Characterization of a *Pachymedusa dactylorhiza*–Sauvagine Analog as a New High-Affinity Radioligand for Corticotropin-Releasing Factor Receptor Studies^S

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ABSTRACT

The corticotropin-releasing factor (CRF) peptide family comprises the mammalian peptides CRF and the urocortins as well as frog skin sauvagine and fish urophysal urotensin. Advances in understanding the roles of the CRF ligand family and associated receptors have often relied on radioreceptor assays using labeled CRF ligands. These assays depend on stable, high-affinity CRF analogs that can be labeled, purified, and chemically characterized. Analogs of several of the native peptides have been used in this context, most prominently including sauvagine from the frog *Phyllomedusa sauvagei* (PS-Svg). Because each of these affords both advantages and disadvantages, new analogs with superior properties would be welcome. We find that a sauvagine-like peptide recently isolated from a different frog species, *Pachymedusa dactylorhiza* (PD-Svg), is a high-affinity agonist whose

radioiodinated analog, [¹²⁵I-Tyr⁰-Glu¹, Nle¹⁷]-PD-Svg, exhibits improved biochemical properties over those of earlier iodinated agonists. Specifically, the PD-Svg radioligand binds both CRF receptors with comparably high affinity as its PS-Svg counterpart, but detects a greater number of sites on both type 1 and type 2 receptors. PD-Svg is also ~10 times more potent at stimulating cAMP accumulation in cells expressing the native receptors. Autoradiographic localization using the PD-Svg radioligand shows robust specific binding to rodent brain and peripheral tissues that identifies consensus CRF receptor-expressing sites in a greater number and/or with greater sensitivity than its PS-Svg counterpart. We suggest that labeled analogs of PD-Svg may be useful tools for biochemical, structural, pharmacological, and anatomic studies of CRF receptors.

Introduction

Corticotropin-releasing factor (CRF) is a neuropeptide first identified as the hypophysiotropic factor that governs the endocrine (pituitary-adrenal) arm of the stress response (Vale et al., 1981). Its distribution and effects in the central nervous system indicated a broader involvement in mediating and/or integrating complementary behavioral and autonomic adaptations to stress (Bale and Vale, 2004). Subsequent work has identified in mammals additional structurally- and functionally-related peptides, the urocortins (Ucn 1–3), which are also active in stress adaptation (Vaughan et al., 1995; Lewis et al., 2001; Reyes et al., 2001). Other nonmammalian members of the CRF peptide family include urotensin 1 from fish urophysis (Lederis et al., 1982) and sauvagine from the skin of the frog, *Phyllomedusa sauvagei* (Montecucchi and Henschen, 1981), which is hereafter referred to as PS-Svg.

CRF and related peptides exert their biologic activities by binding either or both of two G protein-coupled receptors (GPCRs) that are positively coupled to adenylate cyclase. The type 1 (CRF₁) receptor is expressed by anterior pituitary corticotropes and mediates the neuroendocrine actions of the peptide. The CRF₁ receptor is expressed widely in the brain and select peripheral tissues. A structurally related corticotropin-releasing factor receptor 2 (CRF₂) is a product of a different gene and exists in two major forms. One variant, corticotropin-releasing factor receptor 2 splice variant b [CRF_{2(b)}], is expressed in the periphery, including the heart, pancreas, muscle, and choroid plexus, while a second variant, corticotropin-releasing factor receptor 2 splice variant a, is expressed in the brain in a distribution that is distinct from and more limited than that of the CRF₁ subtype. The CRF family of signaling molecules is not only involved in regulating centrally-mediated responses to stress but also plays important roles in adjustments of the cardiovascular, digestive, reproductive, and immune systems to challenges of diverse types.

Full appreciation of the structural, pharmacological, and anatomic bases for signaling at CRF receptors requires the availability of specific high-affinity ligands that can be used as tools with which to characterize these proteins. Such ligands

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ABBREVIATIONS: CRF, corticotropin-releasing factor; CRF₁, corticotropin-releasing factor receptor 1; CRF₂, corticotropin-releasing factor receptor 2; CRF_{2(b)}, corticotropin-releasing factor receptor 2 splice variant b; GPCR, G protein-coupled receptor; PD-Svg, sauvagine from *Pachymedusa dactylorhiza*; PS-Svg, sauvagine from *Phyllomedusa sauvagei*; Ucn, urocortin.

Fig. 1. Comparison of the amino acid sequences of PD-Svg, PS-Svg, CRF, Ucn, and stressin. Amino acid differences between PD-Svg and PS-Svg are boxed in green. Identities to r/m/hCRF are shown in red. Amino acid changes that render PD-Svg more similar to the antagonist stressin are starred in blue. f, D-phenylalanine; h, human; m, mouse; -NH₂, amide; o, ovine; <Q, pyroglutamic acid; r, rat; X, norleucine. The lactam bridge (—) between Glu³⁰ and Lys³³ of stressin is illustrated.

may be radiolabeled and used to identify and quantify binding sites in native tissues, cell lines, and/or isolated membrane preparations and have been instrumental screening tools in the development of small-molecule antagonists (Webster et al., 1996; Tellev et al., 2010). In addition, and in view of the lack of thoroughly validated CRF receptor antisera for immunohistochemical studies, radioligands have provided valuable means of localizing sites of functional receptor binding in situ. Peptides that have been used in this way include analogs of ovine CRF (Perrin et al., 1986), rat Ucn 1 (Perrin et al., 1999), and PS-Svg (Chen et al., 2005) as well as the antagonists, stressin (Gulyas et al., 1995; Rivier and Rivier, 2014) and antisauvagine-30 (Ruhmann et al., 1998). Of these, radioiodinated analogs of PS-Svg, such as [Tyr⁰-Glu¹, Nle¹⁷]-PS-Svg and [Tyr⁰]-PS-Svg, which bind both CRF receptors with relatively high affinity, have come to be most widely used in pharmacological and functional studies (Grigoriadis et al., 1996; Primus et al., 1997; Rominger et al., 1998; Ruhmann et al., 1998; Ardati et al., 1999; Sanchez et al., 1999; Skelton et al., 2000; Bakshi et al., 2002; Lawrence et al., 2002; Maillot et al., 2003; Wigger et al., 2004; Gehlert et al., 2005; Lim et al., 2005; Waser et al., 2006; Silberstein et al., 2009). Recently, a new CRF-related peptide was isolated from a different frog species, *Pachymedusa dancinicolor*, and termed PD-Svg (Zhou et al., 2012). The new peptide differs substantially from its PS-Svg counterpart in primary structure and was found to be more potent in bioassays. Here, we report on studies of a radiolabeled PD-Svg analog aimed at determining whether this compound may offer advantages for studies of CRF receptor localization and function.

