Published in final edited form as:

J Neurochem. 2008 February ; 104(4): 926–936. doi:10.1111/j.1471-4159.2007.05052.x.

Expression, binding, and signaling properties of CRF_{2(a)} receptors endogenously expressed in human retinoblastoma Y79 cells: passage-dependent regulation of functional receptor

Eric Gutknecht^{*,†,1}, Richard L. Hauger^{‡,1}, Ile Van der Linden^{*}, Georges Vauquelin[†], and Frank M. Dautzenberg^{*}

^{*}CNS Research, Johnson & Johnson Research & Development, Beerse, Belgium

[†]Department of Molecular and Biochemical Pharmacology, Institute for Molecular Biology and Biotechnology, Vrije Universiteit Brussel (VUB), Brussel, Belgium

[‡]VA Healthcare System and Department of Psychiatry, University of California, San Diego, California, USA

Abstract

Endogenous expression of the corticotropin-releasing factor type 2a receptor [CRF_{2(a)}] but not CRF_{2(b)} and CRF_{2(c)} was observed in higher passage cultures of human Y79 retinoblastoma cells. Functional studies further demonstrated an increase in CRF_{2(a)} mRNA and protein levels with higher passage numbers (> 20 passages). Although the CRF₁ receptor was expressed at higher levels than the CRF_{2(a)} receptor, both receptors were easily distinguishable from one another by selective receptor ligands. CRF₁-preferring or non-selective agonists such as CRF, urocortin 1 (UCN1), and sauvagine stimulated cAMP production in Y79 to maximal responses of ~100 pmoles/10⁵ cells, whereas the exclusive CRF₂ receptor-selective agonists UCN2 and 3 stimulated cAMP production to maximal responses of ~25–30 pmoles/10⁵ cells. UCN2 and 3-mediated cAMP stimulation was potently blocked by the ~300-fold selective CRF₂ antagonist antisauvagine (IC₅₀ = 6.5 ± 1.6 nmol/L), whereas the CRF₁-selective antagonist NBI27914 only blocked cAMP responses at concentrations > 10 μmol/L. When the CRF₁-preferring agonist ovine CRF was used to activate cAMP signaling, NBI27914 (IC₅₀ = 38.4 ± 3.6 nmol/L) was a more potent inhibitor than antisauvagine (IC₅₀ = 2.04 ± 0.2 μmol/L). Finally, UCN2 and 3 treatment potently and rapidly desensitized the CRF₂ receptor responses in Y79 cells. These data demonstrate that Y79 cells express functional CRF₁ and CRF_{2(a)} receptors and that the CRF_{2(a)} receptor protein is up-regulated during prolonged culture.

Keywords

corticotropin-releasing factor receptor desensitization; corticotropin-releasing factor receptor signaling; cyclic AMP; ligand binding; mRNA expression

Corticotropin-releasing factor (CRF) and its structurally related family members urocortin 1–3 (UCN1–3) potently modulate neuroendocrine, autonomic, and behavioral responses to stress by activating two CRF receptors: CRF₁ and CRF₂ (Dautzenberg and Hauger 2002; Bale and Vale 2004; Grigoriadis 2005; Hauger *et al.* 2006; Steckler and Dautzenberg 2006).

Both receptor subtypes are highly homologous (~70%) and belong to the class B1 subfamily of G protein-coupled receptors (GPCRs) (Dautzenberg *et al.* 2001b; Harmar 2001). Three biologically active splice variants, CRF_{2(a-c)}, have been identified for the CRF₂ receptor, whereas only one high affinity variant of the CRF₁ receptor has been established to be a fully functional GPCR (Hauger *et al.* 2003a).

Corticotropin-releasing factor type 1 and 2 receptors differ strongly in terms of their agonist and antagonist binding preferences. Binding and functional studies in cell lines recombinantly or endogenously expressing CRF₁ receptors revealed a distinct ligand-selective profile: CRF, UCN1, and the non-mammalian CRF agonists fish urotensin I, and frog sauvagine bind with high affinity to the mammalian CRF₁ receptor and stimulate cAMP and calcium signaling pathways (Donaldson *et al.* 1996; Dautzenberg *et al.* 1997, 2001a, 2004b). In contrast, UCN2 and UCN3 do not bind to or activate CRF₁ receptors at physiologically relevant concentrations (Hsu and Hsueh 2001; Lewis *et al.* 2001; Reyes *et al.* 2001; Dautzenberg *et al.* 2004a,b; Grigoriadis 2005). Pharmacological characterization of the CRF₂ receptor splice variants revealed no major differences between CRF_{2(a)}, CRF_{2(b)}, and CRF_{2(c)} receptors (Donaldson *et al.* 1996; Kostich *et al.* 1998; Palchaudhuri *et al.* 1999; Dautzenberg *et al.* 2004b). However, the binding profiles of these three CRF₂ receptors markedly diverge from the binding profile of the CRF₁ receptor (Donaldson *et al.* 1996; Perrin *et al.* 1999; Dautzenberg *et al.* 2001b; Hsu and Hsueh 2001; Lewis *et al.* 2001; Reyes *et al.* 2001). Urotensin I, sauvagine, and UCN1–3 bind with up to 1000-fold higher affinities to the CRF₂ receptor than species homologs of CRF (see Hauger *et al.* 2003a). In agreement with the binding data, a similar rank order of potency is typically observed when these five agonists are used to stimulate cAMP stimulatory G protein (G_s)-coupled cAMP signaling (Donaldson *et al.* 1996; Dautzenberg *et al.* 2001b; Hsu and Hsueh 2001; Lewis *et al.* 2001; Reyes *et al.* 2001) or phospholipase C-mediated transient mobilization of intracellular calcium stores (Dautzenberg *et al.* 2004a). Therefore, UCN2 and UCN3 are generally considered to represent endogenous ligands for mammalian CRF₂ receptor variants, whereas UCN1 is thought to be an endogenous ligand for both CRF receptors.

Pharmacological characterization of CRF₁ and CRF₂ receptors has mainly been completed using recombinant receptor expression systems (Perrin and Vale 2002; Grigoriadis 2005; Hauger *et al.* 2006). However, in a recombinant setting the imbalanced receptor-G protein stoichiometry may strongly influence the receptor signaling properties (see Kenakin 1997). Thus, confirming recombinant GPCR data in an endogenous cellular setting is of high importance. Another aspect of scientific interest is whether or not CRF₁ and CRF_{2(a)} receptors engage in crosstalk or are co-regulated in the CNS. Therefore, identification of cell lines endogenously expressing CRF_{2(a)} receptors alone or together with CRF₁ receptors is critical for gaining further insight into the regulation of CRF receptor signaling.

A large number of brain-derived or neuroendocrine cell lines, including Y79 retinoblastoma, IMR-32 neuroblastoma, CATH.a catecholaminergic, AtT-20 pituitary, PC12 pheochromocytoma, and small lung cell carcinoma NCI-H82 cells endogenously express CRF₁ receptors (Vita *et al.* 1993; Dieterich and DeSouza 1996; Iredale *et al.* 1996; Hauger *et al.* 1997; Kiang *et al.* 1998; Dautzenberg and Hauger 2002; Dermitzaki *et al.* 2007). Despite the widespread expression of CRF₂ receptors in the CNS and the periphery, few cell lines have been found to express endogenously one of the three CRF₂ receptor isoforms (Kiang *et al.* 1998; Hsu and Hsueh 2001; Brar *et al.* 2004; Nemoto *et al.* 2005). The human pancreatic carcinoid BON cell line only expresses the CRF₂ receptor but the splice variant has not been determined (von Mentzer *et al.* 2007). The rat aortic smooth muscle A7r5 cell line exclusively expresses the CRF_{2(b)} receptor that couple to the G_s protein (Hsu and Hsueh 2001; Hoare *et al.* 2005). Although rodent CATH.a catecholaminergic cells express CRF_{2(a)} receptors (Brar *et al.* 2004) and pheochromocytoma PC12 cells express CRF_{2(b)} receptors

(Dermitzaki *et al.* 2007), in addition to CRF₁ receptors, molecular mechanisms regulating CRF receptor signaling have not been characterized in these two cell lines. However, we have extensively studied regulation of the CRF₁ receptor endogenously expressed in human retinoblastoma Y79 cells and found that this cell line provides a valuable system for studying CRF receptor regulation in an endogenous setting (Hauger *et al.* 1997, 2003b; Dautzenberg *et al.* 2001a, 2002a).

