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Human Urocortin II, a Selective Agonist for the Type 2 Corticotropin-Releasing Factor Receptor, Decreases Feeding and Drinking in the Rat

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ABSTRACT

Corticotropin-releasing factor (CRF) has been hypothesized to modulate consummatory behavior through the Type 2 CRF (CRF_2) receptor. However, behavioral functions subserved by the CRF_2 receptor remain poorly understood. Recently, human urocortin II (hUcn II), a selective CRF_2 receptor agonist, was identified. To study the effects of this neuropeptide on ingestive behavior, we examined the effects of centrally infused hUcn II (i.c.v. 0, 0.01, 0.1, 1.0, 10.0 μg) on the microstructure of nosepoke responding for food and water in nondeprived, male rats. Malaise-inducing properties of the peptide were monitored using conditioned taste aversion (CTA) testing. To identify potential sites of action, central induction of Fos protein expression was examined. hUcn II dose dependently reduced the quantity and duration of responding for food and water at doses lower (0.01–1.0 μg) than that forming a CTA (10 μg). Effects were most evident during hours 4 to 6 of the dark cycle. Meal pattern analysis showed that hUcn II potently (0.1 μg) increased the satiating value of food. Rats ate and drank smaller and shorter meals without changing meal frequency. Rats also ate more slowly. hUcn II induced Fos in regions involved in visceral sensory processing and autonomic/neuroendocrine regulation and resembling those activated by appetite suppressants. hUcn II is a promising neuropeptide for investigating the role of the CRF_2 receptor in ingestive behavior.

Corticotropin-releasing factor (CRF) is hypothesized to mediate behavioral, autonomic, endocrine, and immunological responses to stress (Koob and Heinrichs, 1999). Intracerebroventricular (i.c.v.) administration of CRF in rats mimics several behavioral effects of stress, including motor activation, anxiety-like behavior, anorexia, reduced sexual behavior, and altered cognitive performance (Koob et al., 1994). Two genes encoding separate families of G-protein coupled CRF receptors, each having distinct distributions and functional and pharmacological properties (Perrin and Vale, 1999), have been identified (CRF_1 and CRF_2). Whereas molecular and receptor antagonist studies point to activating and anxiogenic-like roles of the CRF_1 receptor (Koob and Heinrichs, 1999), behavioral functions mediated by the CRF_2 receptor have remained obscure.

The discovery of urocortin [Ucn; (Vaughan et al., 1995)], a mammalian CRF paralog with greater affinity than CRF for the CRF_2 receptor, led to the hypothesis that CRF_2 receptor activation may lead to anorectic effects (Spina et al., 1996). Studies with a preferential CRF_2 receptor antagonist (Pelleymounter et al., 2000) and CRF_2 receptor-deficient mice (Bale et al., 2000; Coste et al., 2000) also suggested an anorectic role for the CRF_2 receptor. However, selective agonists for the CRF_2 receptor had not been identified, precluding determination whether CRF_2 receptor activation was sufficient for inducing anorexia.

Recently, urocortin II (Ucn II) and urocortin III (Ucn III) were identified (Lewis et al., 2001; Reyes et al., 2001). Human urocortin II (hUcn II), which was originally identified as human urocortin related peptide, is considered to be the human ortholog to murine urocortin II (mUcn II), a 38-amino

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ABBREVIATIONS: CRF, corticotropin-releasing factor; hUcn II, human urocortin II; D-Phe $CRF_{12–41}$, [D-Phe^{12}, $Nle^{21,38}$ Cα $MeLeu^{37}$] rat/human $CRF_{12–41}$; CTA, conditioned taste aversion; Ucn, urocortin; mUcn II, murine urocortin II; ANOVA, analysis of variance; MED, minimum effective dose; CeA, central nucleus of the amygdala; NTS, nucleus of the solitary tract; SKF 38393, 2,3,4,5-tetrahydro-7,8-dihydroxy-1-phenyl-1*H*-3-benzazepine.

acid neuropeptide in the CRF peptide family. Ucn II is a high-affinity, selective CRF_2 receptor agonist that lacks affinity for the CRF-binding protein. Both hUcn II and mUcn II bind poorly to the CRF_1 receptor ($K_i > 100$ nM), whereas they potently bind to ($K_i < 1$ nM) and induce adenylate cyclase activation via the CRF_2 receptor ($EC_{50} < 1$ nM).

Reyes et al. (2001) recently observed that central infusion of 1 μg mUcn II induced a delayed anorexia. Reduced feeding was likely not due to stress-like disruption of feeding, as hUcn II exhibits delayed anxiolytic-like and mild motor suppressive effects (Valdez et al., 2002). The present experiments were designed to characterize the dose-related effects of central infusion of hUcn II on consummatory behavior in the rat. In addition to examining the time course and magnitude of differences in cumulative food and water intake, changes in the microstructure of feeding behavior were studied, since meal structure analysis can discriminate between drug classes and suggest underlying mechanisms of action (Blundell and Latham, 1978). Potential aversive consequences that could account for ingestive effects of hUcn II were examined using the conditioned taste aversion (CTA) test. Finally, to identify potential sites of action, central induction of Fos protein expression was examined following i.c.v. hUcn II administration.

Materials and Methods

Subjects and Surgery. For behavioral studies, adult male Wistar rats ($n = 59$; 300–350 g at the beginning of experiments) were group-housed in a vivarium at The Scripps Research Institute with 12 h:12 h regular-cycle lighting (on at 6:00 AM) for microstructural analysis of feeding behavior, or reverse-cycle lighting (on at 6:00 PM) for the CTA test. The vivarium was humidity- and temperature-controlled (22°C) with standard rodent chow (LM-485 Diet 7012, Harlan Teklad, Madison, WI) and water available ad libitum, unless stated otherwise. Subjects were acclimated to the vivarium for at least 1 week and tested during their dark cycle.