Materials and Methods

Peptides and Iodination. All the peptides were synthesized using standard methods, and the analogs were radioiodinated using the chloramine-T method and purified by high-performance liquid chromatography. Detailed methods are provided as Supplemental Material. Because the native forms of PS-Svg or PD-Svg contain neither tyrosines nor histidines for radiolabeling, a tyrosine residue [Tyr⁰] was added at the N terminus of each. Consequently, the glutamic acid residue in position 1 of the analogs is not cyclized to pyroglutamic acid, as occurs in the native sauvagine peptides. Additionally, methionine (residue 17) in PS-Svg was replaced with a structurally similar, though non-oxidizable, norleucine residue. Thus, the iodinated analogs used in the present study were

PS-Svg radioligand : [Tyr⁰-Glu¹, Nle¹⁷]-PS-Svg
PD-Svg radioligand : [Tyr⁰-Glu¹]-PD-Svg.

Radioreceptor Assay. All binding assays were carried out in a binding buffer (50 mM Na-HEPES, pH 7.5, 10 mM MgCl₂, 2 mM EGTA, and 0.1% bovine serum albumin) in triplicate using membranes prepared from COSM6 cells transiently expressing either CRF₁ or CRF_{2(b)} receptors. For the saturation studies, 1–10 μg membrane proteins were incubated with increasing doses (0.03–3 nM) of either PD-Svg or PS-Svg radioligands diluted in binding buffer containing 0.02% Triton X-100 in GV microtiter filter plates (Millipore, Billerica,

Fig. 2. The specific binding of PD-Svg radioligand is saturable. Binding of PD-Svg radioligand to membranes from COSM6 cells expressing (A) CRF₁ receptors or (B) CRF_{2(b)} receptors. Total binding (■, solid black line); nonspecific binding (◆, dashed gray line); specific binding (▲, solid gray line). CPM, counts per minute.

Fig. 3. The specific binding of the PS-Svg radioligand is saturable. Binding of PS-Svg radioligand to membranes from COSM6 cells expressing (A) CRF₁ receptors or (B) CRF_{2(b)} receptors. Total binding (■, solid gray line); nonspecific binding (◆, dashed gray line); specific binding (▲, solid black line). CPM, counts per minute.

MA) prewetted with 0.1% polyethyleneimine, followed by washing with binding buffer. The binding reactions were continued to equilibrium at 18°C for 90 minutes. For competitive displacement assays, fixed concentrations (0.2–0.4 nM) of PD-Svg, PS-Svg, or astressin radioligands diluted in binding buffer plus 0.02% Triton X-100 were incubated with the membrane proteins and increasing concentrations of the selected peptides diluted in binding buffer. The competitive displacement assays were performed as described above for saturation assays. Binding was terminated by vacuum aspiration and washing plates twice with binding buffer. Specific binding is taken to be the difference in cpm between the total binding and the binding in the presence of 100 nM unlabeled peptide. Radioactivity on the filters was determined by γ -counter (~90% efficiency). The data were analyzed using GraphPad Prism software (GraphPad Software, La Jolla, CA).

Kinetic Measurements. To measure the association rates, the receptors were incubated with PD-Svg radioligand in the presence or absence of 100 nM unlabeled PD-Svg. The reaction was terminated by filtration, as described above, at the indicated times. The association rate, k_{on} , was calculated from the equation $k_{on} = (k_{ob} - k_{off})/[\text{radioligand}]$. k_{ob} and k_{off} were calculated using GraphPad Prism software. To measure

the dissociation rates, the receptors were incubated with PD-Svg radioligand for at least 120 minutes, at which time dissociation was initiated by the addition of unlabeled PD-Svg in one-fourth incubation volume to a final concentration of 1 μ M. At the indicated times, the reaction was terminated by filtration as described above.

cAMP Assay. Mouse pituitary tumor cells, AtT-20, which express endogenous CRF₁ receptors, or rat aortic smooth muscle cells, A7r5, which express endogenous CRF_{2(b)} receptors, were plated into 48-well dishes and allowed to recover for 24 hours. The cells were then starved overnight in Dulbecco's modified Eagle's medium (Fisher Scientific, Pittsburgh, PA)/0.2% fetal bovine serum/0.1% bovine serum albumin. Cells were preincubated with 0.1 mM 3-isobutyl-1-methylxanthine for 20 minutes and then exposed to peptides for 30 minutes at 37°C. Intracellular cAMP was measured in triplicate from cellular extracts using a radioimmunoassay kit (PerkinElmer, Waltham, MA). Potencies were compared using GraphPad Prism software.

Tissue Autoradiography. All animal procedures were approved by the Institutional Animal Care and Use Committee of the Salk Institute and were conducted in accordance with National Institutes of Health guidelines. Wild-type C57Bl/6 mice (Charles River Laboratories, San Diego, CA), CRF₁ (Smith et al., 1998), and CRF₂ (Bale et al., 2000) receptor null mutants on the same background were maintained on standard mouse chow and water ad libitum, and a 12-hour light/dark cycle with lights on at 6:00 AM. Animals were sacrificed with an overdose of chloral hydrate (350 mg/kg i.p.; Sigma-Aldrich, St. Louis, MO) and briefly perfused via the ascending aorta with ice-cold saline. Tissues of interest were removed quickly and snap frozen on crushed dry ice. Tissues were sectioned at 30 μ m in the coronal plane on a cryostat and mounted onto Superfrost Plus slides (Thermo Scientific, Kalamazoo, MI). Sections were dried and stored at –80°C with desiccant until use.

Before use, slides were equilibrated to room temperature for 30 minutes. Sections were washed 2 $\times$ 3 minutes with binding buffer. Following preincubation, slides were incubated in 5 ml binding buffer containing either 10⁷ cpm (~0.5 nM) of PS-Svg or PD-Svg radioligand at room temperature under gentle agitation for 90 minutes. To determine nonspecific binding, 100 nM unlabeled peptide (PS-Svg or PD-Svg, respectively) was added to the above mixture and applied to slides containing adjacent sections. To determine if PD-Svg radioligand binding was specific for CRF₁ or CRF₂ receptors, brain sections were incubated with radioligand and either 1 μ M antalarmin (selective CRF₁ receptor antagonist) (Webster et al., 1996) or 100 nM astressin-2b (selective CRF₂ receptor antagonist) (Rivier et al., 2002). After incubation, slides were washed 2 $\times$ 5 minutes in binding buffer, dried, and exposed to X-ray film (Kodak Biomax MR; Eastman Kodak, Rochester, NY) for times ranging from 15 hours to 3 days. Sections adjacent to those used for binding were stained with thionin or H&E for reference purposes. Representative tissue autoradiographs were scanned and processed identically in Photoshop (levels tool; Adobe Systems, San Jose, CA) to display their full tonal range for imaging purposes (see Figs. 8–11). For analysis of binding in specific brain areas, unmanipulated autoradiographs were analyzed using ImageJ software (NIH, Bethesda, MD). The mean gray value was measured in each region of interest and expressed in units of optical density. Measurements were taken from sections in the absence (total binding) or presence (nonspecific binding) of the respective unlabeled peptide, and their difference was used as the specific binding measurement. Statistical analyses of PD-Svg and PS-Svg radioligand

TABLE 1

Dissociation binding constant, K_d (nM), and total number of binding sites, B_{max} (pmol/mg), of PD-Svg or PS-Svg radioligand determined from saturation binding to either CRF₁ or CRF_{2(b)} receptor

The data are mean $\pm$ S.E.M.; $n = 3$ per cell.