In the present study, we demonstrate that CRF_{2(a)} receptors are endogenously expressed in Y79 cells, and CRF_{2(a)} receptors can be up-regulated with increasing duration of cell culture (> 20 passages). We also established functional G_s-coupling and cAMP signaling when retinoblastoma CRF_{2(a)} receptors are activated by their selective agonists, UCN2 and UCN3. CRF₁ and CRF₂ receptor signal transduction in Y79 cells could be functionally separated using selective ligands and antagonists. Finally, we provide the first evidence that CRF_{2(a)} receptor function is rapidly regulated by a homologous desensitization mechanism.

Materials and methods

Materials, peptides, reagents, and radiochemicals

All cell culture media and reagents were purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). All peptides (purity > 95) were obtained from Bachem Corporation (Bubendorf, Switzerland) and NBI27914 was obtained from Tocris (Bristol, UK). ¹²⁵I-antisauvagine and ¹²⁵I-sauvagine (both 2000 Ci/mmol) were purchased from Amersham (GE Healthcare, Little Chalfont, UK).

Cell culture

Y79 retinoblastoma cells (American Type Culture Collection (Manassas, VA, USA) No. HTB-18) were grown as suspension cultures in RPMI 1640 medium as described previously (Hauger *et al.* 1997, 2003b; Dautzenberg *et al.* 2001a). Cells were grown at densities ranging from 5×10^7 to 2×10^8 cells/flask in Falcon F-175 flasks and used between passages 5 and 60, depending on the experimental procedure.

cAMP assays

Y79 cells were plated at 80 000 cells/well in assay buffer containing 3-isobutyl-1-methylxanthine (Hank's buffered salt solution supplemented with 3-isobutyl-1-methylxanthine 1 mmol/L, MgCl₂ 10 mmol/L, HEPES 5 mmol/L, and 0.1% bovine serum albumin) in 96 wells black plates (Costar-BD Biocoat, Erembodegem, Belgium). Cells were incubated for 15 min at 37°C with the agonists and lysed following the homogenous time-resolved fluorescence cAMP Dynamic kit two step protocol (Cisbio International, Bagnols/Cèze, France) to determine the production of cAMP. Fluorescence resonance energy transfer was measured in the Discovery reader (Perkin Elmer, Boston, MA, USA). In the desensitization intracellular cAMP levels were measured in nonacetylated cell lysates using a double-antibody radioimmunoassay kit (cAMP[¹²⁵I] assay system, RPA 509; Amersham International, Little Chalfont, UK), as previously described (Dautzenberg *et al.* 2001b; Hauger *et al.* 2003b).

Radioreceptor binding experiments

The preparation of Y79 membrane particulates was essentially as reported previously (Hauger *et al.* 1997). Y79 were harvested and precipitated at 150 g for 10 min. All subsequent steps were performed at 4°C. Cells were washed with ice-cold phosphate-buffered saline and recentrifuged. The pellet was resuspended in ice-cold membrane buffer (50 mmol/L Tris-HCl, pH 7.4) containing 5 mmol/L MgCl₂, 2 mmol/L EGTA, and 100 kIU/mL aprotinin and homogenized with a Polytron (Kinematica, setting 5 for 10 strokes).

The nuclei were precipitated for 5 min at 600 g, the supernatant was removed and stored away, and the pellet was re-extracted as described above. After combining both supernatants they were precipitated at 13 000 g for 30 min.

In siliconized polypropylene tubes (Sigma-Aldrich, Bornem, Belgium) CRF_{2(a)} receptor binding was studied using Y79 membranes (~250 µg of membrane protein for CRF_{2(a)} receptor binding and ~100 µg for CRF₁ receptor binding) using a competitive binding assay between 0.15 nmol/L ¹²⁵I-antisauvagine or ¹²⁵I-sauvagine and increasing concentrations of unlabeled CRF agonists or antagonists (0–10^{−5} mol/L). After incubation at 22°C for 120 min, the tubes were centrifuged at 14 000 g, washed twice (Ruhmann *et al.* 1996) and the radioactivity was counted in a γ-counter (Wallac, Turku, Finland).

RNA isolation and cDNA synthesis

Total RNA was isolated and purified from Y79 cells using the RNeasy kit (Qiagen/Westburg, Leusden, The Netherlands) with Dnase I treatment on the column. First strand cDNA was synthesized from 1 µg total RNA for 1 h at 42°C using Random Hexamer-primers and Superscript II reverse transcriptase (Invitrogen Life Technologies).

Semiquantitative RT-PCR

The three splice variants of the hCRF₂ receptor [CRF_{2(a)}, CRF_{2(b)}, and CRF_{2(c)}] were amplified with the following primer combinations: CRF_{2a}for (5'-GAGCTGCTCTTGGACGGCTGGGGGC-3') and CRF_{2a}rev (5'-CTGCCACAGATACGCAGT-3'); CRF_{2b}for (5'-CAGGCTCCAGTCCCTAAC-3') and CRF_{2b}rev (5'-CAGGTAGTTGACGACAAGG-3'); and CRF_{2c}for (5'-CTGTGCTCAAGCAATCTGCCT-3') and CRF_{2c}rev (5'-CAAAATGGGCTCACACTGTGAG-3'). Primers were designed based on the mRNA sequence find under Ensemble database CRF_{2(a)}, ENST222836 (nucleotides 61–389, 328 bp); CRF_{2(b)}, ENST348438 (nucleotides 36–518, 482 bp); and CRF_{2(c)}, ENST341843 (nucleotides 1–416, 416 bp). Amplification was performed for 35 cycles (94°C for 10 s, 60°C for 20 s, and 72°C for 1 min) with 1 U Taq DNA polymerase and a cDNA equivalent of ~50 ng RNA. The calculated sizes for the amplification products were as follows: CRF_{2(a)} = 310 bp, CRF_{2(b)} = 410 bp, and CRF_{2(c)} = 276 bp. In control amplifications using cloned DNA these sizes were confirmed (not shown). The cDNA for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (NM_002046, nucleotides 208–602, 395 bp) was amplified for 20 cycles with the different cDNAs using the following primer pairs: GAPDH_{for} (5'-CCTTCATTGACCTCAACTAC-3') and GAPDH_{rev} (5'-TGTCATGGATGACCTTGG-3'). The different fragments were sequenced for confirmation of the specific amplification of the various CRF₂ receptor splice variants.

