Subtype-selective corticotropin-releasing factor receptor agonists exert contrasting, but not opposite, effects on anxiety-related behavior in rats

Y. Zhao; G.R. Valdez; E.M. Fekete; J.E. Rivier; W.W. Vale; K.C. Rice; F. Weiss; E.P. Zorrilla

Committee on the Neurobiology of Addictive Disorders (YZ, EMF, EPZ), Harold L. Dorris Neurological Research Institute (YZ, EMF, EPZ) and Molecular and Integrative Neurosciences Department (YZ, FW), The Scripps Research Institute, La Jolla, CA, USA
Department of Psychology, Grand Valley State University, Allendale, MI, USA (GRV)
Institute of Physiology, Pécs University Medical School, 7602 Pécs, Hungary (EMF)
Peptide Biology Laboratory, Salk Institute, La Jolla, CA, USA (JER, WWV)
Chemical Biology Research Branch, National Institute on Drug Abuse, National Institutes of Health, Bethesda, MD, USA (KCR)
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Corresponding author:

Eric P. Zorrilla. (Email: ezorrilla@scripps.edu)

Committee on the Neurobiology of Addictive Disorders, SP30-2400
The Scripps Research Institute
10550 N. Torrey Pines Road
La Jolla, CA 92037 USA
Phone: 858-784-7416
Fax: 858-784-7405

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Abstract

The corticotropin-releasing factor (CRF) system mediates stress responses. Extrahypothalamic CRF1 receptor activation has anxiogenic-like properties, but anxiety-related functions of CRF2 receptors remain unclear. The present study determined the effects of intracerebroventricular administration of a CRF2 agonist, urocortin 3, on behavior of male Wistar rats in the shock-probe, social interaction and defensive withdrawal tests of anxiety-like behavior. Equimole doses of stressin1-A, a novel, CRF1 agonist, were administered to separate rats. The effects of MJL-1-109-2, a CRF1 antagonist, on behavior in the shock-probe test also were studied. Stressin1-A increased anxiety-like behavior in the social interaction and shock-probe tests. Stressin1-A elicited behavioral activation and defensive burying at lower doses (0.04 nmol) but increased freezing, grooming, and mounting at 25-fold higher (1 nmol) doses. Conversely, systemic administration of MJL-1-109-2 (10 mg/kg) had anxiolytic-like effects in the shock-probe test. Unlike stressin1-A or MJL-1-109-2, i.c.v. urocortin 3 infusion did not alter anxiety-like behavior in the shock-probe test across a range of doses that reduced locomotion and rearing and increased grooming. Urocortin 3 also did not decrease social interaction, but decreased anxiety-like behavior in the defensive withdrawal test at a 2 nmol dose. Thus, i.c.v. administration of CRF1 and CRF2 agonists produced differential, but not opposite, effects on anxiety-like behavior. Ucn 3 (i.c.v.) did not consistently decrease or increase anxiety-like behavior, the latter unlike effects seen previously after local microinjection of CRF2 agonists into septum or raphe. With increasing CRF1 activation, however, the behavioral expression of anxiety qualitatively changes from “coping” to “non-coping” and offensive, agonistic behaviors.
Introduction

The corticotropin-releasing factor (CRF) system is a peptide family (CRF and urocortins [Ucns]) and their G-protein coupled receptors (CRF1, CRF2) implicated in stress responses. Since CRF was isolated, three mammalian prohormones that contain CRF-like sequences were identified (Ucn 1, 2 and 3). CRF is a potent CRF1 agonist, but lower affinity CRF2 agonist. Ucn 1 is a potent agonist for both subtypes. In contrast, Ucn 2 and Ucn 3 have much greater potency at membrane CRF2 than CRF1 receptors. CRF and Ucn 1 also bind the corticotropin-releasing factor binding protein (CRF-BP) and soluble CRF2(a) receptor; Ucn 2 and Ucn 3 bind these proteins with less (Ucn 2) or negligible (Ucn 3) affinity. These pharmacological and related primary structure differences led to Ucn 2 and Ucn 3 being termed “type 2 urocortins,” with a gene duplication event putatively originating the “type 1” CRF/Ucn 1 vs. “type 2” Ucn 2/Ucn 3 lineages (Fekete and Zorrilla, 2007).

CRF receptor subtypes show different anatomical distributions and ligand co-distributions, suggesting different isoform functions. Extrahypothalamic CRF1 activation is hypothesized to have arousing and anxiogenic-like properties (Zorrilla and Koob, 2004). However, anxiety-related functions of CRF2 receptors remain unclear. Several results potentially support the hypothesis that brain CRF2 receptor activation has anxiolytic-like effects, perhaps by counterregulating CRF1 action. Intracerebroventricular (i.c.v.) administration of the type 2 Ucns (Valdez et al., 2002; Valdez et al., 2003; Ohata and Shibasaki, 2004; Venihaki et al., 2004; Zorrilla et al., 2004) or low doses of the CRF2
antagonist antisauvagine-30 (Kishimoto et al., 2000) exert anxiolytic-like effects. Also, some, but not all (Coste et al., 2000), studies of CRF₂ knockout mice observed an anxiogenic-like phenotype of mice in the elevated plus-maze (Bale et al., 2000; Kishimoto et al., 2000), emergence (Kishimoto et al., 2000), open field (Bale et al., 2000), or, following stressor exposure, light/dark box tests (Henry et al., 2006, Fig. 6A). In some brain regions, CRF₂ receptor activation has electrophysiologic effects opposite to those of CRF₁ activation (Liu et al., 2004). Finally, CRF₂ knockout mice exhibit increased CRF and Ucn 1 mRNA in the central amygdala and Edinger-Westphal nucleus, respectively (Bale et al., 2000), potential evidence that CRF₂ signaling tonically decreases the threshold for these molecular stress responses (Fekete and Zorrilla, 2007).

