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Corticotropin-releasing factor peptide antagonists: design, characterization and potential clinical relevance

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Abstract

Elusive for more than half a century, corticotropin-releasing factor (CRF) was finally isolated and characterized in 1981 from ovine hypothalami and shortly thereafter, from rat brain. Thirty years later, much has been learned about the function and localization of CRF and related family members (Urocortins 1, 2 and 3) and their 2 receptors, CRF receptor type 1 ($CRFR_1$) and CRF receptor type 2 ($CRFR_2$). Here, we report the stepwise development of peptide CRF agonists and antagonists, which led to the development of the $CRFR_1$ agonist $Stressin_1$; the long-acting antagonists $Astressin_2$-B which is specific for $CRFR_2$; and Astressin B, which binds to both $CRFR_1$ and $CRFR_2$. This analog has potential for the treatment of CRF-dependent diseases in the periphery, such as irritable bowel syndrome.

Keywords

oCRF; h/rCRF; α–hel $CRF_{(9–41)}$; astressin; astressin B; [$DPhe^{12}$] CRF; $astressin\text{-}_2B$; CRF antagonists; $CRFR_1$; $CRFR_2$; CRF; stress; $stressin_1$

A. Introduction

On September 18, 1981, W. Vale, C. Rivier, J. Spiess and J. Rivier reported in Science, the characterization of the 41 residue ovine hypothalamic peptide that stimulates the secretion of adrenocorticotropin hormone (ACTH) and beta-endorphin (Vale et al., 1981). Each member of the team contributed according to his/her own experimental background and expertise. W. Vale headed the overall projects that dealt with the isolation and characterization of ovine hypothalamic (Spiess et al., 1981; Vale et al., 1981) and rat brain (Rivier et al., 1983b) corticotropin-releasing factors (CRF), and provided the critical *in vitro* cultured rat anterior pituitary cells (RAP) assay (Vale et al., 1972a) in collaboration with C. Rivier, who developed a radio-immuno assay specific for rat ACTH (Rivier et al., 1973a). It allowed the detection and identification of the active chromatographic zones (Rivier et al., 1983b; Vale et al., 1981) and was used in the *in vivo* bioassay that demonstrated the ability of the new CRF to release ACTH in rats (Rivier et al., 1973a). J. Rivier developed the reversed phase high performance liquid chromatography (RP-HPLC) systems (column supports, solvent systems, flow rates and temperature) that provided the best separations, reproducibility and

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recoveries for a peptide the size of CRF (Hoeger et al., 1987; Miller and Rivier, 1996; Rivier and McClintock, 1989; Rivier et al., 1982c), while J. Spiess developed the micro-sequencing that provided the putative sequences of ovine and rat CRF (Spiess et al., 1983; Vale et al., 1981). J. Rivier then further used improved protocols of solid phase peptide synthesis, along with preparative RP-HPLC, to obtain the replicate of ovine and rat CRFs and their methionine oxide derivatives (Rivier et al., 1983b; Vale et al., 1981), which confirmed the sequencing results described by Spiess et al. (1983). Each step presented many technical challenges that are well described in the cited literature, and will not be discussed here. Independently, and concomitantly, the structure of sauvagine isolated from the skin of the frog *Phyllomedusa Sauvagei* (Montecucchi and Henschen, 1981) and urotensin I from two teleost fish species (*Cyprinus Carpio* and *Catostomus Commersoni*) (Lederis et al., 1982) were reported.

Immediately upon availability of these sequences, a multi-pronged approach was developed for the extensive characterization of these peptides, including (but not limited to) their distribution in the brain, as well as biological, molecular, chemical, immunological, structural and functional characteristics. Analyses were initiated that used rational, systematic as well as intuitive analoging, a process whereby analogs were designed to fulfill needs that were not satisfied by CRF family members. For example, CRF analogs with higher affinity for their receptor(s) and higher resistance to biodegradation than CRF family members were more potent *in vitro* and longer acting *in vivo* than CRF itself. Some other substitutions resulted in improved chemical stability (for example, substitution of a methionine by norleucine or norvaline).

In the absence of competitive peptide antagonists, we developed specific CRF neutralizing antibodies that helped define, in a first phase, CRF's physiological role (Rivier et al., 1982b). The intravenous administration of a rabbit antiserum to ovine CRF (oCRF) markedly reduced the oCRF-induced rise of plasma ACTH in intact non-stressed adult male rats, while blocking more than 75 percent of the ACTH release observed in rats exposed to ether stress. Furthermore, immunoneutralisation of oCRF significantly lowered ACTH levels in adrenalectomized animals. These results provided evidence that endogenous CRF played an important physiological role in regulating ACTH secretion under a variety of basal and stimulated conditions. Antibodies, however, are not without shortcomings associated with their high molecular weight, species specificity, antigenicity and poor ability to be fully distributed in the brain. In view of the above, designing potent competitive antagonists of CRF became our top priority, so that we could assess the physiologic and patho-physiologic significance of endogenous CRF in experimental animals and, ultimately, in human.

B. Lessons learned from structure-activity relationship (SAR) studies of thyrotropin releasing hormone (TRH), gonadotropin releasing hormone (GnRH) and somatotropin release inhibiting factor (SRIF somatostatin) agonists

1. Agonists

In the late 70's, studies were initiated to establish the SARs of the then known hypothalamic peptides, namely TRH, GnRH, and somatostatin. To this effect, peptides were synthesized by solid phase method (Märki et al., 1981). Purification of the crude synthetic peptides generated after treatment with HF and cleavage from the methyl-benzhydryl-amine (MBHA) and Merrifield resins, respectively, were achieved by preparative RP-HPLC, using three different solvent systems (Gulyas et al., 1995). Peptides were then characterized by

analytical HPLC under various conditions (other than that used for purification), amino acid analysis, capillary zone electrophoresis (CZE), mass spectrometry (MS) and circular dichroism in some cases. The RAP assay (Vale et al., 1972a) was used to determine the potency of our analogs relative to a given concentration of oCRF. The same cells were also used to test analogs for antagonism (Vale et al., 1972b) while *in vivo* experiments were performed in rats (Rivier et al., 1982a; Rivier et al., 1982b).

The first priority in peptide analog design was to identify the shortest bioactive sequence by deleting amino acids (AAs) one or two at a time from the N-, C- (Rivier et al., 1974; Rivier et al., 1973b) or N- and C-termini (Vale et al., 1976), respectively. In the case of GnRH, deletion of the Gly^{10} residue and replacement by an ethylamide resulted in a 3-fold increase in potency (Fujino et al., 1973). However, further deletions resulted in significant, if not total loss of potency. Deletion of two AAs simultaneously from the N- and C-termini of Des-Ala-Gly-somatostatin, led to the discovery of the mini-somatostatins in general (Vale et al., 1976), and octapeptide $DTrp^8$ SRIF (ODT-8) (des-Ala^1-Gly^2, Lys^4, Asn^5, Thr^{12}, Ser^{13}-[$DTrp^8$]-SRIF in particular (Rivier et al., 1975). The second priority was to replace AAs by their optical isomers (Rivier et al., 1993) (or DAla in lieu of Gly) in order to identify the presence of putative turns in GnRH (Monahan et al., 1973) and SRIF (Rivier et al., 1975). Ala scans such as that of SRIF were also commonly used to gain an appreciation of the contribution of each native AA side chain to biological activity and potency (Rivier et al., 1975). Finally, additional substitutions by unnatural amino acids offered the possibility to constrain tertiary structures (Rizo et al., 1996), modulate solubility, [ImBzl-$DHis^6$, Pro^9-NHEt]-GnRH (Karten and Rivier, 1986), and increase potency of TRH analogs, [3-$MeHis^2$]TRH (Rivier et al., 1971), among others.

