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Importance of CRF Receptor-Mediated Mechanisms of the Bed Nucleus of the Stria Terminalis in the Processing of Anxiety and Pain

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Corticotropin-releasing factor (CRF)-mediated mechanisms in the bed nucleus of the stria terminalis (BNST) have a pivotal role in stress-induced anxiety and hyperalgesia. Although CRF is known to activate two receptor subtypes, CRF_1 and CRF_2, attempts to delineate the specific role of each subtype in modulating anxiety and nociception have been inconsistent. Here we test the hypothesis that CRF_1 and CRF_2 receptor activation in the anteriolateral BNST ($BNST_{AL}$) facilitates divergent mechanisms modulating comorbid anxiety and hyperalgesia. Microinfusions of the specific antagonists CP376395 and $Astressin_2B$ into the $BNST_{AL}$ were used to investigate CRF_1 and CRF_2 receptor functions, respectively. We found that CRF_1 and CRF_2 receptors in the $BNST_{AL}$ had opposing effects on exploratory behavior in the elevated plus-maze, somatic mechanical threshold, and the autonomic and endocrine response to stress. However, CRF_1 or CRF_2 receptor antagonism in the $BNST_{AL}$ revealed complementary roles in facilitating the acoustic startle and visceromotor reflexes. Our results suggest that the net effect of CRF_1 and CRF_2 receptor activation in the $BNST_{AL}$ is pathway-dependent and provides important insight into the CRF receptor-associated circuitry that likely underpins stress-induced pathologies.
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INTRODUCTION

Corticotropin-releasing factor (CRF) is a neuromodulator involved in the behavioral and physiological response to stress and is often linked to adversity associated with anxiety and pain disorders, including irritable bowel syndrome (IBS; Chang *et al*, 2009; Dinan *et al*, 2006; Fukudo, 2013). In support, our previous studies and others have investigated the effects of repeated psychological stress in a preclinical model that reproduces the hallmark traits of IBS (Bradesi *et al*, 2005; Myers and Greenwood-Van Meerveld, 2012; Tran *et al*, 2013; Venkova *et al*, 2010). The pivotal finding from these studies indicated that the central mechanisms mediating the stress-induced phenotypes following chronic intermittent psychological stress involved increased CRF, particularly in limbic brain regions (Myers and Greenwood-Van Meerveld, 2012; Tran *et al*, 2013).

Limbic brain regions of IBS patients, including the amygdala and extended amygdala, exhibit hyperactivity in response to stress, negative effect, and colorectal stimuli when compared with controls (Berman *et al*, 2008; Bonaz *et al*, 2002). Interestingly, when IBS patients were challenged with vascular infusions of CRF, there was a greater activation of the extended amygdala in response to colorectal stimuli, suggesting an important role for CRF-mediated mechanisms in the extended amygdala in IBS pathophysiology (Tanaka *et al*, 2011). Although the cause of CRF hypersensitivity in the extended amygdala is unclear, evidence suggests that hyperexcitability of limbic circuitry can be the result of repeated neural stimulation (Rosen and Schulkin, 1998).

Preclinical studies have reported that persistent elevations of systemic corticosteroids (CORT), such as in chronic stress conditions, upregulate CRF in the amygdala (Makino *et al*, 1994; Myers *et al*, 2005; Schulkin *et al*, 1998; Shepard *et al*, 2000, 2003; Tran and Greenwood-Van Meerveld, 2012a) as well as the extended amygdala, specifically the bed nucleus of the stria terminalis (BNST; Makino *et al*, 1994; Shepard *et al*, 2003; Watts and Sanchez-Watts, 1995). Moreover, our previous study demonstrated that the central amygdaloid nucleus (CeA) has a pivotal role in somatic and colonic nociception, through a descending pathway involving the anteriolateral BNST ($BNST_{AL}$; Tran *et al*, 2012c). However, the precise mechanisms of CRF in the $BNST_{AL}$ regulating comorbid anxiety and hyperalgesia in response to a chronic stressor are unclear. Therefore, the goals of the

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present study were to (i) test the hypothesis that chronic intermittent psychological stress induces anxiety and hyperalgesia through sensitized CRF signaling in the $BNST_{AL}$ and (ii) delineate the receptor-mediated mechanisms contributing to CRF sensitization of the $BNST_{AL}$.

