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Neuroanatomical evidence for reciprocal regulation of the corticotrophin-releasing factor and oxytocin systems in the hypothalamus and the bed nucleus of the stria terminalis of the rat: Implications for balancing stress and affect

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Summary

Activation of corticotrophin releasing factor (CRF) neurons in the paraventricular nucleus of the hypothalamus (PVN) is necessary for establishing the classic endocrine response to stress, while activation of forebrain CRF neurons mediates affective components of the stress response. Previous studies have reported that mRNA for CRF2 receptor (CRFR2) is expressed in the bed nucleus of the stria terminalis (BNST) as well as hypothalamic nuclei, but little is known about the localization and cellular distribution of CRFR2 in these regions. Using immunofluorescence with confocal microscopy, as well as electron microscopy, we demonstrate that in the BNST CRFR2-immunoreactive fibers represent moderate to strong labeling on axons terminals. Dual-immunofluorescence demonstrated that CRFR2-fibers co-localize oxytocin (OT), but not arginine-vasopressin (AVP), and make perisomatic contacts with CRF neurons. Dual-immunofluorescence and single cell RT-PCR demonstrate that in the hypothalamus, CRFR2 immunoreactivity and mRNA are found in OT, but not in CRF or AVP-neurons. Furthermore, CRF neurons of the PVN and BNST express mRNA for the oxytocin receptor, while the majority of OT/CRFR2 neurons in the hypothalamus do not. Finally, using adenoviral-based anterograde tracing of PVN neurons, we show that OT/CRFR2-immunoreactive fibers observed in the BNST originate in the PVN. Our results strongly suggest that CRFR2 located on oxytocinergic neurons and axon terminals might regulate the release of this neuropeptide and hence might be a crucial part of potential feedback loop between the hypothalamic oxytocin system and the forebrain CRF system that could significantly impact affective and social behaviors, in particular during times of stress.

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Introduction

Corticotrophin-releasing factor (CRF) containing neurons of the paraventricular nucleus of the hypothalamus (PVN) and the bed nucleus of the stria terminalis (BNST) are critically involved in mediating response to stress (Koob and Heinrichs, 1999; Moore et al., 2000; Shekhar et al., 2005; Hauger et al., 2009). Release of CRF from the PVN activates the hypothalamic-pituitary-adrenal (HPA) axis and initiates the classic endocrine response to stress (Kageyama and Suda, 2009), while CRF BNST neurons mediate the affective response to stressors, including fear and anxiety (Davis, 1998; Alheid, 2003; Walker et al., 2003).

The effects of CRF are mediated by two receptors: CRFR1 and CRFR2 (Chalmers et al., 1995; Lovenberg et al., 1995). In contrast to widely distributed CRFR1, CRFR2 are more regionally restricted and have been found in the lateral septum (LS), ventromedial hypothalamus, raphe nuclei, as well as central amygdala (CeA) and BNST (Chalmers et al., 1995; Chen et al., 2000; Van Pett et al., 2000). Significantly, activation of CRFR1 and CRFR2 appear to mediate opposing behavioral responses (Ji and Neugebauer, 2007; Zhao et al., 2007). Hence, the anxiogenic-like effects of CRF are mediated by CRFR1 (Muller et al., 2003; Nguyen et al., 2006; Sahuque et al., 2006), while CRFR2 activation has been reported to be anxiolytic, anxiogenic, or to have no effect (Bale et al., 2000; Coste et al., 2000; Bale and Vale, 2004; Cooper and Huhman, 2005). This inconsistency suggests that CRFR2 role in modulating the behavioral responses may result from an interaction with other neuromodulatory transmitter systems.

Oxytocin (OT) and arginine-vasopressin (AVP) are nonapeptides synthesized by neurons of the PVN and supraoptic nucleus of the hypothalamus (SON). Both OT and AVP levels are increased in the extended amygdala in response to stress, (Landgraf and Neumann, 2004; Ebner et al., 2005), as a result of release from local fibers (De Vries and Buijs, 1983; Sofroniew, 1983). Like CRFR1 and CRFR2, OT and AVP have generally opposing effects. Hence, AVP acts synergistically with CRF to activate HPA axis and has been shown to enhance anxiety and the consolidation of fear memory (Griebel et al., 2002; Caldwell et al., 2008), while OT acts as an anxiolytic in behavioral paradigms, and dampens the stress response (McCarthy et al., 1996; Windle et al., 1997; Petersson et al., 1999; Neumann et al., 2000; Bale et al., 2001; Amico et al., 2004; Ring et al., 2006; Waldherr and Neumann, 2007).

Intriguingly, the highest levels of OT receptor (OTR) mRNA are found in forebrain regions with high CRF cells density, namely the BNST, CeA, and PVN (Yoshimura et al., 1993; Veinante and Freund-Mercier, 1997). Conversely, CRFR2a mRNA co-localizes with OT mRNA in the rat SON (Arima and Aguilera, 2000), suggesting reciprocal interaction between the CRF and OT systems. However, nothing is known about the relationship between CRFR2 and OT expression in the BNST and hypothalamus, brain regions critically involved in the stress response. In the present study, we addressed this knowledge gap by using immunohistochemical, genetic, and adenoviral-based anterograde tracing techniques to map the regional, cellular, and ultrastructural distribution of CRFR2 in the BNST, PVN and SON in relation to OT, OTR, CRF and AVP.

Methods

Animal subjects

All experiments were performed in tissue from adult (60-70 days old) male, Sprague-Dawley rats (IF, scRT-PCR and WB: Charles River Laboratories, Wilmington, MA; IF and EM - Harlan, Indianapolis, IN). Adenoviral injections were performed on 35 day-old

Sprague-Dawley rats from Charles River. For stereotaxic surgery for colchicine and rAAV-GFP injections, rats were anaesthetized with an IP injection of Dexdormitor (Orion Pharma, Espoo, Finland) and Ketamine hydrochloride (Bioniche Pharma, Bogart, GA, USA) mixture (0.16 mg/kg Dexdormitor and 48 mg/kg Ketamine). All the procedures used were approved by the Institutional Animal Care and Use Committees (IACUC) of Emory University and University of South Carolina School of Medicine, and were in compliance with National Institutes of Health (NIH) guidelines for the care and use of laboratory animals. Animals were housed in same sex groups, 4 animals per cage and were maintained on a 12:12-hr light–dark cycle with ad libitum access to food and water. Animals were housed in animal facility at least 1 week after arrival prior to experiments.