	PD-Svg Radioligand K_d	PS-Svg Radioligand K_d	PD-Svg Radioligand B_{max}	PS-Svg Radioligand B_{max}
CRF ₁	0.48 $\pm$ 0.07	0.49 $\pm$ 0.27	6.8 $\pm$ 0.37	0.5 $\pm$ 0.08
CRF _{2(b)}	0.20 $\pm$ 0.03	0.41 $\pm$ 0.09	12.3 $\pm$ 0.2	4.2 $\pm$ 0.4

Fig. 4. PD-Svg radioligand associates rapidly with and dissociates slowly from CRF receptors. The time course of association of the PD-Svg radioligand to membranes from COSM6 cells expressing (A) CRF₁ receptors or (B) CRF_{2(b)} receptors. The time course of peptide-induced dissociation of PD-Svg radioligand to membranes from COSM6 cells expressing (C) CRF₁ receptors or (D) CRF_{2(b)} receptors. cpm, counts per minute.

binding densities were performed using GraphPad Prism using a two-way analysis of variance with Bonferroni's post hoc test for individual comparisons. For the comparison of background levels, a two-tailed paired *t* test was used. $P < 0.05$ was considered statistically significant.

Results

Figure 1 compares the sequences of PD-Svg and PS-Svg, along with those of other CRF family ligands, including astressin, a synthetic peptide antagonist with high affinity for both receptors (Gulyas et al., 1995). Although some key residues are conserved among all the peptides, PD-Svg shares just a 52% sequence similarity with rat/mouse/human CRF and 62% with PS-Svg (Zhou et al., 2012). The N-terminal portion of the PD-Svg sequence is more similar to CRF, while the C-terminal region is more like PS-Svg. It is also noteworthy that PD-Svg shares a substantially greater structural similarity with astressin than its PS-Svg counterpart.

Radioligand Affinities. The affinity, K_a , of a labeled analog is often represented by its dissociation constant, K_d , which is the reciprocal of K_a . The K_d s are determined from saturation data, in which the receptor binding is determined as a function of increasing amounts of radioligand. To compare the K_d s and number of sites detected by PD-Svg and PS-Svg radioligands, saturation data were obtained for each of the tracers bound to either CRF₁ or CRF_{2(b)} receptors. Figures 2 and 3 show the saturation isotherms for the PD-Svg and PS-Svg radioligands, respectively. It can be seen that the

binding was of high affinity and saturable for both radioligands. For the PD-Svg tracer, the K_d s were 0.42 and 0.19 nM and the corresponding numbers of binding sites were 6.5 and 13 pmol/mg protein for the CRF₁ and CRF_{2(b)} receptors, respectively (Fig. 2). For the PS-Svg tracer, the K_d s were 0.43 and 0.40 nM and the corresponding numbers of binding sites were 0.93 and 6.2 pmol/mg for the CRF₁ and CRF_{2(b)} receptors, respectively (Fig. 3). Averaged data from three experiments are provided in Table 1. Thus, while the affinities of the two analogs for both CRF receptors were comparable, the PD-Svg radioligand detected a greater number of sites than its PS-Svg counterpart.

Kinetics. To characterize the binding kinetics, association rates were determined for the PD-Svg radioligand on each receptor subtype by following specific binding over time. Figure 4, A and B, show representative association curves for the binding of the PD-Svg radioligand to the CRF₁ and CRF_{2(b)} receptors, respectively. The specific binding was calculated as the difference between the total and nonspecific binding. Nonspecific binding was measured in the presence of 100 nM unlabeled PD-Svg. The binding reached equilibrium for both receptors within 2 hours and was stable for at least 4 hours. The k_{on} for CRF₁ was $33 \times 10^7 \text{ min}^{-1}\text{M}^{-1}$. For CRF_{2(b)}, the k_{on} was $8.5 \times 10^7 \text{ min}^{-1}\text{M}^{-1}$.

To determine the rates of dissociation, which were initiated by the addition of excess (1 μM) unlabeled peptide following establishment of equilibrium (~ 2 hours), specific binding was measured as a function of time. As was done for the

Fig. 5. Determination of the relative potencies of some CRF family members by competitive displacement by PD-Svg (■, black), rat/mouse/human CRF (◆, blue), rat/mouse Ucn 1 (▲, purple), mouse Ucn 2 (●, green), and rat/mouse Ucn 3 (▼, red) of PD-Svg radioligand bound to membranes from COSM6 cells expressing (A) CRF₁ receptors or (B) CRF_{2(b)} receptors. CPM, counts per minute.

association data, specific binding was taken to be the difference between the total and nonspecific binding. Analyses of representative dissociation curves for CRF₁ and CRF_{2(b)} receptors (Fig. 4) suggested that the dissociation from each receptor was characterized by two different rate constants. For the experiments shown in Fig. 4, C and D, one dissociation time for the CRF₁ receptor was $t_{1/2} = 0.3$ hours and the other was 4.5 hours. For the CRF_{2(b)} receptor, one $t_{1/2} = 3.5$ hours and the other was 117 hours. These data suggest that the PD-Svg tracer remained bound to both receptors for a very long time and that it may interact with more than one binding site.

Ligand Pharmacology. The affinity of a given ligand may be described by an inhibitory binding constant, K_i , which is a measure of the ability of that compound to compete with a radioligand for binding to cognate receptors. Typical competitive binding isotherms for competition of the PD-Svg radioligand bound to the CRF₁ and CRF_{2(b)} receptors are shown in Fig. 5. The native peptide, rat/mouse/human CRF, was potent in competing for binding to both receptors, as was rat/mouse Ucn 1, whereas mouse Ucn 2 and rat/mouse Ucn 3 were selective for the CRF_{2(b)} receptor. The average K_i s for a series of CRF family ligands are given in Table 2. Analogous competitive binding curves were obtained using the PS-Svg radioligand, and these data are also summarized in Table 2. Interestingly, PS-Svg displayed a different relative affinity when competitively displacing PD-Svg radioligand compared with its affinity in competing binding of the PS-Svg tracer. The K_i of PS-Svg, which was determined from competitive displacement of the bound PD-Svg radioligand, was substantially higher compared with its K_i in displacing the PS-Svg tracer. This shift to the right, i.e., higher K_i in the competitive binding assay, is reminiscent of PS-Svg's limited ability to displace the antagonist astressin (Table 3). It should be noted that the apparent affinities of CRF ligands were dependent not only on the cell line expressing the receptor, but also on the radioligand that was used in the competitive displacement assays.

