Published in final edited form as:

Neuroscience. 2007 June 8; 146(4): 1888–1905.

Chronic low dose ovine corticotropin releasing factor or urocortin II into the rostral dorsal raphe alters exploratory behavior and serotonergic gene expression in specific subregions of the dorsal raphe

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Abstract

Corticotropin releasing factor (CRF) family peptides play key roles in integrating neural responses to stress. Both major CRF receptors have been pharmacologically identified in the dorsal raphe nucleus (DRN), a stress sensitive and internally heterogeneous nucleus supplying many forebrain regions with serotonergic input. Despite the involvement of chronic stress and serotonergic dysfunction in human mood and anxiety disorders, little is known about the effects of chronic CRF receptor activation on the DRN. We infused ovine CRF (1ng/hr), urocortin II (UCNII, 1ng/hr), or vehicle alone into rat DRN over 6 days. During infusion, animals were allowed to freely explore an open field for 15 minutes on each of two days, with the addition of a novel object on the second day. Following behavioral testing, 5-HT_{1A}, 5-HT_{1B}, serotonin transporter (SERT), and tryptophan hydroxylase-2 (*Tph2*) expression were examined through the DRN by in situ hybridization. Ovine CRF infusion resulted in significantly decreased novel object touches, climbs, as well as increased latency to first novel object contact. UCNII had a similar but less dramatic effect, decreasing only climbing behavior. Both ovine CRF and UCNII blunted the decrease in corner time expected on re-exposure to the open field. Both peptides also produced regionally specific changes in gene expression: 5-HT_{1A} expression was increased 30% in the mid-rostral ventromedial DRN, while SERT was decreased by 30% in the mid-caudal shell dorsomedial DRN. There also appeared to be a shift in the relative level of *Tph2* expression between the ventromedial and core dorsomedial DRN at the mid-rostral level. Changes in 5-HT_{1A}, SERT, and relative *Tph2* mRNA abundance were correlated with novel object exploration. These findings suggest chronic intra-DRN administration of CRF agonists decreases exploratory behavior, while producing subregionally limited changes in serotonergic gene expression. These studies may be relevant to mechanisms underlying behavioral changes after chronic stress.

Keywords

dorsomedial; in situ hybridization histochemistry; open field; novel object; serotonin transporter; ventromedial

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Section Editor:

Systems Neuroscience

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Depression and anxiety disorders are among the leading causes of morbidity, mortality, and disability in the United States (Greenberg et al., 2003). They are often comorbid, and both are highly associated with chronic exposure to stress. Multiple lines of evidence suggest that chronic and inescapable stress produce long lasting effects on brain function and behavior that play a prominent role in the development, maintenance, and recurrence of both depression and a variety of anxiety disorders (Gulley and Nemeroff, 1993, Arborelius et al., 1999, Ressler and Nemeroff, 2000).

Altered serotonin neurotransmission appears to be a central mechanism inducing both depressive (Maes and Meltzer, 1995) and anxiety disorders (Ressler and Nemeroff, 2000). The majority of serotonergic fibers to forebrain regions thought to be involved in mood and anxiety regulation arise from the dorsal raphe nucleus (DRN) and median raphe nucleus (MRN) (Azmitia and Segal, 1978, Jacobs and Azmitia, 1992). While projections from DRN and MRN overlap, there is a specific somatotopic organization of these serotonergic projections. In addition, the DRN displays a complex anatomic organization and cellular morphology over its considerable anteroposterior extent. Although the DRN has been recognized as being topographically organized, most previous studies of its function have tended to regard the DRN as a single homogenous structure. DRN can be divided into subregions: ventromedial and dorsomedial regions over much of its length, and dorsolateral wings in the mid-regions (Steinbusch et al., 1981, Descarries et al., 1982, Molliver, 1987). The expression of key genes associated with serotonergic neuronal function differs as a function of subregional localization (Clark et al., 2006). Multiple studies have suggested that these subdivisions differ in cellular and anatomic morphology (Molliver, 1987, Jacobs and Azmitia, 1992) and connectivity (O'Hearn and Molliver, 1984, Peyron et al., 1998, Lee et al., 2003). Furthermore, DRN responds to stress in a complex manner, increasing serotonin release in some terminal fields while decreasing it in others (Kirby et al., 1995).

The effects of stress on the DRN have been hypothesized to involve the stress-related neuropeptide corticotropin releasing factor (CRF). Indeed, neurons in different subregions of the DRN respond to CRF in a divergent manner depending on the subregion and location along the anteroposterior axis (Amat et al., 1998, Price et al., 1998, Kirby et al., 2000, Lowry et al., 2000, Hammack et al., 2002, Price et al., 2002, Pernar et al., 2004). However, with the exception of post-traumatic stress disorder, single acute stressor exposure rarely induces the development of psychiatric illness. Rather, it is chronic stress that is associated with the human anxiety and depressive disorders (Kessler et al., 1985, Gulley and Nemeroff, 1993).

Yet, despite the clinical linkage between chronic stress and depression, almost nothing is known about effects of chronic exposure to CRF on the serotonin system. A single study reported that chronic intracerebroventricular administration of CRF reduced 5-HT response to an inflammatory stressor (Linthorst et al., 1997). Nothing has been reported regarding the effects of chronic CRF receptor activation in the DRN on serotonergic function or behavior. To better understand the role of chronic CRF receptor activation in the DRN on behavior and serotonergic function, we have examined the effect of chronic intra-DRN infusion of very low dose ovine CRF (~8-fold selective CRF₁ agonist), urocortin II (~100-fold selective CRF₂ agonist) or vehicle on behavior in the open field both with and without novel object. We recently used this methodology to assess both thigmotaxis and exploratory drive (Hoplight et al., 2005). Our working hypothesis was that chronic infusion of CRF in the DRN would produce alterations in at least a subset of behaviors related to anxiety and motivational state that have been associated with chronic stress. If true, then chronic CRF exposure in the DRN could be used to develop mechanistic models for behavioral alterations due to chronic stress. Following behavioral testing, expression of four serotonergic genes through the DRN was assessed through quantitative in situ hybridization histochemistry (ISHH). In part because 5-HT levels

in varying DRN terminal fields may show increases, decreases, or no change at all following swim stress (Kirby et al., 1995), we hypothesized that chronic exposure to low-dose CRF agonists would alter expression of tryptophan hydroxylase-2 (*Tph2*), the recently identified brain-specific form of the rate limiting enzyme for serotonin synthesis (Walther et al., 2003, Patel et al., 2004, Zhang et al., 2004), as well as other serotonergic genes in a subregion-specific manner.

Experimental Procedures

Surgery and animal care

Throughout this study, male Sprague-Dawley rats (250 g, Charles River Laboratories, Wilmington, MA, USA) were group housed with lights on at 6AM and off at 6PM local time. Animals were allowed to acclimatize for at least 6 days after arrival prior to surgery. For minipump implantation, rats were anesthetized with isoflurane in oxygen 2–3% (Webster Veterinary Supply, Wilmington, MA, USA), scalp fur shaved, the animal placed in a Stoelting stereotaxic device, and the surgical site cleaned with povidone-iodine solution. In order to reduce deafness due to earbar trauma, 45° earbars were used in all cases. Following scalp incision, skull landmarks were visualized. Between the shoulder blades, a subcutaneous space for the osmotic minipumps was opened using blunt dissection. Intended injection location from bregma was AP –7.5, lateral –0.2, and depth –6.4 approached from 0° angle. A small hole was bored at the site of injection, the superior sagittal sinus was retracted, the dura punctured with a 25G needle, and the cannula slowly advanced to final depth. Two surgical stainless steel screws were placed adjacent to the cannula base and all three were embedded in dental acrylic. Once the dental acrylic hardened, the osmotic minipump connected to the cannula base was placed in the subcutaneous space. The skin was closed with 4-0 nylon monofilament sutures (Ethicon, Somerville, NJ, USA) and surgical methylacrylate glue (VetBond, 3M, St. Paul, MN, USA), given 25µg of buprenorphine (Sigma-Aldrich, St. Louis, MO, USA) subcutaneously once after surgery for pain control, and the rats were watched until they recovered spontaneous movement.

Following behavioral testing, animals were deeply narcotized via CO₂ inhalation, decapitated, and the brains rapidly frozen on dry ice. All animal procedures were approved by this institution's Animal Care Committee and handled in accordance with NIH guidelines. All efforts were made to reduce animal pain, stress, and suffering to the maximum extent practical.

Osmotic minipump preparation

Osmotic minipumps (Alzet model 1007D) delivering 0.5µL/hr for 7 days were prepared 24 hours before use under aseptic conditions. Pumps were filled with oCRF (Phoenix peptides, Burlingame, CA, USA), UCNII (Phoenix peptides), or artificial cerebrospinal fluid (ACSF) vehicle (Price and Lucki, 2001), with the peptides at a concentration of 2ng/µL. In one experiment, pumps were filled with oCRF or ACSF vehicle at a concentration of 200ng/µL for a delivery rate of 100ng/hr. Pumps were connected to 30G infusion cannulae (Plastics One, Roanoke, VA, USA) using prefilled silastic tubing as recommended by the manufacturer (Alzet Osmotic Pumps, Durect Corp., Cupertino, CA, USA) and primed by emersion in sterile 0.9% saline at 37°C overnight. Delivery of drug was confirmed by determining residual volume in the pump at the termination of the experiment.

