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## Time-dependent induction of anxiogenic-like effects after central infusion of urocortin or corticotropin-releasing factor in the rat

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**Abstract** *Rationale:* Corticotropin-releasing factor (CRF) and urocortin (Ucn) belong to the CRF-related family, share a high degree of structural homology and bind to CRF receptors. However, compared with CRF, Ucn was shown to display either weaker or similar anxiogenic-like effects in vivo. *Objective:* To compare the anxiogenic-like responses of rats injected intracerebroventricularly (ICV) with different doses of either rat/human CRF (r/hCRF) or rat Ucn (rUcn) at different intervals after in-

jection. *Methods:* Rats were tested on three validated paradigms of emotional behavior [i.e. elevated plus-maze (EPM), defensive withdrawal (DW) and conflict test (CT)] 5 and 30 min after treatment. *Results:* In the EPM test only r/hCRF, but not rUcn, produced anxiogenic-like effects at the dose of 1.0 µg, when the peptides were injected 5 min before testing. At 30 min after injection, both peptides caused a significant reduction of open arms exploration, rUcn being effective at 0.01 µg. In the DW test both peptides were equally potent in decreasing the exploratory behavior and increasing the time spent in the chamber at the dose of 1.0 µg when tested 30 min after injection. In the CT both rUcn (0.25–1.0 µg) and r/hCRF (0.75–1.0 µg) decreased significantly the responding in the punished component. However, rUcn reduced food responding also in the unpunished component possibly due to its powerful anorectic activity. *Conclusions:* Comparison of anxiogenic-like activities of r/hCRF and rUcn at doses up to 1.0 µg revealed striking differential effects that depended on the time of testing after ICV peptide injection, and on the paradigm of anxiety used. These results suggest that the onset of r/hCRF and rUcn actions related to behavioral responses to anxiety is likely to depend on brain peptide-specific mechanisms including binding properties to CRF-receptors, differential distribution to specific functional brain sites and the distribution and effectiveness of binding-protein interactions.

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Elevated plus-maze · Defensive withdrawal · Conflict test

### Introduction

Corticotropin-releasing factor (CRF) is a 41 amino acid straight chain neuropeptide first isolated from ovine hypothalamus (Vale et al. 1981). CRF is known to be involved in behavioral responses to stress (Sutton et al.

1982; Dunn and Berridge 1990; Owens and Nemeroff 1991; Koob et al. 1994). Central injections of CRF produce a dose-dependent behavioral activation, including behavioral responses to stress and appetite suppression (Britton et al. 1982; Koob and Bloom 1985; Krahn et al. 1988; Menzaghi et al. 1994). Rat urocortin (rUcn) is a 40 amino acid straight chain mammalian neuropeptide, belonging to the CRF family and sharing a 45% sequence identity to rat/human CRF (r/hCRF) (Vaughan et al. 1995). In the rat central nervous system, Ucn-like immunoreactivity (Ucn-LI) was found more restricted than the CRF-like immunoreactivity, in particular in hypothalamic nuclei, septum, Edinger-Westphal nucleus (EW), substantia nigra pars compacta, and ventral tegmental area regions (Kozicz et al. 1998; Yamamoto et al. 1998). Both peptides bind to the 7-transmembrane CRF-receptors 1 and 2 (CRF-R<sub>1</sub> and CRF-R<sub>2</sub>) with some differences: rUcn has a higher affinity to the r/hCRF-R<sub>2</sub>( $\alpha$ ,  $\beta$  and  $\gamma$ ) with respect to CRF (Donaldson et al. 1996; Kostich et al. 1998). In the rat brain, *in situ* hybridization studies indicate that the distribution of CRF-R<sub>1</sub> and R<sub>2</sub> is scarcely overlapping (Lovenberg et al. 1995), the CRF-R<sub>2</sub> mRNA being mainly co-distributed with Ucn-LI (Kozicz et al. 1998). When administered intracerebroventricularly (ICV), both CRF and rUcn elevate plasma ACTH levels, mimicking the endocrine response induced by stress (Vaughan et al. 1995; Turnbull and Rivier 1997). However, Ucn was found to be more powerful in depressing food intake than r/hCRF and produced less anxiogenic-like effects than CRF when tested 5 min after ICV administration (Spina et al. 1996). Recently, two studies have reported that both rUcn and CRF induced comparable anxiogenic-like effects in the rat (Moreau et al. 1997; Jones et al. 1998). Until now, the controversy in the results obtained by the different laboratories on this aspect has not been solved and has been mostly attributed to a methodological problem. However, there is not yet information on how the time after central injection of the two peptides modifies the anxiogenic-like behavior observed by the different research groups.

The aim of the present study was therefore not only to compare the anxiogenic-like effects elicited by ICV injection of r/hCRF and rUcn in rats, but also to investigate further a possible difference in onset and duration of the anxiogenic behavior induced by the two peptides. For this purpose, different time points after central injection were selected for testing the animals, and three widely acknowledged models of anxiety were employed: the elevated plus-maze (EPM) test, the defensive withdrawal (DW) test, and the Geller-Seifter conflict test (CT) modified for incremental shock.

