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Intrathecal Urocortin I in the spinal cord as a murine model of stress hormone-induced musculoskeletal and tactile hyperalgesia

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Abstract

Stress is antinociceptive in some models of pain but enhances musculoskeletal nociceptive responses in mice and muscle pain in patients with fibromyalgia syndrome. To test the hypothesis that urocortins are stress hormones that are sufficient to enhance tactile and musculoskeletal hyperalgesia, we measured von Frey fiber sensitivity and grip force after injection of corticotrophin releasing factor (CRF), urocortin I and urocortin II in mice. Urocortin I (a CRF1 and CRF2 receptor ligand) produced hyperalgesia in both assays when injected intrathecally (i.t.) but not intracerebroventricularly (i.c.v.), and only at a large dose when injected peripherally, suggesting a spinal action. Morphine inhibited urocortin I-induced changes in nociceptive responses in a dose-related fashion, confirming that changes in behavior reflect hyperalgesia rather than weakness. No tolerance developed to the effect of urocortin I (i.t.) when injected repeatedly, consistent with a potential to enhance pain chronically. Tactile hyperalgesia was inhibited by NBI-35965, a CRF1 receptor antagonist, but not astressin 2B, a CRF2 receptor antagonist. However, while urocortin I-induced decreases in grip force were not observed when coadministered i.t. with either NBI-35965 or astressin 2B, they were even more sensitive to inhibition by astressin, a nonselective CRF receptor antagonist. Together these data indicate that urocortin I acts at CRF receptors in the mouse spinal cord to elicit a reproducible and persistent tactile (von Frey) and musculoskeletal (grip force) hyperalgesia. Urocortin I-induced hyperalgesia may serve as a screen for drugs that alleviate painful conditions that are exacerbated by stress.

Keywords

Glucocorticoids; Corticosterone; Pain; corticotropin-releasing hormone; grip force

1. Introduction

Stress alters pain sensitivity. For example, acute restraint of rodents causes thermal antinociception (Imbe *et al.*, 2004) whereas chronic exposure to a daily restraint causes long-lasting thermal hyperalgesia (Okano *et al.*, 1995; Gamaro *et al.*, 1998; da Silva Torres *et al.*, 2003). Forced swims enhance thermal nociception (Quintero *et al.*, 2000; Imbe *et al.*,

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2004; Suarez-Roca *et al.*, 2006a; Suarez-Roca *et al.*, 2006b), chemical nociception (Quintero *et al.*, 2003; Suarez-Roca *et al.*, 2008; Imbe *et al.*, 2010; Quintero *et al.*, 2011) and mechanical nociception in rats (Suarez-Roca *et al.*, 2006a; Okamoto *et al.*, 2012). Corticotropin-releasing factor (CRF) and its related proteins may contribute to these effects as CRF influences nociception (Lariviere & Melzack, 2000; Imbe *et al.*, 2006). Consistent with this possibility, we previously demonstrated that musculoskeletal hyperalgesia is induced in mice by a forced swim and attenuated by a CRF2 receptor antagonist (Abdelhamid *et al.*, 2013).

CRF and its congeners are distributed in pain-relevant sites in the rodent central nervous system (CNS), including the insular cortex, hippocampal formation, thalamus, hypothalamus, periaqueductal gray and locus coeruleus (De Souza *et al.*, 1985; Chalmers *et al.*, 1995; Bittencourt *et al.*, 1999), spinal cord (Merchenthaler *et al.*, 1983; Korosi *et al.*, 2007; Kim *et al.*, 2011) and dorsal root ganglia (Kim *et al.*, 2011). Inhibition of swim stress-induced musculoskeletal hyperalgesia (Abdelhamid *et al.*, 2013) and attenuation of footshock-induced bladder hypersensitivity (Robbins & Ness, 2008) by intrathecally injected CRF2 receptor antagonists suggests that either urocortins or CRF are released from spinal projections in response to stress. Immunofluorescence of CRF- and urocortin I-containing fibers in the spinal cord are consistent with neuronal projections from which these hormones may be released onto neuronal cell bodies expressing both CRF1 and CRF2 receptors (Korosi *et al.*, 2007).

CRF-like ligands have distinct pharmacological profiles for each receptor. CRF binds to CRF1 receptors with high affinity but to CRF2 receptors with significantly lower affinity (Perrin *et al.*, 1995). Urocortin I binds to CRF1 and CRF2 receptors with about equally high affinity (Chen *et al.*, 1993; Lovenberg *et al.*, 1995) but urocortin II and III have high affinity for only CRF2 receptors (Lewis *et al.*, 2001; Reyes *et al.*, 2001). In the CNS, CRF1 and CRF2 receptors frequently have opposite effects (Coste *et al.*, 2001; Reul & Holsboer, 2002) and together shape responses to stress. CRF2 receptor expression along nociceptive pathways is upregulated after neuronal damage that leads to neuropathic pain (Kim *et al.*, 2011). Additionally, in patients with fibromyalgia syndrome CRF is elevated in the cerebrospinal fluid where its concentration correlates with pain sensitivity (McLean *et al.*, 2006).

While the antinociceptive and hyperalgesic effects of CRF have been widely studied, the effect of urocortins on nociception is not well characterized. We determined whether any of three urocortin congeners are sufficient to recapitulate stress-induced hyperalgesia in musculoskeletal and tactile nociceptive assays. Because CRF and urocortins induce release of corticosterone and influence feeding behavior (Zorrilla *et al.*, 2003) and body temperature (Turek & Ryabinin, 2005; Telegdy *et al.*, 2006), we used these parameters to validate doses and delivery routes used.

2. Material and methods

2.1. Animals

Adult Swiss-Webster female mice weighing 20–30 g (Harlan Sprague Dawley Inc., Indianapolis, IN) were used. Animals were housed five per cage, and allowed to acclimate for at least 1 week prior to use. Female mice were used because females are generally more sensitive to pain and to the effect of stress on pain (Fillingim *et al.*, 2009) than males. Estrogen regulates promoter activity of the urocortin gene (Haeger *et al.*, 2006), suggesting that some effects of urocortin may be more prominent in females. In addition, CRF lowers the firing rate of nociceptive-sensitive fibers in females and pre-adult males in locus ceruleus neurons (Borsody & Weiss, 1996).

Mice were allowed free access to food and water, and housed in a room with a constant temperature of 23°C on a 12-h light: 12-h dark cycle; light was on from 6 am to 6 pm. Experiments started around 8 am with baseline measurements; urocortin analogs and other drugs were injected at 9 am and the last behavioral test (8 h after injection) was performed at 5 pm. Animals were used strictly in accordance with the Guidelines of International Association for the Study of Pain and the University of Minnesota Animal Care and Use Committee, and those prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication (NIH) 78-23, revised 1995). All efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to in vivo techniques, if available.

2.2. Drugs and chemicals

CRF (>95% HPLC), urocortin I (>97% HPLC), and urocortin II (>97% HPLC) were purchased from Sigma-Aldrich (St Louis, MO). NBI-35965 (>98% HPLC), astressin 2B (>98% HPLC) and astressin (>90% HPLC) were purchased from Tocris Bioscience (Ellisville, MO). We used 1–10 µg of CRF, urocortin I, and urocortin II to test whether agonists of either CRF1 and/or CRF2 receptors could influence pain when administered peripherally or delivered intrathecally (i.t.) or intracerebroventricularly (i.c.v.) directly into the CNS. This dose range (1–10 µg) was chosen because low doses (3 µg or less) were reported to cause either no effect or analgesia in other modalities of nociception (Lariviere & Melzack, 2000) whereas centrally administered CRF at a dose of 25 µg can cause seizures and/or behavioral problems (Ehlers *et al.*, 1983). However, when we found that 10 µg of urocortin I produced a large effect on mechanical nociception, 3 and 1 µg of urocortin I were also tested to examine the dose-relationship of their responses. The doses of the antagonists used (50 µg NBI-35965, 10 µg astressin 2B and 10 µg astressin) have been previously shown to be centrally active (Martinez *et al.*, 2004b) and were dissolved in double-distilled water.