CRF receptors are members of the GPCR family. As such, it is presumed that high-affinity active conformations that bind agonists are coupled to G proteins, and, therefore, that the binding of agonists should be modulated by guanyl nucleotides whose binding causes dissociation of the G protein from the receptor. Indeed, previous studies have found that guanyl nucleotides inhibit the binding of some CRF agonists (Perrin et al., 1986; Rominger et al., 1998). Therefore, it was of interest to determine the effect of guanyl nucleotides on the binding of the PD-Svg radioligand. We found that the guanyl nucleotide, guanosine 5'-3-*O*-(thio)triphosphate, did not affect binding of this tracer as much as it affected that of its PS-Svg counterpart (Fig. 6). Further, both radioligands showed greater sensitivity to guanosine 5'-3-*O*-(thio)triphosphate when bound to the CRF₁ receptor compared with binding to the CRF_{2(b)} receptor. Interestingly, not all CRF receptor agonist binding is sensitive to the presence of guanyl nucleotides. Specifically, the binding of the labeled agonist, [¹²⁵I-Tyr⁰]-rat Ucn 1, was also found to be insensitive to guanyl nucleotides (Perrin et al., 1999). Together, the data suggest that similar CRF receptor conformations may bind rat Ucn 1

TABLE 2

Inhibitory binding constants, K_i (nM), of representative CRF family ligands determined by competitive binding of PD-Svg or PS-Svg radioligands bound to either CRF₁ or CRF_{2(b)} receptor

Ranges given in parentheses correspond to 95% confidence limits; $n = 3-6$ per cell. Data reproduced from previous publications are indicated

	PD-Svg	PS-Svg	r/hCRF	rUcn 1	mUcn 2	mUcn 3	Astressin
PD-Svg radioligand bound to either CRF ₁ or CRF _{2(b)} receptors							
CRF ₁	4.4 (2.9–6.5)	62 (29–140)	19 (7–49)	3.0 (2.0–4.5)	>300	>300	0.47 (0.42–0.52)
CRF _{2(b)}	1.9 (1.3–2.7)	28 (21–40)	13 (2–16)	4.3 (3.7–4.9)	4.8 (3.3–7.0)	120 (100–140)	0.7 (0.2–3)
PS-Svg radioligand bound to either CRF ₁ or CRF _{2(b)} receptors							
CRF ₁	0.22 (0.18–0.27)	0.75 (0.59–0.94)	1 (0.2–5) ^a	0.32 (0.14–0.77) ^b	>100 ^b	>100 ^b	0.7 (0.3–2) ^a
CRF _{2(b)}	0.40 (0.38–0.42)	3.6 (2.5–5.1)	6 (2–20) ^a	0.6 (0.1–3) ^b	0.7 (0.1–3) ^b	2 (0.8–4) ^b	0.62 (0.49–0.78) ^a

h, human; m, mouse; r, rat.

^aFrom Rivier et al. (2002).

^bFrom Lewis et al. (2001).

TABLE 3

Inhibitory binding constant, K_i (nM), of representative CRF family ligands determined by competitive binding of astressin radioligand bound to either CRF₁ or CRF_{2(b)} receptor

Ranges given in parentheses correspond to 95% confidence limits; $n = 3$ per cell.

	PD-Svg	PS-Svg	Astressin
CRF ₁	9 (3–28)	100 (50–200)	2.3 (1.9–2.9)
CRF _{2(b)}	2.8 (2.5–3.2)	21 (19–23)	1.3 (0.6–2.6)

and PD-Svg, and these conformations may differ from those that recognize PS-Svg.

To compare the functional cellular consequences of PD-Svg and PS-Svg binding, intracellular cAMP accumulation was measured in response to both peptides in cell lines that endogenously express CRF₁ (AtT-20) or CRF_{2(b)} receptors (A7r5) (Fig. 7). The in vitro data are summarized in Table 4 and show that PD-Svg was 4–6 times more potent than PS-Svg in stimulating cAMP accumulation. PD-Svg also had greater efficacy compared with PS-Svg in AtT-20 cells. In addition, a CRF receptor antagonist blocked the cAMP accumulation stimulated by PD-Svg through both receptors (Fig. 7).

Autoradiography. The characteristics of PD-Svg described above suggest that analogs of it may offer advantages as tools for localizing CRF receptors in situ. Thus, we compared the binding of the PD-Svg and PS-Svg radioligands to consensus sites of CRF₁ or CRF₂ receptor expression in the brain and periphery. Here, we summarize some key features of these comparisons. Details of the anatomic distributions will be published separately. Binding of both radioligands in

the brain (Fig. 8) and select peripheral tissues, including the heart (Fig. 9), was found to conform well to prior descriptions of major cellular sites of CRF₁ or CRF₂ receptor mRNA expression (Van Pett et al., 2000) (see Figs. 8–10). These localizations were competed in a dose-related manner by the addition of the corresponding unlabeled peptide over concentrations of 1–100 nM (Fig. 10). In addition, radioligand binding in previously identified CRF₁ or CRF₂ receptor-specific sites of expression was evaluated and confirmed by carrying out binding in null mice lacking expression of either receptor (Fig. 9) and in wild-type brains incubated with CRF receptor-specific antagonists (Fig. 10). In the brain, major sites of PD-Svg radioligand binding included the olfactory bulb and brainstem sensory structures, isocortex and hippocampal cortices, cerebellum and associated cell groups, and limbic forebrain and central autonomic structures among consensus CRF₁ receptor-expressing structures and the choroid plexus, lateral septum, ventromedial nucleus of the hypothalamus, and nucleus of the solitary tract for the CRF₂ receptor (Fig. 8, A–C). While most of these showed good overlap with loci of PS-Svg radioligand binding (Fig. 8, D–F), several prominent sites of cellular CRF₁ receptor mRNA expression that displayed robust PD-Svg tracer binding, including the reticular nucleus of the thalamus, lateral habenula, red nucleus, and pontine gray, all failed to display detectable PS-Svg radioligand binding.

Brain binding of the two radioligands was quantitatively compared in an experiment in which alternate series of sections from the same animals were exposed to either of the two tracers at similar concentrations and labeled to the same

Fig. 6. A comparison of the effects of the guanyl nucleotide, GTP γ S [guanosine 5'-3-O-(thio)triphosphate], on the specific binding of PD-Svg or PS-Svg radioligands. PD-Svg radioligand bound to membranes from COSM6 cells expressing (A) CRF₁ receptors or (B) CRF_{2(b)} receptors or PS-Svg radioligand bound to membranes from COSM6 cells expressing (C) CRF₁ receptors or (D) CRF_{2(b)} receptors. cpm, counts per minute.

Fig. 7. PD-Svg is more potent than PS-Svg at stimulating intracellular cAMP accumulation. PD-Svg (black squares, solid line) and PS-Svg (gray circles, solid line) stimulate cAMP accumulation in (A) AtT-20 cells expressing CRF₁ receptors and (B) A7r5 cells expressing CRF_{2(b)} receptors. The accumulation is blocked by (A) 100 nM nonselective CRF receptor antagonist astressin or (B) 100 nM CRF₂ receptor antagonist astressin₂-B (dashed lines).

specific activities. Under these conditions, overall binding of the PD-Svg radioligand in each of five select brain regions was significantly greater than that of its PS-Svg counterpart (Fig. 11) [$F(1, 40) = 1028$; $P < 0.0001$]. PD-Svg radioligand binding averaged 2.7-fold greater than PS-Svg tracer binding in sites of CRF₁ receptor expression ($P < 0.0001$) but only 0.5-fold more in representative CRF₂ receptor sites ($P < 0.0001$). Nonspecific binding of the PD-Svg tracer was also lower than the PS-Svg radioligand (Fig. 11C) ($P = 0.01$), as assessed in sections incubated with 100 nM of their respective unlabeled peptide.