2. Antagonists

Generation of an antagonist of small (5–15 residues) and middle size (25–45 residues) peptides has generally resulted from some specific AA substitutions (often unnatural or of the D-configuration) (Rivier et al., 1983a), or deletion. Gonadotropin-releasing hormone (GnRH) (Vale et al., 1972b) and human parathyroid hormone (PTH) (Rosenblatt et al., 1977; Rosenblatt et al., 1978) exemplify the former and latter, respectively. In the case of GnRH, systematic deletions of one AA at a time, and of His^2 in particular, allowed us to identify His^2 as a critical amino acid for receptor activation, but less so for binding affinity. This provided the first lead for the development of GnRH antagonists. Substituting His^2 by a D-phenylalanine further improved antagonist potency significantly (Karten and Rivier, 1986). We therefore hypothesized that multiple substitutions might have additive effects such as, in the case of GnRH, increased duration of action, chemical/biological stability in solution (Karten and Rivier, 1986), and reduced toxicity (Karten et al., 1990). These findings were epithomized by the discovery of Fe200486 (Degarelix/Firmagon) (Broqua et al., 2002; Jiang et al., 2001), which blocks gonadotropin secretion upon monthly sc administration, lowers testosterone to castrate levels, and significantly reduces prostate specific antibodies (PSA) within a few days in men with prostate cancer. In the case of PTH, an antagonist resulted from the deletion of six amino acids at the NH_2 terminus of the fully active fragment, yielding the antagonist $PTH_{(7–34)}$-NH_2 (Horiuchi et al., 1983) and its long-acting derivative [$DTrp^{12}$, Tyr^{34}]$bPTH_{(7–34)}$ (Goldman et al., 1988).

C. CRF

1. Receptors

As briefly discussed in the Introduction, in 1982 our laboratory reported the isolation and structure identification of the hypothalamic peptide CRF. This was followed by the report, in 1993, of the primary structure of the CRF receptors type 1 [$CRFR_1$ (Chen et al., 1993)] and

in 1995, that of the CRF receptors type 2 [$CRFR_2$ (Perrin et al., 1995)]. With the discovery of these two receptors, the design of non peptide ligands became possible. Two CRF/Ucn receptors, encoded by different genes and existing in multiple forms as splice variants, have currently been cloned: $CRFR_1$ (Chang et al., 1993; Chen et al., 1993; Vita et al., 1993) and $CRFR_2$ (Kishimoto et al., 1995; Kostich et al., 1998; Lovenberg et al., 1995; Perrin et al., 1995; Stenzel et al., 1995). $CRFR_1$ is the predominant receptor type in the pituitary (Chalmers et al., 1995; Potter et al., 1994) and is also widely distributed throughout the central nervous system. The second CRF receptor, called type 2β (CRFR2β), is expressed not only in the rat brain, but also in peripheral tissues such as the heart, gastrointestinal tract, and epididymis (Perrin et al., 1995). Several reviews have appeared that summarize present knowledge about their distribution and respective roles (Bale and Vale, 2004; Broadbear, 2006; Dautzenberg and Hauger, 2002; Farrokhi et al., 2007; Hauger et al., 2006; Hauger et al., 2009; Krohg et al., 2008; Liapakis et al., 2011; Slominski et al., 2006; Smith et al., 1998; Van Pett et al., 2000; Zorrilla and Koob, 2010).

We will now describe, in chronological order, the development of long-acting agonists and antagonists to $CRFR_1$ and $CRFR_2$ and their potential clinical relevance. Specifically, we retrace the rationale behind the discovery of the main peptide CRF agonists and antagonists that became the workhorses for studies of tissue distribution, pharmacology and physiology of CRF and their receptors.

2. Agonists

CRF release during acute stress is essential for the survival of the organism [for ref., see for example (Gold et al., 1987; Habib et al., 2001; Pecoraro et al., 2006; Sapolsky, 2000)]. However, it is now also well documented that CRF released as a result of exposure to chronic stressors can influence emotions (such as fear and anxiety), and exert negative effects on the homeostasis of most physiological functions including the cardiovascular, gastrointestinal, immune and reproductive systems, the gastrointestinal tract, and the skin. Indeed, these findings have provided fascinating and challenging evidence for the links between emotions and disease (Sternberg, 1997). This review highlights thirty years of CRF peptide SAR studies carried out mostly in our laboratories (yielding ca. 1500 analogs, ca. 20 papers on SAR, and at least 15 different compounds for sale by commercial suppliers of reagents). In addition, Spiess and collaborators contributed two papers on CRF SAR, that dealt with the properties of the CRF receptor (Spiess et al., 1998), and the development of CRFR2β-selective anti-sauvagine-30 (Rühmann et al., 1998). Beyermann et al. achieved signaling selectivity of ligands for $CRFR_1$ by modifying the agonist's signaling domain (Beyermann et al., 2007). Also, using a single-point slight alteration set as a tool for structure-activity relationships, studies of oCRF yielded unique information on the role of each amino acid demonstrating that most analogs, with the exception of the inactive Thr to Ser^7, Arg to Lys^{16}, Glu to Asp^{17} and Gln to Asn^{34}, remained relatively potent (Beyermann et al., 1996). Mazur et al. developed sauvagine analogs selective for $CRFR_2$ while introducing substitutions at positions 35 and 39 (Mazur et al., 2005). Finally, Yamada et al. improved the potencies of the C-terminus 12 AAs of astressin (Yamada et al., 2004) while substituting a number of residues to yield [$DAla^{31}$,Glu^{32},Cha^{38},Asp^{39}]-Ac-$Ast_{(19–30)}$ with a K_i of 3.1 nM as compared to 2.0 nM for astressin.

a) Alpha-helical CRF and α-helical $CRF_{(9–41)}$ (Rivier et al., 1984)—The design of α-helical CRF with increased potency over that of oCRF, sauvagine (Svg) and urotensin I (U_1), allowed us to hypothesize that a fragment could be found that would retain high binding affinity for the CRF receptors without causing their activation. Based on Chou and Fassman's data (Chou and Fasman, 1978; Chou and Fasman, 1987), Montecucchi and Gozzini (Montecucchi and Gozzini, 1982) proposed that sauvagine and CRF assumed a

similar pattern of α-helixes and β-turns. Independently, we presented spectroscopic and physicochemical evidence for such a secondary structure for oCRF, rCRF, sauvagine, and U_1 (Lau et al., 1983; Pallai et al., 1983). Specifically, we hypothesized that such a feature might be essential to receptor recognition and binding. Using Chou and Fassman predictive and statistical analysis (Chou and Fasman, 1978), we designed and synthesized a 41-residue analog with optimized α-helix formation by introduction of the amino acid with the highest P_α value at positions where the aligned, naturally occurring members of the CRF family had nonidentical residues [see Rivier et al. (Rivier et al., 1984) for additional substitutions] (Table 1). When tested in the RAP cells assay, this analog (named α-helical CRF) was 2–3 times more potent than any of the then known members of the CRF family found to be equally potent in releasing ACTH *in vitro* (Vale et al., 1981) (Table 2). Knowing that amidation of the COOH-terminal of CRF was critical for potency, we then investigated the effects of systematic deletion of the NH_2-terminal amino acids of α-helical CRF on its biopotency *in vitro*. Most of the intrinsic activity was conserved even after deletion of residues 1 to 6. Deletion of the next three amino acids, however, led to α-helical $CRF_{(9–41)}$ that showed limited partial agonism with low potency when tested *in vitro*, and inhibited CRF-mediated release of ACTH when tested for antagonism in rats (see Figure 1) (Rivier et al., 1984).

b) α-helical $CRF_{(9–41)}$: Additional *in vivo* studies (Fisher et al., 1991)—Studies were then performed in conscious unrestrained rats to compare the ability of the CRF receptor antagonist α-helical $CRF_{(9–41)}$ to inhibit the actions of CRF in three *in vivo* bioassay systems. When both peptides were administered intracerebroventricularly (icv), an antagonist:agonist ratio between 6:1 and 12:1 was required to abolish CRF-induced elevations of plasma catecholamine levels. When both peptides were administered intravenously (iv), CRF-induced hypotension and tachycardia were completely prevented by an antagonist:agonist ratio of 6:1, while total blockade of CRF-induced elevations of plasma ACTH and β-endorphin levels required an antagonist:agonist ratio of 3,000:1. These results demonstrated a marked difference in the ability of α-helical $CRF_{(9–41)}$ to antagonize various biological actions of CRF, and supported the existence of multiple CRF receptor subtypes. The effectiveness of blocking CRF receptors within the brain was further illustrated by the ability of icv infusion of relatively low doses of α-helical $CRF_{(9–41)}$ to block the central effect of CRF (Lee and Sarna, 1997), or that of swim stress (Coskun et al., 1997) on gastric emptying, as well as stress-induced fighting (Tazi et al., 1987).