## Materials and methods

### Animals

Three hundred and forty-nine male Wistar rats (obtained either from Charles River Laboratories, or bred at The Scripps Research Institute from a stock originally derived from Charles River Labo-

ratories, Kingston, N.Y., USA) weighing about 200 g at the time of arrival, were group-housed in clear plastic cages ( $n=3$ ) and maintained in a temperature and light controlled environment on 12 h light/dark cycle (lights on at 06:00 a.m.). Animals had free access to standard rat chow and tap water, except for the rats trained in the operant CT, which were kept under a regimen of 15–18 g of food per day in addition to that obtained during testing. Under this regimen those animals kept gaining weight steadily during the course of the study.

Rats were allowed a 1-week period of acclimation to the animal room and were handled once before surgery. During the recovery period, to minimize as much as possible any additional anxiousness status that might be caused by unfamiliar situations unrelated to the test itself, rats were handled by all the experimenters involved in the project and habituated to the testing room the day before the experiment. To save on animal use, after exposure to the EPM and with always a minimum of 7 days between experiments, rats were tested on the DW test and received the additional treatment according to a randomized order. All animal facilities and procedures were maintained in accordance with the guidelines of the United States National Institutes of Health regarding the principles of animal care, approved by the Institutional Animal Care and Use Committee of The Scripps Research Institute and of the San Diego VA Medical Center, and assessed by the Association for the Assessment and Accreditation of Laboratory Animal Care.

### Surgery

Animals were anesthetized with halothane and placed in a Kopf stereotaxic instrument. Stainless steel guide cannulae (23 gauge, 7 mm long) were implanted unilaterally, 1 mm above the lateral ventricle (AP  $-0.6$  mm, L+ or  $-2.0$  mm from bregma, and DV  $-3.2$  mm) from the point of entry [according to Pellegrino et al. (1979)]. The cannula was secured to the skull using four stainless steel screws and dental cement and closed with a removable stylet. Animals were allowed a 1-week recovery period prior to testing.

### Peptides

Rat Ucn and r/hCRF were dissolved in double-distilled water (pH 6.7) and sterile saline, respectively. Solutions were prepared fresh just before the start of each experiment, and kept on ice. Peptides were provided by the Clayton Foundation Laboratories for Peptide Biology, The Salk Institute (La Jolla, Calif., USA).

### ICV injections

Rats were injected using an 8.5 mm long 30 gauge injector, connected to calibrated polyethylene 10 tubing.

For the EPM and DW studies, injections were performed by gravity. The tubing was raised above the head of the rat until 2  $\mu$ l of r/hCRF or rUcn were infused. The speed of the injection was controlled by slowly lowering or raising the tube above the height of the cannula, so that the substance was infused over a period of 1 min at a constant flow. The tube was then lowered to the height of the cannula. The injector was kept in place for an additional period of 1 min to prevent back flow leakage. The injector then was removed and replaced by the stylet. These tests were conducted at The Scripps Research Institute, La Jolla.

For the CT experiment, infusions were made via a Hamilton syringe connected to the ICV cannula with a tube, and the liquid was infused over a period of 1 min at a constant rate. As above, the injector was kept in place for an additional period of 1 min and then replaced by a stylet. The r/hCRF dose-response curve was carried out in a different group of animals by the same experimenters and in the same period. In order to minimize the number of animals employed in this project, this curve was used as joint reference data also for another study, and published elsewhere

(Britton et al. 2000). This test was performed at the VA Medical Center, San Diego.

To verify the position of the ICV implanted cannula, the rats were injected ICV with blue dye (Cresyl Violet) by gravity under terminal anesthesia delivered by an overdose of Nembutal administered intraperitoneally. Five animals were removed from the data analysis because of the incorrect position of the cannula or because of formation of severe gliosis around the cannula preventing flow.

#### Elevated plus-maze

An automated version of the EPM described by Pellow et al. (1985) was used. The EPM apparatus was made of Plexiglas and was elevated 50 cm above the ground. It consisted of four arms (50 cm long $\times$ 10 cm wide): two of them had dark walls 40 cm high (enclosed arms) and the other two had only ledges 0.5 cm high (open arms). The two open arms were provided with the same amount of light, 1.5–2.0 lux. Light intensity was kept low in order to balance the amount of time spent by each animal within the open and the closed arms. For testing, rats were placed individually onto the center of the maze facing a closed arm and removed after a 5-min testing period. The apparatus was carefully wiped with a damp sponge and dried after each trial. Time spent on each arm and number of entries for each arm were recorded automatically by photocell beams and a computer program. Each animal was naive and was tested only once on this apparatus.

#### Defensive withdrawal

The DW apparatus was as described in Takahashi et al. (1989). It consisted of a black open field (100 $\times$ 100 $\times$ 40 cm) with 20 $\times$ 20 cm squares marked on the floor. A dark cylindrical glass chamber (16 cm deep $\times$ 10 cm diameter) was placed with the open end 20 cm away from one corner of the open field and the lengthwise running along the side wall. Habituation and testing were run under the same conditions: white noise (60 db) and uniform bright illumination (220–250 lux) provided by a ceiling halogen lamp. Testing was conducted under familiar conditions only. Rats were habituated to the open field without chamber for 10 min, 24 h before the testing day. According to the number of lines crossed, animals were equally distributed in groups to avoid baseline differences. On the testing day, rats were placed inside the chamber, in the open field, and the behavior was recorded manually by two trained observers for 15 min. The following behaviors were scored: time spent in the chamber; number of entries into the chamber (initial placement in the chamber included); number of rearings performed outside of the chamber; number of lines crossed (or crossings, considered only if the rat crossed a line with all four legs). After each testing, both open field and chamber were carefully wiped with a damp sponge and dried.