Subjects were stereotactically implanted with indwelling cannulae directed unilaterally at the lateral ventricle. Anesthetized (halothane, 2–3% in oxygen) subjects were secured in a stereotaxic frame (David Kopf Instruments, Tujunga, CA). Using sterile technique, a straight, stainless steel, 22-gauge guide cannula (Plastics One Inc., Roanoke, VA) was lowered above the lateral ventricle and anchored to the skull with screws and dental cement. With the tooth bar set 5.0 mm above interaural zero, the coordinates were anterior/posterior −0.6 mm, molar concentration per liter ± 2.0 mm relative to bregma, and 3.2 mm ventral from the skull surface (Pellegrino et al., 1979). A dummy stylet (Plastics One Inc.) maintained patency. Subjects were allowed at least 1 week to recover from surgery. Surgical and experimental procedures strictly adhered to the *National Institutes of Health Guide for the Care and Use of Laboratory Animals* (National Institutes of Health Publication number 85-23, revised 1996) and were approved by the Institutional Animal Care and Use Committee of The Scripps Research Institute.

Drugs. hUcn II and [D-Phe^{12}, Nle^{21,38} Cα MeLeu^{37}] rat/human CRF_{12-41} (D-Phe CRF_{12-41}) were synthesized manually using the solid-phase approach, purified using high-pressure liquid chromatography and fully characterized using capillary zone electrophoresis, high-pressure liquid chromatography, and mass spectrometry, as described previously (Reyes et al., 2001). hUcn II was dissolved in sterile 0.5× phosphate-buffered saline (pH = 7.4) immediately before testing and kept on ice. Either hUcn II or vehicle was injected (i.c.v., 5 μl) over 1 min with a Hamilton microsyringe (Hamilton Co., Reno, NV) using a 28-gauge stainless steel injector attached to polyethylene (PE 20) tubing. The injector, which projected 1.3 mm past the end of the cannula, was left in place for 1 min after infusion to allow diffusion. Placement was confirmed histologically.

Microstructural Analysis of Food and Water Responses. In this paradigm, rats were allowed to make nose-poke responses to obtain palatable chow pellets (45 mg precision food pellets, formula A; 60.0% carbohydrate, 3.7% fat, 24.1% protein, 7.0% ash, 5.2% moisture, 370 cal/100 g; P.J. Noyes Company, Inc., Lancaster, NH), from a pellet dispenser (Med Associates Inc., St. Albans, VT). From the other hole of the test cage, rats could make nose-poke responses to obtain 100 μl aliquots of water. Test cages measured 22 cm × 22 cm × 35 cm. Responses were detected by photobeams mounted in the holes and recorded automatically by an IBM PC-compatible personal computer. This procedure allows study of the microstructure of consummatory-directed behavior in nondeprived rats with excellent temporal resolution. Spillage of food pellets in this system was low (M ± S.E.M.: 1.2 ± 0.2% of total responses; $n = 105$ rats).

Prior to testing, rats ($n = 10$) received daily, 15-h sessions spanning their active cycle (−1 h lights off through +2 h lights on) until responding had stabilized (±20% responding for food for 3 consecutive days). Rats were then implanted with i.c.v. cannulae, allowed to recover from surgery and resumed access for at least 1 week, until responding restabilized. For testing, rats were pretreated (30 min prior to testing) with hUcn II at 4:30 PM in a full Latin square design (i.c.v. 0, 0.01, 0.1, 1, or 10 μg) and responses for food and water were monitored for 15 h. No evidence of order, carryover, or conditioning effects were observed in the Latin square design. Test sessions were

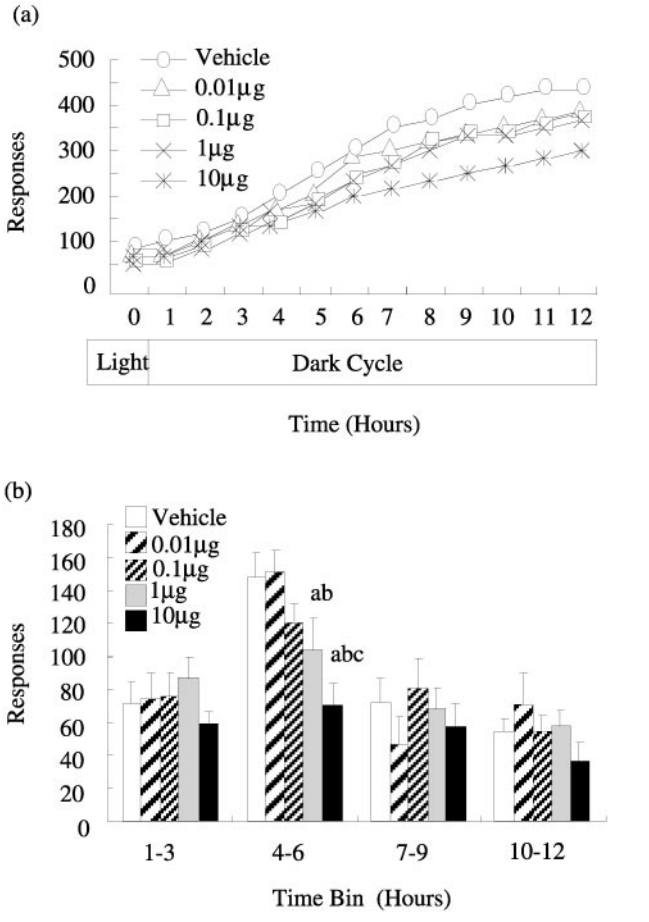

Fig. 1. Effects of i.c.v. infusion of hUcn II on cumulative (a) and incremental (b) responses for food in nondeprived, male Wistar rats ($n = 10$, full Latin square design) during the dark cycle. Nosepoke responses for food are expressed as the mean number of responses ± S.E.M. ○, vehicle; △, 0.01 μg; □, 0.1 μg; ×, 1 μg; ∗, 10 μg of hUcn II. a, b, c, represent significant differences from vehicle, 0.01, 0.1, 1 μg, respectively, $p < 0.05$, Fisher's protected least significant difference test (b).

separated by 4 days. Rats were weighed daily 1 h prior to nose-poke sessions.

Based on statistical burst analysis of our data (E. P. Zorrilla, K. Inoue, G. R. Valdez, unpublished observations), we defined a meal as bursts of responses for food *or* water that contained at least five food-directed responses, with a maximum interresponse interval of 5 min. Duration of eating and drinking within meals was defined separately as the duration of consecutive responses for food or water. Meal sizes for eating and drinking were calculated separately as the average number of food- or water-directed responses during meals. Rates of eating and drinking were calculated by dividing each meal size with its respective duration. Finally, satiety ratio, an index of the satiety time produced by each gram of food consumed, was calculated as the average intermeal interval divided by the average amount of food eaten per meal. Using these criteria, this procedure can distinguish between anorectic agents thought to facilitate meal termination [e.g., satiating agents, such as fenfluramine; (Blundell and Latham, 1978; Burton et al., 1981)], which primarily reduce meal size, from those which act by altering the likelihood of initiating or maintaining feeding [e.g., SKF 38393, a dopamine D_1 receptor agonist (Cooper et al., 1990)], which alters meal frequency.