Discussion

We have characterized an analog of the recently isolated peptide PD-Svg (Zhou et al., 2012), finding that it offers

advantages for studying CRF receptor localization and function, relative to other CRF ligands that have been employed in this context, including PS-Svg analogs. PD-Svg shares key amino acid residues with CRF family ligands that have been implicated in receptor recognition and signal transduction. The current model for CRF ligand-receptor interactions holds that C-terminal residues interact with the receptors' first extracellular domain, docking the peptide on the receptors' binding surface (Grace et al., 2004, 2007, 2010) and placing the N terminus of the peptide in a position to interact with juxtamembrane/transmembrane receptor domains, resulting in signal transduction (Nielsen et al., 2000). Differences in the N-terminal sequences of the two sauvagines may account for the observed differences in their potencies in cAMP signaling assays. As the C-terminal aspect of the peptides govern the initial recognition by CRF receptors (Grace et al., 2004, 2010), the conservation found in this region may account for the peptides' similar affinities. These structural differences may also help explain the greater number of binding sites (i.e., more conformations) recognized by the PD-Svg radioligand.

Interestingly, both radioiodinated Svg peptides displayed higher affinities (lower K_d s) than those determined from competitive displacement assays. It is possible that introducing iodine into the radioligands affects binding relative to that of the native peptide. Testing this would require study of nonradioactive iodinated analogs.

Dissociation data suggest that the binding affinity of the PD-Svg radioligand for each receptor is best described by a two-site model. This may be indicative of two high-affinity sites that were not distinguishable in either the saturation or competitive binding experiments. The values of $t_{1/2}$ for CRF₁ were much smaller than those for the CRF_{2(b)} receptor, suggesting that the radioligand binds the two receptors differently. For most systems, the association between a ligand and cognate receptor(s) is thought to be diffusion controlled, and association rates tend to be similar. Thus, the affinity of a ligand is usually determined by its rate of dissociation. This model has been supported by detailed measurements of ligand association/dissociation to the membrane-associated major histocompatibility complex protein (Kasson et al., 2000). Because of limitations on the concentrations of labeled peptide available for use in the present saturation binding assays, a second high-affinity site may not have been observable. Thus, the affinities determined from the saturation experiments may represent an average, and an even higher affinity site for PD-Svg on CRF receptors may exist. The dissociation rates of the PD-Svg tracer were low relative to those for the PS-Svg tracer reported previously (Grigoriadis et al., 1996; Rominger et al., 1998). It is unclear whether this difference may reflect properties intrinsic to the two peptides or differences in the cellular milieu in which the current (COSM6) and prior (HEK293 or CHO) studies were carried out.

TABLE 4

EC₅₀ (nM) values of representative CRF family ligands for stimulation of intracellular accumulation of cAMP in AtT-20 or A7r5 cells, which express CRF₁ or CRF_{2(b)} receptors, respectively

Ranges given in parentheses correspond to 95% confidence limits. n = minimum 3 per cell.

	PD-Svg	PS-Svg	r/hCRF	rUcn 1	mUcn 2	mUcn 3
AtT-20	0.18 (0.14–0.24)	1.45 (1.2–1.8)	1.5 (1.4–1.7)	0.5 (0.47–0.53)	N.D.	N.D.
A7r5	0.04 (0.02–0.07)	0.30 (0.16–0.58)	N.D.	0.06 (0.03–0.12)	0.06 (0.03–0.14)	0.30 (0.16–0.58)

h, human; m, mouse; N.D., not determined; r, rat.

Fig. 8. The PD-Svg radioligand binds to brain areas that its PS-Svg counterpart does not. Mouse brain sections were incubated with 0.5 nM PD-Svg (A–C) or 0.5 nM PS-Svg (D–F) radioligands in adjacent sections. Films were exposed for 15 hours (PD-Svg) or 3 days (PS-Svg) to achieve comparable levels of exposure. Images were adjusted concurrently in PhotoShop (levels tool) to display their full tonal range. Differences in binding between the PD-Svg and PS-Svg radioligands include the thalamus, where the PD-Svg radioligand bound to the reticular nucleus (RT) and lateral habenula (LH) as well as in the pontine gray (PG) (white arrows), whereas the PS-Svg tracer did not. BLA, basolateral nucleus of the amygdala; CA1, CA1 layer of the hippocampus; CBL, cerebellum; CeA, central nucleus of the amygdala; chp, choroid plexus; Cx, cortex; ECU, external cuneate nucleus; IPN, interpeduncular nucleus; NTS, nucleus of the solitary tract; PAG, periaqueductal gray; SC, superior colliculus; SPV, spinal trigeminal nucleus; VMH, ventromedial nucleus of the hypothalamus. Scale bar = 1 mm.

The relative affinities and selectivities of CRF family ligands determined by competitive binding of the PD-Svg radioligand were generally similar to those determined using the PS-Svg tracer. This included the findings that Ucn 1 was potent on both receptor subtypes and that Ucn 2 and Ucn 3 were selective for the type 2 receptor, although r/hCRF displayed affinities for both receptors that were lower than those found using PS-Svg tracer. The affinities of PS-Svg were

found to be lower than those of PD-Svg when measured with the PD-Svg radioligand relative to the PS-Svg radioligand. Interestingly, the K_i s for PS-Svg that were determined with the PD-Svg tracer were more similar to those determined using an astressin tracer. Both the PD-Svg and astressin radioligands bind to CRF receptors expressed in *Escherichia coli* (Jappelli et al., 2014), whereas the PS-Svg radioligand does not. Further, astressin binds to isolated extracellular

Fig. 9. PD-Svg radioligand binding is comparable to PS-Svg tracer binding in the heart, a CRF_{2(b)} receptor-expressing peripheral tissue. Sections were exposed to either 0.5 nM PD-Svg radioligand (A–D) or 0.5 nM PS-Svg radioligand (E–H) in wild-type mice and exposed to film for 15 hours. Nonspecific binding was obtained by incubating sections with 100 nM of nonlabeled PD-Svg (B) or PS-Svg (F). PD-Svg and PS-Svg radioligand binding in CRF₁ receptor null mice (C and G) was no different from wild-type mice, but binding was abolished in CRF₂ receptor-deficient mice (D and H). At, atrium; V, ventricle. Scale bar = 1 mm.

Fig. 10. Binding of PD-Svg radioligand to CRF receptors is blocked by CRF₁ or CRF₂ receptor-specific antagonists. Adjacent mouse brain sections were incubated with 0.5 nM PD-Svg radioligand (top left) and with a CRF₁ receptor antagonist, antalarmin (top right), or a CRF₂ receptor antagonist, astressin-2B (bottom left). Incubation with the CRF₁ receptor antagonist abolished a majority of the PD-Svg radioligand binding without affecting CRF₂ receptor binding in the lateral hypothalamic area (LHA), medial nucleus of the amygdala (MeA), and especially the choroid plexus (chp, white arrows). In addition, incubation with the unlabeled PD-Svg abolished all radioligand binding. ARH, arcuate nucleus; BLA, basolateral nucleus of the amygdala; CA1, CA1 layer of hippocampus; CeA, central nucleus of the amygdala; Cx, cortex; DG, dentate gyrus; LHA, lateral hypothalamic area; RT, reticular nucleus of the thalamus. Scale bar = 1 mm.

domain 1 and PS-Svg does not. These data, together with the observation that astressin displaced the PD-Svg radioligand with nanomolar affinity, suggest that the agonist PD-Svg binds to conformations of the receptor that are similar to those bound by the antagonist astressin and that these differ from those bound by PS-Svg.