Quantitative RT-PCR

Quantitative RT-PCR (Q-PCR) was performed using an ABI Prism 7700 cyclor (Applied Biosystem, Foster City, CA, USA) using qPCR™ Core Kit w/o dUTP (Eurogentec, Seraing, Belgium). Serial dilutions of the cDNAs were used to generate standard curve for the threshold cycles for hCRF₁, hCRF₂, GAPDH, and β-actin. Samples were diluted 10-fold prior the experiment to ensure that amplification was in the linear part of the standard cDNA curve. Pre-developed Taqman assay reagents from ABI (Applied Biosystem, Warrington, UK) were used for human β-actin and GAPDH. Primers and probes for human CRF₁ and human CRF_{2(a)} were designed with Primer Express software v2.0 (Applied Biosystem, Foster City, CA, USA). The sequences for the CRF₁ and CRF₂ receptor primers used for Q-PCR are given in Table 1. A linear regression line calculated from the standard curves allowed the determination of transcript levels in RNA samples from of Y79 cells on the

different passages. Primers and probes sets for hCRF₁ and hCRF₂ showed very weak [hCRF_{2(a)}] or no (hCRF₁) cross-reactivity for plasmid DNA.

Data reduction and statistical analyses

Data reduction for the binding and cAMP experiments was performed using a log-logit program. IC₅₀, EC₅₀, and maximum values were calculated from the full concentration–response curves for binding, agonist stimulation, and antagonist inhibition of cAMP accumulation using the PRISM™, version 4.0 (GraphPad Software, Inc., San Diego, CA, USA). Statistical significance was determined by ANOVA across experimental groups using PRISM™, version 4.0. If the one-way ANOVA was statistically significant, planned *post hoc* analyses were performed using Bonferroni's multiple comparison tests to determine individual group differences.

Results

Functional expression of CRF_{2(a)} receptors in late passage Y79 cells

We began our study by observing substantial differences in the stimulation of cAMP formation by the CRF₁ receptor-preferring agonist ovine CRF (oCRF) and the CRF₂ receptor-selective agonist UCN3 during the sequence of a profiling of freshly frozen Y79 cells versus cells that had been in culture for more than 30 passages. While the cAMP response to oCRF remained stable (99.6 ± 4.8 pmol/10⁵ cells in passage 4 vs. 95.4 ± 6.1 pmol/10⁵ cells in passage 36), the cAMP response to UCN3 increased from a very low response (2.2 ± 0.8 pmol/10⁵ cells in passage 4; with a basal response of 1.2 ± 0.6 pmol/10⁵ cells) to ~25% of the oCRF responses in passage 36 (24.5 ± 1.9 pmol/10⁵ cells).

Next, RNA was isolated from Y79 cells in passages 5 and 40. After cDNA was synthesized, a semiquantitative RT-PCR analysis was performed in order to identify the CRF₂ receptor splice variant expressed in Y79 cells. No positive PCR amplification for any CRF₂ receptor splice variant was obtained from Y79 cell cDNA isolated in passage 5 (not shown). However, hCRF_{2(a)} cDNA was amplified from cDNA isolated from passage 40 (Fig. 1). Neither hCRF_{2(b)} nor hCRF_{2(c)} cDNA was amplified, whereas using cDNAs isolated from hippocampal or amygdalar RNA revealed successful amplification of both splice variants. Thus, at late passages the higher cAMP response to UCN3 was mediated exclusively by the hCRF_{2(a)} receptor.

Up-regulation of CRF_{2(a)} receptor mRNA and functional responses during long-term cultivation of Y79 cells

We next determined if CRF_{2(a)} receptor expression increased as a function of cell culture duration. After total RNA was isolated from Y79 cells at different passages ranging from passage 5 to 60, Q-PCR analysis was performed. Cells from identical batches were also analyzed for stimulation of cAMP formation by oCRF, UCN3, and the non-selective CRF receptor agonist sauvagine utilizing the homogenous time-resolved fluorescence readout, which is a simple mix-and-measure protocol providing a rapid quantification of cAMP in cellular extracts (<http://www.htf.com/products/gpcr/camp>). As the preliminary functional studies suggested CRF_{2(a)} receptor expression becomes up-regulated while CRF₁ receptor levels remain constant, we determined if expression of CRF_{2(a)} mRNA progressively increases in Y79 cells during longer periods of cell culture. To this end, RNA was isolated from Y79 cells, first strand cDNA synthesis was completed, and the relative mRNA levels of CRF₁ and CRF_{2(a)} receptor were quantified by Q-PCR versus two internal standards, GAPDH and β -actin, which revealed similar results. Q-PCR analyses revealed constant CRF₁ mRNA levels throughout increasing culturing of Y79 cells (Fig. 2b) whereas CRF_{2(a)}

mRNA increased from nearly undetectable levels at passage 5 to maximal levels between passages 26 and 30 (Fig. 2c).

In the cAMP stimulation experiments, oCRF and sauvagine produced similar maximal responses (~100 pmoles/well) in all passages of Y79 cells (Fig. 2a). In contrast, UCN3-mediated cAMP stimulation was significantly increased with greater number of cell passages. At early passages, UCN3-induced CRF_{2(a)} receptor cAMP signaling was minimal based on maximal responses of 2.75 ± 0.23 and 3.28 ± 0.19 pmoles/well at passages 5 and 12, respectively. cAMP responses to UCN3 first became up-regulated at passage 15 (7.34 ± 0.18 pmoles/well), followed by further increases at passages 20 (13.61 ± 0.49 pmoles/well) and 26 (20.53 ± 2.07 pmoles/well). By passage 30, the UCN3-stimulated cAMP accumulation reached a plateau of 25 pmoles/well (Fig. 2a) with no further up-regulation of the cAMP response at passages 55–60 (data not shown).

Functional separation of CRF₁ and CRF_{2(a)} receptors in Y79 cells

Because we observed that endogenous CRF_{2(a)} receptors exhibited maximal cAMP responses in Y79 cells beginning at passage 25, all subsequent pharmacological studies in Y79 cells were performed between passages 25 and 45. To more fully characterize CRF₁ and CRF_{2(a)} receptor signaling, we generated agonist concentration-response curves for a panel of published mammalian and amphibian CRF and UCN peptides. The CRF₁/CRF₂-activating peptides stimulated cAMP responses to a maximum of ~100 pmoles/well (Fig. 3, Table 1), whereas the potencies of those peptides was either close to the values reported for CRF₁ receptors (oCRF and human/rat CRF) or did not allow to discriminate between CRF₁ and CRF₂ receptors (human UCN1 and sauvagine). In contrast to cAMP responses to CRF, UCN1, and sauvagine, significantly smaller stimulation of cAMP accumulation was observed for the CRF₂ receptor-selective agonists UCN2, UCN3 and their N-terminally extended versions stresscopin [SCP; extended version of UCN3, which contains an additional Thr-Lys dipeptide sequence at its N-terminus (see Hsu and Hsueh 2001)] and stresscopin-related peptide [SRP; extended version of UCN2, which contains five additional N-terminal amino acids, His-Pro-Gly-Ser-Arg (see Hsu and Hsueh 2001) (Fig. 3, Table 2)]. These latter four peptides stimulated cAMP production in Y79 cells to a maximum of 25–28 pmoles/well (Fig. 3, Table 2) with potencies in the range reported for recombinant CRF₂ receptors. Therefore, CRF₂-selective agonists exclusively activated CRF₂ receptors in the Y79 cells.