Yet, other findings (Takahashi et al., 2001; Pellemounter et al., 2002; Risbrough et al., 2003; Pellemounter et al., 2004) support the different hypothesis that CRF₂ receptor activation, especially in the lateral septum (Henry et al., 2006) or dorsal raphe (Hammack et al., 2003), has anxiogenic-like effects, promoting defensive responses to uncontrollable stressors. In this view, the anxiogenic-like phenotype of CRF₂ null mutants is not a direct action of CRF₂ deficiency. Rather, their increased expression of anxiogenic-like molecules (CRF/Ucn 1) with affinity for their remaining CRF₁ receptors might (over)compensate for, rather than result from, CRF₂ deficiency. Also, the CRF₂ knockout phenotype might be due to altered maternal rearing by mutant dams (Bale and Vale, 2004).
The net anxiety-related effects of intraventricular (non-site-specific) administration of type 2 Ucns are also of interest because synthetic CRF2 agonists are candidate systemic therapeutics for cardiovascular disease. A limitation of studying Ucn 2 in this context is that it might produce CRF1-mediated actions at pharmacological doses; Ucn 2 is a low affinity, but direct CRF1 agonist \( EC_{50} \approx 360 \text{ nM} \) (Hoare et al., 2005) that also potently binds \( Ki = 4.4 \text{ nM} \) the rodent CRF-BP (Fekete and Zorrilla, 2007), liberating sequestered CRF/Ucn 1. Ucn 3 does not share this CRF1 agonist potential. Of physiologic relevance, Ucn 3 distributes with CRF2 receptors in the lateral septum, medial amygdala and bed nucleus of the stria terminalis (Fekete and Zorrilla, 2007).

To understand better the anxiety-related consequences of central CRF1 vs. CRF2 activation, the present study compared the effects of i.c.v. administration of selective CRF1 and CRF2 agonists. Acute i.c.v. administration of the CRF1 agonist stressin1-A (Rivier et al., 2007), a CRF analog, was hypothesized to increase anxiety-like behavior. Conversely, equimole i.c.v. doses of mouse Ucn 3 (mUcn 3), a CRF2(a) agonist \( EC_{50} = 0.073-14.2 \text{ nM} \) that lacks CRF1 activity \( EC_{50} >> 1500 \text{ nM} \) or CRF-BP affinity \( Ki > 2000 \text{ nM} \), was hypothesized to lack anxiogenic-like activity and perhaps to have anxiolytic-like effects (see Table 1 for comparison of stressin1-A, mUcn 3, and ovine CRF). CRF-related peptides were evaluated in the shock-probe, social interaction, and defensive withdrawal tests. To explore whether Ucn 3 treatment produced behavioral changes like those of a CRF1 antagonist, the effects of systemic administration of MJL-109-2, a novel selective CRF1 antagonist (Jagoda et al., 2003), were determined in the shock-probe test.
Methods:

Animals

Male Wistar rats (Charles River, Raleigh, NC; 200-225 g on arrival) were group-housed (3/cage) in a temperature- and humidity-controlled vivarium on a reversed 12-h light/dark cycle (light off, 10:00 am) with food and water available *ad libitum*. Rats were allowed to acclimate to the vivarium for one week prior to experimental procedures. Behavioral tests were conducted during the dark cycle. All procedures adhered with the *National Institutes of Health Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committee of The Scripps Research Institute.

Intracerebroventricular surgery

For intracranial peptide infusion, rats were surgically implanted with a stainless steel 22-gauge unilateral guide cannula (Plastics One Inc., Roanoke, VA). Anaesthetized (isoflurane, 2-3% in oxygen) rats were secured in a stereotaxic frame (David Kopf Instruments, Tujunga, CA) and implanted, using sterile technique, with the cannula secured 1.2 mm above the final target injection site as follows: AP:-0.6; ML:+ or - 2.0; V:-3.3 mm from the skull, with the interaural bar set at +5.0 mm (Pellegrino L, 1979). Subjects were allowed at least 7 days recovery before testing, during which time they were handled frequently. Cannula placement and tissue integrity also were functionally confirmed at the conclusion of studies by a positive dipsogenic response (>5 ml of water intake in non-deprived rats within 30 min of injection) to i.c.v. angiotensin II (5 µg/5µl).
Drugs and Injections

Murine Ucn 3 (Fekete and Zorrilla, 2007), stressin1-A (cyclo31-34[D-Phe12,MeLeu21,38,Ala22,Arg23,Glu25,39]-Ac-human corticotropin-releasing factor4-41) (Rivier et al., 2007), and angiotensin II were synthesized manually using solid phase methodology, purified using high-pressure liquid chromatography and fully characterized using capillary zone electrophoresis, high-pressure liquid chromatography, and mass spectrometry. Peptides were dissolved in 0.5X phosphate-buffered saline (pH = 7.4) immediately before testing and kept on ice. Intraventricular infusion was performed through a 28-gauge injector that extended 1.2 mm beyond the tip of the guide cannula. The injector was attached via polyethylene (PE-20) tubing to a 10-µl Hamilton microsyringe. Intraventricular treatments were manually injected (5 µl) over 1 min. Injectors were left in place for 1 min after infusion to allow diffusion.

The selective CRF1 antagonist MJL-1-109-2, or pyrazolo[1,5-a]-1,3,5-triazin-4-amine,8-[4-(bromo)-2-chlorophenyl]-N,N-bis(2-methoxyethyl)-2,7-dimethyl-(9Cl); $K_i$ for CRF1 receptors=1.9 nM, cLog$P$ = 3, an analog of DMP696, was synthesized as described previously (Jagoda et al., 2003) and administered intraperitoneally (i.p., 4 ml/kg) in 20% hydroxypropyl-β-cyclodextrin (pH=4.5).

Shock-probe test

The shock-probe test (De Boer and Koolhaas, 2003) was performed under room lighting (~300 lux) in a polycarbonate cage with wood shavings (2") along the bottom and a hole for the shock-probe 1" above the bedding. Rats were kept in the quiet, dark anteroom for
at least 2 h before the beginning of treatments. On the day prior to the test, rats were

group-acclimated to the test cage for 45 min with the probe absent. On the test day, the

probe was inserted and connected to a Coulbourn E13-01 shocker to deliver a 1.5 mA