c) Ala scan of oCRF (Kornreich et al., 1992)—As mentioned earlier, there are precedents for the use of peptide alanine scans in order to gain an appreciation of the critical role played by certain amino acids side chains in a bioactive peptide (Kornreich et al., 1992). oCRF analogs that are monosubstituted by Ala have therefore been synthesized that were from 4.5 times more potent, to <0.001% as potent as native oCRF. The Ala substitutions which showed the greatest loss of potency (<1% of oCRF), consisted in replacing hydrophobic residues ([Ile^6], [Leu^8], [Leu^{14}], and [Leu^{38}]), while the Ala analogs which showed the greatest increase in potency were obtained by replacing hydrophilic residues ([Thr^{22}] and [His^{32}]). The next, and yet unproven, step will be to identify whether some or all of the recognized, beneficial substitutions can be introduced in the same molecule, and be additive in generating potent analogs. As deletion of the first 12 residues will generate peptides that are competitive antagonists, and as all Ala substitutions that yielded more potent analogs than oCRF are beyond residue 19, CRF antagonists that incorporate selected Ala substitutions, might become useful to treat CRF-dependent pathologies in the periphery.

d) Single point D-substitution of oCRF (Rivier et al., 1993)—oCRF analogs with monosubstitutions by D-amino acids were similarly synthesized and found to be between

two times as potent, to less than 0.005% as potent as CRF (Rivier et al., 1993). Out of 37 analogs in this series, three (DPhe^{12}, DGlu^{20} and DAla^{24}) were equipotent to or twice as potent as oCRF, twelve (DLeu^{10}, DLeu^{15}, DGlu^{17}, DVal^{18}, DAsp^{25}, DGln^{26}, DLeu^{27}, DAla^{28}, DGln^{29}, DHis^{32}, DSer^{33}, and DAsp^{39}) had relative potencies in the range from 10 to 60% that of oCRF, twenty-one (DPro^{5}, DIle^{6}, DSer^{7}, DLeu^{8}, DAsp^{9}, DThr^{11}, DHis^{13}, DLeu^{14}, DArg^{16}, DMet^{21}, DThr^{22}, DLys^{23}, DGln^{30}, DAla^{31}, DAsn^{34}, DArg^{35}, DLys^{38}, DLeu^{37}, DLeu^{38}, DLeu^{40}, and DAla^{41}) had potencies ranging from inactive to 10% of that of CRF. From this series, we identified the DPhe^{12} substitution used in further improved agonists and antagonists. Indeed, increased potencies of these molecules *in vitro* led to increased duration of action of both agonists and antagonists (Figures 3 and 4) (Rivier et al., 1993). While such systematic studies lack the luster of hypothesis-directed research, it is still the most efficient approach to identify which residues within the sequence of a biologically active peptide are most sensitive to alteration.

e) [DPhe^{12},Nle^{21,38},C$^{\alpha}$MeLeu^{37}]-r/h CRF$_{(12–41)}$ C$^{\alpha}$MeLeu and Aib partial scan (Hernandez et al., 1993)—The strategy behind the design of [DPhe^{12},Nle^{21,38},C$^{\alpha}$MeLeu^{37}]-r/h CRF$_{(12–41)}$ (also referred to as DPhe^{12} CRF in the literature; Table 1) was to introduce in the same molecule, substitutions known to improve *in vitro* potency [i.e., DPhe^{12} was identified from a D-AA scan of oCRF (Rivier et al., 1993)]. Of interest was the fact that the C$^{\alpha}$MeLeu substitution did not bring detectable conformational changes in the expected way (i.e., stabilization of helical structure as monitored by CD). On the other hand, introduction of N$^{\alpha}$Me amino acids resulted in drastic conformational changes (loss of α-helicity) and biological activity. We used DPhe^{12} CRF as a standard in our *in vitro* RAP assay, and >20 publications showing its inhibitory activity and potency resulted from the use of this analog (Baram et al., 1996; Chen et al., 2012; Curtis et al., 1994; Curtis et al., 1997; Hernandez et al., 1993; Jones et al., 1999; Lowery and Thiele, 2010; Macey et al., 2000; Martinez et al., 1999; Menzaghi et al., 1994; Rodriguez de Fonseca et al., 1996; Tache and Brunnhuber, 2008; Zislis et al., 2007). Nle at positions 21,38 to replace the Methionines conferred stability against oxidation. In addition, we scanned the sequence of [DPhe^{12},Nle^{21,38}]-r/h CRF$_{(12–41)}$ with C$^{\alpha}$MeLeu at positions 14, 15, 37 and Glu^{17} or Aib at positions 20, 22, 24, 26, 28, 30, 31, 32, 33, 34, 39, and 40, respectively. Most analogs retained relative potencies close to that of DPhe^{12} CRF with the exception of Aib^{26}. In addition, we found that C$^{\alpha}$MeLeu^{37} increased local helicity and enzymatic stability. As illustrated in Figure 2, the combined substitutions yielded the first antagonist that inhibited ACTH secretion *in vivo* for more than one hour at the doses tested. This antagonist was also tested in a variety of other *in vivo* systems. For example, the central administration of [DPhe^{12},Nle^{21,38},C$^{\alpha}$MeLeu^{37}]-r/h CRF$_{(12–41)}$ antagonized defensive behavior responses to swim stress (Fonseca et al., 1996), and prevented gastroparesis-induced by surgery (Barquist et al., 1996; Hernandez et al., 1993). [For a more complete discussion see (Stengel and Tache, 2010; Tache et al., 2001) for blockade of visceral responses to stress, and for blockade of various stress-related behaviors, see (Koob, 2008b; a).]

The next contribution from our laboratory was to increase *in vivo* and *in vitro* potencies through the introduction of backbone constraints generated by the use of side chain to side chain bridging, thus constraining the tertiary structure.

f) Cyclo(30–33)[DPhe^{12},Nle^{21,38},Glu^{20},Lys^{23}/Orn^{23}]-r/h CRF$_{(12–41)}$ (Miranda et al., 1994)—With limited literature reference as to where to position lactam bridges as well as their size and chirality, we systematically addressed several hypotheses related to the optimal bridging configuration. First, can bridgeheads be located at positions where a D-residue is allowed, provided that bridge length, chirality, and dipole direction are optimized? Second, can we suggest that any improvement in receptor affinity is mediated through

stabilization of an α- helical structure, a hypothesis that is compatible with the observation that all of these biologically active structures can assume an α-helical conformation in the presence of TFE? Third, by assuming an α-helical bioactive structure for CRF, will a simple predictive tool based on Cα-Cα distances in an ideal polyalanine α-helical model be developed and used with some predictive value? We found an analog with a cycle centered around residue Glu^{20} ([cyclo(20–23)[$DPhe^{12}$,Glu^{20},Lys^{23},$Nle^{21,38}$]h/r$CRF_{(12–41)}$]) with an *in vitro* potency slightly greater than that of $DPhe^{12}$ CRF. This suggested that restricted conformational freedom is compatible with strong antagonist potency (Miranda et al., 1994).