To further confirm the agonist findings, the inhibitory potencies of CRF receptor antagonists on agonist-induced activation of CRF₁ or CRF₂ receptors were assessed. The following three antagonists were chosen: (i) the CRF_{1/2} non-selective antagonist astressin (Gulyas *et al.* 1995); (ii) the CRF₁-specific small molecule antagonist NBI27914 (Chen *et al.* 1996); and (iii) the CRF₂-selective antagonist anti-sauvagine (Ruhmann *et al.* 1998). An oCRF concentration of 10 nmol/L, which is submaximal (EC₈₀) for CRF₁ receptors but only minimally activating CRF₂ receptors (Dautzenberg *et al.* 2001b), was chosen for the CRF₁ receptor antagonism experiments. An EC₈₀ concentration (100 nmol/L) of UCN3, the most selective CRF₂ agonist, was chosen for the CRF_{2(a)} receptor antagonism experiments.

Using oCRF as agonist, astressin and NBI27914 almost equipotently inhibited cAMP production in the nanomolar range (IC₅₀ ~40 nmol/L), whereas only micromolar antisauvagine concentrations showed inhibitory activities (Fig. 4, Table 3). Conversely, using UCN3 as agonist, NBI27914 failed to exert any appreciable antagonist potency. Under these conditions, astressin and antisauvagine inhibited UCN3-mediated cAMP accumulation with IC₅₀ values below 10 nmol/L (Fig. 4, Table 3). Similar antagonist potencies were obtained with UCN2 as agonist (not shown).

CRF_{2(a)} receptor binding in Y79 cells

To further assess the utility of Y79 cells as an endogenous setting for CRF_{2(a)} receptor function, CRF receptor binding studies were completed. ¹²⁵I-antisauvagine was used in these experiments based on our previous data establishing that this radiolabeled ligand is a valuable tool for studying CRF₂ receptors (Higelin *et al.* 2001). Because our cAMP studies indicated that a low number of CRF_{2(a)} receptor binding sites may be expressed in Y79 cells, we used a centrifugation binding method (Dautzenberg *et al.* 1997; Hauger *et al.* 1997). Preliminary binding experiments confirmed that the CRF_{2(a)} receptor protein levels in early passages were too low to obtain appreciable receptor labeling, while in later passages specific receptor binding was observed (not shown). In saturation binding experiments, ¹²⁵I-antisauvagine specifically labeled a maximum of 21.45 ± 0.89 fmoles of CRF₂ receptors per mg protein in membranes prepared from Y79 cells prepared in passages 35 (Fig. 5). The calculated K_d of 220 ± 22 pmol/L (Fig. 5) was close to the K_d value obtained for recombinant CRF_{2(a)} receptors (Dautzenberg *et al.* 2001b; Higelin *et al.*, 2001).

For inhibition binding experiments, either ¹²⁵I-antisauvagine or the CRF_{1/2} non-selective radiolabel ¹²⁵I-sauvagine was used as the radioligand for assessing the competitive binding potencies of the four endogenous CRF₁ or CRF₂ receptor ligands: oCRF and human UCN1–3. When CRF₂ receptors were labeled with ¹²⁵I-antisauvagine, the four peptides competed with a rank order typical for CRF₂ receptors: UCN1 > UCN2 > UCN3 > oCRF (Fig. 6). At high concentrations, the four peptides completely inhibited ¹²⁵I-antisauvagine binding. In contrast, substantial differences were observed when ¹²⁵I-sauvagine was employed as radiolabeled ligand. UCN1 and oCRF were equipotent in inhibiting ¹²⁵I-sauvagine binding (Fig. 6). Moreover, oCRF inhibited ¹²⁵I-sauvagine binding with ~30-fold higher affinity compared with that for ¹²⁵I-antisauvagine. Thus, ¹²⁵I-sauvagine was predominantly labeling CRF₁ receptors in Y79 membranes. In agreement with this observation, UCN2 and UCN3 inhibited less than 25% of ¹²⁵I-sauvagine binding to Y79 cell membranes. The measured K_i values for UCN2 ($K_i = 21.2 \pm 3.6$ nmol/L) and UCN3 ($K_i = 21.2 \pm 3.6$ nmol/L) were close to their agonist potencies in the cAMP experiments.

Desensitization of CRF_{2(a)} receptors in Y79 cells by UCN2 and UCN3

Because we have previously shown that CRF₁ receptors become homologously desensitized via a G protein-coupled receptor kinase 3 (GRK3) mechanism in Y79 cells exposed to oCRF (Hauger *et al.* 1997, 2003b; Oakley *et al.* 2007), we were interested in determining if endogenous retinoblastoma CRF_{2(a)} receptors are also regulated by rapid homologous desensitization. Y79 cells were pre-incubated with near saturating concentrations of UCN2 and UCN3 for 5–60 min, extensively washed to remove bound ligand, and then re-stimulated with 100 nmol/L UCN2. CRF₂ receptor agonist pre-treatment only minimally increased basal cAMP levels in Y79 cells. However, pre-treatment with either UCN2 or UCN3 resulted in a progressive and substantial reduction in cAMP responsiveness to UCN2 re-stimulation. While 100 nmol/L UCN3 pre-treatment gradually decreased maximal CRF_{2(a)} receptor-mediated cAMP responses over a 60-min period to a maximum desensitization of ~70% (Fig. 7b), UCN2 was a more potent desensitizing agonist for retinoblastoma CRF_{2(a)} receptors. A ~80% desensitization of CRF_{2(a)} receptors was observed after a 5-min pre-treatment with 100 nmol/L UCN2 while no CRF₂-receptor-mediated cAMP signal was measured following a 30-min UCN2 pre-incubation (Fig. 7a).

Discussion

The data reported herein indicate that human retinoblastoma Y79 cells endogenously co-express CRF₁ and CRF_{2(a)} receptors. Because we have shown that this cell line replicates co-expression of both CRF receptors in brain neurons modulating anxiety-like, defensive

behavior, and stress responses including the bed nucleus of the stria terminalis, medial, and cortical amygdaloid nuclei, the entorhinal area of the hippocampus, and the ventral tegmental area (Chalmers *et al.* 1996; Sanchez *et al.* 1999; Sahuque *et al.* 2006), we have now established retinoblastoma cells as a well-controlled cellular setting for studying GRK, arrestin, and protein kinase C regulation of signal transduction by CRF_{2(a)} as well as CRF₁ receptors. In our previous research, we have demonstrated that retinoblastoma CRF₁ receptor function is controlled by GRK3 and protein kinase C mechanisms in Y79 cells (Hauger *et al.* 1997, 2003b; Dautzenberg *et al.* 2001a, 2002a,b).

Until now, we had not observed endogenous expression of CRF₂ receptors in this cell line. However, our molecular analyses were only performed using Y79 cells at earlier passages (Dautzenberg *et al.* 2000). When we investigated CRF receptor signaling at very late stage passages (≥ 35 passages in culture) in this study, we detected both CRF₁ and CRF₂ receptor responses in Y79 cells. This finding prompted us to determine if CRF₂ receptor expression levels were initially very low and thus were not detectable in our earlier analyses. Our previous Y79 cell experiments were also performed before the two CRF₂ receptor-specific agonists UCN2 and UCN3 were identified (Hsu and Hsueh 2001; Lewis *et al.* 2001; Reyes *et al.* 2001). Our hypothesis that very low expression of retinoblastoma CRF₂ receptors at early passages would progressively increase during cell culture was supported by our successful amplification using semiquantitative RT-PCR of CRF_{2(a)} cDNA but not of CRF_{2(b)} and CRF_{2(c)} cDNA isolated from late passages (> 40) but not at passage 5. Our detailed functional and QPCR experiments then identified a substantial up-regulation of CRF_{2(a)} receptor mRNA and G_s-coupled cAMP signaling, which initially was close to the detection limit (i.e. $\sim 2\%$ of the total CRF receptor-mediated cAMP responses), slowly increased between passage 10 and 25, and then reached a maximum of $\sim 25\%$ of the total CRF receptor-mediated cAMP responses beginning at passage 30. Similarly, a gradual increase in CRF_{2(a)} receptor mRNA expression was observed from low levels at passage ~ 5 - to 10-fold higher levels at passage 30. The up-regulation of CRF_{2(a)} receptor mRNA levels corresponded well with the observed ~ 10 -fold increases in CRF_{2(a)} receptor-mediated cAMP responses. One study recently failed to detect increases in cAMP levels in Y79 cells incubated with SCP and SRP (0–100 nmol/L) (Hsu and Hsueh 2001) while another study did not observe any stimulation of cAMP accumulation in Y79 cells exposed to 1 nmol/L UCN2 or UCN3 (Radulovic *et al.* 2003). Using Y79 cells at early culture passages when CRF_{2(a)} receptor expression is very low and/or employing insufficient agonist concentration to stimulate CRF_{2(a)} receptor cAMP signaling most likely explains these discrepancies.