A/C shock when contacted. Rats were individually tested in a between-subject design

following pretreatment with equimole doses of mUcn 3 or stressin1-A (0, 0.04, 0.2 or 1

nmol, i.c.v., 10 min before testing; \( n = 8-11 \)/group) or with MJL-109-2 (0, 5 or 10

mg/kg, i.p., 60 min before testing; \( n = 7-9 \)/group). The 10-min pretreatment interval was

chosen based on previous studies that observed behavioral effects of i.c.v. Ucn 3

administration (Valdez et al., 2003; Valdez et al., 2004; Venihaki et al., 2004). To test the

possibility that a longer pretreatment interval might observe other acute effects of Ucn 3

administration, additional rats (\( n = 7 \)/group) received Ucn 3 (1 nmol) or vehicle 30 min

before shock-probe testing. Upon delivery of a shock, the probe was deactivated, and

behavior of the rat was recorded for a further 10 min. Bedding was changed, and the cage

was wiped with water and dried between tests. A rater, unaware of the subject’s
treatment, scored the emission (number of bouts) and duration (time) of the following

behaviors from videotape: defensive burying, freezing, grooming, locomotion, and

rearing. Reflexive shock reactivity also was rated on the following 1 to 4 scale (Pesold

and Treit, 1992): (1) flinch of only head or forepaw; (2) whole body flinch, with or

without slow ambulation away from the probe; (3) whole body flinch and/or jumping

followed by immediate ambulation away from the probe; or (4) whole body flinch and

jump (all 4 paws in the air) followed by immediate and rapid ambulation (i.e., running)

away from the probe. Stressors or anxiogenic-like compounds have been observed to

increase defensive burying and/or freezing in this model (De Boer and Koolhaas, 2003).
Social interaction test

Conditions of the social interaction test were chosen to establish a moderate baseline level of anxiety-like behavior, thereby allowing detection of anxiogenic-like or anxiolytic-like effects, by performing the test in a brightly lit, but familiar arena, as described previously (File and Seth, 2003). Rats were familiarized to the walled test arena (a 106×92×77 cm open field made of black foamed-polyvinylchloride) by placing them individually in the illuminated (~300 lux) arena for 5-min on each of the 2 days prior to social interaction testing. Rats were single-housed for 5 days before the test day to promote social interaction (File and Seth, 2003).

Animals were kept in the quiet, dark anteroom for at least 2 h before the beginning of treatments. On test days, experimental rats were identified with a permanent marker and assigned an experimentally naïve, weight-matched, treatment-free, individually-housed (5 days) male Wistar rat for social interaction testing. Experimental rats (n = 67) were pretreated (-10 min) with equimole doses of mUcn 3 or stressin1-A (0, 0.04, 0.2, or 1 nmol, i.c.v.; n = 8-11/group) in a between-subjects design. The experimental rat and its untreated partner were then placed in opposite corners of the test arena, and the 4.5 min social interaction trial was videotaped and scored naïve to treatment condition for the following classes of behavior: affiliative social interaction (conspecific sniffing, contact, allogrooming), agonistic social interaction (mounting, sidling, attack, threat posture, flee/leave, defensive posture, submission), and non-social behaviors (locomotion, rearing, self-grooming). Only interactions initiated by the experimental rat were scored.
Anxiolytics increase affiliative social interaction, while anxiogenics decrease affiliative social interaction in this model (File and Seth, 2003).

**Defensive withdrawal test**

Defensive withdrawal testing was performed in the identical walled open field as social interaction testing, but containing a cylindrical chamber [a 2-L cylindrical Pyrex beaker (no. 1000)] wrapped in dark brown packing tape. The chamber was open at one end and located 15.0 cm from a corner of the open field, aligned lengthwise, with the open end facing the corner. On the day of testing, rats were kept in the quiet, dark anteroom for at least 2 h before the beginning of treatments. To assess the effect of Ucn 3 on defensive withdrawal behavior in an unfamiliar setting, naive rats \( n = 38 \); 9-10/group) were pretreated with mUcn 3 (0, 0.02, 0.2, or 2 nmol, i.c.v., 10 min before testing) using a between-subjects design. For testing, the animal was placed in the small withdrawal chamber facing the rear of the chamber, and behavior was recorded via a video camera for 10 min. The apparatus was wiped clean with water and dried after each subject. Rats were tested under bright lighting (~300 lux) during their dark (active) cycle. A rater, unaware of treatment condition, scored the following measures from videotape: initial latency to leave the chamber (i.e. placement of all four paws in the open field) and the number and duration of subsequent withdrawals back into the chamber. Anxiolytics and anxiogenics respectively decrease and increase the duration of time spent withdrawn in the chamber in this model (see Takahashi et al., 1990 and references within).
Statistical analysis

Effects of each compound were analyzed by separate one-way between-subjects analyses of variance (ANOVA). Linear contrast ANOVAs were used to identify monophasic dose-responsive effects, as defined by a log-linear dose response function (Rosner, 1995). For the defensive withdrawal test, data were log_{10}-transformed due to skewed and unequal variance between groups. Because of extreme values, data for the defensive burying test still did not meet statistical assumptions of ANOVA after mathematical transformation, so the single most extreme values of each group were Winsorized, yielding homogenous and normal distributions. Significant omnibus tests were followed by Newman-Keuls or Dunnett’s test, as appropriate, to evaluate pairwise group differences between treatment conditions. The statistical package used was SYSTAT 10.0 (SPSS Inc., Chicago, Illinois)
Results:

Shock probe test.

\textit{mUcn 3}. Administration (i.c.v.) of the selective CRF\textsubscript{2} agonist \textit{mUcn 3} did not significantly alter reflexive shock reactivity (\(M \pm \text{SEM}: \text{vehicle: } 1.5 \pm 0.2, \text{0.04 nmol: } 1.7 \pm 0.3, \text{0.2 nmol: } 1.3 \pm 0.2, \text{1 nmol: } 1.4 \pm 0.2\)) or the latency to initiate defensive burying or freezing in the shock-probe test (data not shown). As shown in Fig. 1A-B, \textit{mUcn 3} also did not alter the total duration of defensive burying or freezing behavior. In contrast, \textit{mUcn 3} (0.2, 1 nmol) significantly and in log-linear dose-dependent fashion increased the duration of grooming (Linear ANOVA: \(F(1,26) = 5.59, p < 0.05\)) and reduced the duration of both locomotion (\(F(3,26) = 3.66, p < 0.05\); Linear ANOVA: \(F(1,26) = 8.91, p < 0.01\)) and rearing (\(F(3,26) = 8.43, p < 0.001\); Linear ANOVA: \(F(1,26) = 24.50, p < 0.001\)) (Fig. 1C-1E).