Morphine sulfate (>98% HPLC), obtained from Mallinckrodt (St. Louis, MO) was used for its antinociceptive activity to differentiate the effect of urocortin I on strength, which is insensitive to opiates, from its ability to induce hyperalgesia, which is inhibited by morphine (Kehl *et al.*, 2004). Morphine was given at doses of 3, 10 and 30 mg/kg intraperitoneally (i.p.) 30 min before the von Frey fiber assay as those doses are sufficient to inhibit the

effects of other compounds, like lipopolysaccharides, that induce on musculoskeletal hyperalgesia measured using the grip force assay (Kehl *et al.*, 2004).

2.3. Drug Delivery

Drugs were delivered either subcutaneously in the scruff of the neck and upper back, intraperitoneally in the lower abdominal quadrants, intrathecally or intracerebroventricularly (i.c.v.). Injections made i.t. in mice were delivered at approximately the L5–L6 intravertebral space using a 30-gauge, 0.5 inch disposable needle on a 50 µl Luer tip Hamilton syringe in lightly restrained, unanaesthetized mice (Hylden & Wilcox, 1981). A volume of 5 µL was consistently used to ensure that the drug did not reach the basal cisterns of the brain, ensuring a selective distribution to the spinal cord area (Fairbanks *et al.*, 2003). I.c.v. injections were made freehand in mice that were anesthetized using isoflurane, a drug that allows a rapid recovery from anesthesia. Proficiency in proper placement was ensured via preliminary studies in which the free flow of fluid marks injection into ventricles rather than tissues. Injections were delivered using a 30-gauge needle into the lateral ventricle using anatomical coordinates from bregma (+0.3mm anterior, –1.0mm lateral and –3mm ventral) as described in previous studies (DeVos & Miller, 2013). Drugs delivered centrally were dissolved in 5 µl as this volume was found to be large enough to be easily and accurately measured. CRF and urocortin II were dissolved in saline whereas urocortin I was dissolved in saline that was acidified using acetic acid in a final concentration of 0.25%. Control mice were injected with the same volume of vehicle. Nociception was measured as described below for 3 days prior to injections to establish the baseline response and over a 24–72 h period after injections. This time-course was implemented based on the effects induced by both CRF and urocortins on other parameters widely reported in the literature.

In experiments to determine the involvement of the different CRF receptors in the urocortin I-induced mechanical hyperalgesia, we tested the effect of a CRF1 receptor antagonist (NBI-35965 at 50 µg/5 µl i.t. (Hoare *et al.*, 2003)) and of a CRF2 receptor antagonist (astressin 2B at 10 µg/10 µl i.t.), on urocortin I-induced hyperalgesia (3 µg/5 µl i.t.). 10 µg urocortin I was delivered i.t. in a 5-µl volume 15 min after injection of the antagonist (either NBI-35965 or astressin 2B). Astressin (10 µg/10 µl i.t. or 10 µg/200 µl s.c.), a non-selective antagonist, was injected 30 min before 3 µg or 10 µg of urocortin I.

2.4. Von Frey assay

Mechanical sensitivity to a #4.17 von Frey fiber (1.4 g) was measured in mice daily for at least 3 days prior to injections and typically 4, 8 and 24 h afterwards. Mice were randomized so the average response to the von Frey fiber prior to injection did not differ amongst groups. To test, mice were placed on a wire mesh grating under a glass 6-ounce (177 ml) custard cup, which prevents escape but allows movement of all four limbs and head. The von Frey fiber was applied ten times to the plantar surface of each hind paw, to the point of bending. A positive response was defined as a brisk lifting, shaking or licking of the paw. The number of positive responses out of 20 was recorded as the mechanical sensitivity. We selected the #4.17 fiber as prior tests indicated that this size produced positive responses but of a sufficiently small magnitude that they could still be potentiated to reflect hyperalgesic responses (enhanced nociception). Fiber sizes 4.08 and greater are probably nociceptive as

responses to this size in preliminary studies were inhibited by pretreatment with 10 mg/kg of morphine delivered 30 min prior to von Frey fiber testing. Larger fiber sizes were not used as they produced a sufficiently large nociceptive response that hyperalgesia would not be detected due to the ceiling effect.

2.5. Grip force assay

To assess changes in musculoskeletal hyperalgesia or weakness, forelimb grip force was measured using a grip force analyzer, as described in detail for use with mice (Wacnik *et al.*, 2003; Kehl *et al.*, 2004; Kovacs *et al.*, 2008). Hyperalgesia was differentiated from weakness by the ability of an analgesic compound (morphine) to reverse decreases in grip force caused by enhanced nociception while weakness is unaffected. The apparatus consists of a force transducer that is connected to a wire mesh grid (12 × 7 cm² O.D. with a ~0.5 cm square wire grid) and is positioned on top of an aluminum frame approximately 30 cm above the bench top. During testing, each mouse was held by its tail (close to the base) and gently passed in a horizontal direction over the wire grid until it grasped the grid with its forepaws. The peak force exerted by the forelimbs of each mouse on the grid was recorded by a force transducer. Three grip force measures were obtained, and the average of these measurements was used to represent each animal's forelimb grip force. Animals were familiarized with the grip force apparatus for two days prior to the recording of baseline values and injection of stress hormones on the third day, one hour prior to administration of drugs. The mean grip force values obtained 4, 8 and 24 h after injections are represented for each group.

2.6. Measurement of body weight and temperature

We monitored the efficacy of CRF and urocortin I and II on body weight and temperature to assess their effects on eating and thermoregulation. Body weight was determined using an A&D FX-300i top-loading balance from Cole-Palmer (Vernon Hills, IL). The rectal temperature was measured using a digital thermometer (model Micro Thermo 2-ETI, Braintree Scientific Inc., Braintree, MA).

2.7. Statistical analysis

When comparing two treatments, we used an unpaired, two-tailed Student's *t*-test. Differences in body temperature and body weight at specific time-intervals after drug injection were compared to their appropriate pre-injection control values using a paired two-way Student's *t*-test. Data involving comparisons of more than two groups were analyzed using one-way ANOVA followed by the Newman-Keuls post hoc analysis to determine differences amongst treatment groups tested at the same time or amongst the areas under the curves of multiple treatment groups. Statistical analyses were performed on data derived from the same experiments but figures in some cases depict pooled control values to simplify graphs. Where indicated, the mean grip force values and von Frey fiber responses shown are taken during the peak effect on mechanical sensitivity (where the value for each animal was the average of those taken 4 and 8 h after injections) to capture the optimal hyperalgesic effect of urocortin I. In all analyses, differences were considered significant if the probability that it occurred because of chance alone was less than 5% ($P < 0.05$). All

statistics were completed using Graphpad-Prism Graphpad Software Inc., La Jolla CA, USA.

3. Results

3.1. Change in nociceptive response varies by route of administration

The effects produced by injection of 10 μ g of urocortin I (i.t.) on both von Frey fiber and grip force responses are shown in figure 1 for comparison to its congeners and to other routes of administration. To simplify comparisons between treatments and routes of injection, the effects of urocortin I, urocortin II and CRF were calculated and depicted as areas under the curve from 0 to 24 h compared to that of their respective vehicle-injected control groups (Fig. 1A–H). This time-interval was selected for analysis as it captured the full effect of urocortin I in the grip force assay.

When injected intracerebroventricularly (10 μ g in 5 μ L), urocortin I, urocortin II and CRF each failed to influence either von Frey (urocortin I: $t_{18}=1.62$, $P=0.12$; urocortin II: $t_{12}=0.07$, $P=0.95$; CRF: $t_{12}=0.37$, $P=0.71$) or grip force responses (urocortin I: $t_{18}=1.29$, $P=0.22$; urocortin II: $t_{12}=0.19$, $P=0.86$; CRF: $t_{12}=0.08$, $P=0.93$), as the areas under the curve for von Frey fiber and grip force responses did not differ from their respective vehicle-injected controls. However, when this dose was injected intrathecally, urocortin I significantly increased responses to the von Frey fiber (Fig. 1A; $t_{39}=3.91$, $P=0.0003$) and decreased muscular force exertion as recorded by the grip force assay (Fig. 1E; $t_{55}=3.22$, $P=0.002$). The area under the curve for the longer-lasting effect of 10 μ g of urocortin I on von Frey fibers was also significantly greater than that of controls when analyzed over a 48-h time-interval (Fig. 2A; $t_{61}=3.74$, $P=0.0004$) that captures the full time-course of the effect of this dose on von Frey fiber responses.

