino M, et al, 1997 Adrenocorticotropic hormone-releasing activity of urotensin I and its fragments in vitro. J Pept Res 50: 178-183.
45. Kornreich WD, Galyean R, Hernandez JF, et al, 1992 Alanine series of ovine corticotropin releasing factor (oCRF): a structure-activity relationship study. J Med Chem 35: 1870-1876.
46. Gulyas J, Rivier C, Perrin M, et al, 1995 Potent, structurally constrained agonists and competitive antagonists

of corticotropin-releasing factor. Proc Natl Acad Sci U S A 92: 10575-10579.
47. Chen R, Lewis KA, Perrin MH, Vale WW, 1993 Expression cloning of a human corticotropin-releasing-factor receptor. Proc Natl Acad Sci U S A 90: 8967-8971
48. Harmar AJ, 2001 Family-B G-protein-coupled receptors. Genome Biol 2: REVIEWS3013.
49. Arai M, Assil IQ, Abou-Samra AB, 2001 Characterization of three corticotropin-releasing factor receptors in catfish: a novel third receptor is predominantly expressed in pituitary and urophysis. Endocrinology 142: 446-454.
50. Hillhouse EW, Grammatopoulos DK, 2006 The molecular mechanisms underlying the regulation of the biological activity of corticotropin-releasing hormone receptors: implications for physiology and pathophysiology. Endocr Rev 27: 260-286.
51. Ardati A, Goetschy V, Gottowick J, et al, 1999 Human CRF2 alpha and beta splice variants: pharmacological characterization using radioligand binding and a luciferase gene expression assay. Neuropharmacology 38: 441-448
52. Kostich WA, Chen A, Sperle K, Largent BL, 1998 Molecular identification and analysis of a novel human corticotropin-releasing factor (CRF) receptor: the CRF2gamma receptor. Mol Endocrinol 12: 1077-1085.
53. Rivier J, Gulyas J, Kirby D, et al, 2002 Potent and long-acting corticotropin releasing factor (CRF) receptor 2 selective peptide competitive antagonists. J Med Chem 45: 4737-4747.
54. Rivier J, Gulyas J, Kunitake K, et al, 2007 Stressin1-A, a potent corticotropin releasing factor receptor 1 (CRF1)-selective peptide agonist. J Med Chem 50: 1668-1674
55. Ruhmann A, Bonk I, Lin CR, Rosenfeld MG, Spiess J, 1998 Structural requirements for peptidic antagonists of the corticotropin-releasing factor receptor (CRFR): development of CRFR2beta-selective antisauvagine-30. Proc Natl Acad Sci U S A 95: 15264-15269.
56. Lawrence AJ, Krstew EV, Dautzenberg FM, Ruhmann A, 2002 The highly selective CRF(2) receptor antagonist K41498 binds to presynaptic CRF(2) receptors in rat brain. Br J Pharmacol 136: 896-904.
57. Tezval H, Jahn O, Todorovic C, et al, 2004 Cortagine, a specific agonist of corticotropin-releasing factor receptor subtype 1, is anxiogenic and antidepressive in the mouse model. Proc Natl Acad Sci U S A 101: 9468-9473.
58. Graziani G, Tentori L, Portarena I, et al, 2002 CRH inhibits cell growth of human endometrial adenocarcinoma cells via CRH-receptor 1-mediated activation of cAMP-PKA pathway. Endocrinology 143: 807-813.
59. Haug T, Storm JF, 2000 Protein kinase A mediates the modulation of the slow Ca(2+)-dependent K(+) current, I(sAHP), by the neuropeptides CRF, VIP, and CGRP in hippocampal pyramidal neurons. J Neurophysiol 83: 2071-2079.
60. Papadopoulou N, Chen J, Randeva HS, et al, 2004 Protein kinase A-induced negative regulation of the corticotropin-releasing hormone R1alpha receptor-extracellularly regulated kinase signal transduction pathway: the critical role of Ser301 for signaling switch and selectivity. Mol Endocrinol 18: 624-639.