Tissue processing for immunofluorescence

Two perfusion protocols were employed to facilitate immunohistochemical analysis: 6 rats received a standard 4% paraformaldehyde fixation procedure and a second cohort of 2 rats received intracerebroventricular (icv) colchicine (Sigma Aldrich, 120 µg/2 µl) unilaterally 48 hours prior to perfusion to maximize CRF peptide content in neuronal cell bodies. Perfusion protocol was described in details elsewhere (Dabrowska and Rainnie, 2010).

Immunofluorescence experiments

Immunofluorescence experiment protocols were similar to those previously described (Dabrowska and Rainnie, 2010). Briefly, fluorescent immunohistochemistry was performed using the following primary antibodies: rabbit polyclonal anti-CRFR2 antibody directed against the N-terminal (extracellular) domain (1:1000, ab12964, Abcam, Cambridge, MA), rabbit polyclonal anti-CRFR2 antibody against N-terminal domain (1:1000, NLS3570, Novus Biologicals LLC, Littleton, CO), goat polyclonal anti-CRFR2 antibody against C-terminal domain (1:200, sc-20550, Santa Cruz Biotechnology Inc, Santa Cruz, CA), rabbit polyclonal anti-CRF antibody (1:250, ab11133, Abcam, Cambridge, MA), mouse monoclonal anti-OT antibody, clone 4G11 (1:7500, MAB5296, Chemicon-Millipore, Billerica, MA), rabbit polyclonal anti-AVP antibody (1:7500, AB1565, Chemicon-Millipore, Billerica, MA).

To examine the relative expression of CRFR2, CRF, OT and AVP, we performed immunofluorescence experiments on free-floating serial sections of the rat BNST, PVN and SON. Because CRFR2 mRNA is expressed at high levels in the lateral septum (LS), nucleus accumbens (NAc), and dorsal Raphé nucleus (DRN), these sections were also included in our immunofluorescence experiments as positive controls. Representative sections were taken from Bregma +0.22 mm to -0.4 mm for BNST and LS; from +1.70 to +0.70 mm for NAc; from -1.6 mm to -2.12 mm for PVN and SON; and from -7.30 mm to -8.30 mm for DRN and rinsed 3x (10 min each) in phosphate buffer saline (PBS), permeabilized with 0.5 % Triton-X 100 in PBS, and incubated for 48 hrs at 4°C with the appropriate primary antibodies diluted in 0.5% Triton-X/PBS solution. Sections were rinsed 3x (10 min each) in PBS and then incubated at room temperature for 2 hrs with specific Alexa-Fluor secondary antibodies (1:500, Molecular Probes, Invitrogen, Carlsbad, CA, USA): Alexa-Fluor 488 goat anti-mouse IgG or Alexa-Fluor 488 goat anti-rabbit IgG or Alexa-Fluor 488 donkey anti-goat IgG and Alexa-Fluor 568 goat anti-rabbit IgG, or Alexa-Fluor 568 goat anti-mouse IgG or Alexa-Fluor 568 donkey anti-mouse IgG. Following incubation with secondary antibodies, sections were rinsed 3x (10 min each) in PBS and 1x in 0.05 M phosphate buffer (PB), mounted on gelatin-coated glass slides and coverslipped using Vectashield fluorescence mounting medium (Vector Laboratories, Inc., Burlingame, CA).

Dual-immunofluorescent experiments

Co-expression of CRFR2 with OT; OT with CRF; and OT with AVP in the PVN, SON and BNST was determined by dual-immunofluorescent labeling in the BNST, PVN, and SON. Experimental procedures and antibody dilutions (both primary and secondary) for dual-labeling experiments were identical to single-labeling experiments described above. For each pair, primary antibodies were co-applied for 48 hr and secondary antibodies were co-applied for 2 hr. Confocal spinning disk laser microscopy was used to analyze immunoreactivity patterns and to obtain high-resolution photomicrographs using an Orca R2 cooled CCD camera (Hamamatsu, Bridgewater, NJ) mounted on a Leica DM5500B microscope (Leica Microsystems, Bannockburn, IL) equipped with a CSU10B Spinning Disk (Yokagawa Electronic Corporation, Tokyo, Japan). Semi-quantitative analysis of dual-labeled OT/CRFR2 neurons in the PVN and SON and fibers in the BNST was performed with Simple PCI 6.6 software (Hamamatsu, Sewickley, PA). Six randomly assigned Z-stacks were taken from every section analyzed. Neurons and fibers from 12 sections containing PVN and SON, and 12 sections containing BNST region (from 6 different animals) were counted and analyzed for dual immunolabeling.

CRFR2 antibody specificity

All commercial CRFR2 antibodies used in the study have been previously characterized in the literature (Markovic et al., 2008; Waselus et al., 2009). However, we used additional approaches to determine the specificity of the N-terminal directed antibodies, since they were used for the co-localization study. First, we pre-absorbed each CRFR2 antibody (Abcam and Novus Biologicals) with either a control blocking peptide (CRFR2 antigen), or oxytocin. Second, we performed Western Blot analysis on tissue extracts from the BNST and PVN.

CRFR2 antibody pre-absorption assay—Here, two CRFR2 antibodies were separately incubated for 1 hr with a 20x molar excess of control CRFR2 peptide (1:50, ab80010, Abcam, Cambridge, MA) at room temperature. Pre-incubated antibodies were then applied to representative free-floating sections containing the PVN, SON and BNST, which completely abolished CRFR2 staining in these regions. Based on the substantial overlap between CRFR2 and OT staining (see Results), we performed two additional controls. First, both anti-CRFR2 antibodies were pre-absorbed with 0.07 mM OT solution in 0.5% Triton/PBS (oxytocin acetate, Sigma Aldrich, St. Louis, MO). Pre-incubation of both anti-CRFR2 antibodies with oxytocin acetate solution did not affect CRFR2 staining in the PVN, SON, and BNST, a concentration that completely abolished staining by the anti-OT monoclonal antibody. Second, the OT antibody was pre-absorbed for 1 hr with a 20x molar excess of CRFR2 control peptide. Pre-incubation of the CRFR2 blocking peptide with the anti-OT monoclonal antibody had no effect on OT staining in either region. These findings suggest that the nearly complete overlap between CRFR2 and OT staining is not due to cross-reactivity.