#### Geller-Seifter conflict test

The conflict testing procedures were as those used in previous experiments (Brot et al. 1997; Akwa et al. 1999; Britton et al. 2000). Rats were trained to lever-press for 45 mg Noyes food pellets on a continuous reinforcement schedule in eight sound-attenuated operant chambers equipped with a lever and a food dispenser, and stainless steel bars on the floor through which electric shocks could be delivered (Coulbourn Instruments, Lehigh Valley, Pa., USA). After stabilization of responding, a multiple-schedule conflict test was initiated and consisted of three components: an unpunished (or reward) component, during which responding was reinforced on a random-interval 30-s schedule in a dark chamber, a time-out component, during which responding in the light-illuminated chamber was not reinforced, and a punished or conflict component during which responses signaled by three flashing lights above the lever were both reinforced with food and punished with foot-shock on a continuous reinforcement schedule. Foot-shock

consisted of a scrambled constant current, biphasic square wave produced by an SGS-003 stimulator (BRS/LVE). The electrical current was increased by 0.15 mA with each successive lever press to a maximum of 3.3 mA where it remained constant for the duration of the conflict. A testing session consisted of two cycles of a 5-min reward period, a 2-min time out and a 2-min conflict period, yielding a total daily session duration of 18 min. The number of lever presses during each period was recorded.

#### Experimental design

##### *Experiment 1: elevated plus-maze*

To compare the effects of ICV rUcn or r/hCRF in inducing anxiogenic-like effects on the behavioral response to novelty, rats were tested on the EPM at different times post-injection with vehicle or different doses of peptides. At 5 min, rUcn: 0.01, 0.1, 1.0  $\mu$ g/2  $\mu$ l [number of rats per each dose-group ( $n$ )=8–10] and r/hCRF: 0.1, 0.5 and 1.0  $\mu$ g/2  $\mu$ l ( $n$ =7–8); at 30 min, rUcn: 0.001, 0.01, 0.1, 1.0  $\mu$ g/2  $\mu$ l ( $n$ =6–10) and r/hCRF: 0.1, 0.5 and 1.0  $\mu$ g/2  $\mu$ l ( $n$ =7–11); and at 120 min, rUcn: 0.1 and 1.0  $\mu$ g/2  $\mu$ l ( $n$ =6–7) and r/hCRF: 0.1 and 1.0  $\mu$ g/2  $\mu$ l ( $n$ =5–6). The doses of 0.001, 0.01  $\mu$ g of rUcn and 0.5  $\mu$ g of r/hCRF were added in the tests performed at 5 min and 30 min after ICV injection to extend the dose-response relationships at the early time, and make them comparable to previously published results. Finally, the highest dose (1  $\mu$ g) for both peptides was assessed in the EPM in a group of rats ( $n$ =5–7) 12 hours after ICV injection. Experiments were performed between 6:30 p.m. and 12:00 a.m. A total of 177 rats were used in this paradigm.

##### *Experiment 2: defensive withdrawal paradigm*

To investigate the relative potency of ICV rUcn or r/hCRF in inducing anxiogenic-like effects on the behavioral response to a familiar environment, rats were tested in the DW paradigm. Animals received 2  $\mu$ l of either water pH 6.7 or 0.1, 1  $\mu$ g of rUcn and either saline or 0.1, 1  $\mu$ g of r/hCRF. Tests were performed 5 min ( $n$ =8–10 for rUcn and r/hCRF) and 30 min ( $n$ =8–16 for rUcn and  $n$ =9–14 for r/hCRF) after ICV injection. Experiments were performed between 9:00 a.m. and 4:00 p.m. A total of 102 rats were used in this paradigm.

##### *Experiment 3: Geller-Seifter conflict test*

A dose-response curve was performed for both rUcn and r/hCRF. Rats were injected with either vehicle or 0.25, 0.5, 0.75, 1  $\mu$ g/2  $\mu$ l of rUcn ( $n$ =3–6) or r/hCRF ( $n$ =6–7), 30 min prior to testing. Experiments were performed between 9:00 a.m. and 4:00 p.m. A total of 70 rats were used for this paradigm.

#### Data analysis

##### *Experiment 1: elevated plus-maze*

Data were presented as amount of time spent in the open arms relative to the total amount of time spent in closed and open arms [open/(open+closed) $\times$ 100] and as total number of entries in closed and open arms. The doses of 0.1, 1  $\mu$ g/2  $\mu$ l and vehicle were used, and included in a 3 $\times$ 2 $\times$ 3 factorial design. Among the 177 rats used in this paradigm, nine rats were removed from the study: three rats showed freezing behavior immediately after an initial exploration of the arms, at the very beginning of the test; one rat fell from the maze during the test; five rats leaked fluid immediately after the ICV injection.