Peptide Stability

Osmotic minipumps (Alzet model 1007D) were filled with either oCRF or UCNII at a concentration of 1µg/µL. This concentration was chosen in order to allow direct detection of the peptide by UV absorption, as attempts to use electrochemical detection after derivitization in order to detect peptides at lower concentrations were unsuccessful. After filling, pumps were

placed under sterile conditions at 37°C for 6 days. Aliquots were taken from the pumps at filling and after 6 days, and frozen at -70°C until analysis. HPLC was performed under contract with Phoenix Peptides using a Shimadzu analytical HPLC. Separation was performed on a Develosil 5 ODS-HG 140A 4.6mm × 250mm C18 column using reverse phase advancing from 0% to 60% acetonitrile in 0.1% trifluoroacetic acid over 40 minutes. Detection was performed by absorption at 220nm with oCRF eluting at 30.8 minutes and UCNII eluting at 32.8 minutes. Total area under the curve for the oCRF and UCNII peaks was calculated by Shimadzu analytical software and compared between samples before and after incubation to yield a percent intact after 6 days.

Open field testing with novel object

Apparatus—The open field consisted of a 1 m × 1 m × 25 cm box made of black Plexiglas coated with a rubberized matte black finish to reduce glare. A 4 × 6 cm rectangular hole was located in the center of one wall 1 cm above the floor; this connected to a clear Plexiglas start box (9 × 6 × 6 cm) with a grid floor and two steel guillotine doors on either side (Coulbourn Instruments, Allentown, PA). The door leading to the open field was opened and closed from the adjoining room by a manual pulley system which was baffled to minimize sound transmission. A low-light camera was suspended 200 cm above the field. The camera was attached to a VCR and then to a computer; data was acquired using SMARTTM tracking software program (San Diego Instruments, San Diego, CA). The room was illuminated by a dim (15 W) red light. The computer and animals not being currently tested were kept in separate adjoining rooms.

Procedure—The behavioral testing was a two-day procedure. All animals were tested between 1200 and 1700. On each day the animals were brought up from the vivarium, weighed, and placed in an adjoining room 90 min before testing. The animals were not disturbed during the hour before testing began. The apparatus was cleaned with water and disinfectant (Process NPD; Steris, St. Louis, MO) and completely dried before the start of the test and between each animal. To begin the trial, each animal was placed in the start box for two minutes, after which the guillotine door was opened, allowing entrance to the open field. The latency to exit the start box (sec) was recorded. One animal in the oCRF group did not leave the start box after 15 minutes, it was removed from the start box and excluded from the study. Once the rat entered the open field, the guillotine door shut, and the animal was allowed 15 minutes to explore the field. On the first day the field was empty. The path of the animal was recorded by videotape and by using videotracking software (SMART, San Diego Instruments). The following parameters were calculated from this recording: distance traveled; time spent in the corners, in the center, or in the entry square. The number of fecal boli was manually counted. At the end of the trial, the rat was gently removed and returned to its home cage.

On the second day a small object, a 100-ml orange-capped Pyrex bottle filled halfway with water, was fastened in the open field at the center. Each animal was re-exposed to the open field and measures identical to day 1 were recorded. Time elapsed to first contact as well as the number of investigations of the novel object were recorded by an observer blind to the experimental group of the subject. Novel object contacts were counted as “touches” (examination of the object with the nose and/or a single paw) and “climbs” (climbs onto the object with one or more paws, or examination of the object with both paws simultaneously). Additionally, the differences between days were analyzed by subtracting the values on day 2 from the values on day 1. This provided an index of how each group’s behavior changed on the second day. It should be emphasized that this procedure is not the same as the novel object recognition task that involves multiple objects presented on subsequent days.

In situ hybridization histochemistry

Using a Leica Jung CM3000 cryostat, serial 20µm coronal brain sections across the A-P axis of DRN and MRN were prepared and mounted on Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA, USA). Brains from animals treated with 100ng/hr oCRF were not available for in situ hybridization because the brains were employed for a separate pilot study. The sections from brains treated with 1ng/hr oCRF, UCNII, or vehicle alone were stored at -70°C until processed for in situ hybridization histochemistry. In brief, tissue sections were thawed at room temperature and fixed in cold 4% paraformaldehyde. After rinsing in phosphate-buffered saline, sections were treated with acetic anhydride (0.25% in 0.1 M triethanolamine), dehydrated, delipidated, and air-dried.

In situ hybridization histochemistry for 5-HT_{1A}, 5-HT_{1B}, and SERT was performed using synthetic oligonucleotides as described previously (Neumaier et al., 1996, Neumaier et al., 1997, Neumaier et al., 2000, Clark et al., 2006). For the 5-HT_{1B} studies, three oligonucleotides corresponding to base pairs 1343–1382, 1630–1668, and 1790–1829 of the rat 5-HT_{1B} clone, MG11B (Hamblin et al., 1992) were used. For 5-HT_{1A} probes, one oligonucleotide corresponding to base pairs 1110–1151 was used (Neumaier et al., 2000). For SERT probes, two oligonucleotides corresponding to base pairs 101 to 149 and 570 to 618 of the rat serotonin transporter sequence were used (Blakely et al., 1991). Probes were individually labeled with γ [³³P]-dATP (Amersham Biosciences, Piscataway, NJ, USA) using terminal deoxyribonucleotidyl transferase (Promega, Madison, WI, USA) and purified on NENSORB columns (Dupont NEN Research Products, Boston, MA). Specific activity of each oligonucleotide probe was 3–7 Ci/pmol. Labeled probes were diluted (2 pmol/ml) in a hybridization buffer containing 50% formamide, 10% dextran sulfate, 0.3 M sodium chloride, 10 mM Tris (pH 8.0), 1 mM EDTA, 1x Denhardt's (0.2% each of bovine serum albumin, Ficoll, and polyvinylpyrrolidone), 0.4 mg/ml yeast tRNA, and 10 mM dithiothreitol. Fifty microliters of the hybridization mixture was applied to each slide, and the sections were covered with HybriSlips (Sigma). The slides were incubated in moist, covered trays at 37°C overnight. Following the hybridization reaction, coverslips were removed and the slides were washed twice in 1x SSC (150 mM NaCl in 15 mM sodium citrate) for 30 minutes at 55°C (58°C for SERT), and twice again in 1x SSC at room temperature for 30 minutes. The slides were rinsed in distilled water, dehydrated through a series of graded alcohol rinses containing 300 mM ammonium acetate, and air dried. Autoradiographic signal was detected using a Cyclone storage phosphor scanner (Packard Instruments, Meridian, CT) at 600 dpi resolution and were stored on CD-ROM disks; exposure times were 24–26 h (Neumaier et al., 2000, Clark et al., 2006).

Tph2 in situ hybridization histochemistry was performed essentially as previously described (Clark et al., 2006). Briefly, riboprobes were prepared from a SacI fragment of a partial cDNA clone of *Tph2* kindly provided by Dr. Michael Bader (Max-Delbrück Center, Berlin, Germany). Riboprobes were transcribed using α [³³P]-UTP (Amersham) constituting 65% of total UTP and either T7 (antisense) or SP6 (sense) RNA polymerase as appropriate (both from Promega or New England Biolabs, Ipswich, MA, USA). Labeling probes were purified from unlabeled nucleotides using LiCl precipitation and diluted (1.5 pmol/ml) in a hybridization buffer (50% formamide, 10% dextran sulfate, 0.3 M sodium chloride, 10 mM Tris (pH 8.0), 1 mM EDTA, 1x Denhardt's (0.2% each of bovine serum albumin, Ficoll, and polyvinylpyrrolidone), 0.4 mg/ml yeast tRNA, 200 mM dithiothreitol). Sections were hybridized as described for oligonucleotide probes above. The slides were washed once in RNase wash buffer (500 mM NaCl, 10 mM Tris, 1 mM EDTA, pH 8.0) containing 4µg/ml RNase A for 30 minutes at 37°C, then again for 30 minutes at 37°C in RNase wash buffer without RNase A. Slides were then washed twice in 0.1x SSC (15 mM NaCl in 1.5 mM sodium citrate) for 30 minutes at 60°C, and twice again in 0.1x SSC at room temperature for 30 minutes. The slides were rinsed,

and following air drying, autoradiographic signal was detected, all as described above for oligonucleotide probes.

Densitometry and data analysis

In situ hybridization signal was quantified using a computer-based densitometry system (MCID, Imaging Research, St. Catherine's, ON). DRN subregions and the MRN were examined at six levels on the A-P axis as previously described (Clark et al., 2006): Far-rostral (−7.1mm bregma), rostral (−7.5mm bregma), mid-rostral (−7.8mm bregma), mid-caudal (−8.1mm bregma), and caudal (−8.6mm bregma). Intensity of hybridization signal (measured in arbitrary digital light units) was standardized using ¹⁴C-plastic standard sections co-exposed on each phosphor screen; these yielded a linear relationship between standardized radioactivity and measured signal intensity. Hybridization signal was determined from three to four consecutive brain sections. These were averaged and matching tissue background was subtracted for each region in each brain. Due to loss of tissue during slicing and processing, two animals in the vehicle and oCRF groups and one from the UCNII group that were behaviorally scored were not available for in situ analysis. Due to in situ artifacts, in a few cases it was not possible to accurately sample a particular in situ probe for one or more subregions at a particular A-P level. This is reflected in the statistics presented.