Western blot assay—The Abcam and Novus Biologicals CRFR2 antibodies used in this study were further characterized using Western Blot analysis. Expression of CRFR2 protein was examined in both BNST and PVN tissue samples. Western Blot assay was performed as reported previously (Dabrowska and Rainnie, 2010). Briefly, brain slices (300 μ m) containing the PVN or BNST were prepared using a vibratome (Leica VT 1000S). Subsequently, nitrocellulose membranes were incubated overnight at 4°C with one of the two primary anti-CRFR2 antibodies (1:1000, Abcam and Novus Biologicals) in blocking buffer. On the following day, the immunoblots were incubated with an HRP-labeled specific secondary antibody (peroxidase conjugated anti-rabbit IgG antibody, Vector Labs, 1:2000) for 1 hr at room temperature. The CRFR2 protein in the PVN and BNST samples was

detected using SuperSignal West Chemiluminescence (Pierce Biotechnology) and visualized with an Alpha Innotech Fluorochem imaging system (Alpha Innotech, San Leandro, CA).

OT, AVP, and CRF antibody specificity

The mouse monoclonal OT antibody used in this study has been reported in previous studies to show no cross-reactivity to AVP analogues (AVP, vasotocin, and [Asu1,6,Arg8]-vasopressin) using a competitive ELISA (Liu et al., 2002). The rabbit polyclonal AVP antibody is cited by Chemicon-Millipore to show less than 1% cross-reactivity with OT, but that there is still sufficient specificity to distinguish between OT and AVP producing cells and fibers (Simerly and Swanson, 1987; Markakis et al., 2004). In addition, we have performed pre-absorption assays for both OT and AVP antibodies. Here antibodies were pre-incubated for 1 hr with excess of either OT (0.07 mM oxytocin acetate, Sigma Aldrich, St. Louis, MO) or AVP (0.07 mM [Arg8]-vasopressin acetate, Sigma Aldrich, St. Louis, MO), respectively. Pre-incubation completely abolished respective staining in the PVN, SON, and BNST.

We have demonstrated the specificity of the rabbit polyclonal CRF antibody in a previous study (Martin et al., 2010). In addition, in the current study we also show that CRF staining is restricted to regions previously reported to contain CRF-positive cells and fibers including the PVN, CeA, and BNST.

Anterograde adeno-associated virus-based tracing of the PVN neurons

To trace anterograde projections of PVN neurons and evaluate the connectivity between the PVN and BNST, we expressed green fluorescent protein (GFP) in PVN neurons using a recombinant adeno-associated viral vector (rAAV1-TRUFR-eGFP, estimated titer: 2×10^{12} virus molecules per ml), containing a cytomegalovirus promoter driving GFP expression (Gene Therapy Center Virus Vector Core Facility, the University of North Carolina at Chapel Hill, Chapel Hill, NC, USA) (Kells et al., 2007; Kells et al., 2008). Here, 1 μ l of the rAAV1-GFP was bilaterally injected (per side) into the PVN of two, 35-days old rats, using the following stereotaxic coordinates from Bregma: AP: -1.4 mm; ML: ± 0.5 mm, DV: -7.8 mm (modified from Paxinos and Watson, 1998). Rats were perfused 14 days later following the standard 4% paraformaldehyde fixation protocol described above, and the brains were removed, postfixed, and sliced as described above for immunohistochemistry. Sections containing the PVN and BNST were processed using an immunolabeling for AAV1-GFP and oxytocin or CRFR2 expression in PVN neurons as well as in the BNST as described above. Although the rAAV-CMV-GFP tracer injections were performed on late adolescent, 35 days old rats, these animals were not sacrificed until 14 days later to allow sufficient time for optimal expression of GFP in cell bodies as well as axons and axon terminals in projection sites. Consequently, by the time rats were sacrificed, they were at least 50 days old.

Electron-microscopy immunohistochemistry

Localization of CRFR2 at the ultrastructural level was performed in two rats. Rats were anesthetized with chloral hydrate (350 mg/kg), and perfused intracardially with PBS containing 0.5% sodium nitrite (50 ml), followed by an acrolein/paraformaldehyde mixture (2.0% paraformaldehyde/3.75% acrolein in PB for 1 minute, followed by 2.0% paraformaldehyde in PB for 30 minutes). Brains were then postfixed in 2.0% paraformaldehyde for 2 hrs, and sectioned on a Vibratome in the coronal plane at 60 μ m. Sections were rinsed in 1.0% borohydride in PB for 30 minutes, rinsed thoroughly in several changes of PB for 1 hr, cryoprotected in 30% sucrose in PB, and freeze-thawed three times over liquid nitrogen in order to increase antibody penetration. Sections were rinsed well in PB and then incubated for 36 hrs at 4°C in the rabbit polyclonal anti-CRFR2 primary

antibody (1:2000, Abcam, see above) in PBS containing 1% normal goat serum, and processed using a rabbit ABC kit (Vector Labs) with nickel intensified DAB as the chromogen (Hancock, 1986).

Sections through the BNST were postfixed in 2% osmium tetroxide in 0.16 M sodium cacodylate buffer (pH 7.4) for 1 hr, dehydrated in graded ethanols and acetone, and flat-embedded in Polybed 812 (Polysciences, Warrington, PA) in slide molds, between sheets of Aclar (Ted Pella, Redding, CA). Selected areas of the anterolateral BNST cell group (BNST_{ALG}) were remounted onto resin blanks. Silver thin-sections were collected on formvar-coated slot grids, stained with uranyl acetate and lead citrate, and examined with a JEOL-200CX electron microscope. Micrographs were taken with an AMT XR40 digital camera system (Advanced Microscopy Techniques, Danvers, MA). Figures were then assembled and labeled, and their components' tonal ranges were adjusted and matched by using Adobe Photoshop 6.0.

Data analysis focused on the contacts formed by CRFR2-ir axon terminals, and the identification of CRFR2-ir processes. Areas of BNST that had several segments of darkly stained varicose axons were chosen for ultrastructural analysis. Serial sections were analyzed and often followed on consecutive grids. Serial sections were helpful for verifying label in small and lightly immunoreactive structures and determining the synaptic nature of contacts. Synapses were identified using standard criteria: 1) parallel presynaptic and postsynaptic membranes exhibiting membrane thickenings, 2) a synaptic cleft containing dense material; and 3) clustered synaptic vesicles associated with the presynaptic membrane (Peters A et al., 1991).