### Experiment 2: defensive withdrawal paradigm

Data were presented as mean time spent in the chamber (total time spent in the chamber/number of entries in the chamber), total number of lines crossed and total number of rearings. Among a total of 102 rats used in this paradigm, six rats were taken out of the study: two never came out of the chamber throughout the entire 15 min-period test and one experienced technical problems during testing. Three rats, which showed freezing behavior during the habituation period, were removed from the study and not tested any further on this test.

### Experiment 3: Geller-Seifter conflict test

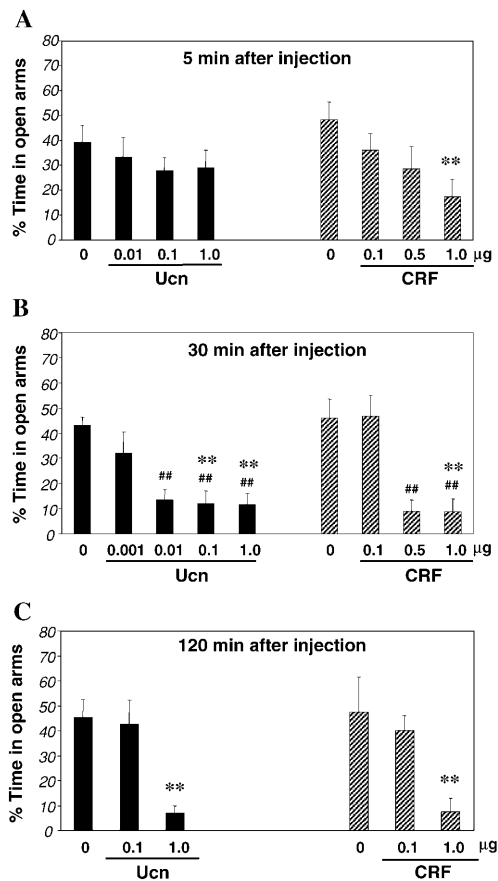
Data were expressed as a percentage of the average of the previous 4 baseline days to reduce within-group variance for both punished and unpunished responding. Among a total of 70 rats used in this paradigm, twelve rats were removed from the study: two rats leaked fluid immediately after ICV injection, ten rats had very low responding (40–50% less than the average responding on the 4 days baseline) in the unpunished component of the test on the testing day. This low responding during the unpunished component, possibly due to anorectic properties induced by the peptides, would make the result obtained in the conflict component difficult to interpret in terms of anxiety-like behavior.

All experimental designs were between-subjects, where each observation was made for each separate animal. Comparisons between the effects of rUcn and r/hCRF over time and over various doses were obtained using a factorial design with the following main factors: Time After Injections, indicating the time when the test was run after the ICV infusion of the peptides; Peptide, consisting of rUcn or r/hCRF; Dose, consisting of the amount of peptide injected ICV, ranging from 0 (vehicle) to 1.0  $\mu$ g. Post-hoc analysis was performed using Newman-Keuls test. In the case of additional doses tested and for analysis of single drug effects, dose-response curves were analyzed by a one-way ANOVA followed by Newman-Keuls post-hoc test. Data are expressed as the mean $\pm$ SEM. For all tests, a significance level of at least  $P < 0.05$  was used.

## Results

### Experiment 1: elevated plus-maze

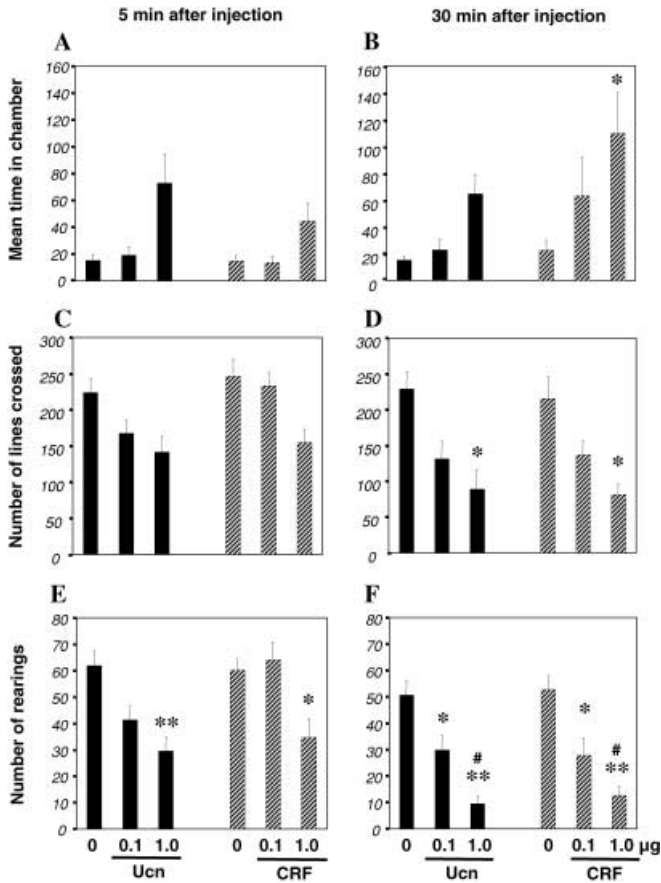
The results of the tests performed in the EPM are reported in Fig. 1. Parametric statistical analysis indicates significant ( $P < 0.001$ ) effects for all the main factors, i.e. Time After Injection, Peptide, and Dose. Significant interactions were found also in the following comparisons: Peptide $\times$ Dose ( $P < 0.05$ ) and Time After Injection $\times$ Peptide $\times$ Dose ( $P < 0.01$ ), indicating that rUcn and r/hCRF anxiogenic-like effect differs according to the time passed between injection and testing, and according to the dose applied. Post-hoc Newman-Keuls comparison indicates that at 5 min after ICV injection rUcn did not affect significantly the percentage of time spent by the rat in the open arm at any of the tested doses, whereas rats injected with r/hCRF showed a significant reduction at the dose of 1  $\mu$ g ( $**P < 0.01$ , Fig. 1A). At 30 min after the ICV injection, both peptides showed clear effects ( $**P < 0.01$ , Newman-Keuls; Fig. 2A) with the 1.0  $\mu$ g dose, but only rUcn showed significant anxiogenic-like effects at the lower dose of 0.1  $\mu$ g. At 120 min after ICV injection, only the 1.0  $\mu$ g dose of both peptides was effective