Both behavioral and in situ hybridization data were analyzed using two way ANOVA with post-hoc testing using Fisher's LSD with $p \leq 0.05$ considered significant between treatment groups. Correlations between novel object exploratory behavior and in situ hybridization results were calculated using a Correlation z-test with $p \leq 0.05$ considered significant. All statistics were performed on commercially available software (StatView 5).

Results

Mean cannula placement and peptide stability

To better understand the role in stress related behavior of chronic exposure to CRF receptor activation in the DRN, very low dose ovine CRF (oCRF, a ~8-fold selective CRF₁ agonist (Lovenberg et al., 1995)), urocortin II (UCNII, a ~100-fold selective CRF₂ agonist (Hsu and Hsueh, 2001, Reyes et al., 2001)) or vehicle were infused for 6 days. Because a single dose of 1ng was found to be the smallest intraraphe dose of oCRF and UCNII effective in altering DRN discharge (Kirby et al., 2000, Pernar et al., 2004) an infusion rate of 1ng/hr was selected for both peptides in order to produce chronic low levels of DRN activation or inhibition.

Using stereotaxic technique, cannulas attached to osmotic minipumps were placed immediately adjacent to the DRN infusing either oCRF, UCNII or vehicle for 6 days (each at 0.5 μ L/hr as this was the smallest infusion volume per hour available in a 7 day minipump). Following in situ hybridization histochemistry, sections were stained with cresyl violet and the location of the infusion cannula (using the atlas of Paxinos and Watson, 1997) determined by an observer blinded to treatment status. Mean cannula placement on the A-P axis did not differ between treatment groups, and the distribution of placements into the DRN was similar (Figure 1). To confirm delivery of the peptide solution, residual volume in each pump was determined and all pumps were found to have functioned normally (data not shown).

In order to determine the degree to which the peptide remained intact over the duration of the infusion, an additional study was performed. Peptide solutions were placed in osmotic minipumps for 6 days at 37°C. HPLC elution profiles of oCRF and UCNII after 6 days of incubation were compared to aliquots frozen before incubation, using total area under the curve for each peptide's elution peak. 61% of the UCN II and 42% of the oCRF remained intact after 6 days. These results suggest that while peptide concentrations decreased by about half over

the course of the infusion, at the end of the 6 days the pumps still contained considerable intact oCRF or UCNII.

Open Field and Novel Object Exploratory Behavior

In order to obtain behavioral data relevant to anxiety with minimal experimental stress, we utilized free exploration of an open field with and without a novel object. This procedure was recently validated as a measure of both anxiety, as assessed by thigmotaxis, as well as exploratory drive (Hoplight et al., 2005). After 5 days of continuous DRN infusion, animals were allowed to freely enter and explore a 1m² open field for 15 minutes. The next day animals were again allowed to enter and freely explore the same open field, to which a novel object had been added. In addition to automated movement tracking, latency to first novel object contact, as well as numbers of touches (touch with nose or paw) and climbs (two or more paws touching), was assessed by a blinded rater. Since the assay assesses the animal's self-initiated and maintained exploration, minimum experimental stress would seem to be involved.

With initial exposure to the open field on day 1, there were no differences between treatment groups for any parameter measured (Figure 2A–F). However, on re-exposure to the open field and a novel object on day 2, oCRF and UCNII treated animals displayed altered behavior. Normally on re-exposure to a familiar open field, rats spend less time in the corners of the open field. In this study, on re-exposure to the open field, vehicle infused rats spent 100 fewer seconds in the corners than on initial exposure. In contrast, on re-exposure to the open field, oCRF and UCNII infused animals spent nearly as much time in the corners as on initial exposure (Figure 2F, Fishers PLSD vs. vehicle, $p < 0.05$). There were no statistically significant differences between treatment groups for day 1, day 2, or the difference between the two for total distance, exit latency, entry square time, fecal boli, or center time (Figure 2A–E).

Behavior in regard to novel object exploration was markedly altered. Compared to animals infused with vehicle, animals chronically infused with oCRF into the rostral DRN displayed a 2.5 fold increase in latency to first novel object contact (Figure 3A, Fisher's PLSD $p < 0.05$), 30% fewer novel object touches (Figure 3B, Fisher's PLSD $p < 0.05$), 50% fewer climbs (Figure 3C, Fisher's PLSD $p < 0.01$), and 35% fewer total novel object contacts (Figure 3D, Fisher's PLSD $p < 0.05$). UCNII had a similar but less dramatic effect, decreasing only the incidence of the more intense exploratory climbs (Figure 3C, Fisher's PLSD $p < 0.01$) while having no effect on simple touches of the novel object (Figure 3B). There was a trend towards a decrease in the total novel object contacts (Figure 3D, Fisher's PLSD $p < 0.10$), though this could have been driven by the consistent decrease in climbing behavior alone. Overall, these results are in keeping with the findings from corner time differences, where UCNII had similar though less profound effects than oCRF.

In situ hybridization histochemistry

Following behavioral testing, brains were sectioned through the rostro-caudal extent of the DRN and in situ hybridization histochemistry for 5-HT_{1A}, 5-HT_{1B}, SERT, and *Tph2* was performed using oligonucleotide probes end labeled with ³³P-dATP (5-HT_{1A}, 5-HT_{1B}, SERT), or a riboprobe incorporating ³³P-UTP (*Tph2*). Signal was detected using phosphorimaging and quantitative densitometry performed using MCID image analysis software (Neumaier et al., 1996, Neumaier et al., 1997). Each subregion of the DRN as well as MRN was examined across five points on the A-P axis as previously described (Clark et al., 2006). Because a recent report suggested that the dorsomedial DRN could be further divided into a core and shell region based on c-fos expression in response to stress (Abrams et al., 2005), we examined these subregions separately.

In the ventromedial DRN at the mid-rostral level, both oCRF and UCNII induced a significant, approximately 30%, increase in 5-HT_{1A} hybridization signal (Figure 4A, ANOVA $F_{(2,31)}=3.71$, $p<0.05$. Fisher's PLSD, oCRF or UCNII vs. Vehicle $p<0.05$). There were no changes in 5-HT_{1A} expression in any other area or A-P level of the DRN or MRN examined (Table 3). SERT hybridization signal displayed similarly regionally restricted alterations. Both oCRF and UCNII decreased SERT signal by almost 30% within the shell of the dorsomedial DRN at the mid-caudal level (Figure 4B, ANOVA $F_{(2,29)}=5.53$, $p<0.01$). Fisher's PLSD oCRF vs. vehicle $p<0.01$, UCNII vs. vehicle $p<0.05$). No difference between treatment groups was evident within the core of the dorsomedial DRN at that level, nor in any other region or level examined (Table 2). There were no differences between the treatment groups in expression of the 5-HT_{1B} receptor in any subregion of DRN or in the MRN (Table 4).

Although *Tph2* signal was not significantly different in any brain region (Table 1), at the mid-rostral level there appeared to be a trend towards decreased signal intensity in the ventromedial DRN and increased signal in the core dorsomedial DRN (Figure 4C–D, Fisher's PLSD $p<0.1$). To determine if these trends were consistent within individual animals or randomly distributed, the ratio of *Tph2* mRNA hybridization signal in ventromedial to dorsomedial DRN was examined. This ratio was found to be reduced by a third in both oCRF and UCNII treated animals, but only in the mid-rostral DRN (Figure 4E, ANOVA $F_{(2,30)}=4.05$, $p<0.05$. Fisher's PLSD, oCRF or UCNII vs. vehicle $p<0.05$). This finding suggests that *Tph2* hybridization signal was decreased in the ventromedial DRN of the same animals in which it was increased in the core dorsomedial DRN, suggesting possible reciprocal regulation.

In regions with identified changes due to oCRF or UCNII infusion, both 5-HT_{1A} and SERT hybridization density were correlated with aspects of novel object behavior. Mid-rostral ventromedial 5-HT_{1A} hybridization signal was negatively correlated with number of climbs (Figure 5A, $r=0.366$, $p<0.05$). Mid-caudal shell dorsomedial SERT hybridization signal was positively correlated with number of touches (Figure 5D, $r=0.360$, $p<0.05$), climbs (Figure 5E, $r=0.383$, $p<0.05$), and total contacts (Figure 5F, $r=0.423$, $p<0.05$). In addition to the relationship between novel object behavior and absolute hybridization signals, there is also a correlation with the ratio of TPH2 mRNA hybridization signal in ventromedial to core dorsomedial DRN at the mid-rostral level. The *Tph2* VM/cDM ratio was positively correlated with both climbs (Figure 5B, $r=0.428$, $p<0.05$), and total novel object contacts (Figure 5C, $r=0.351$, $p<0.05$).