Based on their relatively greater selectivity, we then tested CRF (selective for CRF1) and urocortin II (selective for CRF2) for their effects on von Frey and grip force responses when compared to urocortin I. When injected at the same high dose (10 μ g) and by the same route (i.t.) as that producing a significant and long-term hyperalgesia following urocortin I (Fig. 2), CRF and urocortin II each failed to replicate the hyperalgesic effect of urocortin I when measured using either the von Frey fiber (Fig. 1B; $t_{31}=0.70$, $P=0.49$; Fig. 1C; $t_{31}=1.27$, $P=0.21$) or grip force assay (Fig. 1F; $t_{31}=1.00$, $P=0.32$; Fig. 1G; $t_{31}=0.04$, $P=0.97$). One difference between the injection of urocortin I compared to those of urocortin II or CRF is that the latter two were not injected in an acidified vehicle as they did not require acidification to dissolve. To determine whether the failure of CRF and urocortin II to cause hyperalgesia was due to a lack of co-activation of acid-sensing receptors, we also tested CRF and urocortin II when the same dose was dissolved in acidified vehicle, however, these injections also failed to induce hyperalgesia 8 h after their injection i.t. (data not shown).

3.2. Urocortin I induces hyperalgesia via a spinal site of action

The lowest effective dose of urocortin I (1 μ g i.t.) when administered intrathecally produced no effect on either von Frey fiber sensitivity or grip force responses after systemic (s.c.) administration (von Frey: urocortin I=2.6 $\pm$ 0.6 responses, vehicle=3.1 $\pm$ 0.7 responses ($t_{18}=0.55$, $P=0.59$); grip force: urocortin I=172.1 $\pm$ 5.3 g, vehicle=175.0 $\pm$ 0.6 g ($t_{18}=0.40$,

$P=0.69$)). This suggests that the possible leakage of urocortin I from the spinal cord area to the periphery cannot account for its hyperalgesic effect when injected i.t. It was not until the dose of urocortin I was increased to 10 μg (in 200 μl) that it produced tactile mechanical hyperalgesia when injected peripherally (von Frey fiber assay), peaking 8 h after a s.c. (Fig. 1D; $t_{40}=4.82$, $P<0.0001$) or i.p. injection (Fig. 1D; $t_{39}=2.92$, $P=0.006$). Peripheral administration of urocortin I by the same high dose and routes had no effect on responses measured using the grip force assay (Fig. 1H; s.c. $t_{40}=0.85$, $P=0.40$; i.p. $t_{39}=0.97$, $P=0.34$).

To further confirm the proposed site of action, we injected mice with 10 $\mu\text{g}/5 \mu\text{l}$ i.t. urocortin I 30 min after a s.c. injection of 10 $\mu\text{g}/200 \mu\text{l}$ astressin, a dose that is six times higher than that necessary to antagonize the effect of CRF injected intraperitoneally (Martinez et al., 2002). Astressin failed to prevent the urocortin I-induced increases in either musculoskeletal sensitivity at 4 h (astressin plus urocortin I = 164.2 ± 6.4 responses versus urocortin I alone = 161.7 ± 4.0 responses ($t_{20}=0.33$, $P=0.74$)) or at 8 h (astressin plus urocortin I = 161.9 ± 6.0 responses versus urocortin I alone = 164.7 ± 5.2 responses ($t_{20}=0.35$, $P=0.73$)), or tactile sensitivity at 4 h (astressin plus urocortin I = 9.8 ± 2.2 g versus urocortin I alone = 9.2 ± 2.1 g ($t_{20}=0.20$, $P=0.84$)) or at 8 h (astressin plus urocortin I = 10.5 ± 2.5 g versus urocortin I alone = 8.6 ± 2.2 g ($t_{20}=0.54$, $P=0.59$)), confirming that it is a central rather than peripheral CRF receptor population that mediates hyperalgesia.

3.3. Urocortin I induces hyperalgesia in a dose-related fashion

The long-term effect of urocortin I administered i.t. on von Frey fiber sensitivity was dose-related. Initially, when mice were injected with 1, 3 or 10 $\mu\text{g}/5 \mu\text{l}$ urocortin I, peak effects measured 4–8 h after injection did not differ by dose, but higher doses elicited longer lasting and dose-related increases in von Frey fiber sensitivity, as shown at 24 h after injection (Fig. 2A: $F_{3,110}=11.58$, $P<0.0001$). To confirm that the effect of urocortin I on von Frey fiber responses reflects hyperalgesia, we assessed the effect of morphine on these responses. Morphine, a potent analgesic in the category of a relatively non-selective opioid receptor agonist, was administered at doses of 3, 10 or 30 mg/kg i.p. to mice who had previously (6 h) been injected with urocortin I (10 μg in 5 μl i.t.) or vehicle. Thirty min after its injection, morphine inhibited urocortin I-induced von Frey fiber responses in a dose-related fashion (Fig. 2B; $F_{4,137}=8.07$, $P<0.0001$).

The effect of urocortin I administered i.t. on grip force responses was also dose-related (Fig. 2C). When mice were injected with 1, 3 or 10 $\mu\text{g}/5 \mu\text{l}$ urocortin I, peak effects were again produced 4–8 h after injection, as shown at 4 h ($F_{3,203}=9.46$, $P<0.0001$) and at 8 h ($F_{3,203}=4.44$, $P=0.005$). To confirm that the effect of urocortin I on grip force responses reflects hyperalgesia rather than weakness, we again assessed the effect of morphine on these responses. Morphine was administered at doses of 3, 10 and 30 mg/kg i.p. to mice who had previously (6 h) been injected with urocortin I (10 μg in 5 μl i.t.) or vehicle. Thirty min after its injection, 30 mg/kg of morphine blocked the inhibitory effect of urocortin I on grip force responses (Fig. 2D: $F_{4,137}=8.071$, $P=0.0001$). It is noteworthy that the intrathecal injection of vehicle alone consistently produced a decrease in grip force responses ($t_{99}=9.16$, $P<0.0001$) as well as a transient increase in von Frey fiber responses ($t_{56}=5.11$, $P<0.0001$) 8

h later, consistent with a high sensitivity of these two assays to the stress of even a vehicle injection.

3.4. Repeated intrathecal administration of Urocortin I does not result in tolerance

When urocortin I was injected i.t. daily for 5 days at the lowest effective dose tested (1 µg/5 µl), or for 21 days at 3–4 day intervals with the higher dose (10 µg/5 µl), the maximum effect on von Frey responses observed 8 h after administration of urocortin I were each greater than that of vehicle in terms of area under the curve ($F_{2,38}=6.11$, $P=0.005$) but, while also significantly different than their pre-injection control values, did not differ between doses or from one injection to the next at either 1 µg ($F_{5,12,60}=14.30$, $P<0.0001$) or the 10 µg dose ($F_{3,7,21}=7.54$, $P=0.001$), as illustrated in figure 3A. In contrast, when measured 24 h after its injection, the time-interval most sensitive to its dose-related effects, the number of von Frey fiber responses after each injection of 1 µg of urocortin I increased over time (Fig. 3B; $F_{5,12,60}=8.93$, $P<0.0001$). This indicates no tolerance to the hyperalgesic effect of urocortin I as a longer rather than a shorter duration of action was produced by each injection.

3.5. Systemic effects confirm activation of CRF receptors

In rats, CRF2 receptor activity is known to increase body temperature while CRF1 receptor activity does not (Telegdy *et al.*, 2006). In contrast, injections of urocortin I directly into the dorsal raphe nucleus of mice cause hypothermia (Turek & Ryabinin, 2005). In our study, urocortin I decreased body temperature 4 h (peak effect) after its central injection i.t. (Fig. 4A: $F_{3,51}=7.03$, $P=0.0005$) or i.c.v. (Fig. 4B: $F_{3,38}=8.34$, $P=0.03$) and this effect was mimicked to a much lesser extent by urocortin II, but only when injected i.t. (Fig. 4A).