MATERIALS AND METHODS

Animals

Experiments were performed on male Fischer-344 rats, weighing 240–260 g (Charles Rivers Laboratory, Wilmington, MA). All animals were single-housed to prevent post-surgery complications and maintained on a 12-h light/dark cycle (lights on at 0530 hours) at 21 °C and 70% humidity with *ad libitum* access to food and water. Rats were acclimated to the animal facility for 1 week and to the experimenter and the laboratory for an additional week before experimentation. The experiments were approved by the Oklahoma City Veterans Affairs Medical Center Animal Care and Use Committee (IACUC; protocol no. 0807-004) in accordance with standards established by the *Guide for Care and Use of Laboratory Animals* (1996). All experiments were carried out in accordance with the International Association for the Study of Pain-recommended guidelines of the study of pain.

Stereotaxic Surgeries

Stereotaxic bilateral implantation of cannulae into the $BNST_{AL}$ was performed as previously described (Tran *et al*, 2012c). Briefly, animals were anesthetized with a ketamine (100 mg/kg i.p.)/xylazine (10 mg/kg i.p.) and body temperature was maintained at 37 °C with a homeothermic heating blanket (Harvard Apparatus, Ealing, UK). Rats were placed securely in a stereotaxic surgical frame (Kopf, Tujunga, CA) and a midline incision was made above the skull to expose bregma and lamda. Small holes were made in the skull -0.24 mm posterior to bregma and ± 1.7 mm lateral to midline, and custom designed bilateral guide cannulas (Plastics One, Roanoke, VA) were lowered 6.5 mm from bregma to the $BNST_{AL}$. Four bone screws and skull adhesive were used to secure the cannulas while patency was maintained using parafilm. The incision was sutured and antibiotic/analgesic cream was applied to the wound. The animals recovered for 1 week post surgery before experimentation.

Water Avoidance Stress (WAS)

Repeated WAS is a validated model of psychological stress that induces anxiety, and somatic and visceral hypersensitivity (Bradesi *et al*, 2005; Myers and Greenwood-Van Meerveld, 2012; Tran *et al*, 2013). Rats were placed on a square platform ($8 \times 8 \times 8$ cm) mounted in the center of a white semitransparent plastic container ($50 \times 35 \times 33$ cm) filled with fresh, room temperature water to 1 cm below the surface of the platform. Control animals that received sham-WAS (SHAM) were placed in containers without water. Animals were exposed to WAS or SHAM stress for 60 min each day and a total of 7 days. The fecal-pellet output during the procedure is summarized in Supplementary Figure 1S, showing the difference in autonomic output in response to WAS compared with the SHAM group.

Anxiety-Like Behavior

Anxiety-like behavior was assessed on the elevated plus-maze (EPM) as previously described (Myers and Greenwood-Van Meerveld, 2007, 2010; Tran and Greenwood-Van Meerveld, 2012b). Rats were acclimated to the experimental procedure room for 30 min and were then placed in the center of the EPM facing an open arm. Behavior was recorded using a video camera for 5 min, and the footage was analyzed with the Any-Maze software (Stoelting, Wood Dale, IL). The percentage of time spent in the open arms was used to quantify anxiety-like behavior, with decreased open arm exploration and entries indicating higher anxiety. Total distance traveled was used as an index of locomotor activity.

Acoustic Startle Reflex (ASR) and Prepulse Inhibition (PPI)

Acoustic startle testing was conducted following a previously described protocol (Conti, 2005; Conti *et al*, 2002). Rats were placed in a startle chamber (Med Associates, St Albans, VT) for a 5-min acclimation period before the delivery of any stimulus. All stimuli were presented in the presence of a 70-dB background. In all experiments, the first and last six trials of the session were acoustic startle trials in which a 120-dB, white noise burst was presented for 40 ms. The middle 50 trials consisted of five stimulus types presented in a pseudo-random order: acoustic startle stimuli in the absence of a prepulse stimulus (12 trials); prepulse stimuli, 3, 6, or 12 dB (20 ms) above background, preceding the startle stimulus by 100 ms (10 trials of each prepulse intensity); no stimulus (eight trials). The intertrial intervals were varied and averaged 15 s. The average startle amplitude during the 100 ms following the onset of each startle stimulus was recorded by an accelerometer connected to a computer. Percent PPI was calculated for each rat at each prepulse stimulus intensity as follows: $(1 -$ (startle amplitude after prepulse–pulse pair/startle amplitude after pulse only)) $\times 100$ where *prepulse* is the average startle amplitude on trials in which there was a prepulse stimulus and *startle* is the average amplitude on the trials in which the startle stimulus was presented alone (excluding the first and last six trials of the session). The 24-startle stimulus-alone trials were used to analyze the effect of WAS and CRF receptor antagonists on ASR. Habituation was calculated as the percent change between the first six trials and the last six startle stimulus-alone trials.

