Anxiolytic-Like Effects of Antisauvagine-30 in Mice Are Not Mediated by CRF$_2$ Receptors

Eric P. Zorrilla[1,4]*, Amanda J. Roberts[2], Jean E. Rivier[3], George F. Koob[1]*

1 Committee on the Neurobiology of Addictive Disorders, The Scripps Research Institute, La Jolla, California, United States of America, 2 Molecular and Integrative Neurosciences Department, The Scripps Research Institute, La Jolla, California, United States of America, 3 The Clayton Foundation Laboratories for Peptide Biology and Structural Biology Laboratory, The Salk Institute for Biological Studies, La Jolla, California, United States of America, 4 Department of Neurosciences, University of California San Diego, La Jolla, California, United States of America

Abstract

The role of brain corticotropin-releasing factor type 2 (CRF$_2$) receptors in behavioral stress responses remains controversial. Conflicting findings suggest pro-stress, anti-stress or no effects of impeding CRF$_2$ signaling. Previous studies have used antisauvagine-30 as a selective CRF$_2$ antagonist. The present study tested the hypotheses that 1) potential anxiolytic-like actions of intracerebroventricular (i.c.v.) administration of antisauvagine-30 also are present in mice lacking CRF$_2$ receptors and 2) potential anxiolytic-like effects of antisauvagine-30 are not shared by the more selective CRF$_2$ antagonist astressin$_2$-B. Cannulated, male CRF$_2$ receptor knockout ($n = 22$) and wildtype littermate mice ($n = 21$) backcrossed onto a C57BL/6J genetic background were tested in the marble burying, elevated plus-maze, and shock-induced freezing tests following pretreatment (i.c.v.) with vehicle, antisauvagine-30 or astressin$_2$-B. Antisauvagine-30 reduced shock-induced freezing equally in wildtype and CRF$_2$ knockout mice. In contrast, neither astressin$_2$-B nor CRF$_2$ genotype influenced shock-induced freezing. Neither CRF antagonist nor CRF$_2$ genotype influenced anxiety-like behavior in the plus-maze or marble burying tests. A literature review showed that the typical antisauvagine-30 concentration infused in previous intracranial studies ($\sim$1 mM) was 3 orders greater than its IC_{50} to block CRF$_1$-mediated cAMP responses and 4 orders greater than its binding constants (K_d, K_i) for CRF$_1$ receptors. Thus, increasing, previously used doses of antisauvagine-30 also exert non-CRF$_2$-mediated effects, perhaps via CRF$_1$. The results do not support the hypothesis that brain CRF$_2$ receptors tonically promote anxiogenic-like behavior. Utilization of CRF$_2$ antagonists, such as astressin$_2$-B, at doses that are more subtype-selective, can better clarify the significance of brain CRF$_2$ systems in stress-related behavior.

Competing Interests: The authors have read the journal's policy, and have the following conflicts: Drs. Zorrilla and Koob are coinventors on a patent for the composition and use of non-peptide selective CRF1 antagonists (US20100249138) and Dr. Rivier is the founder of Sentia Medical Sciences, Inc. Sentia has exclusive license from The Salk Institute to develop CRF1 and CRF2 peptide antagonists. Dr. Roberts has no potential conflicts of interest to disclose. This does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials.

* E-mail: ezorrilla@scripps.edu (EPZ); gkoob@scripps.edu (GFK)

Introduction

In mammals, the stress-related peptide corticotropin-releasing factor (CRF) and its paralogs urocortins 1, 2, and 3 (Ucn 1, Ucn 2, Ucn 3), activate two CRF receptor subtypes, CRF$_1$ and CRF$_2$, to varying degrees [1]. CRF$_1$ receptors mediate endocrine, behavioral, and autonomic responses to stress, which has spurred the development of drug-like CRF$_1$ antagonists [2]. In contrast, the role of brain CRF$_2$ receptors in stress responses remains controversial. Studies have implicated anti-stress-like actions, pro-stress-like actions, or a lack of involvement of CRF$_2$ receptors [1]. Part of this uncertainty may reflect that, unlike the case with CRF$_1$ antagonists [2], highly selective ($>$10,000-fold selectivity), small molecule CRF$_2$ antagonists remain unavailable. Researchers have instead used truncated CRF$_2$-preferring (100–1000-fold selectivity) peptide fragments as CRF$_2$ antagonists, principally [D-Phe^{11},His^{12}]sauvagine(11–40)NH$_2$ (antisauvagine-30; [3] and cyclo(31–34)[D-Phe^{11},His^{12},CαMeLeu^{13,39},Nle^{17},Glu^{31},Lys^{34}]Ac-sauvagine$_{(8–40)}$ (astressin$_2$-B; [4]).

Antisauvagine-30 has been described as a selective CRF$_2$ antagonist in the literature (1530 hits in Google Scholar as of August 2012). Antisauvagine-30 potently displaces radioiodinated CRF-related ligands from HEK293 cell membranes expressing recombinant mCRF$_{2b}$ ($K_d = 1.4$ nM; [3], hCRF$_{2a}$ ($K_i = 0.8$ nM; [5], or mCRF$_{2b}$ receptors ($K_i = 0.41$ nM; [6] and has lower affinity for HEK293 membranes expressing CRF$_1$ receptors. Several findings suggest, however, that antisauvagine-30 may block CRF$_1$ receptors at doses that have been used *in vivo*. First, antisauvagine-30 can displace [^{125}I]-oCRF from HEK293-rCRF$_1$ membranes ($Ki = 154$–166 nM; [3,6] and [^{125}I]-sauvagine from HEK293-hCRF$_1$ membranes ($Ki = 100$ nM; [7]. Similarly, antisauvagine-30 competes with [^{125}I]-astressin to bind rat and human uncoupled CRF$_1$ receptors ($Ki = 66$ and 170 nM; [7,8]. Yet, many intracerebroventricular and intracerebral studies have infused antisauvagine-30 at $\sim$4 orders greater concentrations (1–2 mM) (e.g., see Table 1). Moreover, in its original characterization, antisauvagine-30 showed $\sim$30% of the rCRF$_1$ antagonist potency of astressin [3], a potent CRF$_1$ antagonist. Accordingly, anti-