g) Cyclo(30–33)[$DPhe^{12}$,$Nle^{21,38}$,Glu^{30},Lys^{33}]-r/h $CRF_{(12–41)}$ (astressin) (Gulyas et al., 1995)—It took several additional trials before we identified the cyclo (Glu^{i}-Lys^{i+3}), and not (Lys^{i}-Glu^{i+3}), as the best ring size and composition (Gulyas et al., 1995; Miranda et al., 1994) for side chain stabilization, at a time when published work used mostly cyclo(Xaa^{i}-Xbb^{i+4}) scaffolds (Grace et al., 2007a). The discovery of astressin with increased *in vitro* potency (32.5 times that of $DPhe^{12}$CRF or $DPhe^{12}C^{\alpha}MeLeu^{37}$, RAP assay (Table 2) and binding affinity to both $CRFR_1$ and $CRFR_2$ over that of [$DPhe^{12}$,$Nle^{21,38}$]-r/h $CRF_{(12–41)}$ and [$DPhe^{12}$,$Nle^{21,38}$,$C^{\alpha}MeLeu^{37}$]-r/h $CRF_{(12–41)}$ (Table 2), resulted from constraining the structure of [$DPhe^{12}$,$Nle^{21,38}$]-r/h $CRF_{(12–41)}$ further through the introduction of a lactam bridge (see Table 1 and below). We then chose to scan the whole sequence of the CRF antagonist [D-Phe^{12},$Nle^{21,38}$]r/h$CRF_{(12–41)}$ with an i-(i + 3) bridge consisting of the Glu-Xaa-Xbb-Lys lactam scaffold. We found the nonselective $CRFR_1$ and $CRFR_2$ antagonist astressin {cyclo(30–33) [$DPhe^{12}$,$Nle^{21,38}$,Glu^{30},Lys^{33}] r/h$CRF_{(12–41)}$} to be approximately 30 times more potent than [$DPhe^{12}$,$Nle^{21,38}$]r/h$CRF_{(12–41)}$ see Table 2, and 300 times more potent than the corresponding linear analog in an *in vitro* RAP assay. When we used radioiodinated [D-^{125}I-Tyr^{12}]astressin (CRF numbering) as a reliable ligand for binding assays, we observed that astressin had low affinity for the CRF binding protein (Behan et al., 1995) and high affinity (K_i = 2 nM) for the cloned pituitary receptor. *In vivo*, astressin (Figure 3) was found significantly more potent than any previously tested antagonists in reducing ACTH secretion in stressed adrenal-intact, as well as non-stressed adrenalectomized rats, and completely blocked the inhibitory effect of CRF and other members of the CRF family on gastric emptying (Martinez et al., 1998). The observation that by contrast, the $CRFR_1$-selective antagonist NBI-27914 was ineffective in this system, suggested the participation of $CRFR_2$ receptors in mediating gastric empyting. The cyclo(30–33) [Ac-Pro^{4},$DPhe^{12}$,$Nle^{21,38}$,Glu^{30},Lys^{33}]r/h$CRF_{(4–41)}$ agonist and its linear analog are nearly equipotent, while the antagonist astressin and its linear form vary greatly in their potencies. This suggests that the 30–33 lactam cyclization reinstates a structural constraint in the antagonists that is normally induced by the N terminus of the agonist.

Prior generations of CRF antagonists had been administered at high concentrations in the central nervous system, and shown to effectively blunt endogenous CRF actions; however, antagonists that would be potent and long acting on ACTH secretion were still lacking. As illustrated above, astressin was a significant improvement over previously available CRF antagonists due to its high potency, low intrinsic activity, high receptor affinity, and high solubility in neutral aqueous solutions. Because astressin is effective at low doses, the impact of its limited intrinsic activity at high concentrations *in vitro* may be of little consequence *in vivo*. Also, the availability of a high affinity antagonist radioligand has allowed further advances in receptor pharmacology (Perrin et al., 1999). These results illustrated the role that secondary and tertiary structures may play in controlling biological signaling through protein-ligand interactions. To our knowledge, there is no documented evidence that such long distance induction/stabilization (imparted by residues 4–11) of α-helix formation could be restored by a single bridging element 20 residues down the sequence, upon deletion of these residues. We have shown that Glu/Lys i-(i + 3) and i-(i + 4)

lactam bridges may impart the proper geometry for the stabilization of an α-helical backbone (Miranda et al., 1994).

h)Cyclo(30–33)[DPhe^{12},Nle^{21,38},MeLeu^{27,40},Glu^{30},Lys^{33}]-Ac-r/h CRF$_{(9–41)}$ (astressin B) (Rivier et al., 1998)—Next, we identified specific modifications and substitutions of CRF that led to the discovery of antagonists with extended duration of action as compared to that of astressin (Rivier et al., 1998). These additional modifications included elongation of the peptide chain by three residues at the N-terminus, its acetylation, and the [C$^{\alpha}$MeLeu^{37}] substitution to yield cyclo(30–33) [DPhe^{12},Nle^{21},Glu^{30},Lys^{33},C$^{\alpha}$MeLeu^{37},Nle^{38}]Ac-hCRF$_{(9–41)}$. To further increase the efficiency (potency, duration of action, and bioavailability) of this family of antagonists, we introduced two or more C$^{\alpha}$Me-leucine residues at positions shown in earlier studies to be favorable (Hernandez et al., 1993). Whereas the introduction of C$^{\alpha}$Me-leucine residues at positions 27 and either 18, 37, or 40 resulted in dramatic increases in duration of inhibitory action in adx rats after iv injection, the same substitution at positions 27 and either 15, 17, 19, or 41 led to short acting analogs. Other substitutions by C$^{\alpha}$MeLeu at positions 27 and either 10, 13, 14, 21, 24, 36, or 38, yielded analogs with duration of action intermediate between those mentioned above. Cyclo(30–33)[DPhe^{12}, Nle^{21},C$^{\alpha}$MeLeu^{27},Glu^{30},Lys^{33},Nle^{38},C$^{\alpha}$MeLeu^{40}]Ac-hCRF$_{(9–41)}$ (astressin B) was one of the most efficacious analogs of this series (>4 h inhibition of ACTH secretion at 25 μg/adx rat). It was found to be even longer acting via subcutaneous (sc) administration in either an aqueous (>24 h inhibition of ACTH secretion at 100 μg/adx rat) or lipid milieu (DMSO/ peanut oil, >24 h inhibition of ACTH secretion at 30 μg/adx rat) than after iv administration (<12 h inhibition of ACTH secretion at 100 μg/adx rat) (Figure 4). The effectiveness of astressin B was also demonstrated in other systems, such as its ability to significantly interfere with CRF-induced ACTH release in the Rhesus monkey (Broadbear et al., 2004), the prevention of CRF-induced changes in intestinal permeability in the isolated ileum (Overman et al., 2012), or intestinal barrier dysfuntion caused by early weaning in piglets (Smith et al., 2010). These results demonstrated the efficacy of this antagonist in a variety of stress-induced conditions, as well as across species. We concluded that C$^{\alpha}$-methylation at some positions may favor a bioactive conformation while also preventing degradation and/or elimination, resulting in significant extension of duration of action.

i) Short analogs of astressin (Yamada et al., 2004)—An important contribution was made by Yamada et al. (Yamada et al., 2004), who discovered that short C-terminus fragment 19–30 of astressin (astressin numbering) retained high binding affinity. Additional SAR studies of that peptide led to improved binding affinity. The authors assumed that a particular surface of the alpha-helix was important for binding to the receptor. The small peptide containing DAla^{31}, Asn^{34} and cyclohexylalanine (Chx^{38}) on that surface as well as Glu^{32} and Asp^{39} on an adjacent surface was as potent as astressin in binding to the CRF receptor and showed significant ACTH suppression when administered to rats.