The relative insensitivity of PD-Svg radioligand binding to guanyl nucleotides is consistent with parallels between it and the antagonist (Perrin et al., 1999). However, PD-Svg is not an antagonist. It proved to be a more potent agonist than PS-Svg on both receptors in the cAMP signaling assay and more efficacious on AtT-20 cells, which endogenously express CRF₁ receptors. This greater signaling efficacy may reflect binding at a greater number of sites on the cloned receptors. Alternatively, it may be indicative of a greater number of receptor conformations in a high-affinity state that are better able to couple to effector proteins. It is noteworthy in this regard that the binding of the labeled agonist, Ucn 1, is also insensitive to guanyl nucleotides (Perrin et al., 1999) and suggests that PS-Svg and Ucn 1 are recognized by different CRF₁ receptor conformations (Wootten et al., 2013). The current model of GPCRs suggests that the receptors exist in multiple conformations that may couple differentially to

activate different signaling pathways (Wootten et al., 2013; Wisler et al., 2014). The concept of “biased agonism”, which describes the differential signaling capabilities of ligands, is of great interest in both the basic science and clinical (pharmaceutical) arenas (Wisler et al., 2014). Because the recognition domains of the receptors for the sauvagine peptides differ, it is possible that PD-Svg and PS-Svg activate different signaling pathways. It remains to be seen if PD-Svg displays functional selectivity and if so, what its physiologic consequences may be.

PS-Svg radioligands have long been the standard for autoradiographic localization of CRF receptors in a variety of species. Present findings agree that the PS-Svg radioligand binds to major consensus sites of cellular CRF₁ receptor expression in the brain, such as the isocortex, cerebellum, and hippocampus, and to areas enriched in CRF₂ receptors, such as the lateral septum, nucleus of the solitary tract, and choroid plexus, as previously revealed by hybridization histochemistry (Potter et al., 1994; Chalmers et al., 1995; Van Pett et al., 2000). By comparison, however, binding of the PD-Svg tracer was significantly greater than the PS-Svg tracer in all brain areas measured and produced lower levels of nonspecific labeling. This difference was more pronounced in sites of CRF₁ receptor expression, as the PD-Svg ligand displayed robust binding signals in some brain areas identified as sites of CRF₁ receptor mRNA expression and in CRF₁ receptor reporter mice (Justice et al., 2008; Kuhne et al., 2012), but in which we failed to detect above-background binding using the PS-Svg radioligand.

The increased binding of PD-Svg, relative to the PS-Svg, tracer was more pronounced in CRF₁-enriched areas than CRF₂ receptor areas, which may be due to the greater sequence similarity of PD-Svg to CRF₁, which has a higher affinity for CRF₁ over CRF₂ receptors (Reul and Holsboer, 2002). There was no difference in binding of the two radioligands in the heart, which expresses CRF_{2(b)} receptors (Waser et al., 2006), and binding of both radioligands in the heart was abolished in CRF₂ receptor-deleted mice, but undiminished (relative to wild-type) in animals lacking the CRF₁ receptor.

A lingering problem for CRF receptor studies derives from the lack of validated antisera that might be used to detect receptor proteins in situ. Although a substantial number of immunohistochemical studies have been published using antisera raised against synthetic or recombinant CRF receptor fragments, these have described a number of localizations that are at odds with the results of work at the mRNA level (e.g., see supplemental material in Refojo et al., 2011). Transgenic mouse lines that report CRF receptor expression have provided an alternate means of achieving accurate cellular localization of these proteins, though this approach is not immune to artifact (Gong et al., 2003; Justice et al., 2008; Refojo et al., 2011; Kuhne et al., 2012). Moreover, such transgenics can, at best, faithfully report receptor expression, but not subcellular localization, as reporter proteins are unlikely to traffic in the same manner as endogenous receptors. By binding to functional receptors directly, the PD-Svg radioligand can be used (with appropriate controls) to localize CRF₁ or CRF₂ receptor binding sites with high sensitivity, fidelity, and signal to noise or could be used to investigate changes in CRF receptor binding as a result of behavioral, pharmacological, or genetic manipulations. The limited resolution afforded by radioiodinated tracers remains

Fig. 11. PD-Svg radioligand binding was greater than its PS-Svg counterpart in the brain at CRF₁ and CRF₂ receptor sites and produced lower nonspecific binding. (A) Sections were incubated with either 0.5 nM PD-Svg or PS-Svg radioligands and exposed to the same autoradiographic film for 15 hours. (B) Optical density of binding ($n = 5$) was measured in areas of CRF₁ receptor binding (CA1, CA1 hippocampus; CBL, cerebellum; Cx, cortex) and CRF₂ receptor binding (chp, choroid plexus; NTS, nucleus of the solitary tract). (C) Optical density of nonspecific binding in adjacent sections incubated with PD-Svg radioligand ($n = 5$) or PS-Svg radioligand ($n = 5$) and 100 nM of their respective unlabeled peptide. AHN, anterior hypothalamic nucleus; BLA, basolateral nucleus of the amygdala; CeA, central nucleus of the amygdala; chp, choroid plexus; Cx, cortex; HF, hippocampus; RT, reticular nucleus of the thalamus; ZI, zona incerta. Scale bar = 1 mm. ** $P < 0.01$; *** $P < 0.001$.

a serious limitation to their use as anatomic tools. The approach would allow, for example, determination of how CRF₁ receptor binding sites are distributed across somatic and dendritic layers of a laminar structure (like the hippocampus), but not at an individual cellular level. The robustness and low background afforded by the PD-Svg tracer binding may, however, make it possible to achieve cellular resolution by direct fluorescence labeling of the tracer, as has been used in other neuropeptide systems (Sridharan et al., 2014).

In summary, we have characterized a recently described peptide, PD-Svg, as a nonselective CRF receptor agonist, and an analog of it as exhibiting high-affinity binding for both CRF receptors and possessing distinct advantages for studying CRF receptors in cells and tissues.

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Authorship Contributions

Participated in research design: Perrin, Tan, Vaughan, Sawchenko.
Conducted experiments: Perrin, Tan, Lewis, Donaldson.
Contributed new reagents or analytic tools: Vaughan, Miller, Erchegyi, Rivier.
Performed data analysis: Perrin, Tan, Lewis, Donaldson, Sawchenko.

Wrote or contributed to the writing of the manuscript: Perrin, Tan, Vaughan, Donaldson, Erchegyi, Rivier, Sawchenko.