CTA Test. Individually housed rats ($n = 33$) were tested in a 12-day, multiple-pairing, two-bottle taste conditioning procedure adapted from a previous report (Heinrichs et al., 1991). On day 0, colony water bottles were removed at 11:00 AM for the duration of the procedure. Thereafter, limited fluid access was provided in two cage-top, sipper tube bottles, whereby solutions were made available at 11:00 AM for 25 min. On days 1 through 7 at 11:00 AM, subjects had access to distilled water in both bottles. On days 8 and 10, rats had access to one bottle with 0.15% (w/v) saccharin solution and one bottle with water. Immediately following saccharin access, subjects were administered hUcn II in a between-subjects design (i.c.v., 0, 0.1, 1, or 10 μg), receiving the same dose on each day. Only distilled water was available on days 9 and 11. On day 12, each subject again chose between the 0.15% saccharin solution and water in a drug-free state. Initial position and order of presentation of the saccharin bottle were counterbalanced across subjects and alternated daily. To validate this procedure, separate rats ($n = 16$) were administered isotonic (0.15 M) LiCl (Sigma, St. Louis, MO) or NaCl intraperitoneally (volume of 2% body weight) postsaccharin access in lieu of hUcn II. This dose of LiCl is known to induce taste aversion via gastrointestinal toxicosis (Seeley et al., 2000).

Experimental Procedures for Fos Expression Immunohistochemistry. Adult male Sprague-Dawley rats (250–300 g at the beginning of experiments) were housed in a regular-lit (12 h:12 h) colony room at the Salk Institute. For i.c.v. injections, rats were anesthetized with ketamine/xylamine/acepromazine and stereotactically implanted with a 26-gauge guide cannula terminating in a lateral ventricle at the coordinates of anterior/posterior −0.7 mm, molar concentration per liter ± 1.5 mm, and 3.2 mm ventral relative to bregma. Procedures were approved by the Institutional Animal Care and Use Committee of the Salk Institute.

To monitor induced patterns of Fos expression, rats ($n = 2$–3/condition) were injected i.c.v. at 10:00 AM, with hUcn II at doses of 1, 5, or 10 μg (in 2 μl saline), or vehicle alone, and perfused 2 or 6 h later. In antagonist reversal experiments, rats were injected with the nonselective CRF receptor antagonist D-Phe CRF_{12-41} (i.c.v., 10 μg) followed immediately by injection of 5 μg hUcn II to determine the role of CRF receptors in the activational effects of hUcn II.

Animals were deeply anesthetized with chloral hydrate (350 mg/kg, i.p.) and perfused via the ascending aorta with saline followed by ice-cold 4% paraformaldehyde in 0.1% borate buffer at pH 9.5. Brains were postfixed for 16 h and cryoprotected overnight in 10% sucrose in 0.1 M phosphate buffer. Six series of 30-μm thick frozen sections were cut using a sliding microtome, collected in cold ethylene glycol-based cryoprotectant, and stored at −20°C before histochemical processing.

Tissue was pretreated sequentially with 0.3% hydrogen peroxide and 1% sodium borohydride. It was then permeabilized with phosphate-buffered saline/0.3% Triton X-100, and incubated with primary antiserum for 48 h in phosphate-buffered saline/2% blocking serum. Fos immunoreactivity was localized using a polyclonal antiserum raised in rabbits against an N-terminal synthetic fragment of human Fos protein (Santa Cruz Biotechnology, Santa Cruz, CA). Localization was performed using a conventional avidin-biotin immunoperoxidase method with nickel enhancement.

Statistical Analysis. Results are expressed as mean ± S.E.M. For microstructural analysis of ingestive behavior, cumulative nose-poke responses were calculated separately for the light and dark portions of the light cycle. Dose-response analysis of effects of hUcn II on cumulative number and duration of nose-poke responses for food and water were performed using repeated-measures analysis of variance (ANOVA) with dose as the within-subject factor. To determine the time course of the effects of hUcn II, repeated-measures ANOVAs were performed on the incremental number and duration of responses using 3-h time bins (1–3, 4–6, 7–9, and 10–12 h), with dose and time as within-subject factors. Repeated-measures ANOVAs were used to analyze the microstructure (i.e., meal size, meal length, response rate) of eating and drinking for the entire dark cycle period as well as in 6-h time bins, the smallest unit in which almost every rat consumed at least one meal following each dose of hUcn II. Linear contrasts were performed to determine the dose dependence of observed effects. Due to skewed and inhomogeneity of variance for the satiety ratio measure, this parameter was analyzed using Friedman's test, a nonparametric analog of repeated-measures