Somatic Mechanical Threshold

A somatic mechanical threshold was determined using an electronic von Frey (IITC, Woodland Hills, CA) as previously described (Myers and Greenwood-Van Meerveld, 2010; Myers *et al*, 2005; Tran and Greenwood-Van Meerveld, 2012b; Tran *et al*, 2012c). Animals were acclimated to the procedure room for 30 min before unrestrained placement on an elevated mesh floor (12 mm $\times$ 12 mm grid) in a clear plastic enclosure apparatus ($21 \times 27 \times 15$ cm) for an additional 30 min. The apparatus probe (10 μl plastic tip, US Scientific, Orlando, FL.) was applied to the plantar surface of the hindpaw, and the force required to elicit withdrawal of the hind limb was recorded by an experimenter blind to

the treatment protocol. The procedure was repeated three times using the same point on the same paw with 5-min intervals between each measurement, and the trials were averaged into a single *n*-value for each animal.

Visceral Sensitivity

Instrumentation for visceral sensitivity assessment was performed as previously described (Greenwood-Van Meerveld *et al*, 2001; Johnson *et al*, 2012; Myers *et al*, 2005; Tran *et al*, 2013; Tran and Greenwood-Van Meerveld, 2012b; Tran *et al*, 2012c). Briefly, rats were fasted for 16–18 h to ensure that the colon was free of fecal pellets, allowing for the insertion of the colonic balloon catheter. On the morning of the experiment, rats were transported to the testing room and anesthetized with 2–5% isoflurane (Aerrane, Baxter Healthcare, Deerfield, IL). A colonic balloon (5 cm) was inserted 11 cm past the anus into the colon and secured to the base of the tail with tape. Rats were allowed 30 min to recover from anesthesia before colorectal distension (CRD). The conscious, freely moving animals' visceromotor reflex (VMR) to isobaric distension pressures (0–60 mm Hg) was used as an indicator of visceral sensitivity. The colonic balloon catheter was attached to a Distender Series IIR Barostat (G & J Electronics, Toronto, Ontario, Canada), and the VMR was quantified by the number of abdominal contractions resulting in animal arching in response to CRD (Gibney *et al*, 2010; Tran *et al*, 2013). Each constant pressure distension series consisted of a 10-min basal recording period at 0 mm Hg and a 10-min inflation period at 20, 40, and 60 mm Hg separated by 10-min rest periods. To prevent experimental bias, the experimenter was blind to the treatment protocol.

Autonomic Response to a Novel Environment Stressor

Exposure to a novel environment stressor (NES) was used to assess the endocrine response to an acute stressor. In a new well-lit procedure room, the animals were removed from their home cages and placed in a clear empty plastic container (62.230 cm × 45.085 cm × 33.655 cm). The animals were left undisturbed for 30 min. Immediately following the stressor, the animals were killed and blood was collected for analysis of serum CORT and adrenocorticotropic hormone (ACTH).