j) Astressin$_2$-B (Rivier et al., 2002), anti-sauvagine 30 (Rühmann et al., 1998)—We then reported that moving the lactam bridge by two residues toward the C-terminus, led to CRFR$_2$ selectivity (astressin$_2$-B) (Rivier et al., 2002) (Tables 1 and 2), and that therefore members of the CRF family assumed distinct structures when interacting with the CRF$_1$ and CRF$_2$ receptors. Predictive methods, physicochemical measurements, and structure-activity relationship studies have suggested that CRF, its family members, and competitive antagonists such as astressin {cyclo(30–33)-[DPhe^{12},Nle^{21},Glu^{30},Lys^{33},Nle^{38}]hCRF$_{(12–41)}$}, assume an α- helical conformation when interacting with their receptors (Grace et al., 2010; Grace et al., 2007b; Koerber et al., 1998). As indicated earlier, we had shown that α-helical CRF$_{(9–41)}$ and sauvagine exhibited some selectivity for CRF receptors other than that

responsible for ACTH secretion, and later for CRF_2 binding (Perrin et al., 1999). We then suggested the possibility of a helix-turn-helix motif around a turn encompassing residues 30–33 (Koerber et al., 1998), that would confer high affinity for both CRF_1 and CRF_2 (Perrin et al., 1999; Rühmann et al., 1998) in agonists as well as antagonists of all members of the CRF family (Koerber et al., 1998). On the other hand, the substitutions that conferred ca. 100-fold CRF_2 selectivity to the antagonist antisauvagine- 30 {[DPhe^{11},His^{12}]-sauvagine$_{(11–40)}$}, did not confer such property to the corresponding N-terminally extended agonists. We observed that a Glu^{32}-Lys^{35} side chain to side chain covalent lactam constraint in hCRF and the corresponding Glu^{31}-Lys^{34} side chain to side chain covalent lactam constraint in sauvagine, yielded potent ligands that were selective for CRF_2. Additionally, we introduced deletions and substitutions known to increase duration of action to yield antagonists such as cyclo(31–34)[DPhe^{11},His^{12},C$^{\alpha}$MeLeu^{13,39},Nle^{17},Glu^{31},Lys^{34}]Ac-sauvagine$_{(8–40)}$ (astressin$_2$-B) with CRF_2 selectivities greater than 100-fold. CRF receptor autoradiography was performed in rat tissue known to express CRF_2 and CRF_1 in order to confirm that astressin$_2$-B could indeed bind to established CRF_2, but not CRF_1 receptor-expressing tissues. Extended duration of action of astressin$_2$-B versus that of anti-sauvagine-30 was also demonstrated in the CRF_2-mediated animal model, whereby the inhibition of gastric emptying of a solid meal in mice by urocortin administered intraperitoneally at time zero was antagonized by the administration of astressin$_2$-B but not by anti-sauvagine-30 at times -3 and -6 h. Both peptides were effective when given 10 min before urocortin (Martinez et al., 1998).

k) Stressin$_1$ (Rivier et al., 2007)—As described above, the potencies and selectivity of peptide CRF antagonists is increased through structural constraints, suggesting that the resulting ligands assume distinct conformations when interacting with CRF_1 and CRF_2 receptors. To develop selective CRF receptor agonists, we have scanned the sequence -Gln-Ala-His-Ser-Asn-Arg-(residues 30–35 of [DPhe^{12},Nle^{21,38}]Ac-hCRF$_{(4–41)}$) with an *i*-(*i*+3) bridge consisting of the Glu^{i}-Xaa-Xbb-Lys^{i+3} scaffold, where residues *i* = AA^{30}, AA^{31}, and AA^{32}. When *i* = AA^{31}, stressin$_1$, a potent CRF_1 receptor-selective agonist was generated (Table 1). *In vitro* (RAP assay), stressin$_1$ was equipotent to h/rCRF to release ACTH (Table 2). Astressin$_1$ showed a low nanomolar affinity for CRF_1 receptor (K_i = 1.7 nM) and greater than 100-fold selectivity versus CRF_2 receptor (K_i = 222 nM). Stressin$_1$ released slightly less ACTH than oCRF in adult adrenal-intact male rats, with increased duration of action. Interestingly, stressin$_1$, injected intraperitoneally in rats, induced fecal pellet output (a CRF_1 receptor-mediated response) and did not influence gastric emptying and blood pressure (CRF_2 receptor-mediated responses).

D. Pharmacological use of long acting peptide CRF antagonists

As discussed above, CRF receptors are present in many peripheral organs, including the heart, the GI tract, the skin and the gonads, as well as in specialized cells of the immune system. It is therefore reasonnable to predict that a variety of stress-related pathologies might be alleviated by CRF antagonists. Indeed, many investigators have used CRF antagonists in their own system, and collectively this work suggests that these analogs have an important potential as clinical tools. What follows is a short list, without discussion, of some of these findings.

Tache et al. have suggested a role for CRF in functional gastrointestinal disorders (Tache et al., 2009), and for CRF antagonists as a potential drugs for future therapies in gastroenterology (Taché, 2004). More specifically, these investigators have emphasized the role of peripheral CRF signalling pathways in stress-related alterations of gut motility and mucosal function and integrities (Taché and Perdue, 2004). Independently, Risbrough et al. suggest that CRF receptors exert both additive and opposing influences on startle defensive

behavior (Risbrough et al., 2004). Moeser et al. reported that early weaning stress impairs development of mucosal barrier function in the porcine intestine (Smith et al., 2010), while CRF induces intestinal epithelial barrier injury via the release of mast cell proteases and TNF-alpha (Overman et al., 2012). Ferrin et al. showed that astressin B prevents the inhibitory effect of ghrelin on luteinizing hormone pulse frequency in the ovariectomized Rhesus monkey (Vulliémoz et al., 2008), while the nonpeptidic antagonist Antalarmin show differing profiles of activity in Rhesus monkeys (Broadbear et al., 2004).

In humans, mood disorders such as depression are associated with significant pathological changes in the activity of the HPA axis [see for example (Dinan, 2001; Kandel, 1999; Keller et al., 2006; Slattery et al., 2004; Tichomirowa et al., 2005)]. Consequently, it was hypothesized that compounds that would block endogenous CRF activity in the brain might have interesting and useful anti-depressant activity. As a result of years of design of CRF ligands, several of these peptides have become extremely useful reagents in defining the complex role of CRF's family members (Table 1). However, except for α–helCRF$_{(9–41)}$, no CRF peptide antagonist has yet been tested in humans (Fukudo, 2007; Fukudo et al., 1998). We explain this lack of interest by academia for peptide ligands to CRFRs, by the complexity of the syntheses and the significant costs associated with the use of a quantitative *in vitro* assay. While the peripheral administration of peptide CRF analogs offers clinical opportunities listed in part above, it is important to note that these antagonists are not able to penetrate the brain, and therefore do not access CRF neurons in the CNS. An alternate approach would be to use nasal administration to circumvent the blood-brain barrier (BBB) (Bethlehem et al., 2012; Zhu et al., 2012), an approach that has been successfully used for the delivery of oxytocin to patients with schizophrenia (Berardis et al., 2013) and fear extinction deficit (Acheson et al., 2013). On the other hand, the clinical potential of non peptidic CRFR$_1$ antagonists that would be orally active and would penetrate the BBB, has not escaped the pharmaceutical industry, which has pursued the quest for such small molecules very energetically. However to date, non peptidic antagonists capable of targeting the CNS following peripheral administration, have not yet proven clinically useful (Griebel and Holsboer, 2012).

E. Conclusion

The purpose of this brief review was to describe the history of the development of CRF peptidic antagonists, including the rationale used for the synthesis of long-acting analogs. In view of the presence of CRF receptors in many organs, it seems reasonable to predict that these compounds may play a major role in the treatment of chronic stress-induced pathologies in the periphery. Indeed, there is animal (Chatzaki et al., 2013; Moeser et al., 2007; Smith et al., 2009) and clinical (Fukudo, 2007; Fukudo et al., 1998) evidence for the potential of CRF peptide antagonists for the treatment of gastrointestinal dysfunctions such as irritable bowel syndrome, as well as disorders of the skin (Slominski et al., 2013) and reproductive system (Xiao et al., 2007). Additionally, if one can develop intranasal modes of delivery, it is possible that CRF antagonists may also become useful in the treatment of brain pathologies such as post-traumatic stress syndrome and depression. Obviously much remains to be done in this regard, but mounting evidence for the involvement of endogenous CRF in a variety of stress-releated diseases, suggests that developing means to block the receptors that mediate the effects of this peptide, may address as yet unmet clinical needs.