Exploratory behavior following chronic infusion of oCRF at 100ng/hr

As a part of an unrelated small pilot experiment, rats were infused with either 100ng/hr oCRF or ACSF vehicle for 6 days ($n=4$ each group) and underwent behavioral testing exactly as described for animals infused at 1ng/hr. Results were similar to those obtained with oCRF infusions at 1ng/hr. There were no statistically significant differences between treatment groups for day 2, or the difference between the two treatment groups for total distance (Figure 6A), exit latency, entry square time or fecal boli. Nor were there statistically significant differences in any of these measures or in center or corner time on day 1.

On day 2, center time was decreased while corner time was increased in animals given 100ng/hr oCRF. Unlike the smaller differences in animals treated with 1ng/hr oCRF, these differences were statistically significant (Figure 6B and 6C, Students t-test $p<0.05$). This more profound thigmotaxis on re-exposure to the open field was reflected in the statistically significant difference between treatment groups on the difference between day 2 and day 1 center time (Figure 6B and 6D, Students t-test $p<0.05$). As with oCRF at 1ng/hr, animals infused with oCRF at 100ng/hr also displayed less of a decrease in corner time between day 2 and day 1 compared to animals infused with ACSF vehicle (Figure 6C, Students t-test $p<0.01$).

Exploratory behavior towards the novel object presented on day 2 was also markedly altered. Infusion with 100ng/hr oCRF decreased the number of novel object climbs by 85% (Figure 6F, Students t-test $p < 0.01$) and total novel object contacts by 50% (Figure 6G, Students t-test $p < 0.05$) versus vehicle. Although oCRF decreased novel object touches by 20%, this change did not reach statistical significance, and latency to first novel object contact was not statistically different between treatment groups (Figure 6D and 6E).

Discussion

We have found that chronic administration of low doses of CRF receptor agonists markedly alters rat exploratory behavior in the open field. Intraperitoneal administration of the partially selective CRF₁ agonist oCRF for 6 days reduced exploration of a novel object and treated animals did not display the expected decrease in thigmotaxic behavior following re-exposure to a familiar open field. The selective CRF₂ agonist UCNII induced similar though less robust behavioral changes, displaying the same changes in thigmotaxis on re-exposure to the open field, but decreasing only climbing behavior towards a novel object. For both peptides, these behavioral alterations are accompanied by highly regionally-specific alterations in 5-HT_{1A} and SERT hybridization density. The relative levels of *Tph2* hybridization density between the ventromedial and core dorsomedial DRN appear to be similarly altered in a highly specific location along the A-P axis.

Behavioral changes observed following low dose infusions of oCRF at 1ng/hr were maintained following infusions at 100ng/hr. Indeed, this higher dose appeared to produce more substantial behavioral changes, as day 2 center time was decreased and day 2 corner time increased relative to vehicle. Although these trends were present with infusions of 1ng/hr, they did not reach statistical significance. Overall, results following 100ng/hr oCRF infusion replicate our findings at 1ng/hr and add considerable confidence in the validity of our behavioral findings.

This study, does, however have limitations. First, because the higher dose infusions were performed as part of a completely separate study, brain tissue was used for other purposes and was not available for ISHH. Thus, while the behavioral effects of chronic oCRF infusion into the DRN were replicated as a separate study, the ISHH findings were not repeated making the possibility of type I error in this data more concerning. If this were the case however, it would suggest that there were no changes at all in serotonergic gene expression following chronic oCRF or UCNII infusion into the DRN. Such a finding would still be remarkable as it would suggest that CRF agonists might act on the DRN producing substantial changes in behavior independent of modifications in the serotonin system, a surprising result.

In addition, as a result of the chronic nature of this study, the stability of the peptide solutions was a concern. Our investigation found that after 6 days of incubation at 37°C, oCRF and UCNII concentrations decreased to 42% and 61% of their initial concentrations respectively. While this suggests that doses at the end of the 1ng/hr oCRF infusions dropped to about 0.4ng/hr, it is notable that we found essentially identical behavioral changes following infusion of oCRF at an initial rate of 100ng/hr. This higher dose regimen ended with an estimated infusion rate of about 40ng/hr. Based on studies of acute effects of oCRF on DRN discharge (Kirby et al., 2000), it seems highly probable that there should have been more than enough oCRF present at the end of the 100ng/hr study to alter DRN discharge, even if this was in doubt for the 1ng/hr study. Given the near identical behavioral results between 1ng/hr and 100ng/hr oCRF exposure, it is unlikely that our findings are due to either drug withdrawal or a prolonged effect from acute exposure to no longer present CRF agonists. However, future investigations will need to maximize peptide stability, perhaps through use of different vehicle or of agonists with structures that may be more resistant to degradation.

Chronic CRF receptor agonism in the DRN and behavior

In the open field, animals infused with 1ng/hr oCRF or UCNII displayed no differences on initial exposure to an open field, but differed in their behavior during their second exposure. Normally, upon return to a familiar open field, animals will display decreased thigmotaxis. That is, they will spend less time in the corners of the open field on the second exposure than the first. Rats infused intra-DRN with oCRF or UCNII displayed a smaller decrease in thigmotaxis on re-exposure to a familiar open field than vehicle-treated animals.

There are several possible explanations for this effect. Animals may fail to remember the open field as a previously experienced space. Another possibility is that the animals treated with oCRF or UCNII failed to habituate to the open field. A third possibility is that the presence of the novel object on the second day was a sufficiently anxiogenic confound that it produced an increase in thigmotaxis on the second day, resulting in the appearance of failure to habituate to the open field. Favoring the habituation hypothesis, chronic immobilization stress has been reported to reduce habituation to the anxiogenic effects of open field exposure in rats (Dubovicky and Jezova, 2004). Unfortunately, the role of serotonin and the DRN in open field habituation is not clear. While one study found that raphe lesioning (producing about 70% reduction in striatal and hippocampal 5-HT) did not affect habituation of open field behavior (Deakin et al., 1979), chronic fluoxetine increased the rate of habituation to the open field in olfactory bulbectomized rats (Mar et al., 2002). Further study will be needed to establish the mechanisms underlying this apparent failure to habituate to the open field, and to strengthen or weaken alternative hypotheses.

Perhaps the most remarkable behavioral finding in this study was the marked decrease in exploration of a novel object following infusion of oCRF or UCNII into the DRN. These results could represent decreased exploratory drive or alternatively increased neophobic anxiety. The later hypothesis seems unlikely. If the CRF ligands were generally increasing apprehension towards novelty, one would anticipate increased thigmotaxic behavior relative to vehicle on initial exposure to the open field, without the confounding influence of the novel object (Prut and Belzung, 2003). No such finding was observed. Indeed, our results demonstrate no differences between treatment groups in locomotor activity, thigmotaxis, defecation, or exit latency on initial exposure to the open field. This suggests that animals infused with very low dose oCRF and UCNII were not generally more anxious towards a novel environment than animals infused with vehicle. It seems that a more likely hypothesis that rats given chronic intra-DRN injections of oCRF or UCNII experience a decrease in exploratory drive. Yet, if general exploratory drive was decreased by infusion of the CRF receptor agonists, then it might be expected that the tendency of animals to enter the center square on day 1 might be decreased. However, open field behavior is affected by processes other than exploratory drive, and factor analysis has long suggested that central square entry appears to be most effected by anxiety (Whimbey and Denenberg, 1967, Denenberg, 1969). In contrast, while exploration of a novel object is affected by anxiety state, it appears to be more significantly impacted by changes in exploratory drive and novelty seeking (Crawley, 1985, Bevins and Bardo, 1999, Bevins et al., 2002). Thus, novel object exploration would seem to be a more sensitive measure of exploratory drive than center entry in the open field. In this regard, it may be notable that the higher dose of 100ng/hr oCRF appeared to have more of an effect on center time than oCRF at 1ng/hr.

Recent reports in mice chronically stressed by restraint and predator exposure have demonstrated a tight correlation between a lack of exploration towards a novel object (similar to this study) and other behaviors associated with chronic stress exposure. Those animals developing exploratory deficits in response to chronic stress consistently display increased floating during forced swimming and decreased preference for sweet tastes (Berger et al., 2004, Strekalova et al., 2004), behaviors widely used to model depression and anhedonia in

rats and mice (Detke et al., 1995, Crowley et al., 2004). Indeed, in the chronic mild stress model of depression in both rats and mice, decrements in both exploratory behavior and reward seeking are prominent features (Willner et al., 1992, Argyropoulos and Nutt, 1997, Bielajew et al., 2002, Anisman and Matheson, 2005, Bekris et al., 2005, Gronli et al., 2005). Similar results have also been reported following chronic social stress (Rygula et al., 2005). These findings suggest that one important consequence of chronic stress may be reduced novelty and reward seeking behavior, behaviors evocative of the lack of drive and anhedonia commonly seen in human patients with both depression and generalized anxiety (Loas et al., 1992, Carton et al., 1995, Naranjo et al., 2001). The present data suggests that CRF receptors in DRN may be an important mediator of these depressive-like symptoms, especially since the DRN subregions that displayed changes in gene expression project to regions thought to be important in the development of anhedonia, particularly nucleus accumbens (Van Bockstaele et al., 1993, Cabib and Puglisi-Allegra, 1996).