61. Sheng H, Sun T, Cong B, et al, 2008 Corticotropin-releasing hormone stimulates SGK-1 kinase expression in cultured hippocampal neurons via CRH-R1. Am J Physiol Endocrinol Metab 295: E938-946.
62. Kovalovsky D, Refojo D, Liberman AC, et al, 2002 Activation and induction of NUR77/NURR1 in corticotrophs by CRH/cAMP: involvement of calcium, protein kinase A, and MAPK pathways. Mol Endocrinol 16: 1638-1651.
63. Brar BK, Chen A, Perrin MH, Vale W, 2004 Specificity and regulation of extracellularly regulated kinase1/2 phosphorylation through corticotropin-releasing factor (CRF) receptors 1 and 2beta by the CRF/urocortin family of peptides. Endocrinology 145: 1718-1729.
64. Traver S, Marien M, Martin E, Hirsch EC, Michel PP, 2006 The phenotypic differentiation of locus ceruleus noradrenergic neurons mediated by brain-derived neurotrophic factor is enhanced by corticotropin releasing factor through the activation of a cAMP-dependent signaling pathway. Mol Pharmacol 70: 30-40.
65. Melzig MF, Papsdorf G, Loose R, et al, 1998 Effects of long term treatment with corticotropin releasing factor on corticotropic tumor cells in vitro. Regul Pept 74: 35-40.
66. Dautzenberg FM, Gutknecht E, Van der Linden I, et al, 2004 Cell-type specific calcium signaling by corticotropin-releasing factor type 1 (CRF1) and 2a (CRF2(a)) receptors: phospholipase C-mediated responses in human embryonic kidney 293 but not SK-N-MC neuroblastoma cells. Biochem Pharmacol 68: 1833-1844.
67. Punn A, Levine MA, Grammatopoulos DK, 2006 Identification of signaling molecules mediating corticotropin-releasing hormone-R1alpha-mitogen-activated protein kinase (MAPK) interactions: the critical role of phosphatidylinositol 3-kinase in regulating ERK1/2 but not p38 MAPK activation. Mol Endocrinol 20: 3179-3195.
68. Grammatopoulos DK, Randeva HS, Levine MA, Katsanou ES, Hillhouse EW, 2000 Urocortin, but not corticotropin-releasing hormone (CRH), activates the mitogen-activated protein kinase signal transduction pathway in human pregnant myometrium: an effect mediated via R1alpha and R2beta CRH receptor subtypes and stimulation of Gq-proteins. Mol Endocrinol 14: 2076-2091.
69. Dermitzaki E, Tsatsanis C, Gravanis A, Margioris AN, 2002 Corticotropin-releasing hormone induces Fas ligand production and apoptosis in PC12 cells via activation of p38 mitogen-activated protein kinase. J Biol Chem 277: 12280-12287.
70. Markovic D, Punn A, Lehnert H, Grammatopoulos DK, 2008 Intracellular mechanisms regulating corticotropin-releasing hormone receptor-2beta endocytosis and

interaction with extracellularly regulated kinase 1/2 and p38 mitogen-activated protein kinase signaling cascades. Mol Endocrinol 22: 689-706.
71. Refojo D, Echenique C, Muller MB, et al, 2005 Corticotropin-releasing hormone activates ERK1/2 MAPK in specific brain areas. Proc Natl Acad Sci U S A 102: 6183-6188.
72. Karteris E, Hillhouse EW, Grammatopoulos D, 2004 Urocortin II is expressed in human pregnant myometrial cells and regulates myosin light chain phosphorylation: potential role of the type-2 corticotropin-releasing hormone receptor in the control of myometrial contractility. Endocrinology 145: 890-900.
73. Chang CP, Pearse RV 2nd, O'Connell S, Rosenfeld MG, 1993 Identification of a seven transmembrane helix receptor for corticotropin-releasing factor and sauvagine in mammalian brain. Neuron 11: 1187-1195.