As shown in Table 2, \textit{mUcn 3} administration suppressed locomotion and rearing by potently and dose-dependently reducing the number, rather than average duration, of bouts of these behaviors, with a minimum effective dose of 0.2 nmol (for locomotion bouts: \(F(3,26) = 3.39, p < 0.05\); Linear ANOVA: \(F(1,26)=9.43, p < 0.01\); for rearing bouts: \(F(3,26) = 5.58, p < 0.01\); Linear ANOVA: \(F(1,26) = 14.98, p < 0.001\)). The behavioral mechanism by which \textit{mUcn 3} increased grooming was less defined, because ANOVA did not show significant increases in either bout frequency or average bout duration for this behavior.
To explore the possibility that different behavioral outcomes might have resulted from longer Ucn 3 pretreatment intervals, separate rats also were tested in the shock-probe test 30 min after receiving vehicle or 1 nmol mUcn 3. Similar to the acute 10-min pretreatment interval, rats that had received Ucn 3 30 min earlier did not differ in their reflexive shock reactivity (not shown) or duration of burying and freezing behavior, but rather showed significantly less rearing behavior relative to vehicle-treated controls ($M \pm SEM$: 125.8 ± 19.7 vs. 208.1 ± 14.9 sec, $n=7$/group, $p < 0.003$), partly due to fewer bouts of rearing ($M \pm SEM$: 31.7 ± 3.1 vs. 39.4 ± 3.0, $p < 0.02$). The duration of locomotion also tended to be reduced by Ucn 3 ($M \pm SEM$: 169.4 ± 13.2 vs. 199.1 ± 13.0, $p < 0.07$). Unlike the more acute pretreatment interval, grooming was not altered following 30 min pretreatment with Ucn 3 (see also Supplementary Table 1).

$Stressin_1-A$. Administration (i.c.v.) of the selective CRF1 agonist stressin1-A did not significantly affect reflexive shock reactivity ($M \pm SEM$: vehicle: 1.8 ± 0.3, 0.04 nmol: 1.7 ± 0.2, 0.2 nmol: 1.1 ± 0.2, 1 nmol: 2.3 ± 0.4) or the latency to initiate burying or freezing (data not shown). However, unlike mUcn 3, stressin1-A differentially altered the duration of defensive burying ($F(3,41) = 3.35, p < 0.05$) and freezing behavior ($F(3,41) = 2.86, p = 0.05$) in the shock-probe test according to the administered dose (Fig. 1A-B). Linear trend contrast indicated a significant log-linear relation of stressin1-A dose to the duration of freezing behavior ($F(1,41) = 5.66, p < 0.05$). Pairwise comparisons showed that the lowest dose (0.04 nmol) of stressin1-A increased burying but not freezing behavior, whereas the highest dose (1.0 nmol) increased freezing but not burying behavior. As shown in Table 2, stressin1-A administration (0.04, 0.2 nmol) increased the
total duration of burying by significantly increasing the average duration, rather than number, of burying bouts ($F(3,39) = 3.90, p < 0.05$). Conversely, stressin$_1$-A increased the duration of freezing behavior by increasing the number, but not average duration, of freezing bouts ($F(3,39) = 3.03, p < 0.05$). Linear trend contrast analysis indicated a significant log-linear relation of stressin$_1$-A dose to the number of bouts of freezing ($F(1,39) = 5.86, p < 0.05$). Pairwise comparisons indicated significantly more bouts of freezing at the 1 nmol dose.

Stressin$_1$-A (0.2, 1 nmol) also dose-dependently increased the total duration of grooming, $F(3,41) = 3.12, p < 0.01$; Linear ANOVA: $F(1,41) = 13.08, p < 0.01$) (Fig. 1E). Stressin$_1$-A did so by potently increasing the number, but not average duration, of grooming bouts, with a minimum effective dose of 0.04 nmol ($F(3,41) = 6.98, p < 0.001$; Linear ANOVA: $F(1,41) = 19.21, p < 0.001$) (Table 2). In addition, stressin$_1$-A decreased the duration of locomotor activity significantly at the 0.04 nmol dose, but not higher doses, ($F(3,41) = 4.34, p < 0.01$), by decreasing the number, but not average duration, of bouts of locomotion ($F(3,41) = 3.43, p < 0.05$) (Fig. 1C and Table 1). Stressin$_1$-A did not significantly affect rearing behavior (Fig. 1D and Table 2).

**MJL-1-109-2.** Systemic administration of the nonpeptide selective CRF$_1$ antagonist MJL-1-109-2 did not affect reflexive shock reactivity ($M \pm$ SEM: vehicle: 1.8 ± 0.2, 5 mg/kg: 1.7 ± 0.3, and 10 mg/kg: 1.3 ± 0.2) or freezing behavior (Fig. 2B). However, MJL-1-109-2 (10 mg/kg) pretreatment significantly increased the latency to initiate defensive burying ($M \pm$ SEM: vehicle: 166.9 ± 58.3, 5 mg/kg: 155.5 ± 38.0, and 10 mg/kg: 443.3 ± 85.3;
MJL-1-109-2 did not alter the total duration of locomotor activity, rearing, or grooming (Fig. 2C-E). However, whereas the total duration of these behaviors was unaffected, MJL-1-109-2 (10 mg/kg) did significantly reduce the number of bouts of locomotion, rearing, and grooming ($F(2,22) = 4.60$, 10.69, and 4.00, all $p < 0.05$), while reciprocally increasing their bout duration, significantly so for locomotor activity ($F(2,22) = 4.74$, $p < 0.05$). Thus, MJL-1-109-2 pretreatment led to fewer, but more sustained, bouts of these active behaviors (see Table 3).

Social interaction.

mUcn 3. Administration (i.c.v.) of the selective CRF2 agonist mUcn 3 did not significantly alter the duration of affiliative (Fig. 3A, left), non-social (Fig. 3B, left) or agonistic behaviors (which totaled <39.2 sec for all doses, data not shown), in the social interaction test.

Stressin1-A. Unlike mUcn 3, i.c.v. administration of stressin1-A decreased the duration of affiliative social behavior ($F(3,28) = 4.28$, $p < 0.01$) in log-linear, dose-dependent fashion ($F(1,28) = 8.24$, $p < 0.01$). Pairwise comparisons showed significantly reduced affiliative
interaction at the 0.2 and 1 nmol doses (Fig. 3A, right). Conversely, stressin1-A significantly increased the duration of non-social behavior ($F(3,28) = 5.40, p < 0.01$), again in log-linear dose-dependent fashion ($F(1,24)=8.34, p < 0.01$), with pairwise comparisons revealing more non-social behavior in rats treated with each dose of stressin1-A (Fig. 3B, right). The decrease of affiliative social behavior was due to a potent (minimum effective dose=0.04 nmol) reduction in social sniffing ($M \pm SEM$: vehicle 73.3 ± 7.3; 0.04 nmol 43.9 ± 6.8; 0.2 nmol 39.7 ± 3.7; 1 nmol 44.7 ± 6.8), whereas the increase in non-social behavior reflected potent (minimum effective dose=0.04 nmol) increases in locomotor activity ($M \pm SEM$: vehicle 134.3 ± 14.8; 0.04 nmol 174.9 ± 15.2; 0.2 nmol 183.2 ± 3.7; 1 nmol 177.7 ± 10.1). Higher doses of stressin1-A (0.2, 1 nmol) significantly increased the likelihood that a rat exhibited mounting behavior. Whereas only 4% (2/50) of rats treated with vehicle, Ucn 3 or the low dose (0.04 nmol) of stressin1-A attempted to mount their partner, more than one-third of rats treated with higher doses of stressin1-A did so (5/14, or 36%; 29% and 43% at the 0.2 and 1 nmol doses, respectively) (Fisher’s exact test $p = 0.004$).