CRF2 activity also decreases food intake in rats (Yakabi *et al.*, 2011). Consistent with this, urocortin I decreased body weight 24 h after its injection i.t. (Fig. 4E; $F_{3,51}=7.03$, $P=0.0005$) or i.c.v. (Fig. 4F: $F_{3,38}=8.36$, $P=0.0002$). This effect was also mimicked by urocortin II, but only when injected i.c.v. (Fig. 4F). CRF had no effect on body weight or body temperature when injected by either an i.t. or an i.c.v. route. However, 1 h after injection of CRF (10 µg) in a volume that distributes to both brain and spinal cord (20 µl i.t.), CRF increased the concentration of circulating corticosterone (513.9 ± 24.66 ng/ml, $n=8$) when compared to vehicle (166.92 ± 49.1 ng/ml, $n=7$, $t_{13}=6.59$, $P<0.0001$) confirming the activity of this dose of CRF at CRF1 sites along the hypothalamic-pituitary-adrenal pathway.

Injected parenterally, 10 µg of urocortin I decreased body temperature 4 h after its injection s.c. or i.p. when compared to vehicle-injected controls (Fig. 4C: $t_{81}=16.29$, $P<0.0001$), Fig 4D: $t_{39}=5.91$, $P<0.0001$) suggesting that this dose was sufficient to induce a CRF2-mediated effect. In contrast to its effect on body temperature, 10 µg of urocortin I decreased body weight 24 h after a s.c. (Fig. 4G: $t_{81}=10.27$, $P<0.0001$) but not an i.p. injection (Fig. 4H: $t_{39}=0.53$, $P=0.60$). Together, these data confirm that these compounds are biologically active at both CRF1 and CRF2 sites in the CNS in the murine model.

3.6. Urocortin I-Induced tactile hyperalgesia (von Frey fiber) is inhibited by a CRF1 receptor antagonist

To determine whether the hyperalgesic effects of urocortin I were elicited by activation of CRF receptors, we tested the effect of a CRF1 receptor antagonist (NBI-35965 at 50 $\mu\text{g}/5 \mu\text{L}$ i.t.) and of a CRF2 receptor antagonist (astressin 2B at 10 $\mu\text{g}/5 \mu\text{L}$ i.t.), on urocortin I-induced hyperalgesia (3 $\mu\text{g}/5 \mu\text{L}$ i.t.), measured using von Frey fiber sensitivity. When injected i.t. 15 min before 10 μg of urocortin I, NBI-35965 significantly attenuated the increase in von Frey fiber peak responses observed after the injection of urocortin I alone (Fig. 5A: $F_{2,28}=16.16$, $P<0.0001$). In contrast, astressin 2B failed to affect peak von Frey fiber responses detected 4–8 h after urocortin I (Fig. 5B: $F_{2,28}=10.75$, $P=0.0003$), indicating that the hyperalgesia measured using von Frey fiber sensitivity is mediated by CRF1 receptors.

The effect of these antagonists was validated by their ability to influence urocortin I-induced decreases in body temperature (a CRF2 receptor effect) and their ability to attenuate increases in glucocorticoid release (a CRF1 mediated effect). The dose of astressin 2B used was sufficient to prevent the decrease in body temperature induced by 10 μg of urocortin I 4 h after injection (peak effect on temperature) (Fig. 5H: $F_{2,28}=45$, $P=0.003$), whereas NBI-35965 had no effect on urocortin I-induced hypothermia (Fig. 5G: $F_{2,28}=7.25$, $P=0.003$). This is consistent with a CRF2 receptor-mediated hypothermic effect of urocortins on body temperature.

3.7. Urocortin I-Induced musculoskeletal hyperalgesia (grip force) is inhibited by a nonselective CRF receptor antagonist

We then tested the effect of a CRF1 receptor antagonist (NBI-35965) and of a CRF2 receptor antagonist (astressin 2B) on urocortin I-induced hyperalgesia (3 $\mu\text{g}/5 \mu\text{L}$ i.t.), measured using grip force. When injected i.t. 15 min before 10 μg of urocortin I, neither NBI-35965 nor astressin 2B attenuated the decrease in grip force responses observed after the injection of urocortin I alone (Fig. 5D: $F_{2,28}=4.43$, $P=0.021$ and Fig. 5E: $F_{2,28}=3.56$, $P=0.04$). Although the selective CRF antagonists had no significant inhibitory effect on this hyperalgesia, each prevented the statistical significance of the decrease in grip force produced by urocortin I.

Because each selective antagonist appeared to be only partially effective in preventing urocortin I-induced hyperalgesia measured using grip force, we then tested the effect of astressin, a non-selective CRF receptor antagonist (10 $\mu\text{g}/10 \mu\text{L}$ i.t.), on urocortin I-induced hyperalgesia (3 $\mu\text{g}/5 \mu\text{L}$ i.t.). Higher doses of astressin 2B were not used due to observable behavioral effects that we determined might interfere with nociceptive responses. The hyperalgesic effect of urocortin I in the grip force assay was significantly inhibited by pretreatment with astressin delivered 30 min before urocortin I (Fig. 5F: $F_{2,60}=6.48$, $P=0.003$). The dose of astressin used was sufficiently low that it had no effect on CRF1-mediated hyperalgesia measured using von Frey fibers (Fig. 5C: $F_{2,60}=8.03$, $P=0.0008$) or on CRF2-mediated hypothermia (Fig. 5 I: $F_{2,60}=42.69$, $P<0.0001$). Thus, while selective inhibition of either CRF1 or CRF2 receptors has no effect on urocortin I-induced decreases in grip force, inhibition by a nonselective antagonist potently inhibited this same parameter.

To determine the potential synergistic relationship between these two receptors, we explored the effect of both selective antagonists injected together with urocortin I (in series via the same delivery cannula) and found that they produced no greater antagonistic effect on von Frey fiber responses than either antagonist alone (data not shown), indicating a lack of synergy between these two receptors. Rather, the antihyperalgesic action of astressin may be due to an action at other CRF receptors than the CRF1 or CRF2 site.

4. Discussion

Fibromyalgia is a painful condition in which the concentration of CRF is elevated in the cerebrospinal fluid (McLean *et al.*, 2006). The pain of fibromyalgia is exacerbated by stress, which raises the possibility that stress hormones modulate nociception. Our studies show that urocortin I, a stress hormone with both CRF1 and CRF2 affinity, produces a persistent mechanical hyperalgesia when injected intrathecally in the mouse. This hyperalgesia is widespread, dose-dependent and attenuated by CRF receptor antagonists. No tolerance develops to the hyperalgesic effects of urocortin I, consistent with its potential to exacerbate chronic pain conditions.

CRF and its receptors along with urocortins I, II and III are distributed in pain-relevant sites in the brain, spinal cord (Korosi *et al.*, 2007) and periphery (Martinez *et al.*, 2004b) where CRF and its congeners modulate many types of nociception (Lariviere & Melzack, 2000; Imbe *et al.*, 2006). Stress hormones alter pain in a fashion that depends of modality. Whether stress induces antinociception (Lariviere & Melzack, 2000) or hyperalgesia (Imbe *et al.*, 2006) depends on many factors, including the type, length and severity of the stress. The CRF receptor type and distribution relative to each modality of pain contributes to the dichotomous effects of these hormones (Dautzenberg & Hauger, 2002; Ji & Neugebauer, 2008). For example, when injected i.t in mice, CRF produces no effect on thermal nociception (tail flick assay: (Ayesta & Nikolarakis, 1989; Poree *et al.*, 1989; Song & Takemori, 1991)), consistent with the failure of CRF overexpression to influence thermal hyperalgesia (van Gaalen *et al.*, 2002). However, identical injections of CRF are antinociceptive in assays of chemical nociception (abdominal stretch), an effect thought to be mediated by dynorphin and kappa opioid receptors (Przewlocki *et al.*, 1983; Nakazawa *et al.*, 1985; Song & Takemori, 1990). Even different doses of the same compound (e.g. CRF) can produce opposite effects due to differential affinities at CRF receptors (Nijssen *et al.*, 2005). CRF is pronociceptive in models of abdominal pain (Martinez & Tache, 2006) while urocortins exert abdominal antinociceptive effects in mice (Martinez *et al.*, 2004a) and rats (Million *et al.*, 2006).