Sample Collection

Rats were anesthetized with isoflurane (2–5%) followed by rapid decapitation. Blood samples were collected to evaluate CORT and ACTH levels. The time (approximately 0900–1100 hours) was selected to be consistent with our previous experiments investigating the $BNST_{AL}$ (Tran *et al*, 2012c), and is a period that CORT levels are known to be constant and minimal (Allen and Kendall, 1967), which improves resolution when examining stress responses. Approximately 1 ml of trunk blood was collected and centrifuged at 2500 *g* for 10 min. The serum was separated and stored for subsequent CORT and ACTH analyses with enzyme-linked immunosorbent assay using kits purchased from Immuno Diagnostic Systems (Fountain Hills, AZ) and Novus Biologicals (Littleton, CO), respectively. The sensitivity for each kit was 0.55 ng/ml and 1 pg/ml, respectively. Brains were extracted, flash-frozen in pre-chilled 2-methylbutane, and were stored in a plastic container at −80 °C until cryosectioning. In animals that were not treated with infusions, brains were extracted from the skull and a 1-mm coronal section was taken from −0.24 mm posterior to bregma containing the $BNST_{AL}$. A 1-mm hole-punch was used to collect bilateral samples predominantly containing the $BNST_{AL}$ (−6.0 from the dorsal surface of the brain and ±1.7 from midline). Samples were immediately flash-frozen on dry ice and stored at −80 °C until RNA and protein extractions.

RNA and Protein Extraction

Total RNA and protein were extracted from the same tissue preparation with the SurePrep Purification Kit (Fischer BioReagents, Fair Lawn, NJ) using the protocol for RNA and protein extraction. Protein was quantified using the Experion Pro260 system (Bio-Rad, Hercules, CA) and RNA was quantified using the Experion RNA StdSens system (Bio-Rad). The samples were aliquoted and stored at −80 °C for subsequent qRT-PCR and western blot analysis.

CRF Expression

Extraction of RNA was followed by cDNA synthesis using RT^2 First Strand cDNA Kit and qPCR using SYBR Green qPCR Mastermix in a total reaction volume of 25 µl (Qiagen, Valencia, CA). Samples were run in triplicates and 'no template' conditions served as a negative control. Samples were normalized to 28S rRNA and sequences for both primers were described previously (Tran *et al*, 2013). The reaction was performed on an Applied Biosystems StepOne Plus Real-Time PCR System Thermal Cycling Block (Carlsbad, CA) with the initial denaturation at 95 °C for 15 min and subsequent denaturation at 94 °C for 10 s. The samples were annealed at 64.0 °C for 30 s and were extended at 72 °C for 30 s for a total of 40 cycles, with a final extension at 72 °C for 10 min. Melting curves were performed at the end of each experiment from 72 to 95 °C in 90-s intervals that showed only a single peak near 78.9 °C for 28S and 90.09 °C for CRF. The relative quantity of CRF mRNA from each sample was calculated as the difference in C_t for target minus C_t for 28S rRNA (ΔC_t) and calibrated to C_t of SHAM-treated control sample ($\Delta\Delta C_t$). Fold-change in transcription is expressed as $2^{(-\Delta\Delta Ct)}$.

CRF Receptor Expression

Approximately 30 µg of total protein extract was solubilized in Laemmli buffer supplemented with 2-mercaptoethanol and denatured at 95 °C for 5 min. The samples were then resolved on a 4–20% gradient Tris-Glycine polyacrylamide gel (Bio-Rad) using sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Millipore, Billerica, MA). The membranes were blocked with 1% casein in TBS for 1 h. Blots were then incubated for 2 h with primary antibodies anti-CRF_1 receptor (Abcam, Cambridge, MA), anti-CRF_2 receptor (Abcam), or anti-GAPDH (Sigma-Aldrich, St Louis, MO) was used for normalization. Following antibody incubation, the blots

were washed in three changes of TBS-T and incubated for 1 h with horseradish peroxidase (HRP)-conjugated secondary anti-rabbit or anti-mouse antibodies (Millipore). After three more washes in TBS-T, bands were visualized with ECL Western Blot Detection Kit (Amersham, Piscataway, NJ) and imaged using an Omega 12iC chemiluminescent imager (UltraLum, Claremont, CA). Densitometry was performed using the ImageJ software (NIH, Bethesda, MD).

Histology and Retrograde Labeling

Following the last experimental day, animals were infused with 0.5 μl of HRP to determine diffusion radius. The localization and identification of the area of diffusion of the micropellets were verified from post-mortem histological samples. Serial coronal sections (30 μm) were cryosectioned at −20 °C and mounted onto pre-subbed slides. Positive HRP labeling was revealed using the Betazoid DAB Chromogen Kit (Biocare Medical, LLC, Concord, CA). Sections were then counterstained with Hematoxylin and Tacha's Bluing Solution (Biocare Medical) to determine neuronal damage. Visualization of cell bodies and verification of micropellet placement were performed using light microscopy.