Serotonergic gene expression following chronic CRF receptor agonism in the DRN

Several acute stressors have been reported to alter TPH protein (Culman et al., 1984, Boadle-Biber et al., 1989, Chamas et al., 2004) and activity (Singh et al., 1990) levels. The increase in TPH enzyme activity was blocked by intracerebroventricular administration of a nonselective CRF antagonist, and partially reproduced by activation of the amygdala with rat/human CRF (Boadle-Biber et al., 1993). Additionally, TPH1 mRNA was found to be most robustly elevated following repeated immobilization stress, although this isoform of TPH is now known to be expressed primarily outside of brain and is expressed at very low levels in DRN (Chamas et al., 1999, Chamas et al., 2004). This finding supported an early report that chronic immobilization stress increased TPH activity (Culman et al., 1984). Since each of these studies examined the DRN as a whole, they suggested that chronic stress would be expected to produce wide ranging and substantial changes in gene expression in serotonergic neurons of the DRN, particularly for the predominant central neuronal form of TPH, *Tph2*.

However, we have found that chronic administration of low dose oCRF or UCNII into the DRN produced changes in 5-HT_{1A} and SERT expression that were regionally limited. Absolute TPH2 mRNA levels, as reflected by hybridization density, were not significantly altered in any region. However, the relative expression levels between the two DRN subnuclei with the highest *Tph2* expression, the mid-rostral ventromedial and core dorsomedial DRN (Clark et al., 2006), were significantly decreased following intra-DRN infusion of oCRF or UCNII. There are several possible explanations for the limited distribution of mRNA level changes. First, it is possible that serotonergic function is regulated by CRF receptors on levels other than net mRNA abundance, such as translational efficiency, protein stability, or post-translational modification. In addition, prior studies have only demonstrated changes in TPH protein or activity levels after acute CRF administration. The discrepancy between prior findings and the present results may relate to the transition from acute to chronic stress responses. It is also possible that changes in raphe function involve non-5-HT co-transmitters. A variety of neuropeptides are expressed in DRN including NPY (Pau et al., 1998), VIP (Smith et al., 1994, Kozicz et al., 1998), galanin (Hokfelt et al., 1998, Hokfelt et al., 1999, Larm et al., 2003), somatostatin (Araneda et al., 1999), and CRF itself (Commons et al., 2003). These peptides may respond differently under acute and chronic stress exposure than the serotonergic component. The internal anatomical complexity of the DRN has been more clearly appreciated recently (Ratnayake et al., 1999, Commons et al., 2003, Abrams et al., 2004, Abrams et al., 2005, Greenwood et al., 2005, Clark et al., 2006), and discrete changes in serotonergic gene expression in subregions of raphe could be sufficient to induce substantial behavioral changes.

Of particular note, CRF receptor distribution in DRN is complex and varies by region (Day et al., 2004, Pernar et al., 2004). Recent reports have emphasized the discrete subregional nature

of DRN response to both direct and indirect (via the basolateral amygdala) exposure to CRF receptor ligands (Forster et al., 2006, Spiga et al., 2006). The heterogeneous neurochemical anatomy of DRN, especially in relation to CRF receptors, may help explain why a single stress exposure can simultaneously increase or decrease 5-HT levels in forebrain regions that are supplied by different DRN subregions (Kirby et al., 1995, Kirby and Lucki, 1998). For example, increased 5-HT_{1A} expression in the ventromedial DRN at the mid-rostral level would be expected to increase somatodendritic autoreceptor tone within this region, reducing serotonin release in terminal fields supplied by those neurons. Alternatively, the 5-HT_{1A} receptor has been reported on a small number of non-serotonergic neurons in the DRN (Kirby et al., 2003), especially in rostral DRN (Day et al., 2004). If the observed alterations in expression of this receptor reflected changes in this neuronal population, then intra-raphe circuits could also be modulating 5-HT independent of the autoreceptor activity. In any case, mid-rostral ventromedial DRN is well positioned to alter anxiety behavior. Its projection targets include frontal cortex (including medial prefrontal cortex), central nucleus of the amygdala, caudal locus coeruleus, and ventral hippocampus (Imai et al., 1986, Peyron et al., 1998, Lee et al., 2003, Kim et al., 2004). All of these structures have been implicated in modulating behavior in the open field (Velley et al., 1991, Burns et al., 1996, Daenen et al., 2002), making it possible that alterations in serotonergic function in the mid-rostral ventromedial DRN could underlie the changes in exploratory behavior observed.

Subject to similar caveats regarding the relationship between gene expression and protein function, decreased SERT expression in the mid-caudal dorsomedial shell of the DRN could result in decreased serotonin reuptake in terminal fields. Unfortunately little is known about how mid-caudal DRN projections differ from mid-rostral DRN projections, as no anatomic tracing studies have distinguished between the two locations. Nor is there any data regarding distributions of projections from the shell (as opposed to core) dorsomedial DRN. Available data suggests that the dorsomedial DRN near the region in question projects heavily to bed nucleus stria terminalis, central and basolateral nuclei of the amygdala, lateral septum, nucleus accumbens, as well as several cortical regions (Imai et al., 1986, Vertes, 1991, Van Bockstaele et al., 1993). Possible involvement of the bed nucleus stria terminalis is intriguing, as this region has been implicated in recognition of unconditioned aversive environments (Walker and Davis, 1997, Walker et al., 2003), and has been found essential for the development of the animal model of depression referred to as learned helplessness (Hammack et al., 2004).

Our observation of discrete change in gene expression within the shell, as compared to the core, region of the dorsomedial following oCRF or UCNII infusion supports the contention that these two regions differ functionally as proposed by Abrams et al. (2005). Shell dorsomedial DRN has been shown to display greater c-fos expression in response to anxiogenic drugs, as well as providing greater projection to the basolateral amygdala than the core of the dorsomedial DRN and other DRN subregions (Abrams et al., 2005). While direct connectivity between the bed nucleus stria terminalis and the shell of the dorsomedial DRN has not yet been examined, this would seem to be a potentially important area of investigation.

In addition to changes in absolute levels of gene expression, we also found that the relative levels of hybridization signal for *Tph2* in the mid-rostral ventromedial DRN to core dorsomedial DRN appeared to be decreased; these regions have the highest relative *Tph2* expression in the DRN (Clark et al., 2006). If chronic CRF receptor activation increases the relative expression of *Tph2* in the dorsomedial DRN but reduces expression in the ventromedial DRN as suggested by Figure 5, then there would likely be a shift in serotonergic activity in the forebrain targets of these subnuclei. This may help in understanding the differential effects of stress on 5-HT activity in different forebrain regions. For example, during and immediately following forced swimming, extracellular 5-HT levels are increased in the striatum and frontal cortex, terminal fields of the ventromedial DRN; while decreased in the basolateral amygdala

(Kirby et al., 1995). In addition, CRF receptor activation within the raphe has been shown to be a necessary component of many stress effects. Increased *Tph2* activity in dorsomedial DRN neurons could activate one or more structures involved in a particular behavior, while decreased expression in the ventromedial DRN would have an opposing effect. The relative balance of activity between these regions would determine overall behavioral response (e.g. active vs. passive coping (Cryan et al., 2005)). Sena et al. (2003) have suggested that the DRN exerts opposing effects on one-way escape and inhibitory avoidance, suppressing one response when the other is activated (Sena et al., 2003). Johnson et al. (2004) have proposed that control of behavioral response to anxiogenic stimuli may be subregionally specific, with the dorsolateral DRN involved in passive coping; with the ventromedial DRN involved in active escape responses (Johnson et al., 2004). Furthermore, a recent study suggests that in a group of ethanol abusing depressed suicide victims, TPH immunoreactivity was increased in the dorsomedial DRN at the mid-rostral level. While the study quantified immunoreactivity in the dorsomedial DRN as a whole, the figures presented suggest that TPH was increased markedly in the core dorsomedial DRN (Bonkale et al., 2006). Taken collectively, these data suggest that changes in the relative level of *Tph2* expression in the ventromedial and dorsomedial DRN might well play a role in modulating behavior.