References

- Ardati A, Goetschy V, Gottowick J, Henriot S, Valdenaire O, Deuschle U, and Kilpatrick GJ (1999) Human CRF alpha and beta splice variants: pharmacological characterization using radioligand binding and a luciferase gene expression assay. *Neuropharmacology* **38**:441–448.
- Bakshi VP, Smith-Roe S, Newman SM, Grigoriadis DE, and Kalin NH (2002) Reduction of stress-induced behavior by antagonism of corticotropin-releasing hormone 2 (CRH2) receptors in lateral septum or CRH1 receptors in amygdala. *J Neurosci* **22**:2926–2935.
- Bale TL, Contarino A, Smith GW, Chan R, Gold LH, Sawchenko PE, Koob GF, Vale WW, and Lee KF (2000) Mice deficient for corticotropin-releasing hormone receptor-2 display anxiety-like behaviour and are hypersensitive to stress. *Nat Genet* **24**:410–414.
- Bale TL and Vale WW (2004) CRF and CRF receptors: role in stress responsivity and other behaviors. *Annu Rev Pharmacol Toxicol* **44**:525–557.
- Chalmers DT, Lovenberg TW, and De Souza EB (1995) 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* **15**:6340–6350.
- Chen A, Perrin M, Brar B, Li C, Jamieson P, Digruccio M, Lewis K, and Vale W (2005) Mouse corticotropin-releasing factor receptor type 2alpha gene: isolation, distribution, pharmacological characterization and regulation by stress and glucocorticoids. *Mol Endocrinol* **19**:441–458.
- Gehlert DR, Shekhar A, Morin SM, Hipskind PA, Zink C, Gackenhaimer SL, Shaw J, Fitz SD, and Sajdyk TJ (2005) Stress and central Urocortin increase anxiety-like behavior in the social interaction test via the CRF1 receptor. *Eur J Pharmacol* **509**: 145–153.
- Gong S, Zheng C, Doughty ML, Losos K, Didkovsky N, Schambra UB, Nowak NJ, Joyner A, Leblanc G, Hatten ME, et al. (2003) A gene expression atlas of the central nervous system based on bacterial artificial chromosomes. *Nature* **425**: 917–925.
- Grace CR, Perrin MH, DiGraccio MR, Miller CL, Rivier JE, Vale WW, and Riek R (2004) NMR structure and peptide hormone binding site of the first extracellular domain of a type B1 G protein-coupled receptor. *Proc Natl Acad Sci USA* **101**: 12836–12841.
- Grace CR, Perrin MH, Gulyas J, DiGraccio MR, Cantle JP, Rivier JE, Vale WW, and Riek R (2007) Structure of the N-terminal domain of a type B1 G protein-