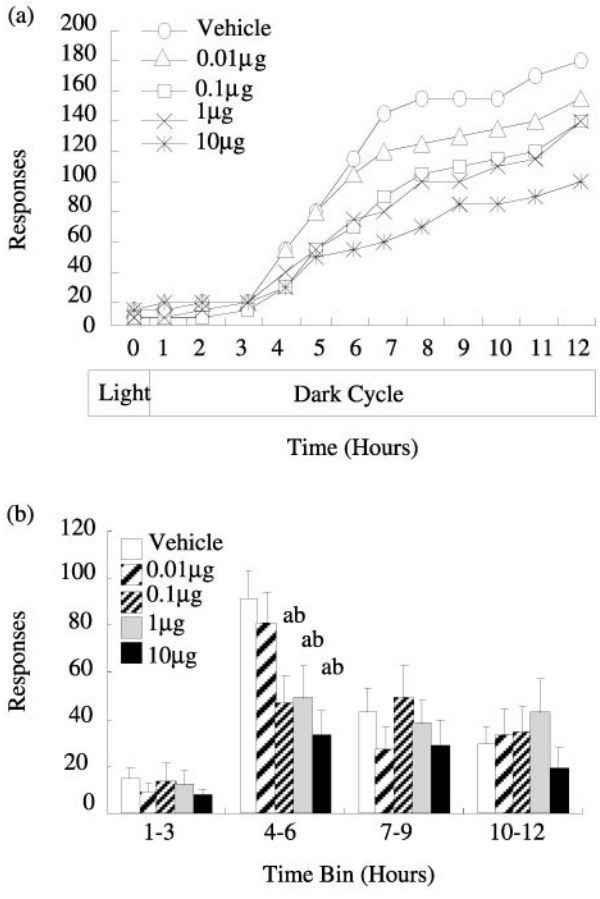

Fig. 2. Effects of i.c.v. infusion of hUcn II on cumulative (a) and incremental (b) responses for water in nondeprived, male Wistar rats ($n = 10$, full Latin square design) during the dark cycle. Nosepoke responses for water are expressed as the mean number of responses ± S.E.M. ○, vehicle; △, 0.01 μg; □, 0.1 μg; ×, 1 μg; ∗, 10 μg of hUcn II. a, b, represent significant differences from Vehicle, 0.01, 0.1, 1 μg, respectively, $p < 0.05$, Fisher's protected least significant difference test (b).

TABLE 1

Effects of intracerebroventricular hUcn II on duration of nose-poke responding for food and water

Effects of i.c.v. pretreatment with hUcn II 90 min prior to the dark cycle on the mean (± S.E.M.) duration of nose-poke responding for food and water during the dark cycle in male Wistar rats (n = 10, full Latin square design).

Parameter	Hours of Dark Cycle				Cumulative
	1–3	4–6	7–9	10–12	
Eating duration, min					
Vehicle	8.1 ± 1.5	16.0 ± 1.6	7.6 ± 1.5	7.2 ± 1.2	38.9 ± 2.4
0.01 μg	8.6 ± 1.9	16.1 ± 1.3	5.2 ± 1.9	8.4 ± 2.3	38.3 ± 3.1
0.1 μg	8.7 ± 1.5	13.2 ± 1.0	8.1 ± 1.9	6.1 ± 1.2	36.3 ± 1.8
1 μg	10.1 ± 1.4	11.9 ± 2.2	7.2 ± 1.2	7.2 ± 1.1	36.3 ± 2.7
10 μg	8.1 ± 0.7	9.1 ± 1.3[a,b]	6.7 ± 1.6	4.6 ± 1.3	28.5 ± 2.6[a,b]
Drinking duration, min					
Vehicle	2.3 ± 0.9	16.9 ± 2.1	8.1 ± 2.2	4.9 ± 1.3	32.1 ± 2.7
0.01 μg	1.8 ± 0.9	11.0 ± 1.9[a]	6.0 ± 2.5	4.9 ± 1.7	23.8 ± 2.2[a]
0.1 μg	2.8 ± 2.0	8.2 ± 2.2[a]	9.1 ± 2.8	4.5 ± 1.6	24.5 ± 3.9[a]
1 μg	1.5 ± 0.9	8.4 ± 2.9[a]	6.9 ± 1.9	7.4 ± 2.5	24.1 ± 3.9[a]
10 μg	1.3 ± 0.5	6.1 ± 2.0[a]	5.9 ± 2.4	2.9 ± 1.1	16.3 ± 3.9[a]

Statistically significant differences: [a,b], significant differences from vehicle and 0.01 μg, respectively, $p < 0.05$, Fisher's protected least significant difference test.

ANOVA. Data from the CTA test were analyzed using mixed ANOVA, with pairings as a within-subject factor and drug as a between-subject factor. For post hoc, pairwise comparisons, Fisher's protected least significant difference was used to interpret significant treatment effects from ANOVAs, and Wilcoxon's signed rank test was used to interpret results from Friedman's test. Sigmoidal analyses using strict criteria for convergence and weighted to minimize the relative distance squared were performed to determine the potency of ingestive effects of hUcn II. The statistical packages used were Systat 10.0 (SPSS, Inc., Chicago, IL), Prism 3.02, and InStat 3.0 (GraphPad, San Diego, CA).

Results

Microstructural Analysis of Food and Water Responses. As shown in Figs. 1 and 2, hUcn II dose-dependently reduced cumulative nose-poke responding for food and water, as reflected in significant Dose (F[4,36] = 6.40, $p < 0.001$ and F[4,36] = 3.27, $p < 0.03$, respectively) and linear contrast effects (F[1,9] = 27.36, $p < 0.001$ and F[1,9] = 16.89, $p < 0.005$, respectively). Table 1 shows that hUcn II also dose-dependently reduced the duration of time spent responding for food and water, as indicated by significant dose (F[4,36] = 4.24, $p < 0.01$ and F[4,36] = 3.33, $p < 0.03$, respectively) and linear contrast effects (F[1,9] = 23.91, $p < 0.001$ and F[1,9] = 16.54, $p < 0.005$, respectively). Post hoc contrasts revealed that hUcn II was more potent at reducing both the cumulative number and duration of nose-poke responses for water than for food (Table 1).

Time course analyses showed that hUcn II reduced nose-poke responding for food and water in a time-dependent fashion, reflected in dose × time effects (for food, F[12,108] = 1.75, $p < 0.07$; for water, F[12,108] = 1.92, $p < 0.05$). Significant effects of time (F[3,27] values = 11.94 and 9.73, $p < 0.001$) reflected that responding for both food and water varied over the dark cycle. Post hoc contrasts revealed that hUcn II selectively reduced responding for food [minimum effective dose (MED) = 1 μg, $p < 0.005$] and water (MED = 0.1 μg, $p < 0.005$) 4–6 h into the dark cycle, the period during which baseline responding was greatest (Figs. 1 and 2). As shown in Table 1, hUcn II also selectively reduced the duration of time spent eating (MED = 10 μg, $p < 0.01$) and drinking (MED = 0.01 μg, $p < 0.01$) during this period. hUcn II pretreatment did not significantly alter the number or duration of responses for food or water during the first postinjection hour, at the end of the light cycle. Sigmoidal analyses revealed that the relative potency (ED_{50} values) of effects of hUcn II on total responses for food and water and total duration of responding for food and water during hours 4 to 6 were 0.46, 0.027, 0.21, and 0.0066 μg, respectively.