74. Kishimoto T, Pearse RV, 2nd, Lin CR, Rosenfeld MG, 1995 A sauvagine/corticotropin-releasing factor receptor expressed in heart and skeletal muscle. Proc Natl Acad Sci U S A 92: 1108-1112
75. Lovenberg TW, Liaw CW, Grigoriadis DE, et al, 1995 Cloning and characterization of a functionally distinct corticotropin-releasing factor receptor subtype from rat brain. Proc Natl Acad Sci U S A 92: 836-840.
76. Perrin M, Donaldson C, Chen R, et al, 1995 Identification of a second corticotropin-releasing factor receptor gene and characterization of a cDNA expressed in heart. Proc Natl Acad Sci U S A 92: 2969-2973.
77. Nabhan C, Xiong Y, Xie LY, Abou-Samra AB, 1995 The alternatively spliced type II corticotropin-releasing factor receptor, stably expressed in LLCPK-1 cells, is not well coupled to the G protein(s). Biochem Biophys Res Commun 212: 1015-1021.
78. Markovic D, Papadopoulou N, Teli T, et al, 2006 Differential responses of corticotropin-releasing hormone receptor type 1 variants to protein kinase C phosphorylation. J Pharmacol Exp Ther 319: 1032-1042.
79. Markovic D, Grammatopoulos DK, 2010 Characterization of structural determinants of type 1 corticotropin releasing hormone (CRH) receptor signalling properties. Methods Mol Biol 634: 285-307.
80. Grammatopoulos DK, Dai Y, Randeva HS, et al, 1999 A novel spliced variant of the type 1 corticotropin-releasing hormone receptor with a deletion in the seventh transmembrane domain present in the human pregnant term myometrium and fetal membranes. Mol Endocrinol 13: 2189-2202.
81. Myers DA, Trinh JV, Myers TR, 1998 Structure and function of the ovine type 1 corticotropin releasing factor receptor (CRF1) and a carboxyl-terminal variant. Mol Cell Endocrinol 144: 21-35.
82. Punn A, Chen J, Delidaki M, et al, 2012 Mapping structural determinants within 3rd intracellular loop that direct signalling specificity of type 1 corticotropin releasing hormone receptor. J Biol Chem 287: 8974-8985.
83. Perrin MH, Sutton S, Bain DL, Berggren WT, Vale WW, 1998 The first extracellular domain of corticotropin releasing factor-R1 contains major binding determinants for urocortin and astressin. Endocrinology 139: 566-570.
84. Perrin MH, Fischer WH, Kunitake KS, et al, 2001 Expression, purification, and characterization of a soluble form of the first extracellular domain of the human type 1 corticotropin releasing factor receptor. J Biol Chem 276: 31528-31534.
85. Perrin MH, DiGruccio MR, Koerber SC, et al, 2003 A soluble form of the first extracellular domain of mouse type 2beta corticotropin-releasing factor receptor reveals differential ligand specificity. J Biol Chem 278: 15595-15600.
86. Klose J, Fechner K, Beyermann M, et al, 2005 Impact of N-terminal domains for corticotropin-releasing factor (CRF) receptor-ligand interactions. Biochemistry 44: 1614-1623.
87. Dautzenberg FM, Wille S, Lohmann R, Spiess J, 1998 Mapping of the ligand-selective domain of the Xenopus laevis corticotropin-releasing factor receptor 1: implications for the ligand-binding site. Proc Natl Acad Sci U S A 95: 4941-4946.
88. Wille S, Sydow S, Palchaudhuri MR, Spiess J, Dautzenberg FM, 1999 Identification of amino acids in the N-terminal domain of corticotropin-releasing factor receptor 1 that are important determinants of high-affinity ligand binding. J Neurochem 72: 388-395.
89. Pioszak AA, Parker NR, Suino-Powell K, Xu HE, 2008 Molecular recognition of corticotropin-releasing factor by its G-protein-coupled receptor CRFR1. J Biol Chem 283: 32900-32912.
90. Grace CR, Perrin MH, Gulyas J, et al, 2007 Structure of the N-terminal domain of a type B1 G protein-coupled receptor in complex with a peptide ligand. Proc Natl Acad Sci U S A 104: 4858-4863.