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I, Jean Rivier, first met Wylie Vale at Rice University in Houston (Texas) for a game of tennis in August 1969. To tell the truth, our skills did not match our aspirations. Wylie's tenacity (and the weather) generally decided the outcome. A year later, we had moved to the Salk Institute in La Jolla and benefitted from Wylie's intimate knowledge of the American way of life, which Catherine and I quickly embraced (he rented a house, we rented an apartment on the beach; he bought a house and so did we). Betty and he, and Catherine and I, also started a family around the same time. On the work front, we quickly learned to collaborate in the laboratory in a way that respected each other's boundaries: for him it was whole animal physiology and neuroendocrinology, while I developed analytical, synthetic and structural peptide expertise. Our combined skills led to fascinating results. I miss a friend, a colleague, and the challenges he would bestow upon the members of the peptide biology laboratories

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Highlights

- CRFs are mediators of gut, heart, skin, immune and CNS homeostasis
- First antagonist α-helical $CRF_{(9–41)}$ is potent in the brain, less though in the periphery
- CRF antagonists will likely play a major role in stress-related diseases
- $Astressin_2$-B was designed to be a $CRFR_2$ selective long acting antagonist
- $Stressin_1$ was designed to be a $CRFR_1$selective long acting agonist

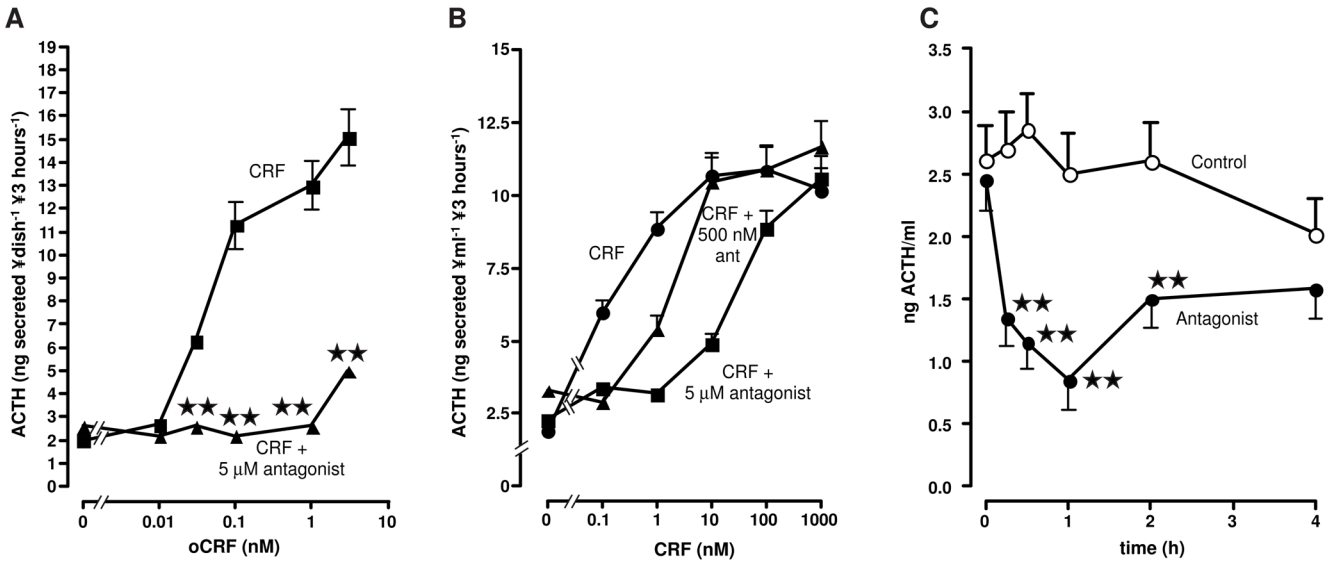

Figure 1.

(A) Interaction between 1 nM CRF (■) and increasing doses of α-helical $CRF_{(9–41)}$ (▲) on ACTH secretion by rat anterior pituitary cells in monolayer culture. (B) Effect of increasing doses of CRF (●) on ACTH release in the presence of 500 nM (▲) or 5 μM (■) α-helical $CRF_{(9–41)}$ on ACTH secretion by rat anterior pituitary cells in monolayer culture. (C) Effect of the iv injection of the vehicle (○) or 1 mg (0.6 μmole/kg) α-helical $CRF_{(9–41)}$ (●) on ACTH release by non-stressed, adrenalectomized male rats. Each point represents the mean + SEM of 6 animals. **, P<0.01 vs. vehicle. Results reproduced from (Rivier et al., 1984) by permission.

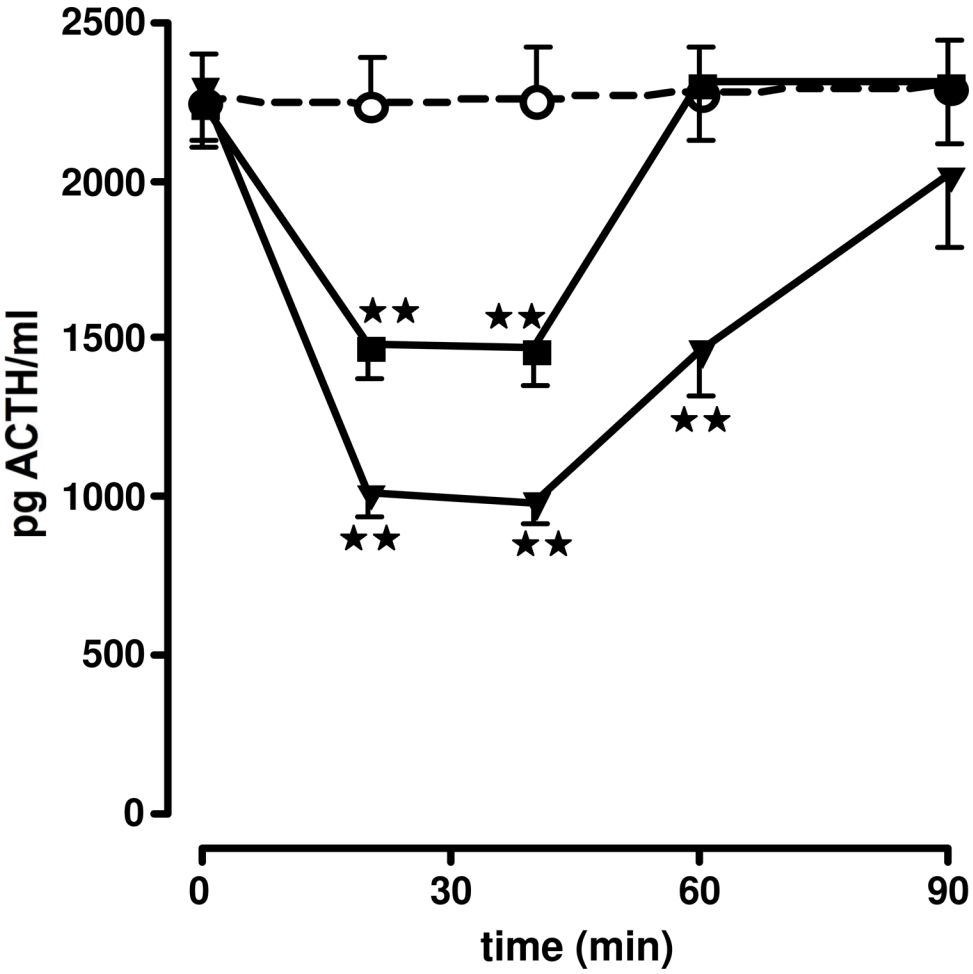

Figure 2.

Effect of the iv injection of the vehicle ○, [DPhe^{12},Nle^{21,38}]h/rCRF$_{12–41}$ ■ or [DPhe^{12},Nle^{21,38},C$^{\alpha}$MeLeu^{37}]h/rCRF$_{12–41}$ ▼, diluted in aqueous solvent, on ACTH secretion in non-stressed adx male rats. Each point represents the mean + SEM of 5–6 animals. **, P<0.01 vs. vehicle. Results reproduced from (Hernandez et al., 1993) by permission.