sauvagine-30 blocks oCRF-induced cAMP accumulation in HEK293-rCRF1 cells [9] and oCRF-induced cAMP responses in human retinoblastoma Y79 cells [10] with $IC_{50s} = 1-2$ µM, concentrations 3 orders lower than those that have been injected. The incomplete selectivity of antisauvagine-30 raises concern that some putative anxiolytic/anti-stress-like actions of antisauvagine-30 previously attributed to antagonism of brain CRF_2 receptors may involve a non-CRF_2 target, such as CRF_1 receptors.

Many antibodies [11] and antagonists [12] were subsequently found to have off-target binding or activity when evaluated in knockout (KO) mice. Here, we tested the hypotheses that any potential anxiolytic-like actions of antisauvagine-30 would 1) be present in mice lacking functional CRF_2 receptors, and 2) not be shared by the more selective CRF_2 antagonist astressin$_2$-B. Astressin$_2$-B binds to CRF_2 receptors in vitro with similar potency as does anti-sauvagine-30 (e.g., displacement of [^{125}I]sauvagine from CHO-hCRF_{2a} membranes ($Ki = 0.49$ vs 0.29 nM), from intrinsic rCRF_{2b} in A7r5 cells ($Ki = 0.17$ vs 0.77 nM), and from CRF_{2a} in rat olfactory bulb ($Ki = 0.50$ vs 0.84 nM) [8]. But, astressin$_2$-B shows one order less affinity for CRF_1 receptors ($Ki > 1000$ nM and 890 nM, respectively) [7,8] than does anti-sauvagine-30 ($Ki = 100$ nM) [3,6,7,8].

A secondary goal of the present study was to evaluate the anxiety-related phenotype of CRF_2 KO mice backcrossed to C57BL/6J background. Previous studies that reported an anxiogenic-like phenotype of CRF_2 knockout mice were performed on a hybrid 129SvJ-C57BL/6J genetic background [13,14]. However, mixed genetic background transgenic mice can lead to spurious or inconsistent results due to the confounding (due to genetic linkage) and interactive influence of mixed genetic background on observed phenotypes [15]. The CRF_2 null mutation was introduced into embryonic stem cells of the 129Sv genetic background. Due to genetic linkage, CRF_2 null mutant mice studied on a hybrid background will overrepresent the 129Sv genetic background as compared to wildtype mice, which will show comparatively more C57BL/6 background [15]. Anxiogenic-like behavior is greater in 129Sv strain mice than in C57BL/6 mice, however [16,17,18,19]. As a result, it is not clear whether the previously reported anxiogenic-like CRF_2 KO phenotype is actually due to the null mutation as opposed to linked 129Sv genetic material. Potentially consistent with the latter possibility, no anxiogenic-like phenotype in elevated plus-maze or open field behavior was seen in CRF_2 KO mice backcrossed 3 generations (~87.5%) to a C57BL/6J background (Coste et al., 2000). Therefore, we here revisit the anxiety-related phenotype of CRF_2 KO mice that were previously reported to show anxiogenic-like behavior on a hybrid background [13], but now studied after being backcrossed extensively (>99.975%) onto a C57BL/6J background.

Materials and Methods

Ethics Statement

Procedures adhered to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publication no. 85–23, 1996) and Principles of Laboratory Animal Care and were approved by the Institutional Animal Care and Use Committee of The Scripps Research Institute (protocol #08-0010). All surgery was performed under isoflurane anesthesia, and all efforts were made to minimize suffering.

Subjects

Subjects were adult (26.5–32.3 g at study onset), male CRF_2 receptor KO ($n = 22$; $Crhr2^{tm1Klee}/Crhr2^{tm1Klee}$; [13] and wildtype littermate mice ($n = 21$; WT, ≥12 generations C57BL/6J backcrossing; ≥99.9755869% consomy) offspring of heterozygote breeding. Mice were group-housed under a reverse 12 h/12 h light/dark cycle in a humidity- (60%) and temperature-controlled (22°C) vivarium with chow (LM-485 Diet 7012, Harlan, Madison, WI) and water available ad libitum.

Table 1. Intracerebroventricular (ICV) studies of antisauvagine-30 effects on stress- or anxiety-related endpoints.

Reference	Minimum effective ICV injection	Concentration (µM)	Dose (nmol)	Result
[14]	400 ng/0.5 µl	219	0.11	INCREASED anxiety-like behavior
[36]	1–5 µg/2 µl	140–680	~0.27–1.37	Reduced anxiety-like behavior
[37]	1–10 nmol/2.5 µl	400–4000	1–10	Reduced CRF-induced anxiety-like behavior and anorexia
[38]	2.2 nmol/2 µl	1100	2.2	Reduced stress-induced weight loss
[39]	10 µg/5 µl	550	2.7	Reduced stress-induced deficits in prepulse inhibition of startle
[40]	10 µg/2 µl	1370	2.7	Reduced burn-induced hypermetabolism
[41]	10 µg/2 µl	1370	2.7	Reduced Ucn 2-induced neuroactivation
[42]	3 nmol/5 µl	600	3	Reduced CRF-induced startle and prepulse inhibition deficits
[43]	3 nmol/5 µl	600	3	Reduced CRF-induced startle
[44]	3 nmol/5 µl	600	3	Reduced anxiety-like behavior
[45]	20 µg/5 µl	1100	5.5	Reduced CRF- and stress-induced neuroactivation
[46]	20 µg/5 µl	1100	5.5	Reduced stress-induced anorexia
[47]	20 µg/5 µl	1100	5.5	Reduced stress- and Ucn 2/Ucn 3-induced HPA-activation
[48]	20 µg/3 µl	1800	5.5	Reduced somatic and noradrenergic responses to opiate withdrawal
[49]	20 µg/3 µl	1800	5.5	Reduced acquisition of conditioned defeat
[50]	6 nmol/10 µl	600	6	Reduced des-acyl ghrelin-induced changes in gastric motor activity
[51]	Osmotic minipump	1200	30/day	Reduced CRF/Ucn 1-induced anorexia and weight loss