91. Hofmann BA, Sydow S, Jahn O, et al, 2001 Functional and protein chemical characterization of the N-terminal domain of the rat corticotropin-releasing factor receptor 1. Protein Sci 10: 2050-2062.
92. Qi LJ, Leung AT, Xiong Y, Marx KA, Abou-Samra AB, 1997 Extracellular cysteines of the corticotropin-releasing factor receptor are critical for ligand interaction. Biochemistry 36: 12442-12448.
93. Grace CR, Perrin MH, DiGruccio MR, et al, 2004 NMR structure and peptide hormone binding site of the first extracellular domain of a type B1 G protein-coupled receptor. Proc Natl Acad Sci U S A 101: 12836-12841.
94. Assil-Kishawi I, Abou-Samra AB, 2002 Sauvagine cross-links to the second extracellular loop of the corticotropin-releasing factor type 1 receptor. J Biol Chem 277: 32558-32561
95. Assil-Kishawi I, Samra TA, Mierke DF, Abou-Samra AB, 2008 Residue 17 of sauvagine cross-links to the first transmembrane domain of corticotropin-releasing factor receptor 1 (CRFR1). J Biol Chem 283: 35644-

35651.
96. Gkountelias K, Tselios T, Venihaki M, et al, 2009 Alanine scanning mutagenesis of the second extracellular loop of type 1 corticotropin-releasing factor receptor revealed residues critical for peptide binding. Mol Pharmacol 75: 793-800.
97. Kraetke O, Holeran B, Berger H, et al, 2005 Photoaffinity cross-linking of the corticotropin-releasing factor receptor type 1 with photoreactive urocortin analogues. Biochemistry 44: 15569-15577.
98. Liaw CW, Grigoriadis DE, Lorang MT, De Souza EB, Maki RA, 1997 Localization of agonist- and antagonist-binding domains of human corticotropin-releasing factor receptors. Mol Endocrinol 11: 2048-2053.
99. Liaw CW, Grigoriadis DE, Lovenberg TW, De Souza EB, Maki RA, 1997 Localization of ligand-binding domains of human corticotropin-releasing factor receptor: a chimeric receptor approach. Mol Endocrinol 11: 980-985.
100. Sydow S, Flaccus A, Fischer A, Spiess J, 1999 The role of the fourth extracellular domain of the rat corticotropin-releasing factor receptor type 1 in ligand binding. Eur J Biochem 259: 55-62.
101. Hoare SR, Fleck BA, Gross RS, et al, 2008 Allosteric ligands for the corticotropin releasing factor type 1 receptor modulate conformational states involved in receptor activation. Mol Pharmacol 73: 1371-1380.
102. Nielsen SM, Nielsen LZ, Hjorth SA, Perrin MH, Vale WW, 2000 Constitutive activation of tethered-peptide/corticotropin-releasing factor receptor chimeras. Proc Natl Acad Sci U S A 97: 10277-10281.
103. Hoare SR, Brown BT, Santos MA, et al, 2006 Single amino acid residue determinants of non-peptide antagonist binding to the corticotropin-releasing factor1 (CRF1) receptor. Biochem Pharmacol 72: 244-255.
104. Liapakis G, Ballesteros JA, Papachristou S, et al, 2000 The forgotten serine. A critical role for Ser-2035.42 in ligand binding to and activation of the beta 2-adrenergic receptor. J Biol Chem 275: 37779-37788.
105. Strader CD, Fong TM, Tota MR, Underwood D, Dixon RA, 1994 Structure and function of G protein-coupled receptors. Annu Rev Biochem 63: 101-132.
106. Lu ZL, Hulme EC, 1999 The functional topography of transmembrane domain 3 of the M1 muscarinic acetylcholine receptor, revealed by scanning mutagenesis. J Biol Chem 274: 7309-7315.
107. Wess J, Maggio R, Palmer JR, Vogel Z, 1992 Role of conserved threonine and tyrosine residues in acetylcholine binding and muscarinic receptor activation. A study with m3 muscarinic receptor point mutants. J Biol Chem 267: 19313-19319.