**Fig. 1A–C** Effects of rUcn and r/hCRF at different time points after administration on the elevated plus-maze. The three panels represent the time spent on open arms (mean $\pm$ SEM) expressed as percentage of time spent in all four arms at 5 min (A), 30 min (B) and 120 min (C) following ICV administration of rUcn or r/hCRF. Groups of rats for both substances were  $n=7-10$  (A),  $n=6-11$  (B) and  $n=5-7$  (C)  $**P < 0.01$  versus 0-dose (vehicle) values; Newman-Keuls test, A–C and  $##P < 0.01$  versus vehicle and versus 0.001  $\mu$ g for rUcn, and  $##P < 0.01$  versus vehicle and 0.1  $\mu$ g for r/hCRF, Newman Keuls test, B)

( $**P < 0.01$ , Newman-Keuls; Fig. 1C). Interestingly, at 12 h after ICV injections, no significant effects on percentage of time spent in all four arms were found at the 1.0 g dose [time spent on open arms (mean $\pm$ SEM): water,  $43.37 \pm 6.38$ ; 1.0  $\mu$ g rUcn,  $50.40 \pm 9.18$ ; saline,  $39.56 \pm 11.05$ ; 1.0  $\mu$ g r/hCRF,  $36.08 \pm 6.26$ ].

When the extended dose-response curve was considered, at 30 min after injection the minimal effective dose for rUcn was 0.01  $\mu$ g ( $*P < 0.01$  versus vehicle and versus the 0.001  $\mu$ g dose, Newman-Keuls; Fig. 1B). Interestingly, r/hCRF showed a significant anxiogenic-like effect only at the dose of 0.5  $\mu$ g ( $##P < 0.01$  versus vehicle and the 0.1  $\mu$ g dose, Newman Keuls; Fig. 1B). Finally, there was no significant difference in the total number of entries in any of the groups and doses tested at any of the time points examined (data not shown).



**Fig. 2A–F** Effects of rUcn and r/hCRF at different time points after injection on the defensive withdrawal paradigm. Effects of ICV infusion of rUcn and r/hCRF on defensive withdrawal and exploratory behavior of rats tested 5 min (A, C and E;  $n=8-10$  for rUcn and r/hCRF) or 30 min (B, D and F;  $n=8-16$  for rUcn and  $n=9-14$  rats for r/hCRF) after treatment. Values represent the mean values ( $\pm$ SEM) of the time spent in the chamber (mean time spent in the chamber; A and B), the total number of lines crossed (number of lines crossed; C and D) and the total number of rearings (number of rearings; E and F). \*\* $P<0.01$ , \* $P<0.05$  versus control values, B, D, E, F, Newman-Keuls test; and # $P<0.05$  versus 0.1  $\mu$ g, F, Newman-Keuls test

### Experiment 2: defensive withdrawal

In the familiar environment of the DW, the mean time spent in the chamber (total time spent in the chamber/total number of entries) of rats was evaluated as a measure of anxiety-like behavior. Results of test performed at 5 min and 30 min after ICV injection are shown in Fig. 2A and B, respectively. Parametric statistical analysis indicated significant effects for the main factors Time after injection ( $P<0.05$ ) and Dose ( $P<0.001$ ), but not for the main factor Peptide. Only the interaction Time After Injection $\times$ Peptide was significant ( $P<0.02$ ), indicating that the two peptides showed the same dose-response profile over time, 1.0  $\mu$ g r/hCRF being significantly different from vehicle at 30 min after ICV injection ( $P<0.01$ , Newman-Keuls; Fig. 2B).