As shown in Figure 1a, implantation of cannulas and infusions did not damage the $BNST_{AL}$. Chromogenic staining for HRP revealed significant staining of the $BNST_{AL}$ and minor outlying regions. Although the use of HRP gives a qualitative estimate of the migration pattern of the infusions, it is unknown how precisely the diffusion properties of HRP match the different drugs used in this study. Importantly, spread into the ventricular system adjacent to the dorsal aspect of the $BNST_{AL}$ is a possibility, but is unlikely to be effective, given the low volume and concentration. To an extent, HRP can also be used as a retrograde label to determine the origin of projections to the $BNST_{AL}$ (Figure 1b). For additional verification of infusion accuracy, sections were collected from the brain regions that project to the BNST (Dong and Swanson, 2004), including the paraventricular nucleus of the hypothalamus (PVN), the CeA, medial nucleus of the thalamus (MDN), the periaqueductal grey (PAG), and nucleus accumbens (NTS). Following cryosectioning and processing with DAB, strong positive staining was revealed in the CeA, mPVN, and NTS. Less intense staining was seen in the PAG and MDN.

Experimental Design

Series 1: two groups of rats were employed in which animals were exposed to WAS or sham-WAS (SHAM; $n=6$/group). Following the procedure, animals were killed and tissue samples were collected from the $BNST_{AL}$ to quantify CRF mRNA and CRF receptor protein expression. Series 2: SHAM control animals were implanted with cannulas ($n=6$/group) localized to the $BNST_{AL}$ to investigate the effect of a selective CRF_1 receptor antagonist (CP376395; 10 mg/ml), a selective CRF_2 receptor antagonist ($Astressin_2B$; 100 μM), or vehicle (VEH; normal saline) infused a rate of 0.1 μl/min (0.5 μl total) directly into each $BNST_{AL}$ ~30 min before each behavior test. A summary of the cannula localization is illustrated in Figure 1c. One animal (VEH) was excluded because of placement issues. Series 3: the effect of CRF antagonism or VEH on WAS-induced anxiety-like behavior, mechanical allodynia, and visceral sensitivity ($n=6$/group) was assessed. Animals were exposed to the 7-day WAS protocol, and 24 h following the last stressor, animals were infused with CP376395, $Astressin_2B$, or VEH and their behaviors were assessed as previously described. Series 4: the effect of CRF antagonism on the ASR and PPI ($n=5$/group) was investigated following the WAS protocol. After the last experimental day, animals from experimental series 2–4 were killed and the post-mortem brains were used for histology and verification of cannula placement.

Drugs and Chemicals

Both CP376395 and $Astressin_2B$ were purchased from Tocris Biosciences (Minneapolis, MN) and dissolved in normal saline (0.9% NaCl). The dose of CP376395, a selective CRF_1 receptor antagonist was chosen to be consistent with our previous studies (Johnson *et al*, 2012; Tran *et al*, 2012c), and the dose of $Astressin_2B$, a selective CRF_2 receptor antagonist, was based on a study by (Ohmura *et al*, 2010). Although biphasic effects are possible with CRF_2 receptor antagonist, the dose of $Astressin_2B$ was carefully selected to avoid such effects. $Astressin_2B$ has a comparable potency to block CRF_2 receptors as the predecessor antisauvagine-30, but with 10-fold less affinity for CRF_1 receptors (Zorrilla *et al*, 2013). In addition to this advantage, the concentration of $Astressin_2B$ used in the present study was approximately fourfold less than the concentration reported to perturb CRF_1 receptor-mediated acoustic startle responses using antisauvagine-30 (Risbrough *et al*, 2004), and approximately sixfold less for gastrointestinal responses (Chen *et al*, 2005).

Data Analysis

Gene expression, anxiety-like behavior, and somatic mechanical sensitivity in response to SHAM or WAS were analyzed using a Student's unpaired *t*-test, and a one-way analysis of variance (ANOVA) was used when comparing antagonist treatments within the SHAM or WAS groups followed by Bonferroni's *post hoc* test. A two-way ANOVA was used to compare main effects of stress protocol and antagonist treatment followed by Tukey's test for multiple comparisons. Owing to repeated measures in the ASR, PPI, and visceral sensitivity assays, these results were analyzed with a two-way ANOVA with repeated measures (ANOVA-RM) with Bonferroni's *post hoc* test. In all tests, significance was determined as $P<0.05$ using the GraphPad Prism Software ver. 6.0b (La Jolla, CA).