108. Donnelly D, 1997 The arrangement of the transmembrane helices in the secretin receptor family of G-protein-coupled receptors. FEBS Lett 409: 431-436.
109. Frimurer TM, Bywater RP, 1999 Structure of the integral membrane domain of the GLP1 receptor. Proteins 35: 375-386.
110. Gether U, 2000 Uncovering molecular mechanisms involved in activation of G protein-coupled receptors. Endocr Rev 21: 90-113.
111. Gkountelias K, Papadokostaki M, Javitch JA, Liapakis G, 2010 Exploring the binding site crevice of a family B G protein-coupled receptor, the type 1 corticotropin releasing factor receptor. Mol Pharmacol 78: 785-793
112. Gkountelias K, Spyridaki K, Giannopoylos P, et al, 2011 Determination of Residues in the Third Membrane-Spanning Segment of CRF1, which are Accessible in the Ligand Binding-Site Crevice of Receptor. The 29th Cyprus-Camerino-Noordwijkerhout Trends in Drug Research Medicinal Chemistry EFMC Symposium, Limassol, Cyprus.
113. Nova Pharmaceutical Corporation, 1990 Corticotropin-releasing factor antagonism compounds. US Patent Office.
114. Beck JP, Curry MA, Chorvat RJ, et al, 1999 Thiazolo[4,5-d]pyrimidine thiones and -ones as corticotropin-releasing hormone (CRH-R1) receptor antagonists. Bioorg Med Chem Lett 9: 1185-1188.
115. Huang CQ, Wilcoxen K, McCarthy JR, et al, 2003 Synthesis and SAR of 8-arylquinolines as potent corticotropin-releasing factor1 (CRF1) receptor antagonists. Bioorg Med Chem Lett 13: 3375-3379.
116. Yoon T, De Lombaert S, Brodbeck R, et al, 2008 The design, synthesis and structure-activity relationships of 1-aryl-4-aminoalkylisoquinolines: a novel series of CRF-1 receptor antagonists. Bioorg Med Chem Lett 18: 891-896.
117. Yoon T, De Lombaert S, Brodbeck R, et al, 2008 2-Arylpyrimidines: novel CRF-1 receptor antagonists. Bioorg Med Chem Lett 18: 4486-4490.
118. Arvanitis AG, Gilligan PJ, Chorvat RJ, et al, 1999 Non-peptide corticotropin-releasing hormone antagonists: syntheses and structure-activity relationships of 2-anilinopyrimidines and -triazines. J Med Chem 42: 805-818.
119. Whitten JP, Xie YF, Erickson PE, et al, 1996 Rapid microscale synthesis, a new method for lead optimization using robotics and solution phase chemistry: application to the synthesis and optimization of corticotropin-releasing factor1 receptor antagonists. J Med Chem 39: 4354-4357.
120. Chen C, Dagnino R, Jr, De Souza EB, et al, 1996 Design and synthesis of a series of non-peptide high-affinity human corticotropin-releasing factor1 receptor antagonists. J Med.Chem. 39: 4358-4360.
121. Chen YL, Obach RS, Braselton J, et al, 2008 2-aryloxy-4-alkylaminopyridines: discovery of novel corticotropin-releasing factor 1 antagonists. J Med Chem 51: 1385-1392.
122. Wustrow DJ, Capiris T, Rubin R, et al, 1998 Pyrazolo[1,5-a]pyrimidine CRF-1 receptor antagonists. Bioorg Med Chem Lett 8: 2067-2070.

123. Chen C, Grigoriadis D 2005 NBI 30775 (R121919), an orally active antagonist of the corticotropin-releasing factor (CRF) type-1 receptor for the treatment of anxiety and depression. In: Drug Dev. Res. (eds), Wiley Subscription Services Inc., A Wiley Company; pp, 216-226.
124. Holsboer F, Ising M, 2008 Central CRH system in depression and anxiety--evidence from clinical studies with CRH1 receptor antagonists. Eur J Pharmacol 583: 350-357.