**Defensive withdrawal.**

Administration (i.c.v.) of mUcn 3 (2 nmol) significantly decreased the total time rats spent withdrawn in the defensive withdrawal chamber ($F(3,26) = 3.82, p < 0.05$) (Fig. 4B). As shown in Fig. 4A-B, mUcn 3 did not reliably alter the latency to emerge from the chamber, but rather, decreased the total duration of subsequent withdrawals back into the enclosure ($F(3,26) = 4.30, p < 0.01$), significantly so at the 2 nmol dose.
Discussion:

The present studies indicate that brain CRF₁ and CRF₂ receptors differentially affect anxiety-like behavior, with the behavioral expression of anxiety qualitatively changing with increasing CRF₁ activation. In the shock-probe test, 40 pmol of stressinin₁-A, a selective CRF₁ agonist, increased defensive burying, whereas 5-to-25-fold higher doses increased freezing and self-grooming. In the social interaction test, 40 pmol of stressinin₁-A decreased social sniffing and increased locomotion, whereas higher doses also increased the frequency of conspecific mounting. The results resemble findings that i.c.v. CRF increases locomotor activation under low-stress conditions, but facilitates freezing and aggression (Tazi et al., 1987; Zorrilla and Koob, 2004) under high-stress conditions. Similarly, CRF increases defensive burying in acclimated rats in the shock-probe test, but decreases burying and increases freezing and grooming in unhandled rats (Diamant et al., 1992). Here, increasing doses of a CRF₁ agonist qualitatively shifted behavior from activation and “active coping” to “non-coping” and even aggressive, agonistic behaviors.

Ucn 3, CRF₂ receptors and anxiety-like behavior

In addition to characterizing effects of a novel CRF₁ agonist, the present study sought to examine the net anxiety-related effects of brain CRF₂ receptor activation. Intracerebroventricular administration of up to 1 nmol of the selective CRF₂ agonist mUcn 3 did not influence anxiety-like behavior in the social interaction or shock-probe tests. A slightly higher dose of Ucn 3 (2 nmol) reduced defensive withdrawal behavior. Ucn 3 also dose-dependently (0.04-1 nmol) decreased locomotion and rearing behavior in the shock-probe test. However, Ucn 3 did not reduce locomotion in the social interaction
test, suggesting that Ucn 3 does not non-specifically alter motor function. Still, suppression of motor activity by Ucn 3 may complicate interpretation of anxiety-like behavior – for example, if Ucn 3 suppressed active behaviors, including burying, during shock-probe testing. Against this hypothesis, Ucn 3 increased self-grooming following a 10-min (but not 30-min) pretreatment interval, a “transition” behavior seen in response to diverse stimuli. Altogether, the results do not support the hypothesis that i.c.v. Ucn 3 potently increases anxiety-like behavior. This conclusion is consistent with previous studies which observed either no effect (Valdez et al., 2004) or decreased anxiety-like behavior of mice and of rats 5-10 min after i.c.v. Ucn 3 pretreatment (Valdez et al., 2003; Venihaki et al., 2004).

This conclusion seems at odds with reports from independent groups that i.c.v. administration of Ucn 2, another CRF₂ agonist, increased acoustic startle reactivity (Risbrough et al., 2003; Risbrough et al., 2004) and plus-maze anxiety-like behavior (Pelleymounter et al., 2002; Pelleymounter et al., 2004). Differences between those studies and the present include the species (rat vs. mouse), pharmacological properties of the injected agonist (Ucn 3 vs. Ucn 2), pretreatment intervals (10-30 min vs. 30 min-2 hr), and specific tests of anxiety-like behavior used. Interestingly, Risbrough and colleagues (2004) concluded that effects of Ucn 2 on startle reactivity partly involved CRF₁ receptors, at which Ucn 3, unlike Ucn 2, lacks any activity. In the same studies, Ucn 3 (i.c.v.), unlike Ucn 2, did not affect plus-maze behavior (up to 3 nmol) (Pelleymounter et al., 2004) or acoustic startle reactivity (up to 2.4 nmol) (Risbrough et al., 2004). In other studies, i.c.v. Ucn 2 did not increase anxiety-like behavior of mice
(1.2 nmol) in the open field, novel object, or light/dark box tests (Henry et al., 2006) or of rats (2.4 nmol) in the plus-maze (Valdez et al., 2002). Altogether, the findings do not strongly support the hypothesis that the singular activation of CRF<sub>2</sub> receptors by intraventricular injection of selective CRF<sub>2</sub> agonists acutely increases anxiety-like behavior.

An important distinction here is that that CRF<sub>2</sub> activation within the dorsal raphe (Hammack et al., 2003) and lateral septum (Henry et al., 2006) reportedly exerts anxiogenic-like effects. Accordingly, intra-raphe/septal Ucn 3 infusion may very well elicit anxiety-like behavior; CRF<sub>2</sub> receptors in those brain regions also may mediate physiological anxiety-like responses. However, the present studies explored the net anxiety-related effects of intraventricular Ucn 3 administration, which also may activate CRF<sub>2</sub> receptors in other brain sites (e.g., amygdala) that potentially produce countervailing (Liu et al., 2004), anxiolytic-like effects. Similar to the present findings that i.c.v. CRF<sub>2</sub> agonist administration did not increase anxiety-like behavior, CRF<sub>2</sub> knockout mice do not exhibit an anxiolytic-like phenotype (Bale et al., 2000; Coste et al., 2000; Kishimoto et al., 2000; Henry et al., 2006).