Note: Not only doses, but also concentrations, are listed because relative dilution of the injected concentration across a given volume of brain is what will determine the local concentration relevant to receptor pharmacodynamics. CRF = corticotropin-releasing factor, HPA = hypothalamic-pituitary-adrenal axis, Ucn = urocortin.
doi:10.1371/journal.pone.0063942.t001

Surgery

Anaesthetized (isoflurane, 1–3%) mice were stereotaxically (David Kopf, Tujunga, CA) implanted with a 27-gauge, 7.5 mm stainless steel guide cannula 1 mm above the lateral ventricle. Coordinates (in mm) were (anterior/posterior: −0.1, medial/lateral: ±1.0 from bregma, dorsal/ventral: −1.5 from skull; [20]. A 30-gauge obturator maintained patency. Mice recovered $\geq$7 days before testing. Cannula placement was inferred from successful gravity injection and from ventricular spread of injected dye in randomly tested mice.

Drugs and injection

Antisauvagine-30 and astressin$_2$-B were synthesized using solid-phase methodology, purified using HPLC and characterized using capillary zone electrophoresis, HPLC and MS [4]. Peptides were dissolved in 0.5× PBS before testing and kept on ice. For intracerebroventricular (i.c.v.) infusions, the 30-gauge injector extended 1 mm beyond the cannula and was attached to tubing (0.01 i.d., 0.03 o.d. inches) from which 2 µl solution was delivered into the ventricle by gravity over 30 sec. The injector was left in place for 60 sec. The pretreatment intervals, during which the mouse was returned to its home cage were 15 min for the marble burying test and 30 min for the plus-maze and shock-induced freezing tests.

Study design

Mice were tested during the dark phase in the marble burying, elevated plus-maze, and shock-induced freezing tests using a between-subjects design for treatment. The same set of mice were subjects in the 3 tests. Experiments involved a 2 (Genotype: WT vs. KO)×3 (Antagonist: vehicle vs. antisauvagine-30 vs. astressin$_2$-B) factorial design. The dose of antisauvagine-30 (i.c.v. ~3 nmol, or 10.7 µg) was representative of doses used in previous studies of stress- or anxiety-related endpoints (Table 1). Astressin$_2$-B was administered at the same dose. Tests were spaced by one week, and mice received a given drug treatment no more than twice across the three tests.

Marble burying

For marble burying testing [21], mice were individually placed in a polycarbonate cage (29×18×12 cm) containing 20 marbles (1.5 cm diameter) evenly spaced on 5-cm deep bedding. Marbles covered at least two-thirds by bedding, an index of anxiogenic-like behavior, were counted 30 min later.

Elevated plus-maze

The plus-maze apparatus has four arms (5×30 cm) at right angles to each other, elevated 30 cm from the floor. Two arms have 16-cm black plastic walls (closed arms), and two arms have 16-cm clear plastic walls (more open arms). Controls tested in this modified apparatus spend 35–40% of their time on the open arms, allowing changes to be detected bidirectionally; mice tested in the original plus-maze (open arms with no wall) typically spend 10–15% of their time on the open arms, making it difficult to detect anxiogenic-like effects. Mice were placed on the center of the maze, and behavior was videorecorded for 5 min. Decreases in % open arm time, calculated as: 100*open arm time/(open arm time+closed arm time) [22], indicate increased anxiety-like behavior. More total arm entries indicate increased locomotor activity [22].

Shock-induced freezing

Mice were placed in a Mouse NIR Video Fear Conditioning System (Med Associates, St. Albans, VT) housed in a sound-proofed box, allowed to habituate for 2 min and then exposed to three 1.5 mA, 1-sec footshocks, separated by 20 sec. Freezing, a CRF/CRF$_1$-dependent defensive response [23], was measured automatically from real-time video recordings (30 frames per second) across 15 min using Video Fear Conditioning Software (Med Associates) that distinguishes between subtle movements, such as whisker twitches, tail flicks and freezing behavior.

Statistics

Analysis of variance (ANOVA) was used to evaluate effects of Genotype, Antagonist and their interaction. Fisher's protected least significant difference tests identified pairwise differences. The software used was Systat 12.0 (SPSS, Chicago, IL).

Results

Figure 1 shows that antisauvagine-30 reduced the duration of shock-induced freezing in both WT and CRF$_2$ KO mice (Antagonist: $F_{2,37} = 4.17$, $p < 0.05$. Antisauvagine-30-treated mice froze less than mice pretreated with either vehicle or astressin$_2$-B (ps<0.05), which did not differ from one another ($p = 0.96$). No Genotype ($F_{1,37} = 0.03$, $p > 0.85$) or Genotype×Antagonist effects ($F_{2,37} = 0.39$, $p > 0.68$) were seen.