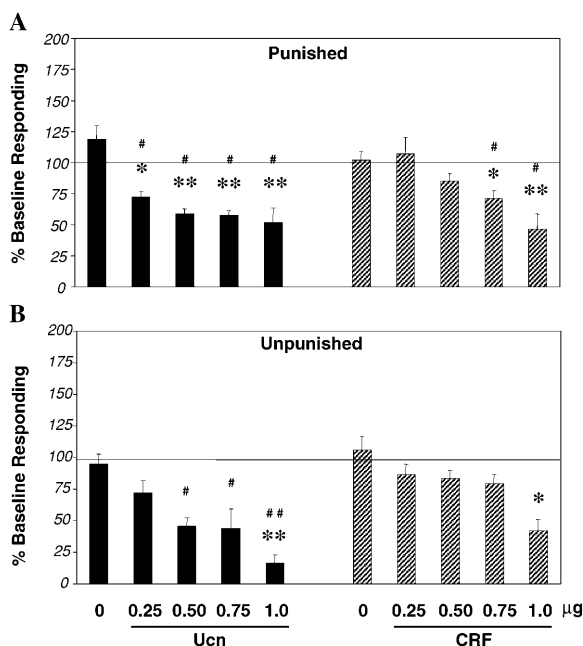
As another measure of anxiogenic-like behavior, the total number of lines crossed was taken into consideration (Fig. 2C and D). Parametric statistical analysis indicated significant effects for the main factors Time After Injection ( $P<0.001$ ) and Dose ( $P<0.001$ ), but not for the main factor Peptide. No interaction was found significant. These results indicate that the effects produced by the two peptides were not different over time or over the various doses used. Both rUcn and r/hCRF significantly decreased the total number of lines crossed at the dose of 1.0  $\mu$ g (\* $P<0.01$ , Newman-Keuls) only in rats tested 30 min after treatment (Fig. 2D).

As an additional measure of exploratory behavior, the total number of rearings was analyzed (Fig. 2 E and F). Parametric statistical analysis indicated significant effects for the main factors Time After Injection ( $P<0.001$ ) and Dose ( $P<0.001$ ), but not for the main factor Peptide. No interaction was found significant. Again, these results indicate that the effects produced by the two peptides were not different over time or over the various doses used. Post-hoc analysis indicated that, at 5 min after ICV injection, rUcn and r/hCRF significantly reduced rearings scores at the dose of 1.0  $\mu$ g (\*\* $P<0.01$  and \* $P<0.05$ , respectively, Newman-Keuls; Fig. 2E). At 30 min after ICV injection, rearings scores were dose-dependently decreased in animals injected with both peptides: the 1.0  $\mu$ g dose was significantly different from vehicle values (\*\* $P<0.01$ , Newman-Keuls; Fig. 2F) as well as from the 0.1 g dose (\* $P<0.05$ , Newman-Keuls; Fig. 2F).

### Experiment 3: Geller-Seifter conflict test

Results obtained in the CT performed 30 min after ICV administration of the peptides is shown in Fig. 3A and B. Parametric analysis of the comparison between the effects of various doses of r/hCRF and rUcn revealed highly significant ( $P<0.01$ ) main factor effects for Dose and Peptide, and a less significant ( $P<0.05$ ) effect for the Response factor and no interaction was found significant. Post-hoc Newman-Keuls test indicated that both peptides induced a significant ( $P<0.01$ ) decrease of the unpunished responding only when the higher dose of 1.0  $\mu$ g was applied. In contrast, punished responding was significantly reduced by rUcn at the dose of 0.25  $\mu$ g, whereas the minimal effective dose of r/hCRF was 1.0  $\mu$ g ( $P<0.05$ ). However, clear but less marked effects were observed also on the unpunished responding. In fact, no significant difference was found between punished and unpunished responding at each of the tested doses for both peptides.

By analyzing the effects of the single peptides with a one-way ANOVA, rUcn was significantly different from the control at the doses of 0.5, 0.75 and 1.0  $\mu$ g in the unpunished response (## $P<0.01$  versus water and 0.25  $\mu$ g; # $P<0.05$  versus water; Newman-Keuls test) and all doses were significantly different from control in the punished response (# $P<0.05$  versus control; Newman-Keuls test).



**Fig. 3** Effects of rUcn and r/hCRF on the Geller-Seifter conflict test. Dose-effect curve of ICV rUcn ( $n=3-6$ ) and r/hCRF ( $n=6-7$ , modified with permission from Britton et al. 2000) on punished (A), and unpunished (B) food-responding in an operant conflict test. Data are expressed as percentage of baseline responding from previous 3 days (mean $\pm$ SEM). Two-way ANOVA (for comparison of the effects produced by both peptides): \*\* $P<0.01$ , \* $P<0.05$ , versus control values, Newman-Keuls post hoc test. One-way ANOVA (for analysis of the effects induced by each peptide): rUcn: ## $P<0.01$  versus water and 0.25  $\mu$ g; # $P<0.05$  versus water; Newman-Keuls test; r/hCRF: # $P<0.05$  versus control; Newman-Keuls test

R/hCRF was significantly different from control only at the dose of 1.0  $\mu$ g in the unpunished response (# $P<0.05$  versus control; Newman-Keuls test) and at the dose of 0.75 and 1.0  $\mu$ g in the punished response (# $P<0.05$  versus control; Newman-Keuls test).