RESULTS

Repeated WAS Sensitizes the CRF and CRF Receptors in the $BNST_{AL}$

As illustrated in Figure 2a, animals exposed to the WAS protocol had a significant 2.06 ± 0.42-fold increase ($t_{(10)}=2.442$; $P=0.02$) in CRF mRNA in tissue samples collected from the $BNST_{AL}$ compared with SHAM controls. On the basis of optical density of western blot bands

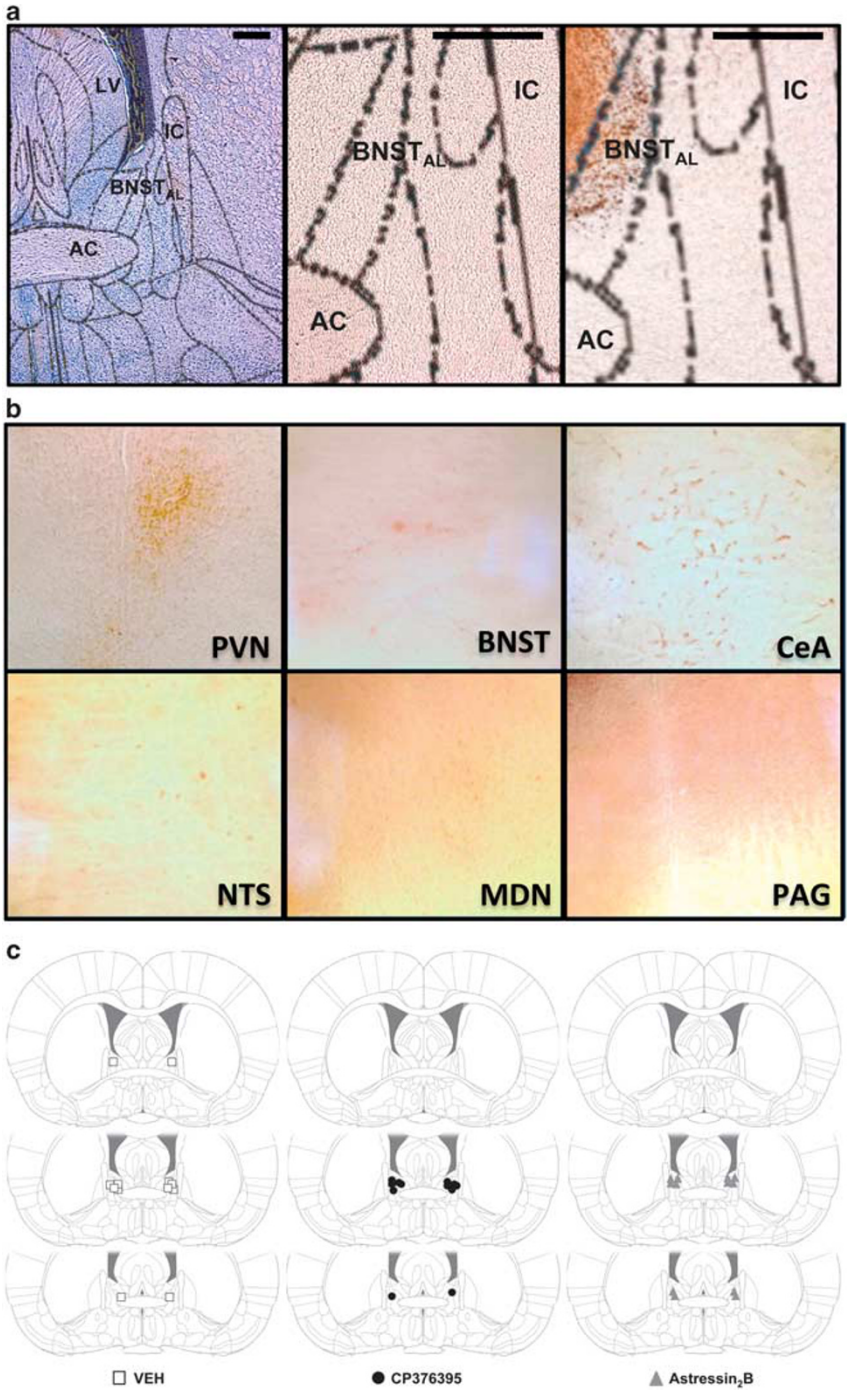

Figure 1 Infusions are restricted to the anteriolateral bed nucleus of the stria terminalis (BNST$_{AL}$). Before being killed, animals were infused with horseradish peroxidase (HRP) to assess diffusion radius. (a) Post-mortem tissue slices were stained with cresyl violet to determine viability (left) and show minimal damage to the BNST. Negative control (middle), and the addition of DAB substrate (right), show that infusions were restricted to the BNST$_{AL}$ and minor outlying areas. (b) The BNST receives inputs from regions associated with sensory and affect processing. To verify infusion accuracy, sections were collected from the regions that project to the BNST including the paraventricular nucleus (PVN), the central amygdala (CeA), medial nucleus (MDN), the periaqueductal grey (PAG), and nucleus accumbens (NTS). Positive staining was revealed in the CeA, PVN, and NTS. Minimal staining was seen in the MDN, and not visible in the PAG. (c) Brain atlas sections for coordinates (from top) bregma −0.12, −0.24, −0.36, respectively, depicting the localization of each cannula placement.

(Figure 2b and c), animals exposed to WAS had higher CRF_1 receptor expression (WAS: 4.19 ± 0.63; SHAM: 1.87 ± 0.16; $t_{(10)} = 3.555$; $P < 0.01$) and CRF_2 receptor expression levels (CRF_2: WAS: 3.70 ± 0.33; SHAM: 1.65 ± 0.19; $t_{(10)} = 5.406$; $P < 0.001$). The ratio between CRF_1 and CRF_2 receptors (Figure 2d) was also increased ($t_{(10)} = 2.282$; $P = 0.03$) in