125. Tellew JE, Lanier M, Moorjani M, et al, 2010 Discovery of NBI-77860/GSK561679, a potent corticotropin-releasing factor (CRF1) receptor antagonist with improved pharmacokinetic properties. Bioorg Med Chem Lett 20: 7259-7264.
126. Beck JP, Arvanitis AG, Curry MA, et al, 1999 Purin-8-ones as corticotropin-releasing hormone (CRH-R1) receptor antagonists. Bioorg Med Chem Lett 9: 967-972.
127. Webster EL, Lewis DB, Torpy DJ, et al, 1996 In vivo and in vitro characterization of antalarmin, a nonpeptide corticotropin-releasing hormone (CRH) receptor antagonist: suppression of pituitary ACTH release and peripheral inflammation. Endocrinology 137: 5747-5750.
128. Deak T, Nguyen KT, Ehrlich AL, et al, 1999 The impact of the nonpeptide corticotropin-releasing hormone antagonist antalarmin on behavioral and endocrine responses to stress. Endocrinology 140: 79-86.
129. Nielsen DM, Carey GJ, Gold LH, 2004 Antidepressant-like activity of corticotropin-releasing factor type-1 receptor antagonists in mice. Eur J Pharmacol 499: 135-146.
130. Habib KE, Weld KP, Rice KC, et al, 2000 Oral administration of a corticotropin-releasing hormone receptor antagonist significantly attenuates behavioral, neuroendocrine, and autonomic responses to stress in primates. Proc Natl Acad Sci U S A 97: 6079-6084.
131. McCarthy JR, Heinrichs SC, Grigoriadis DE, 1999 Recent advances with the CRF1 receptor: design of small molecule inhibitors, receptor subtypes and clinical indications. Curr Pharm Des 5: 289-315.
132. Zorrilla EP, Valdez GR, Nozulak J, Koob GF, Markou A, 2002 Effects of antalarmin, a CRF type 1 receptor antagonist, on anxiety-like behavior and motor activation in the rat. Brain Res 952: 188-199.
133. Briscoe RJ, Cabrera CL, Baird TJ, Rice KC, Woods JH, 2000 Antalarmin blockade of corticotropin releasing hormone-induced hypertension in rats. Brain Res 881: 204-207.
134. Funk CK, Zorrilla EP, Lee MJ, Rice KC, Koob GF, 2007 Corticotropin-releasing factor 1 antagonists selectively reduce ethanol self-administration in ethanol-dependent rats. Biol Psychiatry 61: 78-86.
135. Chu K, Koob GF, Cole M, Zorrilla EP, Roberts AJ, 2007 Dependence-induced increases in ethanol self-administration in mice are blocked by the CRF1 receptor antagonist antalarmin and by CRF1 receptor knockout. Pharmacol Biochem Behav 86: 813-821.
136. Marinelli PW, Funk D, Juzytsch W, et al, 2007 The CRF1 receptor antagonist antalarmin attenuates yohimbine-induced increases in operant alcohol self-administration and reinstatement of alcohol seeking in rats. Psychopharmacology (Berl) 195: 345-355.
137. Stinus L, Cador M, Zorrilla EP, Koob GF, 2005 Buprenorphine and a CRF1 antagonist block the acquisition of opiate withdrawal-induced conditioned place aversion in rats. Neuropsychopharmacology 30: 90-98.
138. Webster E, Barrientos R, Contoreggi C, 2002 Corticotropin releasing hormone (CRH) antagonist attenuates adjuvant induced arthritis: role of CRH in peripheral inflammation. J Rheumatol 29: 1252-1261.
139. Gabry K, Chrousos G, Rice K, 2002 Marked suppression of gastric ulcerogenesis and intestinal responses to stress by a novel class of drugs. Mol Psychiatry 7): 474-483.
140. Greenwood-Van Meerveld B, Johnson A, Cochrane S,2005 Corticotropin-releasing factor 1 receptor-mediated mechanisms inhibit colonic hypersensitivity in rats. Neurogastroenterol Motil 17: 415-422.
141. Martinez V, Taché Y, 2006 CRF1 receptors as a therapeutic target for irritable bowel syndrome. Curr Pharm Des 12: 4071-4088.