Table 2 shows the effects of hUcn II on meal structure. Based on results from the time course analyses, meal structure was analyzed separately for the first and last 6 h of the dark cycle as well as for the entire overnight period. hUcn II did not alter meal frequency (all p values > 0.60). Rather, hUcn II pretreatment reduced the average number of food-directed responses within meals (i.e., "meal size for food"; MED = 1 μg, F[4,36] = 4.61, $p < 0.005$), the average duration of food-directed responding per meal (i.e., "meal length for food"; MED = 10 μg, F[4,36] = 2.76, $p < 0.05$), and the rate of food-directed responding within meals (i.e., "eating rate"; MED = 1 μg, F[4,36] = 3.88, $p < 0.01$). Effects were dose-dependent, as indicated by significant linear contrasts (F values[1,9] = 12.25, 7.83, and 5.89, respectively, all p values < 0.05). Effects of hUcn II on eating rate were more pronounced during the first 6 h of the dark cycle, whereas those on meal size and length were similar throughout. Finally, hUcn II potently increased the satiety ratio (MED = 0.1 μg, Friedman test statistic = 10.0, $p < 0.05$; Table 2).

For meal-related drinking, 2 of 10 rats receiving the 10-μg dose of hUcn II made *no* water-directed responses during the dark cycle. Consequently, the average rate of water-directed responding ("drinking rate") was analyzed using only the eight rats that responded at each dose. Pretreatment with hUcn II reduced both the average meal size (MED = 1 μg, F[4,36] = 5.64, $p < 0.001$) and length (MED = 1 μg, F[4,36] = 3.31, $p < 0.05$) of drinking, but did not alter the drinking rate. Significant effects were dose-dependent, as indicated by linear contrasts (F values[1,9] = 10.64 and 10.89, respectively, p values < 0.01). hUcn II more potently affected the microstructure of meal-related drinking during the first 6 h of the dark cycle (Table 2).

No significant differences were detected in the microstructure of nose-poke responding for food or water on the second post-treatment day.

hUcn II dose-dependently tended to decrease body weight on the first post-treatment day (p = 0.06). No effect on body weight was observed, however, on the second through 4th post-treatment days.

TABLE 2
Effects of intracerebroventricular hUcn II on meal structure

Parameter	Hours of Dark Cycle		Total
	1–6	7–12	
Meal frequency			
Vehicle	4.7 ± 0.8	2.9 ± 0.3	7.6 ± 0.9
0.01 μg	4.8 ± 0.5	2.5 ± 0.3	7.3 ± 0.6
0.1 μg	4.3 ± 0.6	3.1 ± 0.4	7.4 ± 0.8
1 μg	4.5 ± 0.4	3.1 ± 0.2	7.6 ± 0.6
10 μg	3.9 ± 0.5	2.8 ± 0.5	6.7 ± 0.9
Feeding			
Meal size		*food responses*	
Vehicle	54.2 ± 7.0	46.2 ± 4.3	52.1 ± 5.2
0.01 μg	50.6 ± 6.1	49.8 ± 6.1	49.9 ± 5.7
0.1 μg	50.4 ± 6.7	46.1 ± 4.6	47.2 ± 4.6
1 μg	43.0 ± 5.1	42.6 ± 3.0	42.3 ± 3.8[a]
10 μg	37.9 ± 6.2[a,b,c]	33.9 ± 4.3[a,b,c]	35.8 ± 4.6[a,b,c]
Meal length, eating		*min*	
Vehicle	5.9 ± 0.8	5.0 ± 0.3	5.5 ± 0.5
0.01 μg	5.6 ± 0.7	6.0 ± 0.8	5.6 ± 0.7
0.1 μg	5.7 ± 0.7	4.8 ± 0.5	5.2 ± 0.5
1 μg	5.0 ± 0.5	4.9 ± 0.4	4.9 ± 0.4
10 μg	5.1 ± 0.7	4.2 ± 0.5	4.7 ± 0.5[a]
Eating rate		*responses/min*	
Vehicle	9.2 ± 0.2	9.2 ± 0.5	9.1 ± 0.3
0.01 μg	9.2 ± 0.4	8.6 ± 0.4	9.1 ± 0.3
0.1 μg	8.8 ± 0.4	9.5 ± 0.2	9.0 ± 0.3
1 μg	8.5 ± 0.3[a]	8.8 ± 0.3	8.7 ± 0.3
10 μg	7.4 ± 0.5[a,b,c]	8.1 ± 0.5	7.7 ± 0.4[a,b,c,d]
Satiety ratio		*min/g*	
Vehicle	ND	ND	32.3 ± 2.0
0.01 μg	ND	ND	41.0 ± 4.3
0.1 μg	ND	ND	43.3 ± 6.7[a]
1 μg	ND	ND	61.9 ± 17.8[a]
10 μg	ND	ND	71.4 ± 17.7[a]
Drinking			
Meal size		*water responses*	
Vehicle	25.6 ± 3.8	25.5 ± 4.6	24.2 ± 3.0
0.01 μg	19.7 ± 3.6	23.5 ± 4.7	22.9 ± 3.4
0.1 μg	16.0 ± 5.6	28.0 ± 4.1	20.8 ± 4.3
1 μg	13.7 ± 3.5 [a]	27.6 ± 7.5	20.0 ± 3.9
10 μg	10.9 ± 3.1[a,b]	18.1 ± 4.0	16.2 ± 2.8[a,b]
Meal length, drinking		*min*	
Vehicle	4.9 ± 0.7	4.3 ± 0.7	4.3 ± 0.5
0.01 μg	3.0 ± 0.7	3.7 ± 0.8	3.6 ± 0.5
0.1 μg	2.9 ± 1.0	4.3 ± 0.5	3.8 ± 0.7
1 μg	2.2 ± 0.7[a]	4.8 ± 1.2	3.4 ± 0.7
10 μg	2.0 ± 0.6[a]	3.3 ± 0.8	2.9 ± 0.4[a]
Drinking rate		*responses/min*	
Vehicle	ND	ND	5.9 ± 0.5
0.01 μg	ND	ND	6.6 ± 0.6
0.1 μg	ND	ND	6.2 ± 0.9
1 μg	ND	ND	6.2 ± 0.5
10 μg	ND	ND	6.0 ± 0.8

[a,b,c,d] Significant differences from vehicle, 0.01 μg, 0.1 μg, and 1.0 μg, respectively, $p < 0.05$, Fisher's protected least significant difference test; satiety ratio was analyzed with Wilcoxon's signed rank test due to skewness and inhomogeneity of variance.