Single-cell RT-PCR (scRT-PCR)

The procedure used to determine mRNA transcript expression in single cells has been described elsewhere in detail (Martin et al., 2010). Briefly, the cytoplasm from 13 SON neurons, 12 PVN neurons, and 46 BNST Type I – III neurons (Hammack et al., 2007) was aspirated into pipettes containing ~5 µl of RNase-free patch solution by applying gentle negative pressure. The content of the patch pipette were then expelled into a microcentrifuge tube containing 5 µl of the reverse transcription (RT) cocktail: 1x RT Buffer, 10 mM dNTPmix, 1x Random hexanucleotide, Multiscribe RT 5 U/µl, and RNase-free water, and then incubated in a thermal cycler (PTC-200 Peltier, MJ Research). Reverse transcription was performed in a final volume of 10 µl and stored at -20°C before further processing. All reagents for reverse transcription were obtained from Applied Biosystems (Foster City, CA). RT product was amplified in triplicate in 0.2 ml thin walled PCR tubes (3 µl each) as previously described (Martin et al., 2010).

Amplified cDNA from each cell was first screened for the expression of 18S rRNA as a positive control. Amplified cDNA was the subjected to another amplification step using 2 µl of cDNA from each cell as a template and 100 nM of each gene-specific primer. The PCR master mix was comprised of 10x PCR Buffer, 3 mM MgCl₂, 10 mM dNTPs, 2.5 U of Taq DNA Polymerase in a final volume of 20 µl. PCR was performed using a 10 min hot start at 95°C followed by a 40-cycle program for 18S rRNA, CRFR1, CRFR2, OT, AVP, OTR, GAD₆₇ and VGLUT2 (94°C, 40 s; 56°C, 40 s; 72°C, 1 min) and for CRF (95°C, 30 s; 70°C, 1 min; 68°C, 2 min). PCR products were separated by electrophoresis in 1% agarose gel and visualized by staining with ethidium bromide. All primers used in scRT-PCR are summarized in Table 1.

Results

Comparison of CRFR2-, OT-, AVP-, and CRF-immunofluorescence in the hypothalamus and BNST

Hypothalamus—In the hypothalamus, CRFR2-ir showed high somatodendritic labeling in discrete clusters of neurons in the PVN and SON. Notably, multiple axons were seen to originate from the CRFR2-ir cells and course laterally and ventrally towards the pituitary, additional axons coursed dorsally and laterally toward the anterior commissure (Fig. 1A', B'). Both N-terminal anti-CRFR2 primary antibodies demonstrate indistinguishable immunolabeling patterns in all brain areas analyzed but photomicrographs presented in results section were prepared using Abcam anti-CRFR2 antibody. A similar somatodendritic labeling was observed in the PVN using the C-terminal CRFR2 antibody, but the labeling was less intense and restricted to neuronal membranes and proximal dendrites. However, because of the high background observed, it was difficult to differentiate whether observed neuropil-like labeling might refer to CRFR2-labeled processes.

Consistent with previous findings (Caldwell et al., 2008), OT-ir (Fig. 1A, B) and AVP-ir labeling showed high somatodendritic expression in neurons of both the PVN and SON. Here, the majority of the OT- and AVP-ir neurons were found within the magnocellular division of the PVN and SON; however some smaller fusiform-shaped parvocellular neurons were also observed, as described previously (van den Pol, 1982). In contrast, CRF-ir was mainly localized to a population of parvocellular and to lesser extent in magnocellular neurons in the PVN (Fig. 2A', B'), but CRF immunoreactivity was not observed in the SON, in contrast to CRF mRNA expression observed occasionally in the SON in the scRT-PCR experiment (see below).

Significantly, our dual-immunofluorescence showed that 99.5% of CRFR2-ir neurons selectively and mutually co-localize OT-ir in the PVN (Fig. 1A'', B'') and SON (not shown). The mutual co-localization was observed at the level of cell bodies, dendrites and axons. In the cell bodies, OT-ir was observed in both the cytoplasm and the plasma membrane, while CRFR2-ir was mainly observed in the plasma membrane. Co-localization of OT and CRFR2 was also observed with the C-terminal CRFR2 antibody. Dual-immunofluorescence studies further revealed that AVP- and OT-ir localization in magno- and parvocellular neurons of the PVN, for the most part was mutually exclusive, and found in adjacent, but distinct, subpopulations, which parallels previous findings (De Vries and Buijs, 1983; Caldwell et al., 2008) (data not shown). Significantly, the majority of CRF- and OT-ir neurons were also mutually exclusive in the PVN, where both were found in mainly adjacent neuronal populations (Fig. 2A'', B''). However, on several occasions CRF-ir processes were observed to make perisomatic contacts with OT-ir cell somas, and conversely, OT-ir processes were observed to make perisomatic contacts with CRF-ir neurons. Considering that OT-, CRF-, and OT-, AVP-ir neurons were found to be mutually exclusive subpopulations in the PVN and SON, these data suggest that CRFR2-ir neurons do not co-localize AVP or CRF.

BNST—CRFR2 immunoreactive (CRFR2-ir) fibers were observed coursing through distinct regions of the forebrain, including the LS, NAc, DRN, and BNST. Here, CRFR2-ir fibers were characterized by multiple, beaded varicosities, indicative of possible en passant release sites and axon terminals (Fig. 3A, B). Of particular interest was the presence of CRFR2-ir fibers in the BNST where the highest density of labeled fibers was observed in the anterolateral BNST cell group (BNST_{ALG}), a region, which includes the CRF neuron rich oval nucleus.

Consistent with previous studies, both OT-ir and AVP-ir fibers were also found in the BNST, with the high expression of OT-ir fibers being observed in the oval nucleus (Fig.

3A', B'). Significantly, our dual-labeling study showed that nearly all (98%) CRFR2-ir fibers in the BNST also co-localized OT (Fig. 3A'', B''). Similar co-localization of CRFR2 with OT was observed in the NAc, DRN, and LS (data not shown). Moderate to high levels of AVP-ir fibers were also observed in the BNST (Fig. 4A'). AVP-ir fibers were seen to course in parallel to OT-fibers but they never co-localized with OT (Fig. 4A'').

As expected, a large number of CRF-ir neurons were observed in the BNST_{ALG} of colchicine-treated animals, in particular in the oval nucleus, which showed prominent somatodendritic labeling as well as a dense network of CRF-ir fibers (Fig. 4B'). CRF-ir did not co-localize with OT-ir profiles but OT-ir fibers did make broad contacts with the soma of CRF-ir neurons in the BNST_{ALG}, as shown in Fig. 4B''.