In contrast to the CRF_{2(a)} receptor findings, CRF₁ receptor mRNA expression and cAMP signaling remained unaltered from early to prolonged Y79 cell culturing. These findings were unexpected as CRF₁ and CRF₂ receptor gene expression has been reported to be regulated by identical cAMP and calcium signaling cascades, glucocorticoids, and transcription factors (Iredale *et al.* 1996; Iredale and Duman 1997; Xu *et al.* 2001; Nanda *et al.* 2004; Parham *et al.* 2004). Thus, the transcriptional and translational mechanisms governing CRF_{2(a)} receptor expression and its neurobiological significance may differ from those regulating CRF₁ receptor expression. In addition, no effects on cAMP responses were observed when Y79 cells of different passages were challenged with isoproterenol, a β -adrenoceptor agonist (Hauger *et al.* 1997), pituitary adenylate cyclase-activating polypeptide-38, a potent activator for the pituitary adenylate cyclase-activating polypeptide type 1 (PAC₁) receptor (Dautzenberg *et al.* 1999), or forskolin to directly activate adenylate cyclase function (our unpublished observations). The up-regulation of retinoblastoma CRF_{2(a)} receptors during long-term cell culturing thus, may model an important neurobiological process whereby brain neurons may be induced to express higher levels of CRF_{2(a)} receptors during synaptic plasticity.

Having established optimal conditions for promoting CRF_{2(a)} receptor expression in Y79 cells, we completed a careful pharmacological characterization of the two endogenous CRF receptors. Maximal cAMP responses stimulated by mixed CRF agonists (i.e. CRF, UCN1, and sauvagine) and the CRF₂ receptor-selective agonists (i.e. UCN2, UCN3, and the related N-terminally extended peptides SRP and SCP) were clearly separable. Stimulation of cAMP accumulation by mixed CRF receptor agonists was approximately fourfold more efficacious than the CRF₂ receptor-selective ligands. Further confirmation that UCN3 and UCN2 selectively activated endogenous retinoblastoma CRF_{2(a)} receptors was obtained in the functional antagonist experiments. While the non-selective peptide antagonist astressin (Gulyas *et al.* 1995) and the CRF₁-selective antagonist NBI27914 (Chen *et al.* 1996) equipotently inhibited CRF-stimulated cAMP accumulation, the CRF₂-selective antagonist antisauvagine (Ruhmann *et al.* 1998) displayed only a low micromolar antagonist potency. In contrast, antisauvagine and astressin blocked UCN3-stimulated cAMP production in Y79 cells with low nanomolar potencies comparable with their antagonist activity at recombinant CRF_{2(a)} receptors (Dautzenberg *et al.* 2002b). Furthermore, NBI27914 failed to antagonize UCN3-stimulated cAMP accumulation. In complementary saturation binding experiments, ¹²⁵I-antisauvagine bound to CRF_{2(a)} receptors in late passage Y79 cell membranes with an affinity similar to that observed for recombinant CRF_{2(a)} receptors (Higelin *et al.*, 2001). In addition, UCN2, UCN3, and non-selective CRF receptor agonists competed for ¹²⁵I-antisauvagine binding with almost identical affinities at retinoblastoma (see Fig. 6) and recombinant (Dautzenberg *et al.* 2001b) CRF_{2(a)} receptors. UCN1 and CRF fully displaced ¹²⁵I-sauvagine binding with a CRF₁ receptor-like profile while UCN2 and UCN3 only partially competed (~20–25% displacement) for ¹²⁵I-sauvagine binding. Because the UCN2 and UCN3 IC₅₀ values for competition with ¹²⁵I-sauvagine were close to the values obtained for UCN2 and UCN3 displacement of ¹²⁵I-antisauvagine binding, a CRF_{2(a)} receptor-selective binding profile was confirmed. Thus, we conclude that CRF₁ and CRF_{2(a)} receptors endogenously expressed in Y79 cells are fully functional and can be separated from each other by pharmacological tools.

We also provide the first evidence that the human CRF_{2(a)} receptor can be homologously desensitized by exposure to its two selective agonists in a time-dependent manner. CRF_{2(a)} receptor desensitization induced by UCN2 was more rapid in development and greater in magnitude than that caused by UCN3 in accordance with their agonist potencies. These findings are consistent with the established principle that the rate and magnitude of homologous GPCR desensitization and internalization is positively correlated with agonist potency (Clark *et al.* 1999). The maximal level of cAMP accumulation generated by forskolin-induced activation of the adenylyl cyclase was similar in Y79 cells treated with UCN2 and in control cells not exposed to this ligand (data not shown), indicating that CRF₂ receptor desensitization observed in Y79 cells was an homologous, agonist-dependent process. It will be important to determine if homologous desensitization mechanisms regulating CRF₁ and CRF_{2(a)} receptors differ, and if differential molecular regulation of CRF₁ and CRF_{2(a)} receptor signaling modulates anxiety-like defensive behavior.

In conclusion we have characterized endogenous expressed CRF_{2(a)} receptors in human Y79 retinoblastoma cells. CRF_{2(a)} receptors are markedly and progressively up-regulated during prolonged cell culture to ~25% of the total CRF receptor population in Y79 cells. To our knowledge, our study is the first molecular and pharmacological of a cell line endogenously co-expressing both human CRF₁ and CRF_{2(a)} receptors. Y79 cells represent an informative cell model for studying crosstalk between CRF₁ and CRF_{2(a)} receptors and co-regulation of the two CRF receptors. In current studies, we are determining if mechanisms governing homologous CRF_{2(a)} receptor desensitization, internalization, and recycling differ from the GRK3- and β -arrestin2-mediated mechanisms that we have found to regulate CRF₁ receptors (Dautzenberg *et al.* 2001a; Oakley *et al.* 2007). Because limbic brain neurons

implicated in anxiety, depressive, and stress disorders located in the bed nucleus of the stria terminalis, medial and cortical amygdaloid nuclei, the entorhinal area of the hippocampus, and the ventral tegmental area (Hauger *et al.* 2006) co-express both CRF receptors, understanding coordinate and differential regulation of CRF₁ and CRF_{2(a)} receptor signal transduction will provide important insight into the pathophysiology of affective illnesses.

Acknowledgments

Dr. RLH received the support from a Department of Veterans Affairs Merit Review Grant; the VA Center of Excellence for Stress and Mental Health (CESAMH); the VA Mental Illness Research, Education and Clinical Center (MIRECC) of VISN22; NIH/NIA (AG022982) and NIH/NIMH (MH074697) RO1 Grants. We also gratefully acknowledge Sandra Braun for performing the CRF_{2(a)} receptor desensitization experiments and Alan Turken and Tina Smets for completing cAMP assays.