**Stressin<sub>1</sub>-A, CRF<sub>1</sub> receptors and agonistic behavior**

Stressin<sub>1</sub>-A increased mounting behavior in a neutral arena, a context in which mounting is normally rare (McGinnis et al., 2002). Same-sex mounting by an adult male rat is a testosterone-enhanced, threat/attack-associated behavior, commonly interpreted as an “offensive” act of territorial dominance. Tail pinch or being tested with a male
undergoing tail pinch also increases mount frequency in neutral environments. Thus, it has been proposed that arousing stimuli increase the incentive salience of or sensitivity to potential social threats (McGinnis et al., 2002). Perhaps CRF$_1$ receptors mediate the ability of arousing stimuli to facilitate offensive agonistic responses to mildly threatening or ambiguous social stimuli. Supporting this hypothesis, antalarmin, a CRF$_1$ antagonist, reduced aggressive behavior in male rhesus macaques placed in a novel cage adjoining an unfamiliar male (Habib et al., 2000). A CRF$_1$ antagonist (SSR125543A) also decreased dominance/offensive behavior in Syrian hamsters exposed to smaller “intruders” (Farrokhi et al., 2004).

Yet, some data suggest that CRF receptors facilitate submissive/defensive agonistic behavior. Infusion of D-Phe-CRF$_{12-41}$ into the lateral ventricle or extended amygdala (Jasnow et al., 2004) reduced “submissive/defensive behavior” in previously defeated Syrian hamsters encountering an unfamiliar male. Intra-amygdala antalarmin similarly reduced “defensive posturing” in defeated mice exposed to an unfamiliar male (Robison et al., 2004). CRF receptor agonists (i.c.v.) increased defensive behavior and reduced attacks in isolated DBA/2 mice facing a male intruder (Mele et al., 1987).

Several explanations might reconcile the observations. Perhaps CRF$_1$ activation increases the evaluation or salience of social threat, but context or experience guides the specific behavioral response. Relatively innocuous social stimuli would be perceived as surmountable threats (facilitating offensive aggression), known perils would be avoided more readily (facilitating submissive behavior/defensive posturing), and unavoidable,
injurious stimuli would be treated as matters of life and death (facilitating defensive fighting). Accordingly, shock-induced defensive fighting is reduced by CRF antagonists (Tazi et al., 1987). An alternative explanation is that inter-male social conflict behavior falls on a continuum from offensive aggression (at one extreme) through submission/defensive posturing to defensive aggression (at the other extreme), with arousal/anxiety levels influencing the behavior exhibited along this dimension. Accordingly, intra-amygdala CRF increased offensive aggression at lower (10, 50 pmol), but not higher (150 pmol) doses (Elkabir et al., 1990), and antalarmin reduced aggression in rhesus monkeys in relation to its anxiolytic-like activity (Habib et al., 2000). Thus, rather than directly subserving agonistic behavior, perhaps CRF₁ receptors indirectly influence social conflict behavior by modulating the evaluation of social threat or the general level of arousal or anxiety.

Unlike stressin₁-A, Ucn 3 (i.c.v.) did not influence inter-male social interaction, consistent with normal inter-male aggression of CRF₂ knockout mice (Gammie et al., 2005). The results suggest that CRF and stressors reduce social interaction via CRF₁ activation. Indeed, CRF₁ antagonists reverse reductions in social interaction due to ethanol withdrawal, maternal separation, and restraint stress (Zorrilla and Koob, 2004; Gehlert et al., 2005).

Whether CRF₂ receptor simulation alters behavior when CRF₁ receptors are concurrently activated was not addressed in the current studies. CRF₂ stimulation has been proposed by some to elicit anti-CRF/CRF₁-like effects on behavioral outcomes (Valdez et al.,
2002; Ohata and Shibasaki, 2004; Venihaki et al., 2004). Accordingly, functional antagonism between CRF$_1$/CRF$_2$ subtypes was observed in electrophysiological studies of septum and amygdala (Liu et al., 2004). On the other hand, lateral septal CRF$_2$ stimulation produces anxiogenic-like effects in the presence of stressors via a CRF$_1$-dependent mechanism (Henry et al., 2006)(also see Risbrough et al., 2004). The results suggest complexity in the integrated output of these receptor systems that may be brain site- and experience-dependent.

In summary, i.c.v. Ucn 3 increased grooming and reduced locomotion and rearing in the shock-probe test, similar to reported locomotor-suppressing effects of i.c.v. Ucn 2 and Ucn 3 (Valdez et al., 2002; Valdez et al., 2003; Ohata and Shibasaki, 2004; Zorrilla et al., 2004; Henry et al., 2006). At a higher dose, i.c.v. Ucn 3 reduced defensive withdrawal, similar to reported anxiolytic-like effects of Ucn 3 (Valdez et al., 2003; Venihaki et al., 2004), but anxiolytic-like activity was not observed in the social interaction or shock-probe tests up to a 1 nmol dose. Unlike Ucn 3, i.c.v. stressin$_1$-A increased behavioral arousal and “active coping” behaviors at lower doses, while increasing “non-coping” and offensive agonistic behavior at higher doses. MJL-1-109-2, a CRF$_1$ antagonist, reduced anxiety-like behavior in the shock-probe test with a behavioral profile unlike that of Ucn 3. Thus, the behavioral expression of anxiety changes with greater levels of brain CRF$_1$ receptor activation, and stimulation of CRF$_2$ receptors by i.c.v. Ucn 3 does not mimic effects of either a CRF$_1$ agonist or antagonist. The results indicate differential, but not opposing, actions of brain CRF$_1$ and CRF$_2$ receptors on behavioral activation and anxiety-like behavior.
Acknowledgements:

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References:


Footnotes

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Legends for Figures:

Figure 1. Effects of i.c.v. pretreatment (-10 min) with mouse Ucn 3 (mUcn 3) and stressin$_{1}$-A on the ($M \pm$ SEM) total duration of (Panel A) burying, (B) freezing, (C) locomotor, (D) rearing, or (E) grooming behavior in adult male Wistar rats ($n = 8$ / group) tested in a between-subject design in the 10-min shock probe test. Symbols indicate an overall linear effect of Dose, $p < 0.05$, $p < 0.01$; or significant differences from vehicle treatment * $p < 0.05$, ** $p < 0.01$; or the indicated treatment condition, # $p < 0.05$, ## $p < 0.01$ (Newman-Keuls test).