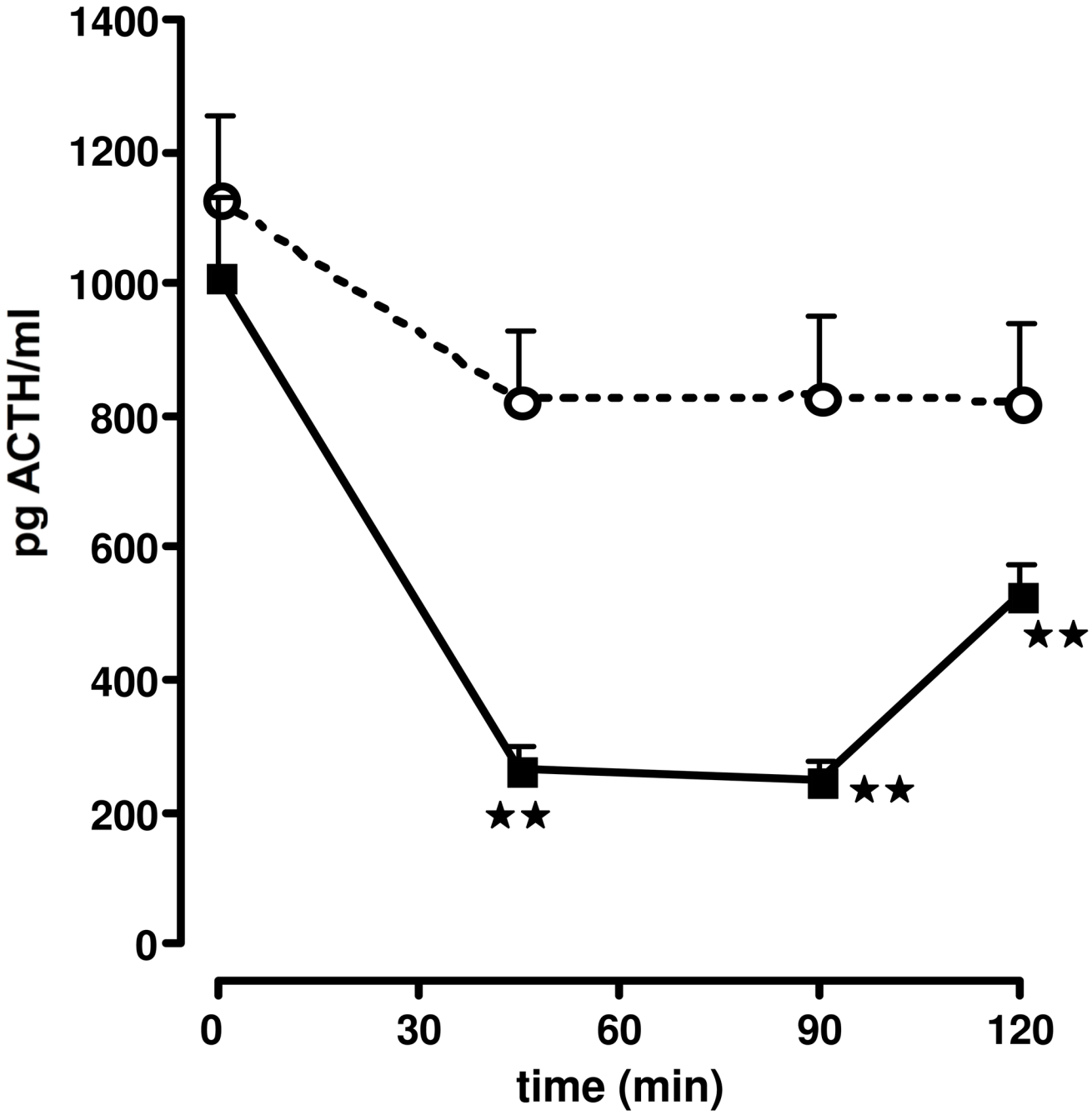

Figure 3.

Effect of the iv injection of the vehicle ○ or astressin ■, diluted in aqueous solvent, on ACTH secretion by non-stress adx male rats. Each point represents the mean + SEM of 5–6 animals. **, P<0.01 vs. vehicle. Results reproduced from (Gulyas et al., 1995) by permission.

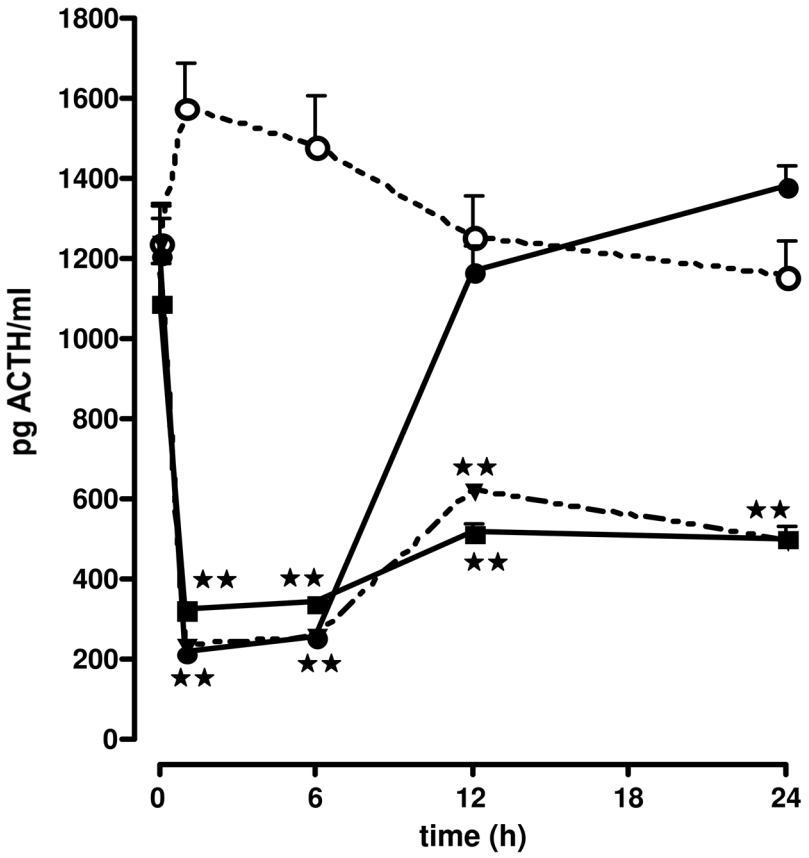

Figure 4.

Effect of the injection of the vehicle ○ or astressin B (●, 400 μg/kg iv in aqueous solvent; ▼, 1.2 mg/kg sc in aqueous solvent; ■, 120 μg/kg sc in oil), on ACTH secretion in non-stressed adx male rats. Each point represents the mean + SEM of 5–6 animals. **, P<0.01 vs. vehicle. Results reproduced from (Rivier et al., 1999) by permission.

Table 1

Sequences of CRF family members and analogs

oCRF	S QEPPISLDLT	FHLLREVLEM	TKADQLAQQA	HSNRKLLDIA-NH_2
h/rCRF	S EEPPISLDLT	FHLLREVLEM	ARAEQLAQQA	HSNRKLMEII-NH_2
hUcn1	DNPSLSIDLT	FHLLRTLLEL	ARTQSQRERA	EQNRIIFDSV-NH_2
hUcn2	IVLSLDVP	IGLLQILLEQ	ARARAAREQA	TTNARILARV-NH_2
hUcn3	FTLSLDVP	TNIMNLLFNI	AKAKNLRAQA	AANAHLMAQI-NH_2
Sauvagine	**U**GPPISIDLS	LELLRKMIEI	EKQEKEKQQA	ANNRLLLDTI-NH_2
$Stressin_1$	**Ac**-PPISLDLT	**f**HLLREVLE**Z**	ARAEQLAQQ**E**	HS**K**RKL**Z**EII-NH_2
Alpha-helical CRF	S QEPPISLDLT	FHLLREMLEM	AKAEQEAEQA	ALNRLLLEEA-NH_2
Alpha-helical $CRF_{(9–41)}$	DLT	FHLLREMLEM	AKAEQEAEQA	ALNRLLLEEA-NH_2
[$DPhe^{12}$,$Nle^{21,38}$]-$hCRF_{(12–41)}$		**f**HLLREVLE**Z**	ARAEQLAQQA	HSNRKL**Z**EII-NH_2
[$DPhe^{12}$,$Nle^{21,38}$,$Leu(Me)^{37}$]-$hCRF_{(12–41)}$		**f**HLLREVLE**Z**	ARAEQLAQQA	HSNRK**XZ**EII-NH_2
Astressin		**f**HLLREVLE**Z**	ARAEQLAQ**E**A	H**K**NRKL**Z**EII-NH_2
Astressin B	**Ac**-DLT	**f**HLLREVLE**Z**	ARAEQ**X**AQ**E**A	H**K**NRKL**Z**E**X**I-NH_2
$Astressin_2$-B	**Ac**-DLS	**f**H**X**LRK**Z**IEI	EKQEKEKQQA	**E**NN**K**LLLD**X**I-NH_2
Anti-Sauvagine-30		**f**HLLRKMIEI	EKQEKEKQQA	ANNRLLLDTI-NH_2