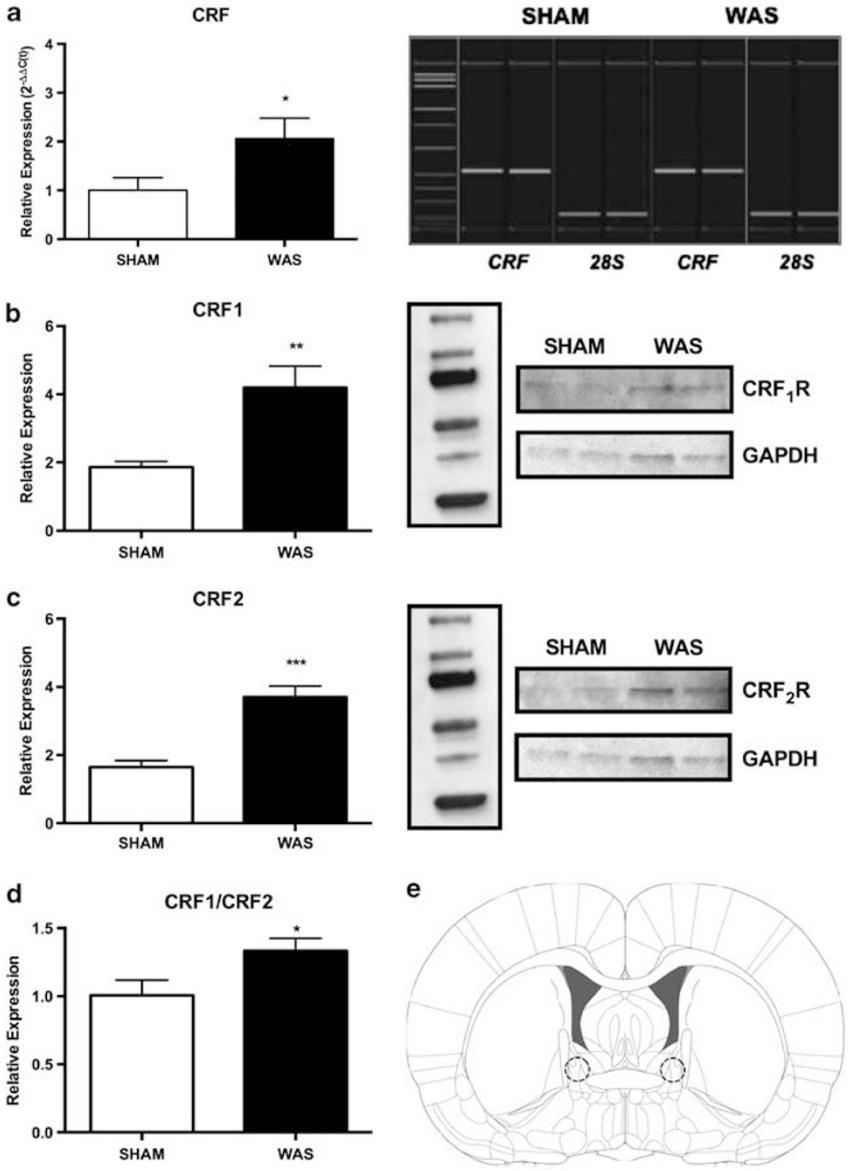

Figure 2 Repeated water avoidance stress (WAS) increases corticotropin-releasing factor (CRF) and CRF receptor expression. CRF expression was assessed using qRT-PCR, whereas CRF_1 and CRF_2 receptors were quantified using western blot analysis. Compared with animals that received SHAM stress, animals that were exposed to chronic WAS expressed higher levels of (a) CRF, (b) CRF_1 receptors, and (c) CRF_2 receptors in the anteriolateral bed nucleus of the stria terminalis ($BNST_{AL}$). (d) The ratio between CRF1 and CRF2 was higher following WAS compared with SHAM-treated animals. (e) The target location of the micropunches is illustrated. Data represent mean ± SEM; $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$ by Student's unpaired *t*-test; $n=6$/group.

animals exposed to WAS (1.33 ± 0.09) compared with SHAM treatment (1.01 ± 0.11). A schematic diagram of the target location of the micropunches is illustrated in Figure 2e.

Effects of WAS on Anxiety, Pain, and HPA Output

Anxiety-like behavior was assessed on the EPM, and percent time spent exploring the open arms is shown (Supplementary Figure 1SA). Animals exposed to WAS spent significantly less time ($t_{(10)}=3.384$; $P<0.01$; $49.8 \pm 3.18\%$) exploring the open arms than SHAM controls ($76.4 \pm 7.19\%$), indicating increased anxiety-like behavior. No changes in locomotor activity were observed (Supplementary Figure 1SB), and there was no significant difference in total distance traveled ($t_{(10)}=0.5283$; $P=0.61$; SHAM: 11.9 ± 1.13 m, WAS: 12.9 ± 1.53 m).