Figure 2. Effects of i.p. pretreatment (-60 min) with MJL-1-109-2 on the ($M \pm$ SEM) total duration of (Panel A) burying, (B) freezing, (C) locomotor, (D) rearing, or (E) grooming behavior in adult male Wistar rats ($n = 7-9$) tested in a between-subject design in the 10-min shock probe test. Symbols indicate significant differences from vehicle treatment * $p < 0.05$, ** $p < 0.01$ (Dunnett’s test).

Figure 3. Effects of i.c.v. pretreatment (-10 min) with mouse Ucn 3 (mUcn 3; $n=8-9$) and stressin$_{1}$-A ($n=6-11$) on the ($M \pm$ SEM) total duration of (Panel A) affiliative social behavior or (B) non-social behavior in adult male Wistar rats tested in a between-subject design with an unfamiliar male conspecific in the 4.5 min social interaction test. Symbols indicate an overall linear effect of Dose, $p < 0.05$, $p < 0.01$; or significant differences from vehicle treatment * $p < 0.05$, ** $p < 0.01$ (Dunnett’s test).
**Figure 4.** Effects of i.c.v. pretreatment (-10 min) with mouse Ucn 3 (mUcn 3) on the (M ± SEM) (Panel A) initial exit latency and (B) subsequent withdrawal duration of adult male Wistar rats (n = 9-10 / group) tested in a between-subject design in the 10-min defensive withdrawal test. Please note logarithmic y-axis scale. Symbol indicates a significant difference from vehicle treatment *p < 0.05 (Dunnett’s test).
**Table 1.** Primary structure and activity of selected CRF-related peptides

### A. Amino acid residue sequence:

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<thead>
<tr>
<th></th>
<th>1</th>
<th>5</th>
<th>10</th>
<th>15</th>
<th>20</th>
<th>25</th>
<th>30</th>
<th>35</th>
<th>40</th>
</tr>
</thead>
<tbody>
<tr>
<td>mUcn 3</td>
<td>FTP</td>
<td>LDSL</td>
<td>DVPT</td>
<td>NI</td>
<td>MNI</td>
<td>LFN</td>
<td>KD</td>
<td>AKN</td>
<td>LR</td>
</tr>
<tr>
<td>oCRF</td>
<td>SQE</td>
<td>PPI</td>
<td>SLDL</td>
<td>TF</td>
<td>HHLE</td>
<td>REV</td>
<td>LE</td>
<td>MT</td>
<td>KAD</td>
</tr>
<tr>
<td>Stressin1-A</td>
<td>Ac-</td>
<td>PPI</td>
<td>SLDL</td>
<td>T1</td>
<td>HHLE</td>
<td>REV</td>
<td>LE</td>
<td>MT</td>
<td>KAD</td>
</tr>
</tbody>
</table>

Note: Numbers indicate amino acid residue position. Boxed regions indicate sequence identity. mUcn 3 = mouse Ucn 3, oCRF = ovine CRF. Abbreviations follow IUPHAR nomenclature, Ac=acetylation. Underlined residues indicate cyclic lactam bridge constraint, 1: f=D-Phe, 2: X=norleucine

### B. Potency of binding affinity and activity at CRF₁ and CRF₂ receptors

<table>
<thead>
<tr>
<th></th>
<th>CRF₁</th>
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<th>CRF₂</th>
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<td>$EC_{50}$ (nM)</td>
<td>$K_i$ (nM)</td>
<td>$EC_{50}$ (nM)</td>
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<tr>
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<td>(binding)</td>
<td>(cAMP)</td>
<td>(binding)</td>
<td>(cAMP)</td>
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<td>&gt;1000 a</td>
<td>5.0 b</td>
<td>0.073 b</td>
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<td>2.2 c</td>
<td>0.42 c</td>
<td>16 d</td>
<td>130 d</td>
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<td>Stressin1-A</td>
<td>1.7 e</td>
<td>ND 1</td>
<td>222 c</td>
<td>ND 1</td>
</tr>
</tbody>
</table>

$K_i$: equilibrium dissociation constant of the unlabeled ligand for the receptor. $K_i$ of oCRF for CRF₁ receptors was calculated per the peptide’s ability to displace $^{125}$I-Tyr⁰-oCRF; other $K_i$ values were calculated per the peptide’s ability to displace $^{125}$I-Tyr⁰-sauvagine. $EC_{50}$: The concentration of the ligand that resulted in 50% of the ligand’s maximal functional response, with all activity potency ($EC_{50}$) estimates based on cAMP accumulation assays. mUcn 3 = mouse Ucn 3, oCRF = ovine CRF. a Data from murine CRF₁ receptors.
expressed in COS-7 cells (Venihaki et al., 2004). Data from rat CRF$_2(a)$ receptors expressed in CHO cells (Lewis et al., 2001). Data from rat CRF$_1$ receptors expressed in HEK cells (Ruhmann et al., 1999). $K_i$ from rat olfactory bulb (CRF$_2(a)$ receptors); $EC_{50}$ from rat CRF$_2(b)$ receptors expressed in A7r5 cells (Hoare et al., 2005). Data from human CRF$_1$ receptors and mouse CRF$_2(b)$ receptors expressed in CHO cells (Rivier et al., 2007). Although cAMP assays have not yet been performed for stressin$_1$-A, it is similarly potent to oCRF in releasing ACTH from dispersed anterior pituitary cells (a CRF$_1$-mediated endpoint) and does not reduce gastric emptying (i.p., 10 µg/kg) or arterial blood pressure (i.v., 10 µg/kg) in vivo (CRF$_2$-mediated endpoints) at a dose that stimulates fecal output and diarrhea (CRF$_1$-mediated endpoints) (Rivier et al., 2007).
Table 2. Effects of i.c.v. administration of urocortin 3 or stressin1-A on bout structure of selected behaviors in the shock-probe test