## Discussion

In this study, the effects of rUcn and r/hCRF were investigated in three different behavioral tests for anxiety, at different time-point intervals after injection of the peptides in rats. The present results of the EPM confirm our previous findings showing that, unlike r/hCRF, rUcn does not produce anxiogenic-like effects when rats are tested 5 min after peptide-injection (Spina et al. 1996). However, rUcn was significantly more potent than r/hCRF in inducing anxiogenic-like effects 30 min after ICV injection, being active at much lower doses compared to r/hCRF on the same paradigm. This evidence corroborates the conclusion drawn from the interaction studies indicating that rUcn is more potent than r/hCRF at 30 min after ICV injection, but not at 5 min, when only r/hCRF was effective. Furthermore, this effect cannot be attributed to a different activation induced by the two

peptides as indicated by a non-significant difference in total number of entries produced by both peptides. These data confirm and extend the results reported by Moreau et al. (1997), showing that CRF and rUcn equally reduced the time spent by rats exploring the open arms of the maze at the dose of 0.5  $\mu$ g.

In contrast to the results obtained with the EPM, ICV infusion of rUcn and r/hCRF did not produce substantially different effects when tested in the DW and CT. Both peptides produced dose-dependent anxiogenic-like effects, supporting the data obtained with the EPM, but they did not differ in potency and efficacy when the two substances were compared. This is not surprising, since these tests are conducted under different conditions and address different kinds of performance.

In the DW, both r/hCRF and rUcn were equally capable of reducing the number of rearings and, less effectively, the number of lines crossed during the 15 min of recording, showing the strongest effects at 30 min after ICV administration. The mean time spent in the chamber was the least sensitive among the DW parameters taken into consideration in this study, showing a non-significant tendency for prolonged peptide-induced occupation of the dark chamber at the lower doses, and a significant effect only following 1.0  $\mu$ g r/hCRF at 30 min after ICV administration. Two important differences between the EPM and the DW paradigms must be considered for explaining the inconsistencies between the two tests: the most relevant is the novelty component intrinsic to the protocol of exposure to the testing apparatus (Montgomery 1955). In fact, this condition plays an important role in the EPM, to which rats are exposed for the first time at the moment of testing, while in the DW paradigm rats were tested in an environment already familiar to them. Moreover, although the selection of the testing times was justified by the results obtained with the elevated plus-maze, the difference in the duration of the two tests (5 min for the EPM versus 15 min for the DW paradigm) might have made it more difficult for the DW test to capture the delayed effect of rUcn versus r/hCRF.

Although rUcn was more efficacious than r/hCRF in decreasing rats' food responding in both punished and unpunished components of the CT at low doses, when the respective anxiogenic-like effects were directly compared r/hCRF and rUcn did not produce any significant difference. In fact, both punished and unpunished behaviors were depressed by both peptides, indicating a lack of discrimination between specific anxiogenic-like effects (i.e. the punished responding) versus non-specific effects on general behavior (i.e. the unpunished responding) as observed previously with other anxiogenic-like treatments (Koob et al. 1986). Since neither r/hCRF nor rUcn produce sedation, it is likely that the peptide-induced decrease in lever pressing for food seen in this test might be related to anorectic properties of these substances that mask the anxiogenic-like effects normally measured with this paradigm. This phenomenon is particularly evident for rUcn, when the response in the unpunished component is considered. In fact, the dose of

0.5  $\mu\text{g}$  decreased food responding dramatically, although rats were not threatened by any noxious stimulus. It has been shown that ICV infusion of Ucn and CRF caused a long-lasting effect in suppressing appetite in rodents (Arase et al. 1988; Morley and Levine 1990; Glowa et al. 1992; Spina et al. 1996; Contarino et al. 2000; Coste et al. 2000), and that the PVN may be a candidate brain area for feeding-regulatory mechanisms (Wang et al. 2001). Interestingly, the suppressive effects of rUcn on feeding were found to last up to 12 h (Spina et al. 1996, 1998), whereas in the present investigation we report that no anxiogenic-like effect was present in the EPM at 12 h after ICV administration. Interestingly, this dissociation is less evident when r/hCRF is centrally administered, showing an almost parallel anorectic/anxiogenic-like effect time-profile (Eckart et al. 1999; Koob and Heinrichs 1999). These observations would support the hypothesis that there is dissociation between the anxiogenic-like effects of rUcn, measurable within a few hours from ICV administration, and its induction of a longer-lasting anorectic effect. However, the different time-duration of the tests (5 min for the EPM versus 18 min for the CT) again might play a role for the inconsistent results obtained in the two paradigms.

The neuropharmacological mechanisms accounting for the differential behavioral effects induced by Ucn compared to CRF are not yet clear. There is evidence in the literature regarding dissimilarities between the effects produced by ICV Ucn and CRF. In fact, Ucn was found to be more potent than CRF in stimulating ACTH release (Vaughan et al. 1995; Turnbull et al. 1999) and to be less potent than CRF in stimulating cAMP accumulation in transfected cells (Ruhmann et al. 1999). A particular combination of differential expression patterns of CRF-binding protein (CRF-BP) and CRF-R<sub>1</sub> and -R<sub>2</sub> distribution could explain the selective functional effects (Potter et al. 1992). In fact, rUcn has a high affinity not only for both CRF receptors but also for the CRF-BP (Kemp et al. 1998; Ruhmann et al. 1999), as well as a slower dissociation rate to this protein (Henriot et al. 1999). The CRF-BP does not seem to have overlapping localization with rUcn (Kemp et al. 1998; Kozicz et al. 1998) whereas the expression of the CRF-BP overlaps with that of CRF and CRF-R<sub>1</sub> in cortical areas (Turnbull and Rivier 1997; Kemp et al. 1998). It is reasonable to suggest that, immediately after ICV infusion, the effective concentration of rUcn available to CRF-R<sub>1</sub> binding would be lower than the concentration of CRF when injected at the same dose. This phenomenon would produce a slower activation of CRF-R<sub>1</sub>-dependent anxiogenic-like behavior by rUcn when compared to CRF, giving a possible explanation for the difference in the onset of the peptide-induced effects. A few minutes later, when the CRF-BP would have been saturated by both peptides, the rate of CRF-R<sub>1</sub> occupancy would be the same, resulting in similar anxiogenic-like effects. Also, the discovery that after injection of the two peptides into the rat brain, CRF is more rapidly metabolized compared to Ucn (Richter and Krause 2000) should be considered.