To confirm that OT-ir neurons of the PVN may innervate the BNST_{ALG}, we used an adeno-associated viral vector expressing GFP under a CMV promoter to selectively label neurons in the PVN. Fourteen days after bilateral rAAV1-CMV-GFP injections into the PVN, animals were sacrificed and the sections were processed for immunohistochemistry. As illustrated in Fig. 5, somatic GFP expression was restricted to neurons in the PVN and GFP-labeled axons were seen coursing ventrally toward the pituitary, and dorso-laterally towards the anterior commissure (Fig. 5A). Significantly, GFP-labeled fibers were observed in multiple forebrain regions, including a large number of fibers in the BNST_{ALG} (Fig. 5B). Our immunofluorescence study revealed that both magno- and parvocellular neurons expressed GFP in the PVN, some of which were OT-positive (Fig. 5A'') and also CRFR2-positive (not shown). Similarly, in the BNST_{ALG} we found GFP-labeled fibers with multiple, beaded varicosities, which also co-localized OT-ir (Fig. 5B'') and CRFR2-ir (Fig. 5C''). These data provide anatomical support for the existence for a direct monosynaptic pathway linking hypothalamic OT-ir neurons with the BNST_{ALG}.

Ultrastructural analysis of CRFR2-ir fibers in the BNST—An ultrastructural electron microscopic (EM) survey of CRFR2 immunoreactivity in the BNST_{ALG} found many labeled axonal profiles, most of which were slender and generally devoid of dense core vesicles (DCV). In fact the majority of CRFR2-ir axons observed in the EM sections were in addition to the relatively sparse, dark varicose axons clearly seen in the light microscope, and probably corresponded to the moderate neuropilar staining observed in the tissue sections. In the electron microscope, CRFR2-ir axons containing DCV varied in thickness and electron density, but were generally broader than the more common CRFR2-ir axons that did not contain DCV. Light to moderate immunoreactivity was also seen in a subset of dendrites (Fig. 6.1A, 6.2). Compared to the number of labeled axonal profiles, synapses were relatively sparse. The vast majority of CRFR2-ir terminals making synaptic contacts were small (<0.5 μ m in diameter) and devoid of DCV (Fig. 6.1B-D, 6.2). These terminals mainly formed synapses with small-caliber (distal) dendrites and spines. Synapses with spines were generally asymmetrical (Fig. 6.1C, D), whereas synapses with dendrites were symmetrical (Fig. 6.1B), or asymmetrical (Fig. 6.1A, 6.2). Large CRFR2-ir terminals with DCV rarely made synaptic contacts (Fig. 6.1A). Some CRFR2-ir axons with DCV made broad contacts with perikarya that did not appear synaptic.

The heterogeneous nature of the synaptic contacts made by CRFR2-ir terminals and the expression of DCV in some CRFR2-ir axon terminals raised the possibility that they may originate from two functionally distinct neuronal populations. Consequently, we next used scRT-PCR to compare and contrast the genetic phenotype of putative OT neurons in the PVN and SON. In addition, we examined the genetic phenotype of Type I – III BNST_{ALG} neurons to determine which, if any, subtype expresses transcripts for either CRF and/or the OT receptor (OTR).

Genetic phenotype of PVN, SON, and BNST neurons—Here, neurons of the PVN, SON, and BNST_{ALG} were screened for their expression of the following mRNA transcripts: OT, AVP, CRF, CRFR1, CRFR2, OTR, a putative marker of glutamatergic neurons - vesicular glutamate transporter type 2 (VGLUT2), as well as a marker of GABAergic neurons, γ -amino-decarboxylase 67 (GAD67). The results of this study are summarized in Table 2.

Consistent with our dual-immunofluorescence study, in the PVN 10/12 neurons sampled expressed mRNA transcripts for OT and of these neurons 8/10 co-expressed transcripts for CRFR2. Significantly, only 1/10 OT mRNA positive neurons co-expressed CRFR1 mRNA. In the PVN, 3/12 neurons sampled expressed transcripts for AVP, of which 2 co-expressed OT transcripts. Importantly, none of AVP-positive neurons co-expressed mRNA transcripts for CRFR2. Moreover, 9/10 OT neurons expressed VGLUT2 transcripts but not GAD67 mRNA suggesting that OT/CRFR2 neurons of the PVN are glutamatergic. Interestingly, only 3/10 OT/CRFR2-positive PVN neurons co-expressed transcripts for the OTR. In contrast, 3/13 PVN neurons sampled expressed transcripts for CRF, of which 2/3 co-expressed transcripts for OTR.

Similarly, 11/13 SON neurons sampled expressed transcripts for OT, and of these 9/11 also co-expressed CRFR2, while only 1/11 co-expressed CRFR1 mRNA and none of them co-expressed AVP. Significantly, 7/11 of these OT/CRFR2 neurons co-expressed transcripts for VGLUT2 and none co-expressed GAD67, suggesting that, like their PVN counterparts, OT/CRFR2-positive neurons of the SON are glutamatergic. Moreover, like the PVN, 2/13 neurons sampled expressed transcripts for CRF and both of these were found to co-express transcripts for OTR, suggesting that local release of oxytocin in the SON and PVN may directly regulate the activity of CRF neurons in these two regions. Conversely, only 2/11 OT-neurons in the SON expressed mRNA for OTR.

Previously, we have described three physiologically distinct cell types in the BNST_{ALG}, type I - III (Hammack et al., 2007). Of the 46 BNST_{ALG} neurons sampled for this study, only 2/15 Type II neurons and 1/16 Type III neurons expressed transcripts for OT, and only 1/15 Type II neurons and 2/16 Type III neurons expressed CRFR2 mRNA. It is important to note that 1/15 Type I neurons, 4/15 Type II neurons, and 2/16 Type III neurons expressed transcripts for CRFR1, and none of these neurons co-expressed CRFR2 transcripts. Significantly, 13/16 Type III neurons were found to express mRNA transcripts for CRF and all of these neurons co-expressed mRNA transcripts for the OTR. In addition, a subpopulation of Type II neurons (6/15) was also found to express transcripts for OTR. Together these data suggest that OT release in the BNST_{ALG} would predominantly modulate the activity of all Type III CRF neurons and a subpopulation of Type II BNST_{ALG} neurons. Notably, none of the 46 Type I – III neurons expressed transcripts for glutamatergic marker, but all of them expressed GAD67 transcripts.