Table 2 shows that there were no significant Genotype, Antagonist or Genotype×Antagonist effects on raw open arm time ($F_{1,37} = 1.07$, $F_{2,37} = 1.22$, $F_{2,37} = 2.41$, all ps>0.1), % open arm time calculated as a function of total arm time ($F_{1,37} = 1.12$, $F_{2,37} = 1.16$, $F_{2,37} = 2.13$, all ps>0.1), or the total number of arm entries in the elevated plus-maze ($F_{1,37} = 0.42$, $F_{2,37} = 1.54$, $F_{2,37} = 0.15$, all ps>0.2). There also were no significant Genotype ($F_{1,37} = 0.35$, $p > 0.55$), Antagonist ($F_{2,37} = 0.12$, ps>0.89) or Genotype×Antagonist ($F_{2,37} = 1.52$, $p > 0.23$) effects on the number of marbles buried in the marble burying test. *A priori* analysis in vehicle-treated mice considered separately also indicated no significant Genotype effect on shock-induced freezing ($p > 0.15$); plus maze measures of % open arm time ($p > 0.15$), open arm time ($p > 0.14$), or total arm entries ($p > 0.72$); or marbles buried ($p > 0.24$).
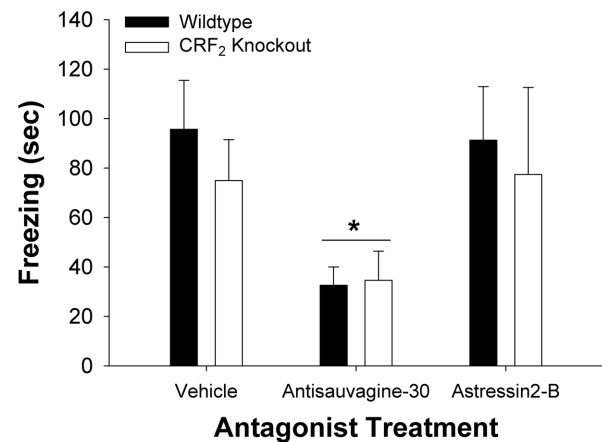

Figure 1. Effects of antisauvagine-30, astressin$_2$-B and CRF$_2$ genotype on shock-induced freezing. The data are expressed as $M \pm$ SEM. Antisauvagine-30 (i.c.v., 3 nmol) significantly and equally reduced the duration of shock-induced freezing in both wildtype and CRF$_2$ knockout mice. In contrast, the same dose of astressin$_2$B, a selective CRF$_2$ antagonist, and CRF$_2$ null genotype did not alter shock-induced freezing ($n = 6$–9/group). *$p < 0.05$, differs from vehicle and astressin$_2$-B-treated mice (Fisher's protected least significant difference test).
doi:10.1371/journal.pone.0063942.g001

Table 2. Effects of genotype and CRF antagonist on behavior in the elevated plus-maze and marble burying tests.

	Vehicle		Antisauvagine-30		Astressin$_2$-B	
	Wildtype (*n*=9)	CRF$_2$ KO (*n*=8)	Wildtype (*n*=6)	CRF$_2$ KO (*n*=7)	Wild type (*n*=6)	CRF$_2$ KO (*n*=7)
Elevated plus-maze						
Open arm time, %	41.1±12.7	18.2±8.0	27.4±9.0	9.4±2.3	25.9±8.0	41.0±11.7
Open arm time, sec	116±37	48±21	71±24	22±5	64±24	110±32
Total arm entries	10.8±2.1	11.9±2.1	15.7±2.6	15.7±2.1	11.8±2.1	14.7±3.7
Marble burying						
Marbles buried	9.9±2.7	11.2±1.8	11.5±2.8	10.0±2.4	6.5±2.8	10.7±2.1

The data are expressed as *M*+SEM. KO = knockout.
doi:10.1371/journal.pone.0063942.t002

Table 3 lists published studies in which antisauvagine-30 was administered site-specifically to discrete brain regions as a CRF$_2$ antagonist. As can be seen, the concentrations that have been infused locally range from 137–2000 µM, on the order of those given i.c.v. previously (Table 1) and in the present study. The median concentration infused, 1050 µM is ~3 orders greater than the reviewed IC$_{50}$ of antisavuagine-30 to block CRF$_1$-mediated cAMP responses (~1–2 µM) and ~4 orders greater than reviewed binding constants (K$_d$, K$_i$~0.066–0.166 µM) of antisauvagine-30 for CRF$_1$ receptors.

Discussion

The present study found that i.c.v. infusion of a dose of antisauvagine-30 intermediate to those used in the literature reduced shock-induced freezing in both wild-type and CRF$_2$ KO

mice, unlike the CRF$_2$ antagonist astressin$_2$-B, which did not mitigate shock-induced freezing in either genotype. The present study also found that neither CRF$_2$ KO nor i.c.v. astressin$_2$-B infusion produced anxiolytic-like effects in 3 tests of anxiety-like behavior. Altogether, the results indicate that increasing doses of antisauvagine-30 lose their specificity and can exert non-CRF$_2$-mediated effects at doses previously used. The collective results do not support the hypothesis that activation of brain CRF$_2$ receptors tonically promotes anxiogenic-like behavior.

Antagonism of CRF$_1$ receptors is a plausible mechanism for the non-CRF$_2$ mediated anxiolytic-like actions of antisauvagine-30 seen here on shock-induced freezing. The low-moderate CRF$_1$ binding affinities (~100 nM) of antisauvagine-30 are not shared by the other widely used CRF$_2$ antagonist, astressin$_2$-B (K$_i$>1000 nM and 890 nM, respectively; [7,8], which is similarly potent to antisauvagine-30 at binding CRF$_2$ receptors (e.g.,

Table 3. Intracerebral (IC) site-specific studies of antisauvagine-30 effects on stress- or anxiety-related endpoints.