Behavioral roles of CRF receptors in the DRN

In this study, we found that oCRF, a partially selective CRF₁ agonist, produced more profound decreases in exploration of a novel object than UCNII, a selective CRF₂ agonist. It is tempting to conclude that CRF₁ is responsible for the observed behavioral and ISHH results. However, given the theoretical selectivity of UCNII from in vitro studies, if the behavioral effects were mediated by CRF₁ and not by CRF₂, then there should have been far less or no effect at all from UCNII. One possible explanation for these results is that UCNII had enough activity at CRF₁ receptors in vivo to produce similar results to oCRF. However, it is also possible that the observed effects were mediated by CRF₂ receptors and differential receptor availability of the peptides produced the apparent discrepancy. Indeed, several studies favor the involvement of CRF₂ receptors in regulating DRN functions, especially in the more caudal regions of the DRN (Hammack et al., 2003, Staub et al., 2006). Additionally, CRF binding protein (CRF-BP) has been reported to modulate the availability of CRF family peptides (Potter et al., 1992, Seasholtz et al., 2002). oCRF and rat UCNII may have different affinities for CRF-BP. oCRF has been reported to have low affinity, with an IC₅₀ in the 300nM range (Jahn et al., 2001). Mouse UCNII, in contrast, has been reported to have a much greater affinity for CRF-BP than oCRF, with an IC₅₀ in the 5nM range (Jahn et al., 2004, Isfort et al., 2006). Unfortunately, binding affinity for rat UCNII (employed in this study) has not been reported, and there is a Tyr (mouse) -> Asn (rat) substitution within a region known to be important for CRF-BP binding (Jahn et al., 2001). Regardless, it is likely that oCRF and rat UCNII have different affinities for CRF-BP. This problem is confounded by the chronic nature of this study with its potential for peptide degradation. A truncation of rat/human CRF, CRF (6–33), acts as an antagonist of CRF-BP binding (Heinrichs and Joppa, 2001). It is possible that breakdown products of oCRF and/or UCNII might also have such activity. If that were the case, then the slightly greater breakdown of oCRF could paradoxically result in a higher local concentrations of oCRF.

As a result of these confounds, little can be concluded from these studies about the differential role of CRF₁ and CRF₂ receptors in the observed results. Future investigations could use more selective CRF₁ and CRF₂ agonists such as stressin₁, a recently available CRF₁ agonist with 100-fold selectivity over CRF₂ (Liu et al., 2004, Rivier et al., 2007), or urocortin III an approximately 1000-fold selective CRF₂ agonist (Lewis et al., 2001). Unfortunately, the in vivo specificity of these agents is no better known than for oCRF and UCNII. And while mouse UCNIII has an IC₅₀ >2000nM for CRF-BP, the binding of stressin₁ has not been reported or

has the CRF-BP affinity of rat UCNIII. Alternatively, to assure complete molecular specificity in vivo and avoidance of confounding effects from CRF-BP, our behavioral observations could be extended into mice allowing the use of CRF receptor knock-outs (Muller and Keck, 2002). Regardless of methodology, additional investigations will be necessary to understand the differential role of CRF receptor subtypes in the DRN.

In summary, we found that chronic infusion of very low dose oCRF or UCNII into the rostral DRN produced reductions in exploratory behavior and increased thigmotaxis on second exposure to an open field, behavioral changes that have been associated with exposure to chronic stress. In addition, following infusion of either CRF agonist, there were behaviorally correlated alterations in serotonergic gene expression within the DRN that were subregionally limited, and include the shell of the dorsomedial DRN, a region identified as being activated by several anxiogenic drugs. In addition, the behavioral findings following infusion of oCRF at 1ng/hr were also observed following infusion at 100ng/hr. To the best of our knowledge, this is the first study to examine the effects of chronic CRF agonist exposure on the DRN. Because of the similarity between the behavioral phenotype produced by chronic stress and the effects described here from chronic intra-DRN CRF agonist infusion, this study may have considerable relevance to the mechanisms by which chronic stress promotes depression and anxiety disorders. Unfortunately, our results do not provide strong evidence to favor the role of one CRF receptor over another in their actions on serotonergic neurons and behavior described in this paper. Furthermore, CRF binding protein is present in the DRN and could modulate the availability of CRF family neuropeptides there (Potter et al., 1992, Seasholtz et al., 2002) thus altering local peptide concentrations. More specific pharmacologic and transgenic experiments should help clarify the roles of each receptor type as well as CRF-BP in the DRN. In addition, future studies will be needed to more fully characterize the behavioral and neurochemical effects of chronic CRF agonist infusion into the DRN, as well as to determine the limits of its utility as a mechanistic model for the biology of chronic stress exposure. Such an understanding of the role of chronic CRF receptor activation in the DRN upon behaviors so closely related to both anxiety disorders and depression should be helpful in the development of new strategies to interfere with the effects of chronic stress.

Acknowledgements

This work was supported by NIMH (MH 63303 to J.F.N., and MH 66548 to M.S.C.) as well as a NARSAD Young Investigator Award to M.S.C.

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List of Abbreviations

DRN	dorsal raphe nucleus
MRN	median raphe nucleus
A-P	anteroposterior
CRF	corticotropin releasing factor
oCRF	ovine corticotropin releasing factor
UCNII	urocortin II
SERT	serotonin transporter
ACSF	artificial cerebrospinal fluid

Figure 1. Infusion cannula tip placement

After in situ hybridization histochemistry, sections were stained with cresyl violet and the location of the infusion cannula tip determined by a blinded observer. Line drawings of sample coronal sections are adapted from Paxinos and Watson (Paxinos and Watson, 1997). The anteroposterior location of each row of sections is given at the left in distance from bregma. Black circles on the leftmost column indicate tip locations on vehicle treated animals (-7.47 ± 0.07 mm from bregma), white circles on the middle column indicate location on ovine corticotropin-releasing factor (CRF) infused animals (-7.38 ± 0.07 mm), and grey circles on the rightmost column indicate tip locations for urocortin II (UCNII) infused animals (-7.43 ± 0.09 mm). Mean cannula placement on the anteroposterior (AP) axis did not differ between treatment groups, and the distribution of placements into the DRN is similar. Vehicle $n=12$, oCRF $n=12$, UCNII $n=11$. All data are presented as means $\pm$ SEM.

Figure 2. Open field behavior

A. Total distance traveled in cm is shown for day 1 (vehicle: 2496±131, oCRF: 2333±107, UCNII: 2464±155), day 2 (vehicle: 2713±202, oCRF: 2510±131, UCNII: 2768±204) and the difference between days (vehicle: 217±105, oCRF: 115±76, UCNII: 304±103). **B.** Latency to exit the start box in seconds is shown for day 1 (vehicle: 140±51, oCRF: 212±70, UCNII: 143±31), day 2 (vehicle: 101±24, oCRF: 101±28, UCNII: 91±24) and the difference between days (vehicle: -39±42, oCRF: -58±26, UCNII: -52±12). **C.** Total time spent in the entry square in seconds is shown for day 1 (vehicle: 325±16, oCRF: 326±21, UCNII: 327±22), day 2 (vehicle: 271±14, oCRF: 304±18, UCNII: 231±20) and the difference between days (vehicle: -54±20, oCRF: -33±17, UCNII: -96±10). **D.** Number of fecal boli per animal is shown for day 1

(vehicle: 1.6 ± 0.8 , oCRF: 0.9 ± 0.4 , UCNII: 1.2 ± 0.5), day 2 (vehicle: 1.5 ± 0.7 , oCRF: 1.3 ± 0.6 , UCNII: 1.2 ± 0.6) and the difference between days (vehicle: -0.1 ± 0.8 , oCRF: 0.7 ± 0.7 , UCNII: 0 ± 0.3). **E.** Time spent in the center is shown in seconds for day 1 (vehicle: 64 ± 8 , oCRF: 63 ± 11 , UCNII: 55 ± 6), day 2 (vehicle: 219 ± 21 , oCRF: 171 ± 19 , UCNII: 180 ± 24) and the difference between days (vehicle: 156 ± 18 , oCRF: 103 ± 18 , UCNII: 125 ± 24). **F.** Time spent in the corners is shown in seconds for day 1 (vehicle: 359 ± 17 , oCRF: 366 ± 28 , UCNII: 371 ± 26), day 2 (vehicle: 260 ± 25 , oCRF: 328 ± 37 , UCNII: 351 ± 39) and the difference between days (vehicle: -99 ± 21 , oCRF: -14 ± 30 , UCNII: -20 ± 32). * $p < 0.05$ (Fisher's PLSD vs. vehicle). Vehicle $n=14$, oCRF $n=14$, UCNII $n=12$. All data are presented as means $\pm$ SEM. Treatment groups are coded as shown below panels E and F, vehicle: black, oCRF: white, UCNII: grey.

Figure 3. Novel object behavior

A. Latency in seconds to first touch of the novel object is shown. Vehicle: 79 ± 21 , oCRF: 196 ± 68 , UCNII: 88 ± 27 . * $p < 0.05$ (Fisher's PLSD vs. vehicle). **B.** Total novel object touches (one paw or nose touch) are indicated for each treatment group. Vehicle: 23.5 ± 1.6 , oCRF: 16.6 ± 2.1 , UCNII: 21.6 ± 2.2 . * $p < 0.05$ (Fisher's PLSD vs. vehicle). **C.** Total novel object climbs (two paw touch or climbing on novel object) are shown for each treatment group. Vehicle: 15.4 ± 2.0 , oCRF: 8.0 ± 1.4 , UCNII: 8.4 ± 1.6 . # $p < 0.01$ (Fisher's PLSD vs. vehicle). **D.** Total contacts (touches + climbs) are shown for each treatment group. Vehicle: 38.9 ± 3.0 , oCRF: 24.6 ± 3.0 , UCNII: 30.0 ± 3.3 . * $p < 0.05$, ^ $p < 0.1$ (Fisher's PLSD vs. vehicle). Vehicle $n = 14$, oCRF $n = 14$, UCNII $n = 12$. All data are presented as means $\pm$ SEM. Treatment groups are coded as shown below panels C and D, vehicle: black, oCRF: white, UCNII: grey.