- coupled receptor in complex with a peptide ligand. *Proc Natl Acad Sci USA* **104**: 4858–4863.
- Grace CR, Perrin MH, Gulyas J, Rivier JE, Vale WW, and Riek R (2010) NMR structure of the first extracellular domain of corticotropin-releasing factor receptor 1 (ECD1-CRF-R1) complexed with a high affinity agonist. *J Biol Chem* **285**: 38580–38589.
- Grigoriadis DE, Liu XJ, Vaughn J, Palmer SF, True CD, Vale WW, Ling N, and De Souza EB (1996) 125I-Tyro-sauvagine: a novel high affinity radioligand for the pharmacological and biochemical study of human corticotropin-releasing factor 2 alpha receptors. *Mol Pharmacol* **50**:679–686.
- Gulyas J, Rivier C, Perrin M, Koerber SC, Sutton S, Corrigan A, Lahrichi SL, Craig AG, Vale W, and Rivier J (1995) Potent, structurally constrained agonists and competitive antagonists of corticotropin-releasing factor. *Proc Natl Acad Sci USA* **92**:10575–10579.
- Jappelli R, Perrin MH, Lewis KA, Vaughan JM, Tzitzilonis C, Rivier JE, Vale WW, and Riek R (2014) Expression and functional characterization of membrane-integrated mammalian corticotropin releasing factor receptors 1 and 2 in *Escherichia coli*. *PLoS ONE* **9**:e84013.
- Justice NJ, Yuan ZF, Sawchenko PE, and Vale W (2008) Type 1 corticotropin-releasing factor receptor expression reported in BAC transgenic mice: implications for reconciling ligand-receptor mismatch in the central corticotropin-releasing factor system. *J Comp Neurol* **511**:479–496.
- Kasson PM, Rabinowitz JD, Schmitt L, Davis MM, and McConnell HM (2000) Kinetics of peptide binding to the class II MHC protein I-Ek. *Biochemistry* **39**:1048–1058.
- Kühne C, Puk O, Graw J, Hrabě de Angelis M, Schütz G, Wurst W, and Deussing JM (2012) Visualizing corticotropin-releasing hormone receptor type 1 expression and neuronal connectivities in the mouse using a novel multifunctional allele. *J Comp Neurol* **520**:3150–3180.
- Lawrence AJ, Krstew EV, Dautzenberg FM, and Rühmann A (2002) The highly selective CRF(2) receptor antagonist K41498 binds to presynaptic CRF(2) receptors in rat brain. *Br J Pharmacol* **136**:896–904.
- Lederis K, Vale W, Rivier J, MacCannell KL, McMaster D, Kobayashi Y, Suess U, and Lawrence J (1982) Urotensin I - a novel CRF-like peptide in *Catostomus commersoni* urophysis. *Proc West Pharmacol Soc* **25**:223–227.
- Lewis K, Li C, Perrin MH, Blount A, Kunitake K, Donaldson C, Vaughan J, Reyes TM, Gulyas J, Fischer W, et al. (2001) Identification of urocortin III, an additional member of the corticotropin-releasing factor (CRF) family with high affinity for the CRF2 receptor. *Proc Natl Acad Sci USA* **98**:7570–7575.
- Lim MM, Nair HP, and Young LJ (2005) Species and sex differences in brain distribution of corticotropin-releasing factor receptor subtypes 1 and 2 in monogamous and promiscuous vole species. *J Comp Neurol* **487**:75–92.
- Mailhot C, Wang L, Million M, and Taché Y (2003) Intraperitoneal corticotropin-releasing factor and urocortin induce Fos expression in brain and spinal autonomic nuclei and long lasting stimulation of colonic motility in rats. *Brain Res* **974**:70–81.
- Montecucci PC and Henschen A (1981) Amino acid composition and sequence analysis of sauvagine, a new active peptide from the skin of *Phyllomedusa sauvagei*. *Int J Pept Protein Res* **18**:113–120.
- Nielsen SM, Nielsen LZ, Hjorth SA, Perrin MH, and Vale WW (2000) Constitutive activation of tethered-peptide/corticotropin-releasing factor receptor chimeras. *Proc Natl Acad Sci USA* **97**:10277–10281.
- Perrin MH, Haas Y, Rivier JE, and Vale WW (1986) Corticotropin-releasing factor binding to the anterior pituitary receptor is modulated by divalent cations and guanyl nucleotides. *Endocrinology* **118**:1171–1179.
- Perrin MH, Sutton SW, Cervini LA, Rivier JE, and Vale WW (1999) Comparison of an agonist, urocortin, and an antagonist, astressin, as radioligands for characterization of corticotropin-releasing factor receptors. *J Pharmacol Exp Ther* **288**:729–734.
- Potter E, Sutton S, Donaldson C, Chen R, Perrin M, Lewis K, Sawchenko PE, and Vale W (1994) Distribution of corticotropin-releasing factor receptor mRNA expression in the rat brain and pituitary. *Proc Natl Acad Sci USA* **91**:8777–8781.
- Primus RJ, Yevich E, Baltazar C, and Gallagher DW (1997) Autoradiographic localization of CRF1 and CRF2 binding sites in adult rat brain. *Neuropsychopharmacology* **17**:308–316.
- Refojo D, Schweizer M, Kuehne C, Ehrenberg S, Vogl AM, Dedic N, Schumacher M, von Wolff G, Avrabs C, et al. (2011) Glutamatergic and dopaminergic neurons mediate anxiogenic and anxiolytic effects of CRHR1. *Science* **333**: 1903–1907.
- Reul JM and Holsboer F (2002) Corticotropin-releasing factor receptors 1 and 2 in anxiety and depression. *Curr Opin Pharmacol* **2**:23–33.
- Reyes TM, Lewis K, Perrin MH, Kunitake KS, Vaughan J, Arias CA, Hogenesch JB, Gulyas J, Rivier J, Vale WW, et al. (2001) Urocortin II: a member of the corticotropin-releasing factor (CRF) neuropeptide family that is selectively bound by type 2 CRF receptors. *Proc Natl Acad Sci USA* **98**:2843–2848.
- Rivier J, Gulyas J, Kirby D, Low W, Perrin MH, Kunitake K, DiGrucio M, Vaughan J, Reubi JC, Waser B, et al. (2002) Potent and long-acting corticotropin releasing factor (CRF) receptor 2 selective peptide competitive antagonists. *J Med Chem* **45**: 4737–4747.
- Rivier JE and Rivier CL (2014) Corticotropin-releasing factor peptide antagonists: design, characterization and potential clinical relevance. *Front Neuroendocrinol* **35**:161–170.
- Rominger DH, Rominger CM, Fitzgerald LW, Grzanna R, Largent BL, and Zaczek R (1998) Characterization of [125I]sauvagine binding to CRH2 receptors: membrane homogenate and autoradiographic studies. *J Pharmacol Exp Ther* **286**:459–468.
- Rühmann A, Bonk I, Lin CR, Rosenfeld MG, and Spiess J (1998) Structural requirements for peptidic antagonists of the corticotropin-releasing factor receptor (CRFR): development of CRFR2beta-selective antisauvagine-30. *Proc Natl Acad Sci USA* **95**:15264–15269.
- Sánchez MM, Young LJ, Plotsky PM, and Insel TR (1999) Autoradiographic and in situ hybridization localization of corticotropin-releasing factor 1 and 2 receptors in nonhuman primate brain. *J Comp Neurol* **408**:365–377.
- Silberstein S, Vogl AM, Refojo D, Senin SA, Wurst W, Holsboer F, Deussing JM, and Arzt E (2009) Amygdaloid pERK1/2 in corticotropin-releasing hormone over-expressing mice under basal and acute stress conditions. *Neuroscience* **159**: 610–617.
- Skelton KH, Nemeroff CB, Knight DL, and Owens MJ (2000) Chronic administration of the triazolobenzodiazepine alprazolam produces opposite effects on corticotropin-releasing factor and urocortin neuronal systems. *J Neurosci* **20**:1240–1248.
- Smith GW, Aubry JM, Dellu F, Contarino A, Bilezikjian LM, Gold LH, Chen R, Marchuk Y, Hauser C, Bentley CA, et al. (1998) Corticotropin releasing factor receptor 1-deficient mice display decreased anxiety, impaired stress response, and aberrant neuroendocrine development. *Neuron* **20**:1093–1102.
- Sridharan R, Zuber J, Connelly SM, Mathew E, and Dumont ME (2014) Fluorescent approaches for understanding interactions of ligands with G protein coupled receptors. *Biochim Biophys Acta* **1838**:15–33.
- Tellew JE, Lanier M, Moorjani M, Lin E, Luo Z, Slee DH, Zhang X, Hoare SR, Grigoriadis DE, St Denis Y, et al. (2010) Discovery of NBI-77860/GSK561679, a potent corticotropin-releasing factor (CRF1) receptor antagonist with improved pharmacokinetic properties. *Bioorg Med Chem Lett* **20**:7259–7264.
- Vale W, Spiess J, Rivier C, and Rivier J (1981) Characterization of a 41-residue ovine hypothalamic peptide that stimulates secretion of corticotropin and beta-endorphin. *Science* **213**:1394–1397.
- Van Pett K, Viau V, Bittencourt JC, Chan RK, Li HY, Arias C, Prins GS, Perrin M, Vale W, and Sawchenko PE (2000) Distribution of mRNAs encoding CRF receptors in brain and pituitary of rat and mouse. *J Comp Neurol* **428**:191–212.
- Vaughan J, Donaldson C, Bittencourt J, Perrin MH, Lewis K, Sutton S, Chan R, Turnbull AV, Lovejoy D, Rivier C, et al. (1995) Urocortin, a mammalian neuropeptide related to fish urotensin I and to corticotropin-releasing factor. *Nature* **378**: 287–292.
- Waser B, Rehmann R, Rivier J, Vale W, and Reubi JC (2006) CRF receptors in the rodent and human cardiovascular systems: species differences. *Peptides* **27**: 3029–3038.
- Webster EL, Lewis DB, Torpy DJ, Zachman EK, Rice KC, and Chrousos GP (1996) In vivo and in vitro characterization of antalarmin, a nonpeptide corticotropin-releasing hormone (CRH) receptor antagonist: suppression of pituitary ACTH release and peripheral inflammation. *Endocrinology* **137**:5747–5750.
- Wigger A, Sánchez MM, Mathys KC, Ebner K, Frank E, Liu D, Kresse A, Neumann ID, Holsboer F, Plotsky PM, et al. (2004) Alterations in central neuropeptide expression, release, and receptor binding in rats bred for high anxiety: critical role of vasopressin. *Neuropsychopharmacology* **29**:1–14.
- Wisler JW, Xiao K, Thomsen AR, and Lefkowitz RJ (2014) Recent developments in biased agonism. *Curr Opin Cell Biol* **27**:18–24.
- Wootton D, Lindmark H, Kadmiel M, Willcockson H, Caron KM, Barwell J, Drmotá T, and Poyner DR (2013) Receptor activity modifying proteins (RAMPs) interact with the VPAC2 receptor and CRF1 receptors and modulate their function. *Br J Pharmacol* **168**:822–834.
- Zhou Y, Jiang Y, Wang R, Bai B, Zhou M, Chen T, Cai J, Wang L, and Shaw C (2012) PD-sauvagine: a novel sauvagine/corticotropin releasing factor analogue from the skin secretion of the Mexican giant leaf frog, *Pachymedusa dancicolor*. *Amino Acids* **43**:1147–1156.

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