Reference	Minimum effective intracerebral injection	Dose (pmol)	Concentration (µM)	Result
[52]	0.25 µg/0.5 µl	68.5	137	Reduced alcohol-induced increases in dynorphin levels
[53]	0.25 µg/0.5 µl	68.5	137	Reduced alcohol-induced increases in β-endorphin levels
[54]	0.4 µg/0.5 µl	110	220	Reduced stress-enhanced fear conditioning and Mek-1/2-dependent signaling
[55]	0.4 µg/0.5 µl	110	220	Reduced stress/CRF-induced anxiety-like behavior and cognitive deficits
[56]	0.4 µg/0.5 µl	110	220	Reduced stress-induced anxiety-like behavior and fear conditioning deficits
[57]	0.2 nmol/0.5 µl	200	400	Reduced acquisition of a CRF-induced conditioned place aversion
[58]	0.5 µg/0.2 µl	137	685	Reduced the expression of conditioned defeat
[59]	0.5 nmol/0.5 µl	500	1000	Reduced inescapable shock-induced shuttlebox escape deficits
[60]	55 pmol/0.05 µl	55	1100	Reduced ethanol-induced hypothermia
[61]	2 µg/0.5 µl	550	1100	Reduced isolation-induced anxiety-like behavior
[62]	2 µg/0.5 µl	550	1100	Reduced CRF-induced CeA serotonin efflux in amphetamine pre-treated rats
[63]	2 µg/0.5 µl	550	1100	Reduced heightened anxiety-like behavior in amphetamine pre-treated rats
[64]	2 µg/0.5 µl	550	1100	Reduced CRF- or CeA-activation-induced mPFC serotonin efflux
[65]	2 µg/0.5 µl	550	1100	Reduced CRF-induced increases in NAc serotonin efflux
[66]	1 nmol/0.5 µl	1000	2000	Reduced stress-induced anorexia
[67]	1 nmol/0.5 µl	1000	2000	Reduced Ucn 2-induced BLA serotonin efflux and neuroactivation.

Note: Not only doses, but also concentrations, are listed because relative dilution of the injected concentration across a given volume of brain is what will determine the local concentration relevant to receptor pharmacodynamics. Mek-1/2 = Mitogen-activated extracellular signal-regulated kinases; CeA = central nucleus of the amygdala; BLA = basolateral amygdala; mPFC = medial prefrontal cortex; NAc = nucleus accumbens; CRF = corticotropin-releasing factor; Ucn 2 = urocortin 2.
doi:10.1371/journal.pone.0063942.t003

displacement of [^{125}I]sauvagine from CHO-hCRF$_{2a}$ membranes [$K_i = 0.49$ vs. 0.29 nM], from intrinsic rCRF$_{2b}$ in A7r5 cells [$K_i = 0.17$ vs. 0.77 nM], and from CRF$_{2a}$ in rat olfactory bulb [$K_i = 0.50$ vs. 0.84 nM]; [8]. Accordingly, the i.c.v. dose of astressin$_2$-B used here, which can block anorexia induced by urocortin 3, a selective CRF$_2$ agonist [24], did not reduce shock-induced freezing. The results suggest that astressin$_2$-B is more CRF$_2$-selective than antisauvagine-30.

Many previous studies using antisauvagine-30 have interpreted that its effects were not CRF$_1$ mediated because central administration of small molecule, selective CRF$_1$ antagonists did not produce the same effects. Unfortunately, these comparisons have involved excessively lipophilic CRF$_1$ antagonists, such as NBI27914, CP-154,526, or antalarmin, which are water insoluble, precipitate upon central administration and may therefore not diffuse to target sites or be available for pharmacological activity. Better controls would involve less hydrophobic, recently developed CRF$_1$ antagonists more suitable for intracerebral administration, such as NBI-35965, GW-876008, pexacerfont or BMS-561,388.

Neither CRF$_2$ KO nor selective CRF$_2$ antagonism via astressin$_2$-B altered behavior in three anxiety models, suggesting that CRF$_2$ signaling is not a key modulator of anxiety-like behavior under basal conditions. Two previous studies that reported a basal anxiogenic-like phenotype of CRF$_2$ knockout mice were performed on a hybrid 129SvJ-C57BL/6J genetic background [13,14]. In contrast, similar to the present results in mice fully backcrossed onto a C57BL/6J background, no significant anxiety-like phenotype was seen in CRF$_2$ knockout mice backcrossed 3 generations toward a C57BL/6J background [25]. Thus, because 129Sv and C57BL/6J mice differ in anxiety-like behavior [16,17,18,19], genetic background may have interacted with the effect of CRF$_2$ null mutation on behavioral measures in previous studies [15]. However, these results should not be prematurely concluded to mean that CRF$_2$ receptors do not modulate anxiety-like behavior. Consistent with an anxiolytic-like action of CRF$_2$ activation, i.c.v. administration of type 2 urocortins, selective CRF$_2$ agonists, can produce anxiolytic-like and anti-stress-like behavioral effects [26,27,28,29,30,31, 32,33,34]. Perhaps CRF$_2$ receptors are normally quiescent under basal conditions, but are recruited in compensatory opposition to high or more sustained stress, as brought out following stressors or the anxiogenic-like 129Sv genetic background. Consistent with

this hypothesis, CRF$_2$ KO mice previously showed an anxiogenic-like phenotype in the light-dark box test following 30-min immobilization stress, but not under basal conditions (see Fig. 6A in [35]). Under this view, the stressful aspects of the 3 tests used in the present study (novelty, brief shock) may have been too brief in duration (<5 min), mild in magnitude, or initiated too soon before the behavioral assessment to allow a putative compensatory CRF$_2$ response to be observed. Finally, it cannot be ruled out that a larger sample size might have led to a statistically significant *p*-value. For example, a trend for an anxiogenic-like effect of CRF$_2$ null mutation, as reported previously [13,14], was present in vehicle-treated subjects of the elevated plus-maze that, if considered separately, would have attained significance with a sample size of 16/group (standardized Cohen's $d = -0.73$).