Western Blot—Finally, our Western Blot analysis for CRFR2 revealed two distinct bands at approximately 40 and 48 kDa in the BNST and PVN tissue extracts. The 48-kDa band corresponds to the full-length CRFR2 α protein, which has been calculated from the published sequence (Miyata et al., 2001). However, a soluble splice variant of CRFR2 has also been reported with a molecular weight of 40 kDa (Chen et al., 2005). Importantly, the soluble splice variant includes most of the N-terminal domain, the portion of the protein against which the CRFR2 antibodies were targeted. The soluble splice variant can bind CRF but not CRFR2 specific ligands (Miyata et al., 1999; Miyata et al., 2001; Chen et al., 2005), suggesting that the splice variant may selectively inhibit CRF modulation of the OT system and spare UCN modulation (Fig. 7).

Discussion

The results of our study have revealed, for the first time, a potential feedback loop between the hypothalamic oxytocin system and the forebrain CRF system. Hence, in the forebrain, CRFR2 are expressed by neurons of the PVN and SON and in both regions CRFR2-ir is found in OT-, but not AVP or CRF-ir neurons. Our scRT-PCR study confirmed that hypothalamic neurons, which co-express mRNA for OT and CRFR2, do not express CRF or AVP mRNA. Conversely, expression of the OTR in the hypothalamus is restricted to a subpopulation of putative CRF-, but not OT/CRFR2-neurons, which suggest that OTR activation might regulate excitability of CRF neurons in the PVN. ScRT-PCR further demonstrated that OT neurons of both the PVN and SON express mRNA for glutamatergic but not GABA-ergic markers.

In the BNST_{ALG}, we showed that CRFR2-ir was restricted to fibers with multiple beaded varicosities, suggesting axon terminals. This observation was confirmed by our EM study, which revealed two distinct CRFR2-ir profiles; 1) small caliber axon terminals that lacked DCV and made primarily asymmetric synapses with the spines of BNST_{ALG} neurons (possibly glutamatergic terminals (Shepherd, 1996)), 2) larger caliber axon terminals containing DCV (possibly peptidergic terminals (Zhang et al., 2009)), which occasionally contacted BNST_{ALG} neurons without making specific synapses. Significantly, CRFR2-ir fibers were co-localized with OT-ir fibers, and OT-ir fibers made broad perisomatic contacts with CRF-ir neurons in the oval nucleus of the BNST_{ALG}. Consistently, scRT-PCR study revealed that all Type III BNST_{ALG} neurons sampled, which expressed CRF, co-expressed mRNA transcripts for the OTR, suggesting that CRF neurons would be responsive to local OT release. Moreover, unlike the OT neurons of the hypothalamus, all of the BNST neurons sampled express GAD67, therefore appeared to be GABAergic. Finally, a discrete subpopulation of Type II BNST_{ALG} neurons expressed OTR but not CRF mRNA. The neuropeptide phenotype of these GABAergic neurons has yet to be determined.

The somatodendritic expression of CRFR2 peptide in the PVN and SON is consistent with previous *in situ* hybridization studies for CRFR2 mRNA expression in the hypothalamus (Chalmers et al., 1995; Van Pett et al., 2000). However, discrepancies were observed between these early *in situ* hybridization studies and our current immunohistochemical study. Hence, strong CRFR2 *in situ* hybridization signals were observed in the BNST, LS and DRN, whereas in our study CRFR2-ir in these regions was confined to fibers and no somatic CRFR2-ir was observed. However, in agreement with the *in situ* studies our scPCR study also revealed that ~7% of BNST_{ALG} neurons express CRFR2 mRNA. One potential explanation for this apparent inconsistency is that any CRFR2 peptide expressed by BNST_{ALG} neurons may be rapidly shipped out from cell bodies to downstream projection sites. Alternatively, the similarity in the N-terminal amino acids sequence between CRFR2 and CRFR1 raises the possibility that the observed CRFR2-ir might result from non-specific staining of CRFR1. However, it seems unlikely for several reasons; 1) CRFR1 and CRFR2 expression patterns are substantially different (Radulovic et al., 1998; Chen et al., 2000), 2) similar patterns of staining were observed with antibodies directed against N- and C-terminal fragments of CRFR2 in our immunolabeling experiment, and 3) our scPCR study confirmed that transcripts for the CRFR2 but not CRFR1 were expressed by OT neurons in the PVN and SON.

Previous studies have suggested that connections between the BNST and the PVN were mostly unidirectional, with the BNST acting as a relay center to regulate the flow of information from the limbic structures to the PVN (Sullivan et al., 2004; Choi et al., 2007). Indeed, afferent fibers originating from rich in CRF neurons, the oval and fusiform BNST nuclei (Swanson et al., 1983), have been shown to innervate the magnocellular region of the

PVN (Dong et al., 2001). Here, we show that CRFR2-ir co-localizes with OT-ir in the magnocellular neurons of the hypothalamus, and also OT fibers of the BNST, suggesting that hypothalamic nuclei may reciprocally innervate the BNST via CRFR2/OT-ir fibers. Our viral vector tracing study indeed showed that OT-ir fibers in the BNST_{ALG} originate, at least partially, from OT neurons of the PVN, which parallels findings by Ross et al. (2009) in the NAc.

Outside the hypothalamus, previous *in situ* hybridization and ligand-binding studies had reported CRFR2 to localize within distinct subcortical nuclei, including the BNST, LS, and DRN (Chalmers et al., 1995; Van Pett et al., 2000; Lim et al., 2005), and it had been assumed that postsynaptic activation of CRFR2 within these nuclei would directly modulate neuronal populations. Instead, our immunofluorescence study has revealed that CRFR2 labeling in these regions was situated on OT-ir fibers coursing through the neuropil, suggesting a more indirect modulation of neural activity in these regions. At the ultrastructural level, CRFR2-labeling was observed in two types of axonal profile. At first glance these data suggested that the CRFR2 antibody might label two distinct afferent inputs to the BNST_{ALG}. However, our scRT-PCR study of PVN and SON neurons revealed that OT/CRFR2 neurons expressed mRNA transcript for VGLUT2, which is consistent with previous studies (Kawasaki et al., 2006; Iremonger et al., 2010). In the NAc, OT-ir axospinous synapses also display ultrastructural features of excitatory glutamatergic contacts (Ross et al., 2009); suggesting the CRFR2/OT fibers in the BNST_{ALG} and NAc may originate from a common glutamatergic source. In support of a glutamatergic/OT pathway from the PVN to the BNST, electrical stimulation of the PVN has been shown to evoke an orthodromic excitatory response in the majority of BNST neurons recorded (Saphier and Feldman, 1986). Together, these data suggest that OT neurons of the hypothalamus may utilize glutamate to make precise point-to-point synaptic contacts with BNST_{ALG} neurons, whereas OT may regulate neural activity in a more paracrine fashion (Hu et al., 2001). Consistent with this premise, our EM study revealed that large caliber CRFR2 axonal profiles containing DCV appeared to contact BNST_{ALG} neurons, but most of them did not form distinct synapses. Furthermore, at the LM level, OT-ir fibers were observed to make perisomatic contacts with CRF-containing BNST_{ALG} neurons, and our scRT-PCR study revealed that these neurons (Type III) routinely express OTR mRNA transcripts, suggesting that they would be responsive to local OT release.