<table>
<thead>
<tr>
<th>Peptide dose (nmol)</th>
<th>Behavior</th>
<th></th>
<th></th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Number of bouts</td>
<td>Burying</td>
<td>Freezing</td>
<td>Grooming</td>
<td>Locomotion</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mUcn 3</td>
<td>Vehicle</td>
<td>7.8 ± 1.7</td>
<td>3.4 ± 0.8</td>
<td>3.3 ± 0.8</td>
<td>60.4 ± 3.0</td>
</tr>
<tr>
<td></td>
<td>0.04</td>
<td>7.4 ± 1.5</td>
<td>2.3 ± 0.6</td>
<td>2.7 ± 0.6</td>
<td>56.3 ± 2.1</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>8.1 ± 2.3</td>
<td>4.5 ± 1.0</td>
<td>5.1 ± 0.8</td>
<td>48.9 ± 2.3</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>5.0 ± 2.4</td>
<td>1.4 ± 0.5</td>
<td>3.7 ± 0.6</td>
<td>47.4 ± 5.6</td>
</tr>
<tr>
<td>Stressin1-A</td>
<td>Vehicle</td>
<td>7.5 ± 2.1</td>
<td>3.6 ± 0.9</td>
<td>1.6 ± 0.5</td>
<td>57.2 ± 3.9</td>
</tr>
<tr>
<td></td>
<td>0.04</td>
<td>13.9 ± 3.2</td>
<td>4.7 ± 1.2</td>
<td>4.0 ± 0.7</td>
<td><strong>44.3 ± 3.4</strong></td>
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<tr>
<td></td>
<td>0.2</td>
<td>6.9 ± 2.3</td>
<td>4.3 ± 0.7</td>
<td>4.2 ± 0.7</td>
<td>51.6 ± 3.5</td>
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<tr>
<td></td>
<td>1.0</td>
<td>5.6 ± 1.5</td>
<td><strong>7.1 ± 1.5</strong></td>
<td>6.9 ± 1.2</td>
<td><strong>53.9 ± 3.0</strong></td>
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</table>

Duration per bout (sec)

<table>
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<tr>
<th>Peptide dose (nmol)</th>
<th>Behavior</th>
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<th></th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mUcn 3</td>
<td>Vehicle</td>
<td>4.0 ± 0.74</td>
<td>0.9 ± 0.11</td>
<td>10.8 ± 3.94</td>
<td>3.2 ± 0.22</td>
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<tr>
<td></td>
<td>0.04</td>
<td>4.2 ± 1.0</td>
<td>1.5 ± 0.22</td>
<td>20.8 ± 3.94</td>
<td>3.6 ± 0.18</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>5.2 ± 0.83</td>
<td>4.5 ± 1.0</td>
<td>11.0 ± 1.90</td>
<td>3.5 ± 0.27</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>3.9 ± 1.39</td>
<td>1.4 ± 0.31</td>
<td>18.1 ± 3.20</td>
<td>3.61 ± 0.34</td>
</tr>
<tr>
<td>Stressin1-A</td>
<td>Vehicle</td>
<td>3.0 ± 0.38</td>
<td>1.5 ± 0.13</td>
<td>19.5 ± 7.90</td>
<td>3.5 ± 0.27</td>
</tr>
</tbody>
</table>
Effects of i.c.v. pretreatment (-10 min) with mouse Ucn 3 (mUcn 3) or stressin1-A on the number (top) and average duration (bottom) of bouts of selected behaviors in adult male Wistar rats (n = 8 / group) tested in a between-subjects design in the 10-min shock probe test. Symbols indicate significant differences from vehicle treatment * p < 0.05, ** p < 0.01 (Dunnett’s test).

<table>
<thead>
<tr>
<th>Dose (μg)</th>
<th>Number (M ± SEM)</th>
<th>Duration (M ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.04</td>
<td>6.1 ± 0.81*</td>
<td>11.7 ± 2.80</td>
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<tr>
<td>0.2</td>
<td>5.4 ± 0.98*</td>
<td>17.6 ± 4.20</td>
</tr>
<tr>
<td>1.0</td>
<td>4.1 ± 0.55</td>
<td>16.1 ± 3.60</td>
</tr>
</tbody>
</table>
Table 3. Effects of i.p. administration of MJL-1-109-2 on bout structure of selected behaviors in the shock probe test.

<table>
<thead>
<tr>
<th>MJL-1-109-2 dose (mg/kg)</th>
<th>Burying</th>
<th>Freezing</th>
<th>Grooming</th>
<th>Locomotion</th>
<th>Rearing</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Number of bouts</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>21.3 ± 4.9</td>
<td>14.8 ± 3.2</td>
<td>3.9 ± 0.8</td>
<td>61.6 ± 3.4</td>
<td>41.4 ± 2.9</td>
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<tr>
<td>5</td>
<td>14.0 ± 4.1</td>
<td>9.3 ± 3.0</td>
<td>2.6 ± 0.6</td>
<td>64.1 ± 2.7</td>
<td>43.0 ± 2.2</td>
</tr>
<tr>
<td>10</td>
<td>3.6 ± 2.9**</td>
<td>11.1 ± 4.5</td>
<td>1.1 ± 0.6**</td>
<td>43.3 ± 8.9*</td>
<td>23.9 ± 4.4**</td>
</tr>
<tr>
<td><strong>Duration per bout (sec)</strong></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>4.7 ± 0.8</td>
<td>1.8 ± 0.4</td>
<td>6.1 ± 1.3</td>
<td>3.2 ± 0.3</td>
<td>4.5 ± 0.4</td>
</tr>
<tr>
<td>5</td>
<td>4.3 ± 0.7</td>
<td>1.9 ± 0.4</td>
<td>10.7 ± 3.7</td>
<td>2.8 ± 0.2</td>
<td>4.3 ± 0.2</td>
</tr>
<tr>
<td>10</td>
<td>2.6 ± 1.1</td>
<td>3.1 ± 0.9</td>
<td>12.8 ± 1.8</td>
<td>6.6 ± 1.9*</td>
<td>5.5 ± 1.5</td>
</tr>
</tbody>
</table>

Note: Effects of i.p. pretreatment (-60 min) with MJL-1-109-2 on the (M ± SEM) number (top) and average duration (bottom) of bouts of selected behaviors in adult male Wistar rats (n = 7-9) tested in a between-subjects design in the 10-min shock probe test. Symbols indicate significant differences from vehicle treatment * p < 0.05, ** p < 0.01 (Dunnett’s test).
Figure 1
Figure 2

(A) Burying Duration (sec) for MJL-1-109-2 with three treatment groups: 0 mg/kg (open bars), 5 mg/kg (stippled bars), and 10 mg/kg (solid bars).

(B) Freezing Duration (sec) for MJL-1-109-2.

(C) Locomotor Duration (sec) for MJL-1-109-2.

(D) Rearing Duration (sec) for MJL-1-109-2.

(E) Grooming Duration (sec) for MJL-1-109-2.
Figure 3

A

Affiliative Social Behavior (sec)

- 0 nmol
- 0.04 nmol
- 0.2 nmol
- 1 nmol

B

Non-social Behavior (sec)

- mUcn 3 (CRF₂)
- Stressin₁-A (CRF₁)

** and $$$ indicate statistical significance.