The hypothesis that the rUcn-induced anxiogenic-like effects are mediated via CRF-R<sub>1</sub>, is supported also by the effects on social interaction of bilateral injection of rUcn into the basolateral amygdala, an important anatomical structure in the stress/anxiety response (Sanders and Shekhar 1995; Sajdyk and Shekhar 1997) containing a large majority of CRF-R<sub>1</sub> (Bittencourt and Sawchenko 2000). Indeed, rUcn induced significantly stronger anxiogenic-like effects compared to CRF, as measured by the dose-dependent decrease of social interaction time (Sajdyk et al. 1999). The relevance of CRF-R<sub>1</sub> in anxiogenic-like behavior mediated by Ucn is supported indirectly also by data obtained in CRF-knockout mice. These mice show normal (Weninger et al. 1999) or reduced (Smith et al. 1998; Timpl et al. 1998) stress-induced behaviors, which are blocked by the non-selective CRF antagonist  $\alpha$ -helicalCRF<sub>(9-41)</sub> and by the selective CRF-R<sub>1</sub> antagonist CP-154,526 suggesting that a CRF-like peptide, with the likely candidate Ucn, is compensating for the missing CRF in mediating anxiogenic-like responses (Weninger et al. 1999).

Alternatively, the higher affinity of rUcn for the CRF-R<sub>2</sub> may partly explain some of the differences between rUcn and CRF in their central effects (Vaughan et al. 1995; Donaldson et al. 1996). Recent observations on null mutation of CRF-R<sub>2</sub> in mice showed enhanced spontaneous anxiogenic-like behavior (Bale et al. 2000; Coste et al. 2000; Kishimoto et al. 2000), suggesting that CRF-R<sub>2</sub> activation can exert effects opposite to those of CRF-R<sub>1</sub> activation (Kishimoto et al. 2000). The delayed effects of rUcn also could be related to an initial prevalent activation of the CRF-R<sub>2</sub> that would counterbalance the activation of CRF-R<sub>1</sub>, which will in turn become prevalent for both peptides over time. Differences of substrates expressing CRF-R<sub>1</sub> and -R<sub>2</sub> receptors and involved in controlling different components of emotional behavior also could play a role (Vaughan et al. 1995; Chalmers et al. 1996; Donaldson et al. 1996; Liebsch et al. 1999; Radulovic et al. 2000). Interestingly, the Fos induction pattern stimulated by ICV injection of Ucn has been shown to be more restricted than that of CRF and displays more activation of brain sites showing CRF-R<sub>2</sub> expression (Bittencourt and Sawchenko 2000). Indeed, central administration of 1.0  $\mu\text{g}$  rUcn and CRF induces a similar but non-overlapping pattern of Fos expression at 2 h after administration, with stronger effects of CRF than Ucn (Bittencourt and Sawchenko 2000). In fact, rUcn produced higher effects on Fos expression in the lateral nucleus of the septum, but less effects than CRF in all layers of the isocortex and olfactory bulbs, and various nuclei of the brainstem. Moreover, both peptides produced the same effects on cell groups that are involved in autonomic control but express neither CRF receptors (Bittencourt and Sawchenko 2000). These data indicate that both peptides are able to directly activate Fos expression in neurons expressing CRF receptors, but also to recruit indirectly autonomic structures involved in the integrated response to stress and, most likely, in the anxiogenic-like behavior investigated in the present study.

In conclusion, this study confirms that central injections of rUcn and r/hCRF induce an anxiety-like state in the rat that varies between the two peptides according to the testing conditions and to the time of testing considered after treatment. In fact, when compared to r/hCRF, ICV rUcn tested 30 min after treatment had not only a higher potency in decreasing exploratory behavior in the EPM, as indicated by the differences in threshold dose of rUcn inducing this effect, but also a delayed onset. Under the familiar conditions of the DW and the CT paradigms, with a significantly more powerful decrease in lever-pressing induced by rUcn in the latter test, there was a lack of significant difference in the comparison between anxiogenic-like responses induced by both peptides. In this context, it is likely that the effects induced by rUcn and r/hCRF may vary according to the brain structures involved in mediating emotional and stress-coping behavior. Furthermore, the ability of these two peptides to access those target areas, binding the different subtypes of CRF receptors present, and the different conditions and performances required in the tests used in this investigation, need to be taken into consideration. Ultimately, it can be hypothesized that the two members of the CRF family tested in this study possibly maintain an independent physiological role in non-stressful conditions where they might interact, and their roles overlap, during activating/stressful events.

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