Abbreviations used

CRF	corticotropin-releasing factor
CRF₁	CRF type 1 receptor
CRF₂	CRF type 2 receptor
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GPCR	G protein-coupled receptor
GRK	G protein-coupled receptor kinase
G_s	cAMP stimulatory G protein
oCRF	ovine CRF
Q-PCR	quantitative RT-PCR
SCP	stresscopin
SRP	stresscopin-related peptide
UCN	urocortin

References

- Bale TL, Vale WW. CRF and CRF receptors: role in stress responsivity and other behaviors. *Annu. Rev. Toxicol.* 2004; 44:525–557.
- Brar BK, Chen A, Perrin MH, Vale W. Specificity and regulation of extracellularly regulated kinase-1/2 phosphorylation through corticotropin-releasing factor (CRF) receptors 1 and 2b by the CRF/urocortin family of peptides. *Endocrinology.* 2004; 145:1718–1729. [PubMed: 14670995]
- Chalmers DT, Lovenberg TW, De Souza EB. Localization of novel corticotropin-releasing factor receptor (CRF2) mRNA expression to specific subcortical nuclei in rat brain: comparison with CRF1 receptor mRNA expression. *J. Neurosci.* 1996; 15:6340–6350. [PubMed: 7472399]
- Chen C, Dagnino R Jr, De Souza EB, et al. Design and synthesis of a series of non-peptide high-affinity human corticotropin-releasing factor1 receptor antagonists. *J. Med. Chem.* 1996; 39:4358–4360. [PubMed: 8893829]
- Clark RB, Knoll BJ, Barber R. Partial agonists and G protein-coupled receptor desensitization. *Trends Pharmacol. Sci.* 1999; 20:279–286. [PubMed: 10390646]
- Dautzenberg FM, Hauger RL. The CRF peptide family and their receptors: yet more partners discovered. *Trends Pharmacol. Sci.* 2002; 23:71–77. [PubMed: 11830263]
- Dautzenberg FM, Dietrich K, Palchaudhuri MR, Spiess J. Identification of two corticotropin-releasing factor receptors with high ligand selectivity from *Xenopus laevis*: unusual pharmacology of the type 1 receptor. *J. Neurochem.* 1997; 69:1640–1649. [PubMed: 9326293]

- Dautzenberg FM, Mevenkamp G, Wille S, Hauger RL. N-terminal splice variants of the type I PACAP receptor: isolation, characterization and ligand binding/selectivity determinants. *J. Neuroendocrinol.* 1999; 11:941–949. [PubMed: 10583729]
- Dautzenberg FM, Higelin J, Teichert U. Functional characterization of corticotropin-releasing factor type 1 receptor endogenously expressed in human embryonic kidney 293 cells. *Eur. J. Pharmacol.* 2000; 390:51–59. [PubMed: 10708706]
- Dautzenberg FM, Braun S, Hauger RL. GRK3 mediates desensitization of CRF₁ receptors: a potential mechanism regulating stress adaptation. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 2001a; 280:R935–R946. [PubMed: 11247813]
- Dautzenberg FM, Py-Lang G, Higelin J, Fischer C, Wright MB, Huber G. Different binding modes of amphibian and human CRF type 1 and type 2 receptors: evidence for evolutionary differences. *J. Pharmacol. Exp. Ther.* 2001b; 296:113–120. [PubMed: 11123370]
- Dautzenberg FM, Higelin J, Brauns O, Butscha B, Hauger RL. Five amino acids of the *Xenopus laevis* CRF₂ receptor mediate differential binding of CRF ligands in comparison to its human counterpart. *Mol. Pharmacol.* 2002a; 61:1132–1139. [PubMed: 11961131]
- Dautzenberg FM, Wille S, Braun S, Hauger RL. GRK3 regulation during CRF- and urocortin-induced CRF₁ receptor desensitization. *Biochem. Biophys. Res. Commun.* 2002b; 298:303–308. [PubMed: 12413940]
- Dautzenberg FM, Gutknecht E, Van der Linden I, Olivares-Reyes JA, Durrenberger F, Hauger RL. 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.* 2004a; 68:1833–1844. [PubMed: 15450949]
- Dautzenberg FM, Higelin J, Wille S, Brauns O. Molecular cloning and functional expression of the mouse CRF2(a) receptor splice variant. *Regul. Pept.* 2004b; 121:89–97. [PubMed: 15256278]
- Dermitzaki E, Tsatsanis C, Minas V, et al. Corticotropin-releasing factor (CRF) and the urocortins differentially regulate catecholamine secretion in human and rat adrenals, in a CRF receptor type-specific manner. *Endocrinology.* 2007; 148:1524–1538. [PubMed: 17194738]
- Dieterich KD, DeSouza EB. Functional corticotropin-releasing factor receptors in human neuroblastoma cells. *Brain Res.* 1996; 733:113–118. [PubMed: 8891255]
- Donaldson C, Sutton S, Perrin MH, Corrigan AZ, Lewis KA, Rivier J, Vaughan JM, Vale WW. Cloning and characterization of human urocortin. *Endocrinology.* 1996; 137:2167–2170. [PubMed: 8612563]
- Grigoriadis DE. The corticotropin-releasing factor receptor: a novel target for the treatment of depression and anxiety-related disorders. *Expert Opin. Ther. Targets.* 2005; 9:651–684. [PubMed: 16083336]
- Gulyas J, Rivier C, Perrin M, Koerber SC, Sutton S, Corrigan A, Lahrichi SL, Craig AG, Vale W, Rivier J. Potent, structurally constrained agonists and competitive antagonists of corticotropin-releasing factor. *Proc. Natl Acad. Sci. USA.* 1995; 92:10575–10579. [PubMed: 7479843]
- Harmar AJ. Family-B G-protein-coupled receptors. *Genome Biol.* 2001; 2:3013. Reviews.
- Hauger RL, Dautzenberg FM, Flaccus A, Liepold T, Spiess J. Regulation of corticotropin-releasing factor receptor function in human Y-79 retinoblastoma cells: rapid and reversible homologous desensitization but prolonged recovery. *J. Neurochem.* 1997; 68:2308–2316. [PubMed: 9166723]
- Hauger RL, Grigoriadis DE, Dallman MF, Plotsky PM, Vale WW, Dautzenberg FM. International union of pharmacology. XXXVI. Current status of the nomenclature for receptors for corticotropin-releasing factor and their ligands. *Pharmacol. Rev.* 2003a; 55:21–26. [PubMed: 12615952]
- Hauger RL, Olivares-Reyes JA, Braun S, Catt KJ, Dautzenberg FM. Mediation of CRF₁ receptor phosphorylation and desensitization by protein kinase C: a possible role in stress adaptation. *J. Pharmacol. Exp. Ther.* 2003b; 306:794–806. [PubMed: 12734388]
- Hauger RL, Risbrough V, Brauns O, Dautzenberg FM. Corticotropin releasing factor (CRF) receptor signaling in the central nervous system: new molecular targets. *CNS Neurol. Disord. Drug Targets.* 2006; 5:453–479. [PubMed: 16918397]