Figure 4. In situ hybridization histochemistry

A. 5-HT_{1A} hybridization density in the mid-rostral ventromedial DRN is shown. Vehicle: 0.41 ± 0.04 , oCRF: 0.55 ± 0.05 , UCNII: 0.53 ± 0.03 . ANOVA $F(2,31)=3.71$, $p<0.05$. Fisher's PLSD, oCRF or UCNII vs. Vehicle $*p<0.05$. **B.** SERT hybridization density in the mid-caudal shell dorsomedial DRN is shown. Vehicle: 4.41 ± 0.36 , oCRF: 3.24 ± 0.22 , UCNII: 3.34 ± 0.22 . ANOVA $F(2,29)=5.53$, $p<0.01$. Fisher's PLSD oCRF vs Vehicle $\#p<0.01$, UCNII vs. vehicle $*p<0.05$. **C.** *Tph2* hybridization density in the mid-rostral ventromedial DRN is shown. Vehicle: 6.27 ± 0.52 , oCRF: 4.82 ± 0.45 , UCNII: 5.35 ± 0.50 . **D.** *Tph2* hybridization density in the mid-rostral core dorsomedial DRN is shown. Vehicle: 2.33 ± 0.21 , oCRF: 3.07 ± 0.41 , UCNII: 2.86 ± 0.22 . **E.** The relative levels of *Tph2* expression between the ventromedial and

dorsomedial DRN are expressed as the ratio between the two are shown across the A-P axis. The only portion of the DRN to demonstrate an effect of CRF agonists was the mid-rostral region. Vehicle: 2.88 ± 0.32 , oCRF: 1.94 ± 0.22 , UCNII: 1.95 ± 0.26 . ANOVA $F(2,30)=4.05$, $p<0.05$. Fisher's PLSD, oCRF or UCNII vs Vehicle $*p<0.05$. All values are shown as mean $\pm$ SEM. All density values are represented in units of $\mu\text{Ci/g}$. Vehicle $n=12$, oCRF $n=10-11$, UCNII $n=9-11$. Treatment groups are coded as shown below panels C and D, vehicle: black, oCRF: white, UCNII: grey.

Figure 5. Correlations between gene expression and novel object behavior

A. 5-HT_{1A} hybridization signal in the mid-rostral ventromedial DRN correlates to climbs. $r=0.366$, $p<0.05$. **B.** The ratio of TPH2 mRNA hybridization signal in ventromedial to core dorsomedial DRN at the mid-rostral level correlates to climbs. $R=0.428$, $p<0.05$. **C.** The ratio of TPH2 mRNA hybridization signal in ventromedial to core dorsomedial DRN at the mid-rostral level correlates to total novel object contacts. $R=0.351$, $p<0.05$. **D.** SERT hybridization signal in the mid-caudal shell dorsomedial DRN correlates to the number of touches. $R=0.360$, $p<0.05$. **E.** SERT hybridization signal in the mid-caudal shell dorsomedial correlates to the number of climbs. $R=0.383$, $p<0.05$. **F.** SERT hybridization signal in the mid-caudal shell dorsomedial correlates to the number of total contacts. $R=0.383$, $p<0.05$.

Figure 6. Behavior following infusion of 100ng/hr oCRF

A. Total distance traveled in cm is shown for day 1 (vehicle: 2496±131, 100ng/hr oCRF: 2333 ±107, day 2 (vehicle: 2476±116, 100ng/hr oCRF: 2359±150) and the difference between days (vehicle: 342±80, 100ng/hr oCRF: -10.8±140). **B.** Time spent in the center is shown in seconds for day 1 (vehicle: 96±16, 100ng/hr oCRF: 39±8, day 2 (vehicle: 252±16, 100ng/hr oCRF: 81 ±20) and the difference between days (vehicle: 156±14, 100ng/hr oCRF: 27±15). **C.** Time spent in the corners is shown in seconds for day 1 (vehicle: 282±25, 100ng/hr oCRF: 364±35, day 2 (vehicle: 234±10, 100ng/hr oCRF: 411±39) and the difference between days (vehicle: -48.3±30, 100ng/hr oCRF: 53±12). **D.** Latency in seconds to first touch of the novel object is shown. Vehicle: 71±31, 100ng/hr oCRF: 49±25. **E.** Total novel object touches (one paw or nose touch) are indicated for each treatment group. Vehicle: 23.5±2.5, 100ng/hr oCRF: 18.8 ±2.3. **F.** Total novel object climbs (two paw touch or climbing on novel object) are shown for each treatment group. Vehicle: 17.5±2.9, 100ng/hr oCRF: 2.8±1.5. **G.** Total contacts (touches + climbs) are shown for each treatment group. Vehicle: 41±3.6, 100ng/hr oCRF: 21.5±2.7. *p<0.05 (Fisher's PLSD vs. vehicle). Vehicle n=4, 100ng/hr oCRF n=4. All data are presented as means ± SEM. Treatment groups are coded as shown between panels A–C and D–G, vehicle: black, 100ng/hr oCRF: white.

Table 1***Tph2* in situ hybridization histochemistry results**

Data are shown for *Tph2* hybridization densities for each DRN subregion and the MRN at 5 levels on the A-P axis, as previously defined (Clark et al., 2006). All values are shown as mean $\pm$ SEM in units of $\mu\text{Ci/g}$. Vehicle n=12, oCRF n=10–11, UCNII n=9–11. N/A= not applicable, region not defined at this level on the A-P axis.

<i>Tph2</i> region sampled		Far-rostral –7.15mm	Rostral –7.5mm	Mid-rostral –7.8mm	Mid-caudal –8.1mm	Caudal –8.5mm
ventromedial DRN	Vehicle	1.29 $\pm$ 0.18	4.78 $\pm$ 0.67	6.27 $\pm$ 0.52	4.26 $\pm$ 0.54	4.30 $\pm$ 0.41
	OCRf	1.19 $\pm$ 0.13	4.18 $\pm$ 0.45	4.82 $\pm$ 0.45	4.72 $\pm$ 0.54	4.48 $\pm$ 0.61
	UCNII	1.11 $\pm$ 0.08	3.87 $\pm$ 0.47	5.35 $\pm$ 0.50	4.30 $\pm$ 0.51	4.00 $\pm$ 0.32
core dorsomedial DRN	Vehicle	1.82 $\pm$ 0.29	2.36 $\pm$ 0.33	2.33 $\pm$ 0.21	2.44 $\pm$ 0.38	2.35 $\pm$ 0.30
	OCRf	1.85 $\pm$ 0.33	2.34 $\pm$ 0.20	3.07 $\pm$ 0.41	2.60 $\pm$ 0.25	2.14 $\pm$ 0.21
	UCNII	1.76 $\pm$ 0.25	2.23 $\pm$ 0.24	2.86 $\pm$ 0.22	2.27 $\pm$ 0.21	2.09 $\pm$ 0.14
shell dorsomedial DRN	Vehicle	N/A	N/A	1.62 $\pm$ 0.18	2.15 $\pm$ 0.32	N/A
	OCRf	N/A	N/A	1.91 $\pm$ 0.32	2.29 $\pm$ 0.28	N/A
	UCNII	N/A	N/A	1.95 $\pm$ 0.17	2.12 $\pm$ 0.18	N/A
dorsolateral DRN	Vehicle	N/A	N/A	2.55 $\pm$ 0.23	1.40 $\pm$ 0.16	N/A
	OCRf	N/A	N/A	2.55 $\pm$ 0.23	1.62 $\pm$ 0.22	N/A
	UCNII	N/A	N/A	2.31 $\pm$ 0.18	1.11 $\pm$ 0.32	N/A
median raphe	Vehicle	1.16 $\pm$ 0.27	3.46 $\pm$ 0.67	4.27 $\pm$ 0.46	2.20 $\pm$ 0.32	1.26 $\pm$ 0.20
	OCRf	1.64 $\pm$ 0.43	3.32 $\pm$ 0.82	4.44 $\pm$ 0.50	2.33 $\pm$ 0.25	1.00 $\pm$ 0.11
	UCNII	0.71 $\pm$ 0.11	2.18 $\pm$ 0.38	4.04 $\pm$ 0.61	2.30 $\pm$ 0.31	1.14 $\pm$ 0.16

Table 2**SERT in situ hybridization histochemistry results**

Data are shown for SERT hybridization densities for each DRN subregion and the MRN at 5 levels on the A-P axis, as previously defined (Clark et al., 2006). All values are shown as mean $\pm$ SEM in units of $\mu\text{Ci/g}$. Vehicle n=12, oCRF n=10–11, UCNII n=9–11. N/A= not applicable, region not defined at this level on the A-P axis.

*p<0.05 (Fisher's PLSD vs. vehicle).