Based on the conflicting effects of CRF receptor activity on various modalities of nociception, their effects specifically on musculoskeletal and tactile nociception cannot be extrapolated from those previously reported. To address this, the current study examined the influence of these compounds and their routes of administration specifically on musculoskeletal and tactile nociception. Our studies demonstrate that intrathecal injections of urocortin I produce mechanical hyperalgesia in both the von Frey fiber and grip force assays.

Hyperalgesia produced by i.t. injections of urocortin I are consistent with the presence of neuronal projections containing CRF and urocortin I throughout the mouse spinal cord. These proteins are dense in the intermediate zone and surrounding the central canal (Korosi *et al.*, 2007) but also present in lamina I and II of the dorsal horn (Bittencourt *et al.*, 1999; Korosi *et al.*, 2007; Kim *et al.*, 2011). CRF1 and CRF2 receptor mRNA is also expressed throughout the spinal gray matter where CRF2 receptor mRNA exceeds that of CRF1 in the thoracic and lumbar spinal cord (Korosi *et al.*, 2007; Kim *et al.*, 2011). Small dorsal root ganglia cells contain CRF protein together with only weak CRF2 receptor mRNA (Kim *et al.*, 2011), but their expression increases in a rat model of neuropathic pain (Kim *et al.*, 2011). This suggests a greater modulatory capacity of stress along damaged nociceptive pathways.

The hyperalgesic effect of urocortin I on grip force responses and von Frey fiber sensitivity was reversed by morphine in a dose-related fashion, consistent with an effect of urocortin I on nociception. The relatively high dose of morphine required to antagonize urocortin I-induced hyperalgesia (30 mg/kg i.p.) usually suggests that mu opioid receptors, for which morphine has a relatively high affinity, do not mediate this antinociception. In fact, CRF receptors are known to antagonize the antinociceptive effect of morphine on thermal nociception (Ayesta & Nikolarakis, 1989; Poree *et al.*, 1989; Song & Takemori, 1991). A similar antagonism may account for the attenuated action of morphine on urocortin I-induced mechanical hyperalgesia.

Grip force was only decreased by urocortin I when injected i.t. In contrast, von Frey fiber sensitivity was increased by all routes except when injected i.c.v., indicating that the location of receptors capable of inducing tactile hyperalgesia appear to be more widespread than those influencing grip force. However, the lowest effective dose of urocortin I (1 µg) only increased von Frey fiber sensitivity when injected i.t., suggesting that nociceptive processing in the spinal cord area is a more sensitive target for urocortin I than the peripheral receptor populations present in skin, skeletal muscle and adipose tissue. In addition, the inability of peripherally injected antagonists to inhibit the hyperalgesic effects of urocortin I injected i.t. suggest that leakage of drug from the spinal area to the periphery does not likely account for its hyperalgesic action.

Both assays of mechanical hyperalgesia appear to involve a CRF receptor, however, the CRF receptor population for grip force appears to differ from that involved in von Frey fiber sensitivity. Tactile hyperalgesia (von Frey) was inhibited by NBI-35962, a CRF1 receptor antagonist. This is consistent with previous reports that CRF1 receptors mediate tactile pain in rats (Hummel et al 2010).

In contrast, the musculoskeletal hyperalgesic effect of urocortin I (grip force) was inhibited by astressin, the non-selective antagonist, and prevented by astressin 2B, the CRF2 receptor antagonist. This is consistent with the contribution of CRF2 receptors to the mediation of swim stress-induced musculoskeletal hyperalgesia in mice (Abdelhamid *et al.*, 2013). Although astressin 2B prevented the development of musculoskeletal hyperalgesia, its failure to completely block the decrease in grip force induced by urocortin may be due to a shorter time-course of action or a lower efficacy in spite of higher selectivity for the CRF2

receptor. Thus, selective inhibition of either CRF 1 or CRF 2 had no significant effect on urocortin I-induced decrease in grip force while a nonselective antagonist did. This may indicate that urocortin I also influences hyperalgesia via an as yet undescribed CRF receptor that confounds the interpretation of our results.

Repeated administration of CRF intrathecally leads to tolerance to its antinociceptive effect (Song & Takemori, 1990; 1991), but in the present study no tolerance developed to the peak (8 h) mechanical hyperalgesia following injections of urocortin I at 3–4 day intervals. Rather, the magnitude of hyperalgesia 24 h after injection increased resulting in longer hyperalgesic episodes. This enduring pronociceptive action of urocortin I may contribute to the persistent hyperalgesia in response to repeated stress in rodents (Abdelhamid *et al.*, 2013) as well as in many painful conditions in humans.

It is somewhat perplexing that urocortin I-induced hyperalgesia was not mimicked by either CRF or urocortin II. The failure of CRF and urocortin II to produce hyperalgesia cannot be attributed to an insufficient dose, as these injections were biologically active. For example hypothermia, known to be induced by activation of CRF2 sites (Turek & Ryabinin, 2005), was evident after injection of urocortin I by all routes (i.c.v., i.t., s.c., i.p.) and to a lesser extent after centrally injected urocortin II (i.t.). This hypothermia was prevented by astressin 2B but not by NBI-35962, indicating a classic CRF2-mediated effect. In addition, urocortin I (i.c.v., i.t., s.c.) and urocortin II (i.c.v.) each decreased food intake, likely by interrupting feeding and digestion (CRF1 receptor) or by postponing appetite and digestion (CRF2 receptor) (Zorrilla *et al.*, 2003). While CRF had no effect in our study on either body temperature or weight, the dose used increased corticosterone, indicating physiologic activity along the hypothalamic-pituitary-adrenal axis.

It is possible that the distribution of exogenously administered urocortin I differs from that of CRF and urocortin II allowing it better access to the relevant CRF receptor population. Alternatively, the unique hyperalgesic effects of urocortin I may be, in part, due to its association with CRF binding proteins, molecules that have a higher affinity for urocortin I. This binding protein can either chaperone urocortin I to its target (Westphal & Seasholtz, 2006) or inhibit CRF-mediated activation of CRF1 receptors (Manuel *et al.* 2014). In contrast, urocortin II can displace CRF agonists from these binding proteins (Jahn *et al.*, 2005).

Fibromyalgia is a painful condition in which the concentration of CRF is elevated in the cerebrospinal fluid (McLean *et al.*, 2006). The pain of fibromyalgia is exacerbated by stress. In addition, fibromyalgia is frequently comorbid with sensitizing disorders including Crohn's disease (49%) (Buskila *et al.*, 1999), ulcerative colitis (19%) (Buskila *et al.*, 1999), irritable bowel syndrome (about 70%) (Veale *et al.*, 1991; Sivri *et al.*, 1996; Barton *et al.*, 1999; Sperber *et al.*, 1999), interstitial cystitis (Alagiri *et al.*, 1997; Clauw *et al.*, 1997), and endometriosis (Nothnick, 2004). In conditions like ulcerative colitis, immunoreactivity for urocortin I is increased and correlates with the severity of inflammation in colonic mucosa (Saruta *et al.*, 2004). While urocortin I is antinociceptive in viscera, the present study predicts that urocortin I originating from the skin or gut might simultaneously cause tactile mechanical hyperalgesia similar to that after a peripheral injection. Furthermore, if the

expression of CRF2 receptors increases in these conditions, as it does in a rat model of neuropathic pain (Kim *et al.*, 2011), the mechanical hyperalgesia induced by endogenously released urocortin I might be enhanced even more than in healthy individuals.

Now that the ability of urocortin I to induce hyperalgesia is established by the present study and the importance of CRF2 receptors in stress-induced musculoskeletal nociception known (Abdelhamid *et al.*, 2013), further characterization of these CRF receptor populations is warranted in future studies. This model may elucidate the roles of CRF receptors in stress-induced mechanical hyperalgesia and serve as a screen for drug targets in painful conditions that are exacerbated by stress.