- Higelin J, Py-Lang G, Paternoster C, Ellis GJ, Patel A, Dautzenberg FM. 125 I-antisauvagine-30; a novel and specific high-affinity radioligand for the characterization of corticotropin-releasing factor type 2 receptors. *Neuropharmacology*. 2001; 40:114–122. [PubMed: 11077077]
- Hoare SR, Sullivan SK, Fan J, Khongsaly K, Grigoriadis DE. Peptide ligand binding properties of the corticotropin-releasing factor (CRF) type 2 receptor: pharmacology of endogenously expressed receptors, G-protein-coupling sensitivity and determinants of CRF2 receptor selectivity. *Peptides*. 2005; 26:457–470. [PubMed: 15652653]
- Hsu SY, Hsueh AJW. Human stresscopin and stresscopin-related peptide are selective ligands for the type 2 corticotropin-releasing hormone receptor. *Nat. Med.* 2001; 7:605–611. [PubMed: 11329063]
- Iredale PA, Duman RS. Glucocorticoid regulation of corticotropin-releasing factor1 receptor expression in pituitary-derived AtT-20 cells. *Mol. Pharmacol.* 1997; 51:794–799. [PubMed: 9145917]
- Iredale PA, Terwilliger R, Widnell KL, Nestler EJ, Duman RS. Differential regulation of corticotropin-releasing factor1 receptor expression by stress and agonist treatments in brain and cultured cells. *Mol. Pharmacol.* 1996; 50:1103–1110. [PubMed: 8913341]
- Kenakin T. Differences between natural and recombinant G protein-coupled receptor systems with varying receptor/G protein stoichiometry. *Trends Pharmacol. Sci.* 1997; 18:456–464. [PubMed: 9458693]
- Kiang JG, Ding XZ, Gist ID, Jones RR, Tsokos GC. Corticotropin-releasing factor induces phosphorylation of phospholipase C-gamma at tyrosine residues via its receptor 2beta in human epidermoid A-431 cells. *Eur. J. Pharmacol.* 1998; 363:203–210. [PubMed: 9881591]
- Kostich WA, Chen A, Sperle K, Largent BL. Molecular identification and analysis of a novel human corticotropin-releasing factor (CRF) receptor: the CRF $_{2\gamma}$ receptor. *Mol. Endocrinol.* 1998; 12:1077–1085. [PubMed: 9717834]
- Lewis K, Li C, Perrin MH, et al. 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. USA*. 2001; 98:7570–7575. [PubMed: 11416224]
- von Mentzer B, Murata Y, Ahlstedt I, Lindstrom E, Martinez V. Functional CRF receptors in BON cells stimulate serotonin release. *Biochem. Pharmacol.* 2007; 73:805–813. [PubMed: 17184738]
- Nanda SA, Roseboom PH, Nash GA, Speers JM, Kalin NH. Characterization of the human corticotropin-releasing factor2(a) receptor promoter: regulation by glucocorticoids and the cyclic adenosine 5'-monophosphate pathway. *Endocrinology*. 2004; 145:5605–5615. [PubMed: 15331578]
- Nemoto T, Mano-Otagiri A, Shibasaki T. Urocortin 2 induces tyrosine hydroxylase phosphorylation in PC12 cells. *Biochem. Biophys. Res. Commun.* 2005; 330:821–831. [PubMed: 15809070]
- Oakley RH, Olivares-Reyes JA, Hudson CC, Flores-Vega F, Dautzenberg FM, Hauger RL. Carboxyl terminal and intracellular loop sites for CRF $_1$ receptor phosphorylation and β -arrestin2 recruitment: a mechanism regulating stress and anxiety responses. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 2007; 293:R209–R222. [PubMed: 17363685]
- Palchoudhuri MR, Hauger RL, Wille S, Fuchs E, Dautzenberg FM. Isolation and pharmacological characterization of two functional splice variants of corticotropin-releasing factor type 2 receptor from *Tupaia belangeri*. *J. Neuroendocrinol.* 1999; 11:419–428. [PubMed: 10336722]
- Parham KL, Zervou S, Karteris E, Catalano RD, Old RW, Hillhouse E. Promoter analysis of human corticotropin-releasing factor (CRF) type 1 receptor and regulation by CRF and urocortin. *Endocrinology*. 2004; 145:3971–3983. [PubMed: 15142984]
- Perrin, MH.; Vale, W. Chapter 25: corticotropin-releasing factor receptors. In: Pangalos, MN.; Davies, CH., editors. *Understanding G Protein-Coupled Receptors and Their Role in the CNS*. Oxford University Press; New York: 2002. p. 505-526.
- Perrin MH, Sutton SW, Cervini LA, Rivier JE, Vale WW. Comparison of an agonist, urocortin, and an antagonist, astressin, as radioligands for characterization of corticotropin-releasing factor receptors. *J. Pharmacol. Exp. Ther.* 1999; 288:729–734. [PubMed: 9918582]

- Radulovic M, Hippel C, Spiess J. Corticotropin-releasing factor (CRF) rapidly suppresses apoptosis by acting upstream of the activation of caspases. *J. Neurochem.* 2003; 84:1074–1085. [PubMed: 12603831]
- Reyes TM, Lewis K, Perrin MH, et al. 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. USA.* 2001; 98:2843–2848. [PubMed: 11226328]
- Ruhmann A, Kopke AK, Dautzenberg FM, Spiess J. Synthesis and characterization of a photoactivatable analog of corticotropin-releasing factor for specific receptor labeling. *Proc. Natl Acad. Sci. USA.* 1996; 93:10609–10613. [PubMed: 8855226]
- Ruhmann A, Bonk I, Lin CR, Rosenfeld MG, Spiess J. Structural requirements for peptidic antagonists of the corticotropin-releasing factor receptor (CRFR): development of CRFR2beta-selective antisauvagine-30. *Proc. Natl Acad. Sci. USA.* 1998; 95:15264–15269. [PubMed: 9860957]
- Sahuque LL, Kullberg EF, Mcgeehan AJ, Kinder JR, Hicks MP, Blanton MG, Janak PH, Olive MF. Anxiogenic and aversive effects of corticotropin-releasing factor (CRF) in the bed nucleus of the stria terminalis in the rat: role of CRF receptor subtypes. *Psychopharmacology (Berl.)*. 2006; 186:122–132. [PubMed: 16568282]
- Sanchez MM, Young LJ, Plotsky PM, Insel TR. Autoradiographic and in situ hybridization localization of corticotropin-releasing factor 1 and 2 receptors in nonhuman primate brain. *J. Comp. Neurol.* 1999; 408:365–377. [PubMed: 10340512]
- Steckler T, Dautzenberg FM. Corticotropin-releasing factor receptor antagonists in affective disorders and drug dependence – an update. *CNS Neurol. Disord. Drug Targets.* 2006; 5:147–165. [PubMed: 16611089]
- Vita N, Laurent P, Lefort S, Chalon P, Lelias JM, Kaghad M, Le Fur G, Caput D, Ferrara P. Primary structure and functional expression of mouse pituitary and human brain corticotrophin releasing factor receptors. *FEBS Lett.* 1993; 335:1–5. [PubMed: 8243652]
- Xu G, Rabadan-Diehl C, Nikodemova M, Wynn P, Spiess J, Aguilera G. Inhibition of corticotropin releasing hormone type-1 receptor translation by an upstream AUG triplet in the 5' untranslated region. *Mol. Pharmacol.* 2001; 59:485–492. [PubMed: 11179443]

Fig. 1. Semiquantitative RT-PCR amplification of cDNAs encoding different CRF₂ receptor splice variants from human brain tissue and Y79 cells. Amplification for 35 cycles was performed using different primer sets (see Materials and methods). In control reactions, GAPDH cDNA was amplified from all tissues for 20 cycles; L, DNA standard; H, hippocampus; A, amygdala; NC, negative control (H₂O).

Fig. 2.