CTA Test. As expected, postaccess treatment with LiCl reduced markedly the preference ratio for 0.15% saccharin inducing a CTA after one pairing that was maintained with two pairings (preference ratios: 4.9 and 53.1% for isotonic LiCl and NaCl, respectively, following the first postpairing, and 3.5 and 59.9% following the second postpairing). Using this procedure, hUcn II also induced a CTA, as evidenced by a dose × pairing effect ($F[6,58] = 3.64$, $p < 0.005$). Sigmoidal analysis revealed that the ED_{50} of the effects of hUcn II following the second postpairing was 3.51 μg. As shown in Fig. 3, the CTA was evident after a single pairing with preference ratios differing significantly from vehicle only at the highest (10-μg) dose. The 10-μg dose of hUcn II produced aversion for saccharin comparable in magnitude to that induced by LiCl. With repeated pairing, the high-dose aversion was maintained, and lower doses showed no decrement in saccharin preference ratios. Subsequent to hUcn II treatment, rats receiving 10 μg, but not lower, doses of hUcn II also significantly reduced their total fluid intake under drug-free conditions on both water only ($p < 0.001$ versus vehicle-treated rats after second postpairing, 9.5 ± 1.8 versus 18.5 ± 1.5 ml) and saccharin choice ($p < 0.05$ versus vehicle-treated rats after second postpairing, 13.9 ± 1.6 versus 21.3 ± 1.7 ml) access days.

Fos Expression. Intracerebroventricular injection of saline resulted in Fos expression solely in those areas where constitutive expression is commonly observed in nonmanipulated animals (i.e., paraventricular nucleus of thalamus, supramammillary nucleus). Each of the three i.c.v. doses of hUcn II (1, 5, or 10 μg; $n = 2$–3 per condition) gave rise to a

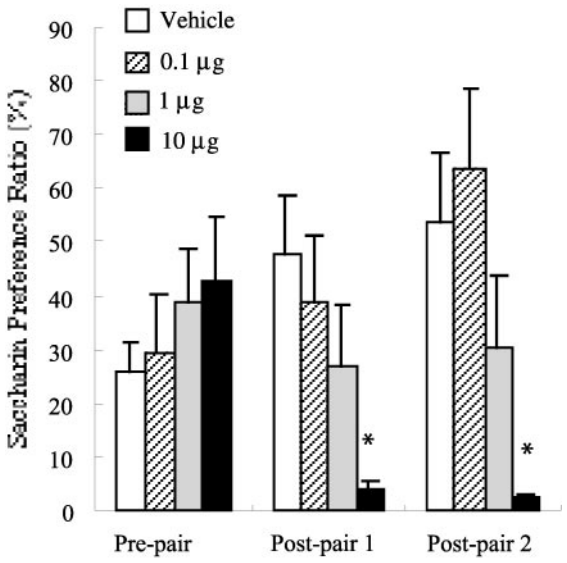

Fig. 3. Effects of i.c.v. infusion of hUcn II on the formation of a conditioned taste aversion. Mean (± S.E.M.) preference ratio for 0.15% saccharin solution over water in subjects prior to or 48 h following a first or second postpairing of hUcn II immediately following access to the previously novel saccharin solution, *, $p < 0.05$ versus respective vehicle.

similar pattern of Fos induction in the brain, with only a modest tendency for higher doses to provoke more robust labeling. Fos induction 2 h postinfusion was similar in topography and magnitude to that observed 6 h postinfusion. Cell groups that consistently showed the most pronounced induction of Fos included a set of highly interconnected structures known to be involved in the processing of visceral sensory information and in regulating autonomic and neuroendocrine function (Fig. 4). These included discrete aspects of the bed nucleus of the stria terminal (oval subnucleus), the paraventricular nucleus of the hypothalamus (medial parvocellular part), the central nucleus of the amygdala (lateral division, CeA), the lateral parabrachial nucleus (external lateral subnucleus), and the nucleus of the solitary tract (medial subnucleus, NTS). Additionally, weak to moderate activational responses were observed with lesser consistency in scattered cells of a handful of other regions, including the isocortex, caudoputamen, and lateral septum. Apart from labeling of ostensibly nonneuronal elements of the ependyma and immediately adjoining regions, Fos induction was not observed in the locus coeruleus, a cell group commonly noted as being responsive in certain (emotional) stress paradigms. Pretreatment with 10-μg injections of the nonselective CRF receptor antagonist, D-Phe CRF_{12-41}, markedly attenuated Fos induction observed throughout the brain in response to a 5-μg dose of hUcn II (data not shown). Control animals showed negligible expression of Fos in hUcn II-responsive regions (Fig. 4).

Discussion

Central infusion of hUcn II, a selective CRF_2 receptor agonist, dose-dependently reduced ingestive behavior at doses that did not elicit signs of visceral illness. hUcn II reduced the number and duration of nose-poke responses for food and water in nondeprived rats, especially during hours 4 to 6 of the dark cycle. Meal pattern analysis revealed that i.c.v. hUcn II potently (MED = 0.1 μg) increased the satiating value of food. Rats ate and drank smaller and shorter meals without changing meal frequency. Rats also ate more slowly. hUcn II induced Fos expression in cell groups recognized as nodes for visceral sensory processing and central autonomic/neuroendocrine control. Thus, Ucn II is a mammalian neuropeptide with central satiation-like properties.

Recently, we reported that i.c.v. hUcn II had delayed anxiolytic-like effects and mild locomotor suppressive effects (Valdez et al., 2002). The effects of hUcn II on ingestion are likely not due to sedation or motor impairment, however. First, hUcn II did not suppress motor behavior 5 to 6 h postinjection during the dark cycle, the period during which its anticonsummatory effects were evident (Valdez et al., 2002). Second, hUcn II reduced intake without affecting the *rate* of responding for water. Therefore, motor impairment cannot likely account for its ingestive effects.