It is possible that CRFR2 expression on large caliber OT fibers may provide a mechanism, by which CRF release in the BNST_{ALG} could function to regulate paracrine release of OT into the milieu surrounding CRF neurons. In the central amygdala (CeA), Stoop and colleagues have elegantly shown that OT acts to excite a subpopulation of GABAergic neurons in the lateral CeA, which then act to inhibit output neurons of the medial CeA by increasing tonic GABA release (Huber et al., 2005; Viviani and Stoop, 2008). Like the BNST_{ALG}, the lateral CeA also contains a high density of CRF-containing neurons and it is possible that these are the same cell population that is excited by OT. If OT also excites CRF neurons of the BNST, presynaptic CRFR2 on OT terminals may act in a feedback fashion to modulate OT release in the BNST_{ALG}. Furthermore, small-caliber CRFR2-ir axonal terminals observed in EM study, which presumably are glutamatergic and make asymmetric axo-spinous synapses, might preferentially target local circuit GABAergic interneurons, or neurons containing peptide neurotransmitters other than CRF, such as enkephalin, somatostatin, or neuropeptide Y (Arluison et al., 1990; Walter et al., 1991). Activation of this cell population may then inhibit the CRF cell population.

However, it should not be overlooked that a small population of Type II and Type III BNST neurons expressed CRFR2 mRNA transcripts. This observation might correlate with CRFR2-ir observed occasionally in dendrites at the EM level, and potentially also with axon

terminals forming symmetrical synapses, what might correspond to a third type of small, potentially OT-negative CRFR2-fibers, which may originate from local or distant GABAergic neurons in contrast to the majority of OT/CRFR2 glutamatergic terminals originating from the hypothalamus. CRFR2-ir observed on distal dendrites and spines suggests a possible role in modulation of intrinsic membrane properties of subpopulations of BNST neurons.

While neither OT nor CRFR2 are thought to exert strong control over the basal homeostatic set point for HPA axis activation, both seem to act to dampen HPA axis activation in response to environmental stressors. For example, CRFR2^{-/-} mice have normal basal ACTH and CORT levels, but overproduce CRF and show a more rapid and longer lasting elevation of ACTH and CORT after a stressor, resulting in an extended stress response (Bale et al., 2000; Coste et al., 2000). Similarly, OT^{-/-} mice show higher CORT levels in response to psychogenic stressors compared to wild-type controls (Mantella et al., 2005; Amico et al., 2008). Although the activation of the HPA axis by the PVN is primarily dependent on the activity of CRF-synthesizing, parvocellular neurons, which project directly into the pituitary, the results of our study raise some interesting points about possible reciprocal interactions between magnocellular OT neurons and CRF parvocellular neurons in the PVN. Nearly all of the PVN CRF neurons probed in our single-cell PCR study expressed oxytocin receptor (OTR) mRNA, which suggests that local OT release could directly impact excitability of CRF-synthesizing neurons in the PVN and, therefore, directly modulate activity of the HPA axis. Indeed, OT has been shown to inhibit both basal and stress-induced HPA axis activity (Windle et al., 1997; Neumann et al., 2000; Bale et al., 2001; Amico et al., 2004). Significantly, the inhibitory effect of OT on the basal activity of the HPA axis was mainly attributed to OT action within the PVN because local infusion of an OT receptor antagonist in the PVN significantly increased the basal release of ACTH (Neumann et al., 2000). The results from our current study extend this understanding, and further suggest that these two interrelated circuits could act to functionally regulate both the central and the peripheral response to stress. Hence, local CRF release arising from either parvocellular neurons or BNST neurons could activate CRFR2 on magnocellular OT neurons to induce somatodendritic OT release in the PVN itself, as well as terminal OT release in the BNST. Our single-cell RT-PCR data further suggests that, in turn, CRF containing neurons in both regions would be highly responsive to local OT release and therefore this potential feedback inhibitory circuit could act to curtail prolonged central and HPA axis activation in response stress.

An interaction between the CRFR2 and OT systems would also reconcile data on social behavior regulation. Hence, OT is known to facilitate partner preference formation (Insel and Hulihan, 1995), and microinjection of CRF into the NAc accelerates partner preference formation in male prairie voles, an effect mediated by CRFR2 (Young and Wang, 2004; Lim and Young, 2006). Our data would strongly suggest that the similarities in the behavioral responses observed following CRFR2 and OT manipulations are because they are potentially impacting a common, reciprocally linked, neuroanatomical circuit (see schematic - Fig. 8).

Intriguingly, CRF is not the only endogenous ligand for CRFR2; two other members of the CRF peptide family, urocortin 2 (UCN2) and urocortin 3 (UCN3) are potent, selective ligands for CRFR2 (Hsu and Hsueh, 2001; Lewis et al., 2001). Neurons containing UCN3 are also found in the BNST (Wittmann et al., 2009), and central administration of either UCN2 or UCN3 is reported to modulate delayed anxiolytic-like behavior (Valdez et al., 2002; Valdez et al., 2003). The results of our study suggest that this delayed effect may be consistent with an indirect activation of the OT system (Windle et al., 1997; Labuschagne et al., 2010).