Stimulation of cAMP production by sauvagine, oCRF and UCN3 (a) and Q-PCR for CRF₁ (b) and CRF₂ (c) mRNA in Y79 cells at increasing cell culture passages. (a) Y79 cells in different culture passages (80 000 per well) were incubated with increasing concentrations of sauvagine, oCRF, and UCN3 (300 nmol/L) for the indicated time. By ANOVA, there were significant differences across the groups ($F = 396.3$, $p < 0.0001$). The following *post hoc* differences were found to be statistically significant between groups: ^a $p < 0.05$ versus passages 12 and 15; ^b $p < 0.0001$ versus passage 5; ^c $p < 0.002$ versus passage 20; ^d $p < 0.0001$ versus passages 5, 12, and 15; ^e $p < 0.0001$ versus passage 20. (b) Q-PCR amplification of CRF₁ cDNA from first strand cDNA synthesized from total RNA isolated from Y79 at different culture passages. (c) Q-PCR amplification of CRF₂ cDNA from first strand cDNA synthesized from total RNA isolated from Y79 at different culture passages. The results are representatives of three independent experiments performed in quadruplicate and normalized against GAPDH. Similar results were obtained with β -actin cDNA as internal standard (not shown). By ANOVA, there were significant differences across the groups ($F = 86.39$, $p < 0.0001$). The following *post hoc* differences were found to be statistically significant between groups: ^f $p < 0.0001$ versus passage 5; ^g $p < 0.0005$ versus passage 12; ^h $p < 0.0001$ versus passage 12; ⁱ $p < 0.002$ versus passage 15; ^j $p < 0.0001$ versus passage 15; ^k $p < 0.0001$ versus passage 20.

Fig. 3.

Stimulation of cAMP production in Y79 cells by various CRF receptor agonists. Cells (80 000 per well) were incubated with increasing agonist concentrations (0.01 nmo/L to 1 μ mol/L) for 15 min at 25°C and cAMP accumulation was measured by homogenous time-resolved fluorescence readout as described in the Materials and methods section. Data are representative of eight independent stimulations performed in quadruplicate.

Fig. 4.

Antagonist-mediated inhibition of CRF₁ or CRF_{2(a)} receptor-mediated stimulation of cAMP accumulation in Y79 cells. Y79 cells were incubated with increasing concentrations of astressin, NBI27914 and antisauvagine (0.1 nmol/L to 10 μ mol/L each) in the presence of 10 nmol/L oCRF (CRF₁) or 100 nmol/L UCN3 [CRF_{2(a)}]. The results are representatives of five independent antagonist experiments performed in quadruplicate.

Fig. 5. Saturation binding of ^{125}I -antisauvagine to membranes prepared from Y79 cells. Membrane proteins (250 μg per data point) were incubated with increasing concentrations of ^{125}I -antisauvagine (1 pmol/L to 2 nmol/L) for 2 h at 22°C. Bound radiolabel was separated from the free radioactivity by rapid centrifugation in a table top centrifuge at 4°C and two washing steps. Non-specific binding was determined by a large molar excess of unlabeled UCN3 (10 $\mu\text{mol/L}$). The results are representative of three independent experiments performed in triplicate.

Fig. 6.

Inhibition of ^{125}I -antisauvagine and ^{125}I -sauvagine binding to membranes of Y79 cells by various natural CRF agonists. Membranes were incubated at 22°C for 2 h with the two radiolabels and increasing concentrations of various CRF agonists (0.1 nmol/L to 10 $\mu\text{mol/L}$). The results are representative of four independent experiments performed in triplicate.

Fig. 7.

Comparison of time course for homologous CRF₂ receptor desensitization induced by UCN2 or 3. After pre-treatment with 100 nmol/L UCN2 (a) or UCN3 (b) for 5 min to 1 h was completed, Y79 cells were extensively washed and maximally re-stimulated with 100 nmol/L UCN2 for 15 min. Data are mean $\pm$ SEM of values expressed as picomoles of cAMP generated by 10⁶ cells that was collected in five independent experiments ($n = 10$ replicates per group). By ANOVA, there were significant differences across the groups ($F = 48.58$, $p < 0.0001$). The following *post hoc* differences were found to be statistically significant between UCN2-stimulated cAMP responses in individual groups: ^a $p < 0.001$ versus control; ^b $p < 0.001$ versus 5 min UCN3; ^c $p < 0.01$ versus 15 min UCN3; ^d $p < 0.001$ versus 15 min UCN3; ^e $p < 0.05$ versus 5 min UCN2.

Table 1Sequence of Q-PCR primers for the amplification of CRF₁ and CRF₂ receptors

Primer	CRF ₁ NM_004382 (1131–1188)	CRF ₂ NM_004382 (292–331)
Forward	5'-CACGTCTGAGACCATCA-3'	5'-TGTGAGCCCATTTTGATGA-3'
Reverse	5'-GGGCAGCAGCACCAGAGT-3'	5'-AAGGGCGATGCGGTAGTG-3'
Probe	5'-ACAGGAAGGCTGTGAAA-3'	5'-AAGCAGAGGAAGTATGACC-3'

CRF₁, CRF type 1 receptor; CRF₂, CRF type 2 receptor.

Table 2

Potencies of various CRF peptides to stimulate cAMP accumulation in Y79 retinoblastoma cells

Peptide	EC ₅₀ (nmol/L)	E _{max} (pmol over basal)
oCRF	1.61 ± 0.59 ^a	102.8 ± 8.8 ^e
h/rCRF	4.25 ± 1.09 ^b	105.2 ± 9.6 ^e
hUCN1	2.67 ± 0.77 ^c	101.2 ± 6.42 ^e
hUCN2	15.5 ± 3.1 ^d	26.6 ± 3.2
SRP	9.69 ± 2.13 ^d	28.6 ± 5.1
hUCN3	39.7 ± 4.1	24.6 ± 4.8
mUCN3	37.5 ± 12.3	25.6 ± 4.4
SCP	52.1 ± 8.3	28.6 ± 5.6
Sauvagine	1.57 ± 0.28 ^a	105.8 ± 7.8 ^e

The data are means ± SEM of four to seven independent stimulation experiments performed in quadruplicate. Statistical differences

^a $p < 0.0001$ versus hUCN2, hUCN3, mUCN3, SCP, and SRP

^b $p < 0.02$ versus hUCN2, hUCN3, mUCN3, SCP, and SRP

^c $p < 0.002$ versus hUCN2, hUCN3, mUCN3, SCP, and SRP

^d $p < 0.001$ versus hUCN3, mUCN3, and SCP

^e $p < 0.0001$ versus hUCN2, hUCN3, mUCN3, SCP, and SRP.

CRF, corticotropin-releasing factor

oCRF, ovine CRF

h/rCRF, human/rat CRF

UCN, urocortin

hUCN, human UCN

SCP, stresscopin.

Table 3

Antagonist potencies of antisauvagine, astressin, and NBI27914 on cAMP accumulation in Y79 cells stimulated with either a CRF₁-selective (oCRF, 10 nmol/L) or a CRF₂-selective (UCN3, 100 nmol/L) agonist

Antagonist	CRF ₁ (oCRF) IC ₅₀ (nmol/L)	CRF ₂ (UCN3) IC ₅₀ (nmol/L)
Antisauvagine	2040 ± 179	6.46 ± 1.62
Astressin	36.7 ± 9.2	5.47 ± 2.01
NBI27914	38.4 ± 3.6	> 10 000

The data are means ± SEM of three to six independent stimulation experiments performed in quadruplicate. CRF, corticotropin-releasing factor; CRF₁, CRF type 1 receptor; CRF₂, CRF type 2 receptor; UCN, urocortin.