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References

- Abdelhamid RE, Kovacs KJ, Pasley JD, Nunez MG, Larson AA. Forced swim-induced musculoskeletal hyperalgesia is mediated by CRF2 receptors but not by TRPV1 receptors. *Neuropharmacology*. 2013; 72:29–37. [PubMed: 23624287]
- Alagiri M, Chottiner S, Ratner V, Slade D, Hanno PM. Interstitial cystitis: unexplained associations with other chronic disease and pain syndromes. *Urology*. 1997; 49:52–57. [PubMed: 9146002]
- Ayesta FJ, Nikolarakis KE. Peripheral but not intracerebroventricular corticotropin-releasing hormone (CRH) produces antinociception which is not opioid mediated. *Brain Res*. 1989; 503:219–224. [PubMed: 2605516]
- Barton A, Pal B, Whorwell PJ, Marshall D. Increased prevalence of sicca complex and fibromyalgia in patients with irritable bowel syndrome. *Am J Gastroenterol*. 1999; 94:1898–1901. [PubMed: 10406256]
- Bittencourt JC, Vaughan J, Arias C, Rissman RA, Vale WW, Sawchenko PE. Urocortin expression in rat brain: evidence against a pervasive relationship of urocortin-containing projections with targets bearing type 2 CRF receptors. *J Comp Neurol*. 1999; 415:285–312. [PubMed: 10553117]
- Borsody MK, Weiss JM. Influence of corticotropin-releasing hormone on electrophysiological activity of locus coeruleus neurons. *Brain Res*. 1996; 724:149–168. [PubMed: 8828564]
- Buskila D, Odes LR, Neumann L, Odes HS. Fibromyalgia in inflammatory bowel disease. *J Rheumatol*. 1999; 26:1167–1171. [PubMed: 10332985]
- Chalmers DT, Lovenberg TW, De Souza EB. Localization of novel corticotropin-releasing factor receptor (CRF2) mRNA expression to specific subcortical nuclei in rat brain: comparison with CRF1 receptor mRNA expression. *J Neurosci*. 1995; 15:6340–6350. [PubMed: 7472399]
- Chen R, Lewis KA, Perrin MH, Vale WW. Expression cloning of a human corticotropin-releasing-factor receptor. *Proc Natl Acad Sci U S A*. 1993; 90:8967–8971. [PubMed: 7692441]
- Clauw DJ, Schmidt M, Radulovic D, Singer A, Katz P, Bresette J. The relationship between fibromyalgia and interstitial cystitis. *J Psychiatr Res*. 1997; 31:125–131. [PubMed: 9201654]
- Coste SC, Murray SE, Stenzel-Poore MP. Animal models of CRH excess and CRH receptor deficiency display altered adaptations to stress. *Peptides*. 2001; 22:733–741. [PubMed: 11337086]
- da Silva Torres IL, Cucco SN, Bassani M, Duarte MS, Silveira PP, Vasconcellos AP, Tabajara AS, Dantas G, Fontella FU, Dalmaz C, Ferreira MB. Long-lasting delayed hyperalgesia after chronic restraint stress in rats-effect of morphine administration. *Neurosci Res*. 2003; 45:277–283. [PubMed: 12631463]
- Dautzenberg FM, Hauger RL. The CRF peptide family and their receptors: yet more partners discovered. *Trends Pharmacol Sci*. 2002; 23:71–77. [PubMed: 11830263]

- De Souza EB, Insel TR, Perrin MH, Rivier J, Vale WW, Kuhar MJ. Corticotropin-releasing factor receptors are widely distributed within the rat central nervous system: an autoradiographic study. *J Neurosci.* 1985; 5:3189–3203. [PubMed: 3001239]
- DeVos SL, Miller TM. Direct intraventricular delivery of drugs to the rodent central nervous system. *J Vis Exp.* 2013; (75)
- Ehlers CL, Henriksen SJ, Wang M, Rivier J, Vale W, Bloom FE. Corticotropin releasing factor produces increases in brain excitability and convulsive seizures in rats. *Brain Res.* 1983; 278:332–336. [PubMed: 6605787]
- Fillingim RB, King CD, Ribeiro-Dasilva MC, Rahim-Williams B, Riley JL 3rd. Sex, gender, and pain: a review of recent clinical and experimental findings. *J Pain.* 2009; 10:447–485. [PubMed: 19411059]
- Fu Y, Neugebauer V. Differential mechanisms of CRF1 and CRF2 receptor functions in the amygdala in pain-related synaptic facilitation and behavior. *J Neurosci.* 2008; 28:3861–3876. [PubMed: 18400885]
- Gamaro GD, Xavier MH, Denardin JD, Pilger JA, Ely DR, Ferreira MB, Dalmaz C. The effects of acute and repeated restraint stress on the nociceptive response in rats. *Physiol Behav.* 1998; 63:693–697. [PubMed: 9523917]
- Haeger P, Andres ME, Forray MI, Daza C, Araneda S, Gysling K. Estrogen receptors alpha and beta differentially regulate the transcriptional activity of the Urocortin gene. *J Neurosci.* 2006; 26:4908–4916. [PubMed: 16672665]
- Hoare SR, Sullivan SK, Pahuja A, Ling N, Crowe PD, Grigoriadis DE. Conformational states of the corticotropin releasing factor 1 (CRF1) receptor: detection, and pharmacological evaluation by peptide ligands. *Peptides.* 2003; 24:1881–1897. [PubMed: 15127940]
- Hylden JL, Wilcox GL. Intrathecal substance P elicits a caudally-directed biting and scratching behavior in mice. *Brain Res.* 1981; 217:212–215. [PubMed: 6167328]
- Imbe H, Iwai-Liao Y, Senba E. Stress-induced hyperalgesia: animal models and putative mechanisms. *Front Biosci.* 2006; 11:2179–2192. [PubMed: 16720304]
- Imbe H, Murakami S, Okamoto K, Iwai-Liao Y, Senba E. The effects of acute and chronic restraint stress on activation of ERK in the rostral ventromedial medulla and locus coeruleus. *Pain.* 2004; 112:361–371. [PubMed: 15561392]
- Imbe H, Okamoto K, Donishi T, Senba E, Kimura A. Involvement of descending facilitation from the rostral ventromedial medulla in the enhancement of formalin-evoked nocifensive behavior following repeated forced swim stress. *Brain Res.* 2010; 1329:103–112. [PubMed: 20226771]
- Jahn O, Radulovic J, Stiedl O, Tezval H, Eckart K, Spiess J. Corticotropin-releasing factor binding protein - A ligand trap? *Mini-Rev Med Chem.* 2005; 5:953–960. [PubMed: 16250837]
- Ji G, Neugebauer V. Pro- and anti-nociceptive effects of corticotropin-releasing factor (CRF) in central amygdala neurons are mediated through different receptors. *J Neurophysiol.* 2008; 99:1201–1212. [PubMed: 18171711]
- Kehl LJ, Kovacs KJ, Larson AA. Tolerance develops to the effect of lipopolysaccharides on movement-evoked hyperalgesia when administered chronically by a systemic but not an intrathecal route. *Pain.* 2004; 111:104–115. [PubMed: 15327814]
- Kim EH, Ryu da H, Hwang S. The expression of corticotropin-releasing factor and its receptors in the spinal cord and dorsal root ganglion in a rat model of neuropathic pain. *Anat Cell Biol.* 2011; 44:60–68. [PubMed: 21519550]
- Korosi A, Kozicz T, Richter J, Veening JG, Olivier B, Roubos EW. Corticotropin-releasing factor, urocortin 1, and their receptors in the mouse spinal cord. *J Comp Neurol.* 2007; 502:973–989. [PubMed: 17444496]
- Kovacs KJ, Papic JC, Larson AA. Movement-evoked hyperalgesia induced by lipopolysaccharides is not suppressed by glucocorticoids. *Pain.* 2008; 136:75–84. [PubMed: 17686584]
- Lariviere WR, Melzack R. The role of corticotropin-releasing factor in pain and analgesia. *Pain.* 2000; 84:1–12. [PubMed: 10601667]
- Lewis K, Li C, Perrin MH, Blount A, Kunitake K, Donaldson C, Vaughan J, Reyes TM, Gulyas J, Fischer W, Bilezikjian L, Rivier J, Sawchenko PE, Vale WW. Identification of urocortin III, an