In conclusion, we demonstrated a reciprocal neuroanatomical relationship between CRF-containing neurons of the BNST_{ALG} and OT-containing neurons of the hypothalamus. Our finding, that CRFR2 co-localizes OT in both hypothalamic neurons and axons terminals throughout the BNST_{ALG}, suggests that activation of CRFR2 could modulate not only the excitability of hypothalamic OT neurons, but also the release of this neuropeptide in the BNST. Conversely, OTR mRNA expression on CRF neurons suggests that OT might modulate their excitability. Given the critical role played by the OT and CRF systems in regulating social and affective behaviors, understanding how this apparent feedback loop modulates behavior will be crucial to our understanding of psychopathological conditions such as generalized anxiety disorder and autism.

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Figure 1.

A-A'' Photomicrographs showing high level co-localization of CRFR2 (**A'**, red) and OT (**A**, green) in the PVN (**A''**, merged, 10x, scale bar 100 μ m). Both CRFR2 (**A'**, red) and OT (**A**, green) demonstrate high somatodendritic immunoreactivity in the PVN neurons. **B-B''** As seen at higher magnification (63x, scale bar 10 μ m), all CRFR2-immunoreactive neurons (**B'**, red) co-localize OT (**B**, green) at the level of soma, dendrites and axons in the PVN (**B''**, merged; 3V - 3rd ventricle).

Figure 2.

A-A'' Photomicrographs showing adjacent and mainly non-overlapping somatodendritic immunoreactivity of OT (**A**, green) and CRF (**A'**, red) in the PVN (**A''**, merged, magnification 10x, scale bar 100 μ m, 3V – 3rd ventricle). **B-B''** At higher magnification (63x, scale bar 10 μ m) it can be seen that CRF (**B'**, red, closed arrows) and OT (**B**, green, open arrows) represent exclusive neuronal populations in the PVN (**B''**, merged). Occasionally, OT-positive cells demonstrate immunolabeling for CRF as indicated by double arrows.

Figure 3.

A-A'' Photomicrographs showing high level co-localization of CRFR2-immunoreactive fibers (**A**, green) and OT-positive fibers (**A'**, red) in the anterolateral BNST group (BNST_{ALG}, **A''**, 20x, scale bar 100 μm). **B-B''** As seen at higher magnification (63x, scale bar 10 μm), both CRFR2- (**B**, green) and OT- (**B'**, red) immunoreactive fibers are characterized by multiple, beaded varicosities, indicative of possible en passant release sites and axon terminals and the great majority of CRFR2-immunopositive fibers co-localize OT in the BNST_{ALG} (**B''**, merged, LV – lateral ventricle, CP – Caudate Putamen). All of the photomicrographs shown above represent compiled Z-stack images taken at 50 μm resolution through the z-axis of the tissue and processed using Simple PCI 6.6 software.

Figure 4.

A-A'' Photomicrographs showing exclusive immunoreactivity of OT (**A**, green) and AVP-positive fibers (**A'**, red) in the BNST_{ALG}. Both AVP and OT-immunoreactive fibers are highly expressed in the BNST_{ALG} but they are exclusively localized with each other (**A''**, merged, 63x, scale bar 10 μ m). **B-B''** Photomicrographs showing exclusive OT- (**B**, green) and CRF- (**B'**, red) immunoreactivity in the BNST_{ALG}. High somatodendritic immunoreactivity of CRF is seen in the BNST_{ALG} with the highest expression in the oval nucleus division. As indicated by double arrows, OT-positive fibers make perisomatic contacts with CRF-immunoreactive cells in the BNST_{ALG} (**B''**, merged, 63x, scale bar 10 μ m).

Figure 5.

Figure 5. A-A'' Photomicrographs showing high level co-localization of GFP (**A**, green) with OT (**A'**, red) in the PVN fourteen days after rAAV1-CMV-GFP was injected into the PVN. GFP expression was restricted to the PVN neurons and some of GFP-immunoreactive neurons co-localized OT (**A''**, merged) and CRFR2 (not shown, 10x, scale bar 100 μ m). **B-B''-C-C''** Significantly, GFP-labeled fibers (**B**, **C**, green), with beaded varicosities were also observed in the BNST_{ALG} and some of these fibers co-localized OT (**B''**, merged) and CRFR2 (**C''**, merged, double arrows, 63x, scale bar 10 μ m).

Figure 6.1.

Electron micrographs of CRFR2-immunoreactivity in the BNST_{ALG}. **A:** A CRFR2-ir axon terminal (C+ T) makes asymmetrical synaptic contact (arrow) with a lightly-immunoreactive small caliber dendrite (C+ SD). The postsynaptic density appears thicker in serial sections. Two dense core vesicles (DCV) in the labeled terminal are indicated. **B:** A CRFR2-ir axon terminal (C+ T) makes symmetrical synaptic contact (arrow) with an unlabeled small caliber dendrite (USD). **C, D:** CRFR2-ir terminals (C+ T) make asymmetrical synaptic contacts (arrows) with dendritic spines (sp). Scale bars = 0.5 μ m: Scale in B is for A and B; scale in D is for C and D.

Figure 6.2.

Electron micrograph of a CRFR2-ir terminal (C+ T) making asymmetrical synaptic contact (arrow) with an unlabeled small caliber dendrite (USD) in the BNST_{ALG}. This unlabeled dendrite also receives an asymmetrical synapse (arrowhead) from an unlabeled terminal (UT). The unlabeled terminal also makes asymmetrical synaptic contact (arrowhead) with a spine (sp) emerging from a lightly immunoreactive dendrite (C+ SD). Scale bar = 0.5 μ m.

Figure 7. Expression of CRFR2 protein in both PVN and BNST tissue extracts was confirmed by presence of two distinct bands in the immunoblots at ~ 48 and 40 kDa, which correspond, respectively, to full-length CRFR2 protein and soluble splice variant of CRFR2.

Table 1

PCR primers used in the study

Genes	Accession #	Product size (bp)
18S rRNA	X01117	563
CRF	NM031019	394
CRFR2	NM022714	307
CRFR1	NM030994	248
OT	X59496	463
OTR	AY338194	186
AVP	X59496	440
VGLUT2	AF271235	342
GAD67	M76177	426

Table 2
Summary of scRT-PCR results from PVN and SON neurons and type I-III BNST neurons.

	Total	CRF	CRFR2	CRFR1	OT	OTR	AVP	VGLUT2	GAD67
PVN	12	3	8	1	10	5	3	10	0
SON	13	2	9	2	11	4	0	7	0
BNST type I	15	7	0	1	0	1	0	0	6
BNST type II	15	8	1	4	2	6	0	0	15
BNST type III	16	13	2	2	1	13	0	0	16