Note: Z = Nle; X = Leu(Me); Ac = acetyl; U = pGlu.

hCRF differs from oCRF in that residues Gln^2, Thr^{22}, Lys^{23}, Asp^{25}, Leu^{38}, Asp^{39}, Ala^{41} in oCRF are substituted by the corresponding Amino Acids Glu^2, Ala^{22}, Arg^{23}, Glu^{25}, Met^{38}, Glu^{39} and Ile^{41} in hCRF.

$Stressin_1$ differs from hCRF in that residues 1 to 3 in hCRF are deleted and replaced by an acetyl group. In addition, Phe^{12}, Met^{21}, Ala^{31}, Asn^{34}, Met^{38} in hCRF are substituted by the corresponding $DPhe^{12}$, Nle^{21}, Glu^{31}, Arg^{34} and Nle^{38} in $stressin_1$. Substitution of Met by Nle precludes oxydation of Met to Met (O) therefore adding to overall chemical stability. In addition, a lactam bridge between Glu^{31} and Lys^{34} has been formed to lock in a specific turn favorable for $CRFR_1$ selectivity.

Overall rationale in the design of α-helical-CRF was to optimize α-helicity resulting from the substitution of amino acids in oCRF by α-helical inducing residues found in other members of the CRF family (sauvagine, urotensins, hCRF) with greater P values.

α-helical $CRF_{(9–41)}$ is a C-terminal 30 amino acid fragment of α-helical CRF. Other fragments of active linear human sequences include [$DPhe^{12}$,$Nle^{21,38}$]-$hCRF_{(12–41)}$ and [$DPhe^{12}$,$Nle^{21,38}$,$Leu(Me)^{37}$]-$hCRF_{(12–41)}$ with known substitutions such as $DPhe^{12}$,Nle^{21},Nle^{38} and $C\alpha MeLeu^{37}$ that increased potency in the rat anterior pituitary cells in culture (RAP) assay.

Astressin differs from [$DPhe^{12}$,$Nle^{21,38}$]-$hCRF_{(12–41)}$ in that a lactam bridge encompassing residues -Glu^{30}-Ala-His-Lys^{33}- was introduced in lieu of -Gln^{30}-Ala-His-Ser^{33}- in the parent [$DPhe^{12}$,$Nle^{21,38}$]-$hCRF_{(12–41)}$.

Astressin B, a non-selective, potent, long-acting antagonist, differs from astressin, a nonselective, potent, short-acting antagonist, in that astressin B was elongated at the N-terminal by Ac-Asp-Leu-Thr that may confer resistance to amino peptidases and $C\alpha MeLeu^{27,40}$ that may confer additonal conformational stability.

$Astressin_2$-B, an 8–40 fragment of sauvagine and a $CRFR_2$ selective potent, long-acting antagonist, differs from sauvagine in that residues Leu^{12}, Leu^{14}, Met^{18}, Ala^{32}, Arg^{35}, Thr^{40} (CRF numbering) are substituted by the corresponding $DPhe^{12}$, $C\alpha MeLeu^{14}$, Nle^{18}, Glu^{32}, Lys^{35}, $C\alpha MeLeu^{40}$. We hypothesized that positioning of the lactam bridge (Glu^{32}, Xaa, Xbb, Lys^{35}] is in part responsible for $CRFR_2$ selectivity.

Anti-sauvagine-30 differs from sauvagine (12–41) (CRF numbering) in that Leu^{12}-Glu^{13} are substituted by $DPhe^{12}$-His^{13}.

Table 2

Binding affinities and *in vitro* relative potencies

	hCRF R1 EC_{50} nM	mCRF R2β EC_{50} nM	Relative Potencies[a,b] RAP assay
oCRF[a]	1.2 (0.9 – 1.6) (Rivier et al., 2007)	52 (21 – 128) (Rivier et al., 2007)	1.0
r/hCRF[a]	1.0 (0.2 – 4.6) (Rivier et al., 2007)	6.2 (2.0 – 19) (Rivier et al., 2007)	1.0 (Gulyas et al., 1995)
hUcn 1[a]	01. (0.1 – 0.2)*	0.5 (0.3 – 0.7)*	ND
hUcn 2[a]	>100 (Lewis et al., 2001)	0.5 (0.2 – 1.2) (Lewis et al., 2001)	ND
hUcn 3[a]	>100 (Lewis et al., 2001)	13.5 (9.2 – 19.7) (Lewis et al., 2001)	ND
Sauvagine[a]	0.9 (0.5 – 1.7) (Rivier et al., 2002)	1.6 (0.7 – 3.8) (Rivier et al., 2002)	ND
$Stressin_1$[a]	1.7 (0.5 – 6.2) (Rivier et al., 2007)	222 (137 – 361) (Rivier et al., 2007)	1.1(0.53 – 2.2) (Rivier et al., 2007)
α-helical $CRF_{(1–41)}$[a]	2.5 (1.1 – 5.5)*	0.4 (0.3 – 0.6)	2.0 – 3.0 (Rivier et al., 1984)
α-helical $CRF_{(9–41)}$[b]	19 (5.5 – 66) (Rivier et al., 2002)	1.1 (1.0 – 1.3) (Rivier et al., 2002)	0.03(0.02–0.05) (Gulyas et al., 1995)
[$DPhe^{12}$,$Nle^{21,38}$]-$hCRF_{(12–41)}$[b]	19.2 (13.4 – 27.5) (Rivier et al., 2002)	4.4 (2.0 – 9.6) (Rivier et al., 2002)	1.0 (Gulyas et al., 1995)
[$DPhe^{12}$,$Nle^{21,38}$,$C^{\alpha}Me$ Leu^{37}]-$hCRF_{(12–41)}$[b]	2.7 (1.2–5.9)* 7.7 (6.0–9.8)	2.8 (2.0–3.9)* 4.9 (3.7–6.5)	1.0 (0.5 – 2.0) (Hernandez et al., 1993)
Astressin[b]	0.7 (0.3 – 1.8) (Rivier et al., 2002)	0.6 (0.5 – 0.8) (Rivier et al., 2002)	32.5(12–82) (Gulyas et al., 1995)
Astressin B[b]	0.3 (0.1 – 0.8)*	1.2 (0.9 – 1.6)*	1.1 (0.50 – 2.5) (Rivier et al., 1999)
$Astressin_2$-B[b]	>500 (Rivier et al., 2002)	1.3 (1.0 – 1.7) (Rivier et al., 2002)	ND
Anti-sauvagine 30[b]	400 (Rivier et al., 2002)	1.1 (0.6 – 2.2) (Rivier et al., 2002)	ND

[a] CRF agonists were tested in an *in vitro* assay measuring stimulation of ACTH release by rat anterior pituitary cells in culture (RAP assay) when using hCRF or oCRF as the standard compound (Gulyas et al., 1995; Rivier et al., 2007).

[b] CRF antagonists were tested measuring alteration of CRF-induced release of ACTH by rat anterior pituitary cells in culture relative to that of [$DPhe^{12}$,$Nle^{21,38}$]-h/r$CRF_{(12–41)}$ as the standard compound (Hernandez et al., 1993).

* Unpublished data; assay carried out by C. Miller under the supervision of Dr. M. Perrin.