SERT region sampled		Far-rostral –7.15mm	Rostral –7.5mm	Mid-rostral –7.8mm	Mid-caudal –8.1mm	Caudal –8.5mm
ventromedial DRN	Vehicle	1.96 $\pm$ 0.24	6.82 $\pm$ 0.79	7.92 $\pm$ 0.75	6.58 $\pm$ 0.54	6.65 $\pm$ 0.76
	oCRF	2.52 $\pm$ 0.27	7.50 $\pm$ 0.64	7.87 $\pm$ 0.62	6.12 $\pm$ 0.74	6.20 $\pm$ 0.41
	UCNII	2.46 $\pm$ 0.34	7.55 $\pm$ 0.75	8.75 $\pm$ 0.75	6.12 $\pm$ 0.63	7.35 $\pm$ 0.65
core dorsomedial DRN	Vehicle	2.49 $\pm$ 0.26	3.94 $\pm$ 0.47	5.43 $\pm$ 0.84	4.34 $\pm$ 0.36	3.32 $\pm$ 0.54
	oCRF	2.29 $\pm$ 0.22	3.97 $\pm$ 0.26	4.54 $\pm$ 0.33	4.75 $\pm$ 0.82	3.38 $\pm$ 0.23
	UCNII	2.29 $\pm$ 0.46	3.38 $\pm$ 0.77	5.34 $\pm$ 1.80	4.27 $\pm$ 0.92	3.85 $\pm$ 0.80
shell dorsomedial DRN	Vehicle	N/A	N/A	3.35 $\pm$ 0.36	4.41 $\pm$ 0.36	N/A
	oCRF	N/A	N/A	2.93 $\pm$ 0.23	*3.24 $\pm$ 0.22	N/A
	UCNII	N/A	N/A	3.56 $\pm$ 0.41	*3.34 $\pm$ 0.22	N/A
dorsolateral DRN	Vehicle	N/A	N/A	2.55 $\pm$ 0.23	1.40 $\pm$ 0.16	N/A
	oCRF	N/A	N/A	3.84 $\pm$ 0.21	2.62 $\pm$ 0.23	N/A
	UCNII	N/A	N/A	3.81 $\pm$ 1.06	2.41 $\pm$ 0.39	N/A
median raphe	Vehicle	1.29 $\pm$ 0.25	3.47 $\pm$ 0.67	3.25 $\pm$ 0.26	2.57 $\pm$ 0.31	1.70 $\pm$ 0.22
	oCRF	1.51 $\pm$ 0.39	2.88 $\pm$ 0.44	4.20 $\pm$ 0.40	2.81 $\pm$ 0.49	1.48 $\pm$ 0.17
	UCNII	1.22 $\pm$ 0.79	2.77 $\pm$ 2.18	3.92 $\pm$ 1.64	2.99 $\pm$ 1.38	1.53 $\pm$ 0.40

Table 3**5-HT_{1A} in situ hybridization histochemistry results**

Data are shown for 5-HT_{1A} hybridization densities for each DRN subregion and the MRN at 5 levels on the A-P axis, as previously defined (Clark et al., 2006). All values are shown as mean $\pm$ SEM in units of μ Ci/g. Vehicle n=12, oCRF n=10–11, UCNII n=9–11. N/A= not applicable, region not defined at this level on the A-P axis.

*p<0.05 (Fisher's PLSD vs. vehicle). Note that in some cases, hybridization densities and SEM values between different treatment groups are identical within the number of significant figures, and this is not a typographical error.

5-HT _{1A} region sampled		Far-rostral –7.15mm	Rostral –7.5mm	Mid-rostral –7.8mm	Mid-caudal –8.1mm	Caudal –8.5mm
ventromedial DRN	Vehicle	0.13 $\pm$ 0.03	0.28 $\pm$ 0.02	0.41 $\pm$ 0.04	0.24 $\pm$ 0.02	0.26 $\pm$ 0.03
	oCRF	0.10 $\pm$ 0.02	0.31 $\pm$ 0.04	*0.55 $\pm$ 0.05	0.29 $\pm$ 0.03	0.25 $\pm$ 0.01
	UCNII	0.10 $\pm$ 0.02	0.34 $\pm$ 0.03	*0.53 $\pm$ 0.03	0.28 $\pm$ 0.03	0.27 $\pm$ 0.02
core dorsomedial DRN	Vehicle	0.08 $\pm$ 0.02	0.17 $\pm$ 0.02	0.18 $\pm$ 0.02	0.19 $\pm$ 0.02	0.11 $\pm$ 0.01
	oCRF	0.08 $\pm$ 0.01	0.18 $\pm$ 0.02	0.22 $\pm$ 0.02	0.20 $\pm$ 0.02	0.13 $\pm$ 0.01
	UCNII	0.09 $\pm$ 0.01	0.19 $\pm$ 0.02	0.22 $\pm$ 0.02	0.18 $\pm$ 0.02	0.11 $\pm$ 0.02
shell dorsomedial DRN	Vehicle	N/A	N/A	0.16 $\pm$ 0.01	0.16 $\pm$ 0.02	N/A
	oCRF	N/A	N/A	0.17 $\pm$ 0.01	0.15 $\pm$ 0.02	N/A
	UCNII	N/A	N/A	0.16 $\pm$ 0.01	0.16 $\pm$ 0.02	N/A
dorsolateral DRN	Vehicle	N/A	N/A	0.19 $\pm$ 0.04	0.51 $\pm$ 0.04	N/A
	oCRF	N/A	N/A	0.23 $\pm$ 0.04	0.11 $\pm$ 0.01	N/A
	UCNII	N/A	N/A	0.23 $\pm$ 0.04	0.11 $\pm$ 0.01	N/A
median raphe	Vehicle	0.18 $\pm$ 0.01	0.17 $\pm$ 0.01	0.16 $\pm$ 0.01	0.12 $\pm$ 0.01	0.13 $\pm$ 0.01
	oCRF	0.16 $\pm$ 0.02	0.16 $\pm$ 0.01	0.18 $\pm$ 0.01	0.11 $\pm$ 0.01	0.11 $\pm$ 0.01
	UCNII	0.17 $\pm$ 0.02	0.20 $\pm$ 0.02	0.18 $\pm$ 0.01	0.12 $\pm$ 0.01	0.11 $\pm$ 0.01

Table 4**5-HT_{1B} in situ hybridization histochemistry results**

Data are shown for 5-HT_{1B} hybridization densities for each DRN subregion and the MRN at 5 levels on the A-P axis, as previously defined (Clark et al., 2006). All values are shown as mean $\pm$ SEM in units of μ Ci/g. Vehicle n=12, oCRF n=10–11, UCNII n=9–11. N/A= not applicable, region not defined at this level on the A-P axis.

5-HT _{1B} region sampled		Far-rostral –7.15mm	Rostral –7.5mm	Mid-rostral –7.8mm	Mid-caudal –8.1mm	Caudal –8.5mm
ventromedial DRN	Vehicle	0.26 $\pm$ 0.08	0.95 $\pm$ 0.18	1.03 $\pm$ 0.07	0.64 $\pm$ 0.06	0.68 $\pm$ 0.05
	OCRf	0.21 $\pm$ 0.06	0.82 $\pm$ 0.09	1.06 $\pm$ 0.09	0.52 $\pm$ 0.03	0.49 $\pm$ 0.15
	UCNII	0.31 $\pm$ 0.07	0.83 $\pm$ 0.11	1.11 $\pm$ 0.11	0.69 $\pm$ 0.09	0.67 $\pm$ 0.05
core dorsomedial DRN	Vehicle	0.39 $\pm$ 0.06	0.65 $\pm$ 0.22	0.38 $\pm$ 0.05	0.42 $\pm$ 0.03	0.39 $\pm$ 0.03
	OCRf	0.39 $\pm$ 0.10	0.56 $\pm$ 0.05	0.40 $\pm$ 0.05	0.41 $\pm$ 0.03	0.39 $\pm$ 0.03
	UCNII	0.39 $\pm$ 0.09	0.44 $\pm$ 0.08	0.50 $\pm$ 0.07	0.43 $\pm$ 0.07	0.28 $\pm$ 0.02
shell dorsomedial DRN	Vehicle	N/A	N/A	0.37 $\pm$ 0.03	0.41 $\pm$ 0.04	N/A
	OCRf	N/A	N/A	0.48 $\pm$ 0.06	0.50 $\pm$ 0.07	N/A
	UCNII	N/A	N/A	0.48 $\pm$ 0.19	0.50 $\pm$ 0.07	N/A
dorsolateral DRN	Vehicle	N/A	N/A	0.70 $\pm$ 0.05	0.51 $\pm$ 0.04	N/A
	OCRf	N/A	N/A	0.59 $\pm$ 0.06	0.36 $\pm$ 0.06	N/A
	UCNII	N/A	N/A	0.70 $\pm$ 0.05	0.50 $\pm$ 0.04	N/A
median raphe	Vehicle	0.38 $\pm$ 0.07	0.75 $\pm$ 0.16	0.51 $\pm$ 0.05	0.24 $\pm$ 0.03	0.23 $\pm$ 0.03
	OCRf	0.40 $\pm$ 0.05	0.45 $\pm$ 0.11	0.70 $\pm$ 0.13	0.31 $\pm$ 0.05	0.20 $\pm$ 0.05
	UCNII	0.44 $\pm$ 0.06	0.49 $\pm$ 0.06	0.50 $\pm$ 0.06	0.38 $\pm$ 0.08	0.20 $\pm$ 0.03