- additional member of the corticotropin-releasing factor (CRF) family with high affinity for the CRF2 receptor. *Proc Natl Acad Sci U S A*. 2001; 98:7570–7575. [PubMed: 11416224]
- Lovenberg TW, Liaw CW, Grigoriadis DE, Clevenger W, Chalmers DT, De Souza EB, Oltersdorf T. Cloning and characterization of a functionally distinct corticotropin-releasing factor receptor subtype from rat brain. *Proc Natl Acad Sci U S A*. 1995; 92:836–840. [PubMed: 7846062]
- Manuel R, Metz JR, Flik G, Vale WW, Huising MO. Corticotropin-releasing factor-binding protein (CRF-BP) inhibits CRF- and urotensin-I-mediated activation of CRF receptor-1 and -2 in common carp. *Gen Comp Endocrinol*. 2014; 202:69–75. [PubMed: 24769042]
- Martinez V, Tache Y. CRF1 receptors as a therapeutic target for irritable bowel syndrome. *Curr Pharm Des*. 2006; 12:4071–4088. [PubMed: 17100612]
- Martinez V, Wang L, Million M, Rivier J, Tache Y. Urocortins and the regulation of gastrointestinal motor function and visceral pain. *Peptides*. 2004a; 25:1733–1744. [PubMed: 15476940]
- Martinez V, Wang L, Rivier J, Grigoriadis D, Tache Y. Central CRF, urocortins and stress increase colonic transit via CRF1 receptors while activation of CRF2 receptors delays gastric transit in mice. *J Physiol*. 2004b; 556:221–234. [PubMed: 14755002]
- Martinez V, Wang L, Rivier JE, Vale W, Tache Y. Differential actions of peripheral corticotropin-releasing factor (CRF), urocortin II, and urocortin III on gastric emptying and colonic transit in mice: role of CRF receptor subtypes 1 and 2. *J Pharmacol Exp Ther*. 2002; 301:611–617. [PubMed: 11961064]
- McLean SA, Williams DA, Stein PK, Harris RE, Lyden AK, Whalen G, Park KM, Liberzon I, Sen A, Gracely RH, Baraniuk JN, Clauw DJ. Cerebrospinal fluid corticotropin-releasing factor concentration is associated with pain but not fatigue symptoms in patients with fibromyalgia. *Neuropsychopharmacology*. 2006; 31:2776–2782. [PubMed: 16936702]
- Merchenthaler I, Hynes MA, Vigh S, Shally AV, Petrusz P. Immunocytochemical localization of corticotropin releasing factor (CRF) in the rat spinal cord. *Brain Res*. 1983; 275:373–377. [PubMed: 6194860]
- Million M, Wang L, Wang Y, Adelson DW, Yuan PQ, Maillot C, Coutinho SV, McRoberts JA, Bayati A, Mattsson H, Wu V, Wei JY, Rivier J, Vale W, Mayer EA, Tache Y. CRF2 receptor activation prevents colorectal distension induced visceral pain and spinal ERK1/2 phosphorylation in rats. *Gut*. 2006; 55:172–181. [PubMed: 15985561]
- Nakazawa T, Ikeda M, Kaneko T, Yamatsu K. Analgesic effects of dynorphin-A and morphine in mice. *Peptides*. 1985; 6:75–78. [PubMed: 2859574]
- Nielsen M, Ogenae N, Meulemans A, Coulie B. Divergent role for CRF1 and CRF2 receptors in the modulation of visceral pain. *Neurogastroenterol Motil*. 2005; 17:423–432. [PubMed: 15916630]
- Nothnick WB. Novel targets for the treatment of endometriosis. *Expert Opin Ther Targets*. 2004; 8:459–471. [PubMed: 15469395]
- Okamoto K, Tashiro A, Chang Z, Thompson R, Bereiter DA. Temporomandibular joint-evoked responses by spinomedullary neurons and masseter muscle are enhanced after repeated psychophysical stress. *Eur J Neurosci*. 2012; 36:2025–2034. [PubMed: 22519876]
- Okano K, Kuraishi Y, Satoh M. Effects of repeated cold stress on aversive responses produced by intrathecal excitatory amino acids in rats. *Biol Pharm Bull*. 1995; 18:1602–1604. [PubMed: 8593488]
- Perrin M, Donaldson C, Chen R, Blount A, Berggren T, Bilezikjian L, Sawchenko P, Vale W. Identification of a second corticotropin-releasing factor receptor gene and characterization of a cDNA expressed in heart. *Proc Natl Acad Sci U S A*. 1995; 92:2969–2973. [PubMed: 7708757]
- Poree LR, Dickenson AH, Wei ET. Corticotropin-releasing factor inhibits the response of trigeminal neurons to noxious heat. *Brain Res*. 1989; 502:349–355. [PubMed: 2819471]
- Przewlocki R, Stala L, Greczek M, Shearman GT, Przewlocka B, Herz A. Analgesic effects of mu-, delta- and kappa-opiate agonists and, in particular, dynorphin at the spinal level. *Life Sci*. 1983; 33(Suppl 1):649–652. [PubMed: 6141505]
- Quintero L, Cuesta MC, Silva JA, Arcaya JL, Pinerua-Suhaibar L, Maixner W, Suarez-Roca H. Repeated swim stress increases pain-induced expression of c-Fos in the rat lumbar cord. *Brain Res*. 2003; 965:259–268. [PubMed: 12591144]

- Quintero L, Moreno M, Avila C, Arcaya J, Maixner W, Suarez-Roca H. Long-lasting delayed hyperalgesia after subchronic swim stress. *Pharmacol Biochem Behav.* 2000; 67:449–458. [PubMed: 11164071]
- Quintero RA, Quintero LF, Chmait R, Gomez Castro L, Korst LM, Fridman M, Kontopoulos EV. The quantitative lung index (QLI): a gestational age-independent sonographic predictor of fetal lung growth. *Am J Obstet Gynecol.* 2011; 205:544 e541–548. [PubMed: 21944224]
- Reul JM, Holsboer F. Corticotropin-releasing factor receptors 1 and 2 in anxiety and depression. *Curr Opin Pharmacol.* 2002; 2:23–33. [PubMed: 11786305]
- Reyes TM, Lewis K, Perrin MH, Kunitake KS, Vaughan J, Arias CA, Hogenesch JB, Gulyas J, Rivier J, Vale WW, Sawchenko PE. Urocortin II: a member of the corticotropin-releasing factor (CRF) neuropeptide family that is selectively bound by type 2 CRF receptors. *Proc Natl Acad Sci U S A.* 2001; 98:2843–2848. [PubMed: 11226328]
- Rivier J, Gulyas J, Kirby D, Low W, Perrin MH, Kunitake K, DiGruccio M, Vaughan J, Reubi JC, Waser B, Koerber SC, Martinez V, Wang L, Tache Y, Vale W. Potent and long-acting corticotropin releasing factor (CRF) receptor 2 selective peptide competitive antagonists. *J Med Chem.* 2002; 45:4737–4747. [PubMed: 12361401]
- Robbins MT, Ness TJ. Footshock-induced urinary bladder hypersensitivity: role of spinal corticotropin-releasing factor receptors. *J Pain.* 2008; 9:991–998. [PubMed: 18632307]
- Saruta M, Takahashi K, Suzuki T, Torii A, Kawakami M, Sasano H. Urocortin 1 in colonic mucosa in patients with ulcerative colitis. *J Clin Endocrinol Metab.* 2004; 89:5352–5361. [PubMed: 15531481]
- Sivri A, Cindas A, Dincer F, Sivri B. Bowel dysfunction and irritable bowel syndrome in fibromyalgia patients. *Clin Rheumatol.* 1996; 15:283–286. [PubMed: 8793261]
- Song ZH, Takemori AE. Involvement of spinal kappa opioid receptors in the antinociception produced by intrathecally administered corticotropin-releasing factor in mice. *J Pharmacol Exp Ther.* 1990; 254:363–368. [PubMed: 2166788]
- Song ZH, Takemori AE. Antagonism of morphine antinociception by intrathecally administered corticotropin-releasing factor in mice. *J Pharmacol Exp Ther.* 1991; 256:909–912. [PubMed: 1848633]
- Sperber AD, Atzmon Y, Neumann L, Weisberg I, Shalit Y, Abu-Shakrah M, Fich A, Buskila D. Fibromyalgia in the irritable bowel syndrome: studies of prevalence and clinical implications. *Am J Gastroenterol.* 1999; 94:3541–3546. [PubMed: 10606316]
- Suarez-Roca H, Leal L, Silva JA, Pinerua-Shuhaibar L, Quintero L. Reduced GABA neurotransmission underlies hyperalgesia induced by repeated forced swimming stress. *Behav Brain Res.* 2008; 189:159–169. [PubMed: 18255166]
- Suarez-Roca H, Quintero L, Arcaya JL, Maixner W, Rao SG. Stress-induced muscle and cutaneous hyperalgesia: differential effect of milnacipran. *Physiol Behav.* 2006a; 88:82–87. [PubMed: 16624346]
- Suarez-Roca H, Silva JA, Arcaya JL, Quintero L, Maixner W, Pinerua-Shuhaibar L. Role of mu-opioid and NMDA receptors in the development and maintenance of repeated swim stress-induced thermal hyperalgesia. *Behav Brain Res.* 2006b; 167:205–211. [PubMed: 16214233]
- Telegdy G, Adamik A, Toth G. The action of urocortins on body temperature in rats. *Peptides.* 2006; 27:2289–2294. [PubMed: 16650509]
- Turek VF, Ryabinin AE. Ethanol versus lipopolysaccharide-induced hypothermia: involvement of urocortin. *Neuroscience.* 2005; 133:1021–1028. [PubMed: 15964490]
- van Gaalen MM, Stenzel-Poore MP, Holsboer F, Steckler T. Effects of transgenic overproduction of CRH on anxiety-like behaviour. *Eur J Neurosci.* 2002; 15:2007–2015. [PubMed: 12099906]
- Veale D, Kavanagh G, Fielding JF, Fitzgerald O. Primary fibromyalgia and the irritable bowel syndrome: different expressions of a common pathogenetic process. *Br J Rheumatol.* 1991; 30:220–222. [PubMed: 2049586]
- Wacnik PW, Kehl LJ, Trempe TM, Ramnaraine ML, Beitz AJ, Wilcox GL. Tumor implantation in mouse humerus evokes movement-related hyperalgesia exceeding that evoked by intramuscular carrageenan. *Pain.* 2003; 101:175–186. [PubMed: 12507712]