While antisauvagine-30 exerted non-CRF$_2$ actions at the tested dose, this does not mean that it is intrinsically non-selective. Lower *in vivo* doses or concentrations might be shown via a KO control study to be adequately selective for functional studies. Indeed, the finding that a low central dose of antisauvagine-30 (i.c.v., 400 ng) previously produced an anxiogenic-like effect, opposite to those seen with increasing doses of the antagonist (see Table 1), is consistent with the interpretation that antisauvagine-30 may lose specificity with increasing doses. The present result with a 3 nmol dose of antisauvagine-30 suggests that many (if not most) previous intracranial administration studies used a dose that can exert non-CRF$_2$ mediated effects, complicating their interpretation (Table 1). Utilization of CRF$_2$ antagonists at doses validated to be subtype-selective in knockout mice can help further clarify the biological significance of brain CRF$_2$ systems in stress-related behavior.

Acknowledgments

Author Contributions

Conceived and designed the experiments: EPZ GFK AJR. Performed the experiments: AJR. Analyzed the data: EPZ GFK AJR. Contributed reagents/materials/analysis tools: JER AJR. Wrote the paper: EPZ GFK AJR JER.

References

1. Fekete EM, Zorrilla EP (2007) Physiology, pharmacology, and therapeutic relevance of urocortins in mammals: ancient CRF paralogs. Front Neuroendocrinol 28: 1–27.
2. Zorrilla EP, Koob GF (2010) Progress in corticotropin-releasing factor-1 antagonist development. Drug Discov Today 15: 371–383.
3. Ruhmann A, Bonk I, Lin CR, Rosenfeld MG, Spiess J (1998) Structural requirements for peptidic antagonists of the corticotropin-releasing factor receptor (CRFR): development of CRFR2beta-selective antisauvagine-30. Proc Natl Acad Sci U S A 95: 15264–15269.
4. Rivier J, Gulyas J, Kirby D, Low W, Perrin MH, et al. (2002) Potent and long-acting corticotropin releasing factor (CRF) receptor 2 selective peptide competitive antagonists. J Med Chem 45: 4737–4747.
5. Higelin J, Py-Lang G, Paternoster C, Ellis GJ, Patel A, et al. (2001) 125I-Antisauvagine-30: a novel and specific high-affinity radioligand for the characterization of corticotropin-releasing factor type 2 receptors. Neuropharmacology 40: 114–122.
6. Brauns O, Brauns S, Zimmermann B, Jahn O, Spiess J (2002) Differential responsiveness of CRF receptor subtypes to N-terminal truncation of peptidic ligands. Peptides 23: 881–888.
7. Hoare SR, Sullivan SK, Schwarz DA, Ling N, Vale WW, et al. (2004) Ligand affinity for amino-terminal and juxtamembrane domains of the corticotropin releasing factor type I receptor: regulation by G-protein and nonpeptide antagonists. Biochemistry 43: 3996–4011.
8. Hoare SR, Sullivan SK, Fan J, Khongsaly K, Grigoriadis DE (2005) 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 26: 457–470.
9. Brauns O, Liepold T, Radulovic J, Spiess J (2001) Pharmacological and chemical properties of astressin, antisauvagine-30 and alpha-helCRF: significance for behavioral experiments. Neuropharmacology 41: 507–516.
10. Gutknecht E, Hauger RL, Van der Linden I, Vauquelin G, Dautzenberg FM (2008) Expression, binding, and signaling properties of CRF2(a) receptors endogenously expressed in human retinoblastoma Y79 cells: passage-dependent regulation of functional receptors. J Neurochem 104: 926–936.
11. Refojo D, Schweizer M, Kuehne C, Ehrenberg S, Thoeringer C, et al. (2011) Glutamatergic and dopaminergic neurons mediate anxiogenic and anxiolytic effects of CRHR1. Science 333: 1903–1907.
12. Della-Zuana O, Revereault L, Beck-Sickinger A, Monge A, Caignard DH, et al. (2004) A potent and selective NPY Y5 antagonist reduces food intake but not through blockade of the NPY Y5 receptor. Int J Obes Relat Metab Disord 28: 628–639.
13. Bale TL, Contarino A, Smith GW, Chan R, Gold LH, et al. (2000) Mice deficient for corticotropin-releasing hormone receptor-2 display anxiety-like behaviour and are hypersensitive to stress. Nat Genet 24: 410–414.
14. Kishimoto T, Radulovic J, Radulovic M, Lin CR, Schrick C, et al. (2000) Deletion of crhr2 reveals an anxiolytic role for corticotropin-releasing hormone receptor-2. Nat Genet 24: 415–419.
15. Gerlai R (1996) Gene-targeting studies of mammalian behavior: is it the mutation or the background genotype? Trends Neurosci 19: 177–181.

16. Contet C, Rawlins JN, Deacon RM (2001) A comparison of 129S2/SvHsd and C57BL/6JOlaHsd mice on a test battery assessing sensorimotor, affective and cognitive behaviours: implications for the study of genetically modified mice. Behav Brain Res 124: 33–46.

17. Voikar V, Koks S, Vasar E, Rauvala H (2001) Strain and gender differences in the behavior of mouse lines commonly used in transgenic studies. Physiol Behav 72: 271–281.

18. Dockstader CL, van der Kooy D (2001) Mouse strain differences in opiate reward learning are explained by differences in anxiety, not reward or learning. J Neurosci 21: 9077–9081.

19. Rodgers RJ, Boullier E, Chatzimichalaki P, Cooper GD, Shorten A (2002) Contrasting phenotypes of C57BL/6JOlaHsd, 129S2/SvHsd and 129/SvEv mice in two exploration-based tests of anxiety-related behaviour. Physiol Behav 77: 301–310.