Reyes et al. (2001) also observed that mUcn II had delayed anorectic effects compared with agonists with CRF_1 receptor affinity. Similarly, nonselective CRF receptor agonists show preserved delayed-onset anorexia in CRF_1-null mutant mice (Bradbury et al., 2000; Contarino et al., 2000), but not in CRF_2 knockouts (Coste et al., 2000). The delayed effects of hUcn II on ingestive behavior were similar in time course to its reported anxiolytic-like effects. Fos induction by hUcn II 2 h postinfusion did not differ in topography or magnitude from that 6 h postinfusion, however. Further studies may clarify whether the time-dependence is related to hUcn II per se, the biological actions or neuroanatomical location of its putative targets, or baseline levels of intake/anxiety.

hUcn II also suppressed water intake potently. Indeed, the minimum effective doses and ED_{50} values for its hypodipsic effects were approximately one log-order lower than those for its anorectic effects. Hypodipsia following i.c.v. CRF or Ucn has been reported in rats (Spina et al., 1996) and mice (Bradbury et al., 2000). Pair-feeding experiments suggest that effects on fluid intake in sheep are secondary to reduced feeding (Weisinger et al., 2000). In contrast, i.c.v. CRF in rabbits increases sodium appetite, water intake, and water excretion, consistent with a primary role in osmotic regulation (Tarjan et al., 1991). The rat supraoptic nucleus expresses both Ucn II (Reyes et al., 2001) and CRF_2 mRNA (Van Pett et al., 2000). However, central hUcn II infusion did not induce marked Fos expression there. Whether the hypodipsic effects of hUcn II in the rat reflect primary osmoregulatory mechanisms or secondary reductions in periprandial drinking remains to be determined.

hUcn II induced the formation of a significant CTA at a dose 10- to 1000-fold higher than effective anorectic and antidipsogenic doses. Moreover, the 0.1 and 1 μg doses of hUcn II did not decrease preference for the conditioned stimulus from the first to second pairing, as would be predicted from repeated presentation of a mildly aversive unconditioned stimulus (Grote and Brown, 1971). Thus, we did not find that the effects of hUcn II on the quantity or quality of intake at low to moderate doses could be accounted for by malaise. Similarly, we recently found that hUcn II, unlike LiCl, did not stimulate kaolin clay intake (E. P. Zorrilla, K. Inoue, R. Lintz, unpublished observations), an unconditioned behavior reflecting gastrointestinal malaise (Takeda et al., 1993). These findings are consistent with the observation that Ucn, which has affinity for both CRF_1 and CRF_2 receptors, elicits anorexia at doses lower than that required to form a CTA (Spina et al., 1996). Neither Ucn nor Ucn II

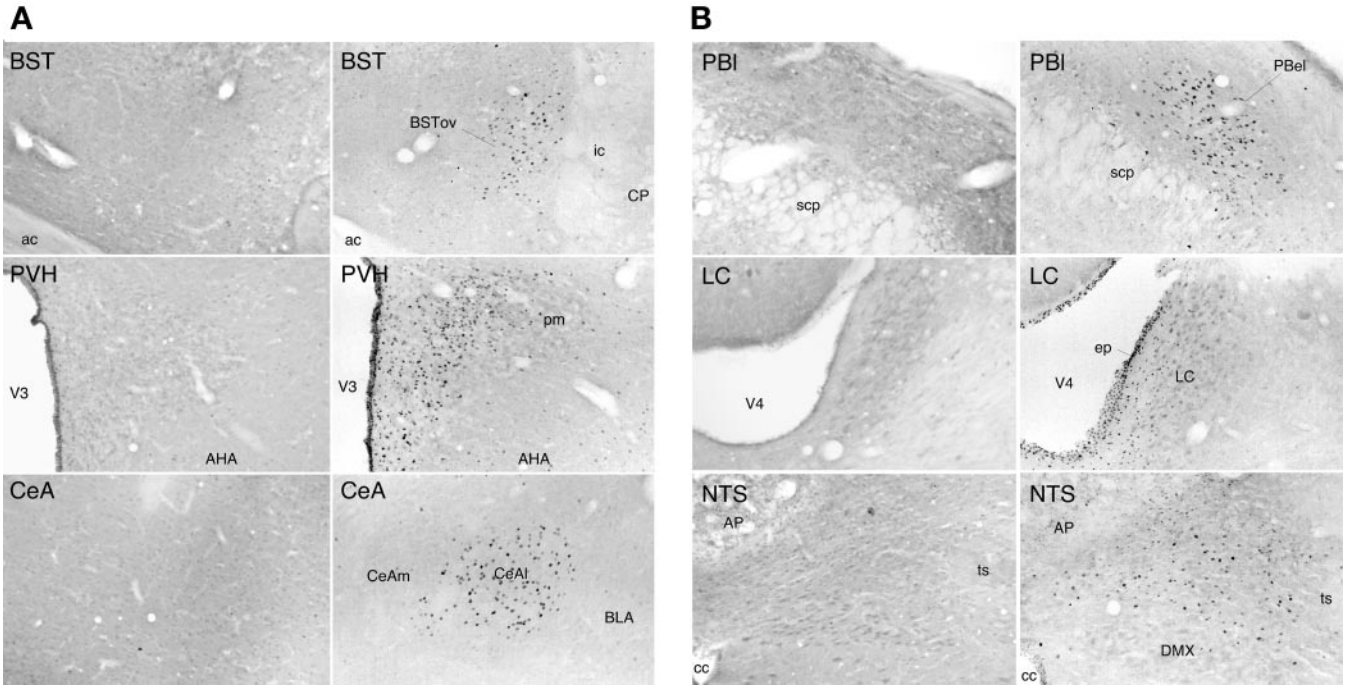

Fig. 4. Brightfield photomicrographs of immunoperoxidase preparations of rat brain, showing areas of Fos expression 2 h following i.c.v. injection of (left panels) vehicle or (right) 1 μg of hUcn II. The topography and magnitude of Fos induction 6 h postinfusion resembled that shown here. The principal site of Fos induction seen in this material comprised an interconnected set of cell groups known to be involved in visceral sensory processing and central autonomic and neuroendocrine control. These included (Fig. 4a) a discrete aspect (oval subnucleus) of the bed nucleus of the stria terminalis (BSTov), the parvocellular division of the paraventricular nucleus of the hypothalamus (PVH), the lateral part of the central nucleus of the amygdala (CeA), (Fig. 4b) the external lateral subnucleus of the lateral parabrachial nucleus (PBI) and the medial division of the nucleus of the solitary tract (NTS). This pattern was similar for peptide doses ranging from 1–10 μg/rat. Fos induction was not reliably observed in the locus coeruleus (LC), apart from labeling seen in the ependyma (ep) and the immediately adjoining regions, which are presumably nonspecific effects of i.c.v. injections. Magnification for all photomicrographs is 65×. Abbreviations: ac, anterior commissure; AHA, anterior hypothalamic area; AP, area postrema; BLA, basolateral nucleus of the amygdala; cc, central canal; CP, caudoputamen; DMX, dorsal motor nucleus of vagus; ic, internal capsule; pm, posterior magnocellular part (PVH); scp, superior cerebellar peduncle; ts, solitary tract; V3 (V4), third (fourth) ventricle.