- Westphal NJ, Seasholtz AF. CRH-BP: the regulation and function of a phylogenetically conserved binding protein. *Front Biosci.* 2006; 11:1878–1891. [PubMed: 16368564]
- Yakabi K, Noguchi M, Ohno S, Ro S, Onouchi T, Ochiai M, Takabayashi H, Takayama K, Harada Y, Sadakane C, Hattori T. Urocortin 1 reduces food intake and ghrelin secretion via CRF(2) receptors. *Am J Physiol Endocrinol Metab.* 2011; 301:E72–82. [PubMed: 21540451]
- Zorrilla EP, Tache Y, Koob GF. Nibbling at CRF receptor control of feeding and gastrocolonic motility. *Trends Pharmacol Sci.* 2003; 24:421–427. [PubMed: 12915052]

Fig. 1.

The effects produced by 10 μ g of either urocortin I, urocortin II or CRF compared to their respective vehicle-injected controls. The area under the curve of von Frey fiber responses (panels A–D) and grip force (panels E–H) in response to CRF analogs from 0 to 24 h are summarized in these bar graphs to allow easier comparison of the magnitude of their effects when delivered by different routes. The effect of urocortin I (10 μ g) at a dose that was active when injected i.t., is compared to that when injected (in 200 μ l) subcutaneously (s.c.) or intraperitoneally (i.p.). The effect of these systemic urocortin I injections on von Frey fiber (D) and grip force (H) responses peaked 8 h after injection and resolved by 24 h. An asterisk indicates a significant difference ($P < 0.05$) in the area under the curve when that group is compared to its appropriate vehicle-injected control using Student's t-test with a cutoff for significance of $P < 0.05$. The number of mice/group is indicated at the bottom of each column.

Fig. 2.

Dose-response relationships of urocortin I on von Frey fiber responses, grip force and the ability of morphine to inhibit these effects. Each panel depicts the change in magnitude of the value compared to each group's control (baseline) values prior to injection. Panels on the left depict the mean ($\pm$ SEM) responses to von Frey fibers (A) and grip force (C) following three doses of urocortin I (1, 3, and 10 μ g) compared to 5 μ l of vehicle. Although each dose and its respective control group were examined on different days, vehicle-injected mice did not differ from one experiment to the next and were, therefore, pooled for graphical representation. An asterisk indicates a significant difference ($P < 0.05$) when compared to its own vehicle-injected controls as determined using one-way ANOVA followed by the Newman-Keuls post hoc analysis at specific times. The panels on the right show the ability of increasing doses of morphine (injected 6 h after 10 μ g of urocortin I) to inhibit the effect of urocortin I on von Frey fiber responses (B) and grip force (D) when tested 30 min after its injection. An asterisk indicates a significant difference ($P < 0.05$) between the treatments indicated as determined using one-way ANOVA followed by the Newman-Keuls post hoc

analysis. The number of mice/group is indicated in the legend (A, C) or at the bottom of the columns (B, D).

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Fig. 3.

Responses measured in the von Frey fiber assay to repeated injections of urocortin I. Urocortin I was injected daily at 1 μ g i.t., or at a dose of 10 μ g i.t. at 3–4 day intervals, as indicated by arrows. The number of von Frey fiber responses was depicted at 8 h (A) and at 24 h (B) after every injection to monitor the peak and duration of effects of urocortin I after repeated injections. An asterisk by the curve indicates a significant difference ($P < 0.05$) in responses between that treatment group and its vehicle-injected control group when comparing the areas under their curves using one-way ANOVA and the Newman-Keuls post hoc analysis. The pound sign (#) indicates a significant difference between the response after the first and fifth injection of 1 μ g of urocortin I (B). Controls did not differ from each other on the two test days and were therefore pooled for graphical representation. The number of mice/group is indicated in the legend.

Fig. 4.

Effects of urocortin I, urocortin II and CRF injected either intracerebroventricularly (i.c.v.), intrathecally (i.t.), subcutaneously (s.c.) or intraperitoneally (i.p.) on the mean ($\pm$ SEM) body temperature (4 h, at peak effect) and body weight (24 h, at peak effect). Each panel depicts the change in magnitude of the value compared to each group's control (baseline) values prior to injection. Control and the three treatment groups were compared statistically using one-way ANOVA followed by the Newman-Keuls post hoc analysis to determine significant differences between treatment groups (A, B, E, F) or using Student's unpaired t-test (C, D, G, H). An asterisk indicates a significant difference ($P < 0.05$) when compared to vehicle-injected controls. The number of mice/group is indicated at the bottom of each column.

Fig. 5.

Effects of CRF antagonists on von Frey fiber responses (A, B, C), grip force (D, E, F) and body temperature (G, H, I). Each panel depicts the change in magnitude of the value compared to each group's control (baseline) values prior to injection. The mean grip force and von Frey fiber responses shown are taken during the peak effect on mechanical sensitivity (where the value for each animal was the average of those taken at 4 and 8 h after injection) to capture the optimal hyperalgesic effect of 3 or 10 μ g of urocortin I when injected with non-selective or selective antagonists, respectively. Body temperature is shown at 4 h after injection, the time of its optimal hypothermic effect. Preliminary studies showed that 10 μ g of astressin, a non-selective CRF receptor antagonist, 50 μ g of NBI-35965, a CRF1 antagonist and 10 μ g of astressin 2B, a CRF2 antagonist had no effect when injected alone at this dose and by this route. The effects of CRF antagonists were analyzed using one-way ANOVA followed by the Newman-Keuls post hoc analysis. An asterisk indicates a significant difference between the groups indicated ($P < 0.05$). The number of mice/group is indicated at the bottom of each column.