20. Franklin K, Paxinos G (2007) The Mouse Brain in Stereotaxic Coordinates, 3rd Edition San Diego: Academic Press.

21. Njung'e K, Handley SL (1991) Evaluation of marble-burying behavior as a model of anxiety. Pharmacol Biochem Behav 38: 63–67.

22. File SE (2001) Factors controlling measures of anxiety and responses to novelty in the mouse. Behav Brain Res 125: 151–157.

23. Kalin NH, Sherman JE, Takahashi LK (1988) Antagonism of endogenous CRH systems attenuates stress-induced freezing behavior in rats. Brain Res 457: 130–135.

24. Fekete EM, Inoue K, Zhao Y, Rivier JE, Vale WW, et al. (2007) Delayed satiety-like actions and altered feeding microstructure by a selective type 2 corticotropin-releasing factor agonist in rats: intra-hypothalamic urocortin 3 administration reduces food intake by prolonging the post-meal interval. Neuropsychopharmacology 32: 1052–1068.

25. Coste SC, Kesterson RA, Heldwein KA, Stevens SL, Heard AD, et al. (2000) Abnormal adaptations to stress and impaired cardiovascular function in mice lacking corticotropin-releasing hormone receptor-2. Nat Genet 24: 403–409.

26. Valdez GR, Inoue K, Koob GF, Rivier J, Vale W, et al. (2002) Human urocortin II: mild locomotor suppressive and delayed anxiolytic-like effects of a novel corticotropin-releasing factor related peptide. Brain Res 943: 142–150.

27. Valdez GR, Zorrilla EP, Rivier J, Vale WW, Koob GF (2003) Locomotor suppressive and anxiolytic-like effects of urocortin 3, a highly selective type 2 corticotropin-releasing factor agonist. Brain Res 980: 206–212.

28. Zorrilla EP, Reinhardt LE, Valdez GR, Inoue K, Rivier JE, et al. (2004) Human urocortin 2, a corticotropin-releasing factor (CRF)2 agonist, and ovine CRF, a CRF1 agonist, differentially alter feeding and motor activity. J Pharmacol Exp Ther 310: 1027–1034.

29. Zhao Y, Valdez GR, Fekete EM, Rivier JE, Vale WW, et al. (2007) Subtype-selective corticotropin-releasing factor receptor agonists exert contrasting, but not opposite, effects on anxiety-related behavior in rats. J Pharmacol Exp Ther 323: 846–854.

30. Ohata H, Shibasaki T (2004) Effects of urocortin 2 and 3 on motor activity and food intake in rats. Peptides 25: 1703–1709.

31. Venihaki M, Sakihara S, Subramanian S, Dikkes P, Weninger SC, et al. (2004) Urocortin III, a brain neuropeptide of the corticotropin-releasing hormone family: modulation by stress and attenuation of some anxiety-like behaviours. J Neuroendocrinol 16: 411–422.

32. Telegdy G, Kadar K, Toth G (2011) Anxiolytic action of urocortin 3 fragments in mice. Behav Brain Res 222: 295–298.

33. Tanaka M, Kadar K, Toth G, Telegdy G (2011) Antidepressant-like effects of urocortin 3 fragments. Brain Res Bull 84: 414–418.

34. Tanaka M, Telegdy G (2008) Antidepressant-like effects of the CRF family peptides, urocortin 1, urocortin 2 and urocortin 3 in a modified forced swimming test in mice. Brain Res Bull 75: 509–512.

35. Henry B, Vale W, Markou A (2006) The effect of lateral septum corticotropin-releasing factor receptor 2 activation on anxiety is modulated by stress. J Neurosci 26: 9142–9152.

36. Takahashi LK, Ho SP, Livanov V, Graciani N, Arneric SP (2001) Antagonism of CRF(2) receptors produces anxiolytic behavior in animal models of anxiety. Brain Res 902: 135–142.

37. Pelleymounter MA, Joppa M, Carmouche M, Cullen MJ, Brown B, et al. (2000) Role of corticotropin-releasing factor (CRF) receptors in the anorexic syndrome induced by CRF. J Pharmacol Exp Ther 293: 799–806.

38. Miragaya JR, Harris RB (2008) Antagonism of corticotrophin-releasing factor receptors in the fourth ventricle modifies responses to mild but not restraint stress. Am J Physiol Regul Integr Comp Physiol 295: R404–416.

39. Sutherland JE, Conti LH (2011) Restraint stress-induced reduction in prepulse inhibition in Brown Norway rats: role of the CRF2 receptor. Neuropharmacology 60: 561–571.

40. Chance WT, Dayal R, Friend LA, Thomas I, Sheriff S (2007) Mediation of burn-induced hypermetabolism by CRF receptor-2 activity. Life Sci 80: 1064–1072.

41. Staub DR, Evans AK, Lowry CA (2006) Evidence supporting a role for corticotropin-releasing factor type 2 (CRF2) receptors in the regulation of subpopulations of serotonergic neurons. Brain Res 1070: 77–89.

42. Risbrough VB, Hauger RL, Roberts AL, Vale WW, Geyer MA (2004) Corticotropin-releasing factor receptors CRF1 and CRF2 exert both additive and opposing influences on defensive startle behavior. J Neurosci 24: 6545–6552.

43. Risbrough VB, Hauger RL, Pelleymounter MA, Geyer MA (2003) Role of corticotropin releasing factor (CRF) receptors 1 and 2 in CRF-potentiated acoustic startle in mice. Psychopharmacology (Berl) 170: 178–187.