has produced a conditioned taste preference at any dose tested. In contrast, CRF, which has somewhat preferential affinity for the CRF_1 receptor (Vaughan et al., 1995), forms a CTA at anorectic doses [e.g., i.c.v. 0.5–5.0 mg; (Heinrichs et al., 1991; Benoit et al., 2000)], and forms a conditioned taste preference at lower doses (Heinrichs et al., 1991). These findings suggest that CRF_1 and CRF_2 receptor activation elicit different internal states and that CRF_2 receptor activation may produce anorexia without CTA-forming consequences.

Ingestive behavior usually occurs in discrete bursts of eating and drinking (or "meals") and can be better understood by considering aspects of this microstructure, including meal frequency, size, duration, and eating/drinking rate. The effects of hUcn II on the microstructure of feeding were similar to those of serotonin-related agents that reduce meal size and eating rate, without affecting meal initiation. These compounds, such as fenfluramine, are hypothesized to act by facilitating satiation, or the process of terminating feeding (Blundell, 1986; Kaplan et al., 1997). Likewise, peripheral cholecystokinin (Ritter et al., 1999) and intrahepatic-portal vein glucose infusions (Langhans et al., 2001) decrease meal size and increase satiety ratio, without affecting meal frequency. In contrast, many other anorectic treatments reduce meal frequency in the rat. These include SKF 38393, a D_1 receptor agonist (Cooper et al., 1990), amphetamine (Grinker et al., 1980), inescapable shock stress (Dess and Vanderweele, 1994), and malaise-inducing agents, such as LiCl (West et al., 1987). Unlike hUcn II, i.c.v. Ucn and ovine CRF, which have predominant affinity for the CRF_1 receptor, both act in part by reducing meal frequency (Spina et al., 1996). These findings suggest that central CRF_2, but not CRF_1, receptor activation may have satiation-like effects on meal structure.

Ucn II-induced Fos expression was restricted to cell groups involved in central autonomic/neuroendocrine control and visceral sensory processing, including the CeA, PVN of the hypothalamus, oval subnucleus of the bed nucleus of the stria terminal, NTS, and the lateral parabrachial nucleus. These areas did not constitutively express Fos, consistent with prior findings under our experimental conditions (Chan and Sawchenko, 1994; Li and Sawchenko, 1998; Bittencourt and Sawchenko, 2000). A similar pattern of Fos expression has been observed following nocturnal i.c.v. administration of hUcn II (Reyes, unpublished observations). Of prominent hUcn II-responsive regions, only the NTS richly expresses the CRF_2 receptor. The basis for activation of the other regions is unclear, although they all receive projections from the NTS and are extensively interconnected. Pretreatment with D-Phe CRF_{12-41}, a nonselective CRF receptor antagonist, prevented Fos induction, suggesting CRF receptor mediation. The relative absence of Fos induction by hUcn II in other CRF_2-rich regions (e.g., lateral septum, ventromedial hypothalamus) is puzzling and remains to be explained. In particular, the extent to which Fos serves as a sensitive marker of CRF_2 activation has not been systematically ex-

plored. This pattern of Fos induction is similar to that elicited by mUcn II (Reyes et al., 2001) and supports the proposal that hUcn II is the human ortholog of mUcn II (Lewis et al., 2001). The pattern of Fos induction differs, however, from that seen following CRF or Ucn administration (Bittencourt and Sawchenko, 2000). Whereas both CRF and Ucn provoke activational responses in the same hUcn II-responsive central autonomic structures, CRF also gives rise to widespread Fos induction in CRF_1-rich cell groups, whereas Ucn activates both CRF_1- and CRF_2-expressing targets (Benoit et al., 2000; Bittencourt and Sawchenko, 2000). Different Fos induction patterns thus partly reflect these peptides' receptor specificities, but also may indicate a complex interplay between the systems recruited during CRF_1 or CRF_2 activation, alone or in concert.

Several hUcn II-responsive regions, notably the NTS, parabrachial nucleus, PVN, and CeA, are activated by recognized appetite suppressants, including leptin (Van Dijk et al., 1996), melanocortin receptor agonists (Thiele et al., 1998; Benoit et al., 2000), peripheral cholecystokinin (Day et al., 1994) and *d*-fenfluramine (Li and Rowland, 1993). Accordingly, this activational pattern may reflect a substrate related to feeding regulation. Notably, hUcn II did not induce Fos protein in the area postrema, a chemoreceptor trigger zone for nausea where Fos is seen after diverse, malaise-inducing anorectic stimuli (Van Dijk et al., 1996; Sakai and Yamamoto, 1997; Thiele et al., 1998), and which is a critical substrate for the formation of CTAs (Sakai and Yamamoto, 1997).

Peripheral administration of hUcn II and stresscopin-related peptide also reduce food intake, raising the question of whether the present results are centrally mediated (Hsu and Hsueh, 2001; Million et al., 2002). At doses comparable with those used herein, intravenously administered mUcn II was minimally effective in inducing brain Fos in the rat and significantly less so than i.c.v. administration (Reyes et al., 2001). Moreover, the intravenous doses previously observed to achieve anorexia were at least one log order higher than those used in the present study. Therefore, a central site of action for the present effects of hUcn II is likely.

In summary, central infusion of hUcn II produced satiation-like changes in meal structure, reducing intake at doses that did not induce signs of malaise. Central hUcn II administration activated a network suggesting a role in visceral sensory processing and autonomic/neuroendocrine regulation and resembling that activated by recognized appetite suppressants. Ucn II is a promising, putative neuropeptide for investigating the role of the CRF_2 receptor in ingestive behavior.

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