44. Pelleymounter MA, Joppa M, Ling N, Foster AC (2002) Pharmacological evidence supporting a role for central corticotropin-releasing factor(2) receptors in behavioral, but not endocrine, response to environmental stress. J Pharmacol Exp Ther 302: 145–152.

45. Takahashi C, Ohata H, Shibasaki T (2011) Corticotropin-releasing factor (CRF) receptor subtypes in mediating neuronal activation of brain areas involved in responses to intracerebroventricular CRF and stress in rats. Peptides 32: 2384–2393.

46. Sekino A, Ohata H, Mano-Otagiri A, Arai K, Shibasaki T (2004) Both corticotropin-releasing factor receptor type 1 and type 2 are involved in stress-induced inhibition of food intake in rats. Psychopharmacology (Berl) 176: 30–38.

47. Maruyama H, Makino S, Noguchi T, Nishioka T, Hashimoto K (2007) Central type 2 corticotropin-releasing hormone receptor mediates hypothalamic-pituitary-adrenocortical axis activation in the rat. Neuroendocrinology 86: 1–16.

48. Navarro-Zaragoza J, Nunez C, Ruiz-Medina J, Laorden ML, Valverde O, et al. (2011) CRF(2) mediates the increased noradrenergic activity in the hypothalamic paraventricular nucleus and the negative state of morphine withdrawal in rats. Br J Pharmacol 162: 851–862.

49. Cooper MA, Huhman KL (2010) Blocking corticotropin-releasing factor-2 receptors, but not corticotropin-releasing factor-1 receptors or glucocorticoid feedback, disrupts the development of conditioned defeat. Physiol Behav 101: 527–532.

50. Chen CY, Inui A, Asakawa A, Fujino K, Kato I, et al. (2005) Des-acyl ghrelin acts by CRF type 2 receptors to disrupt fasted stomach motility in conscious rats. Gastroenterology 129: 8–25.

51. Cullen MJ, Ling N, Foster AC, Pelleymounter MA (2001) Urocortin, corticotropin releasing factor-2 receptors and energy balance. Endocrinology 142: 992–999.

52. Lam MP, Gianoulakis C (2011) Effects of corticotropin-releasing hormone receptor antagonists on the ethanol-induced increase of dynorphin A1-8 release in the rat central amygdala. Alcohol 45: 621–630.

53. Lam MP, Gianoulakis C (2011) Effects of acute ethanol on corticotropin-releasing hormone and beta-endorphin systems at the level of the rat central amygdala. Psychopharmacology (Berl) 218: 229–239.

54. Sananbenesi F, Fischer A, Schrick C, Spiess J, Radulovic J (2003) Mitogen-activated protein kinase signaling in the hippocampus and its modulation by corticotropin-releasing factor receptor 2: a possible link between stress and fear memory. J Neurosci 23: 11436–11443.

55. Radulovic J, Ruhmann A, Liepold T, Spiess J (1999) Modulation of learning and anxiety by corticotropin-releasing factor (CRF) and stress: differential roles of CRF receptors 1 and 2. J Neurosci 19: 5016–5025.

56. Todorovic C, Radulovic J, Jahn O, Radulovic M, Sherrin T, et al. (2007) Differential activation of CRF receptor subtypes removes stress-induced memory deficit and anxiety. Eur J Neurosci 25: 3385–3397.

57. Sahuque LL, Kullberg EF, McGeehan AJ, Kinder JR, Hicks MP, et al. (2006) 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) 186: 122–132.

58. Cooper MA, Huhman KL (2007) Corticotropin-releasing factor receptors in the dorsal raphe nucleus modulate social behavior in Syrian hamsters. Psychopharmacology (Berl) 194: 297–307.

59. Hammack SE, Schmid MJ, LoPresti ML, Der-Avakian A, Pellymounter MA, et al. (2003) Corticotropin releasing hormone type 2 receptors in the dorsal raphe nucleus mediate the behavioral consequences of uncontrollable stress. J Neurosci 23: 1019–1025.

60. Turek VF, Ryabinin AE (2005) Ethanol versus lipopolysaccharide-induced hypothermia: involvement of urocortin. Neuroscience 133: 1021–1028.

61. Bledsoe AC, Oliver KM, Scholl JL, Forster GL (2011) Anxiety states induced by post-weaning social isolation are mediated by CRF receptors in the dorsal raphe nucleus. Brain Res Bull 85: 117–122.

62. Scholl JL, Vuong SM, Forster GL (2010) Chronic amphetamine treatment enhances corticotropin-releasing factor-induced serotonin release in the amygdala. Eur J Pharmacol 644: 80–87.

63. Vuong SM, Oliver HA, Scholl JL, Oliver KM, Forster GL (2010) Increased anxiety-like behavior of rats during amphetamine withdrawal is reversed by CRF2 receptor activation. Behav Brain Res 208: 278–281.

64. Forster GL, Pringle RB, Mouw NJ, Vuong SM, Watt MJ, et al. (2008) Corticotropin-releasing factor in the dorsal raphe nucleus increases medial prefrontal cortical serotonin via type 2 receptors and median raphe nucleus activity. Eur J Neurosci 28: 299–310.

65. Lukkes JL, Forster GL, Renner KJ, Summers CH (2008) Corticotropin-releasing factor 1 and 2 receptors in the dorsal raphe differentially affect serotonin release in the nucleus accumbens. Eur J Pharmacol 578: 185–193.

66. Ohata H, Shibasaki T (2011) Involvement of CRF2 receptor in the brain regions in restraint-induced anorexia. Neuroreport 22: 494–498.

67. Amat J, Tamblyn JP, Paul ED, Bland ST, Amat P, et al. (2004) Microinjection of urocortin 2 into the dorsal raphe nucleus activates serotonergic neurons and increases extracellular serotonin in the basolateral amygdala. Neuroscience 129: 509–519.