Following anxiety assessment, pain behaviors were examined by quantifying somatic mechanical threshold (Supplementary Figure 1SC) and the VMR to graded pressures of CRD (Supplementary Figure 1SD). Somatic mechanical sensitivity was assessed by quantifying the mechanical force required to illicit a reflexive withdrawal of the hindpaw. Animals exposed to WAS showed a significant decrease in somatic mechanical threshold ($t_{(10)}=11.82$; $P<0.001$; 52.8 ± 1.68 g) compared with SHAM controls

(76.7 ± 1.12 g), suggesting an increase in somatic sensitivity. Moreover, the animals exposed to the WAS protocol exhibited an increased sensitivity to visceral stimuli. Specifically, in response to CRD there was a significant effect of distension pressure ($F_{(3,\ 30)} = 506.9$; $P < 0.001$), a significant effect of stress protocol ($F_{(1,\ 10)} = 5.510$; $P = 0.04$), and a significant interaction ($F_{(3,\ 30)} = 3.421$; $P = 0.03$).

One day following the last behavioral assessment, the animals were exposed to NES before being killed to assess HPA output. As shown in Supplementary Figure 1SE and F, compared with SHAM, animals that were previously exposed to WAS had elevated serum CORT ($t_{(10)} = 3.504$; $P < 0.01$; SHAM: 595.25 ± 86.538 ng/ml, WAS: 1184.7 ± 144.26 ng/ml) and serum ACTH ($t_{(10)} = 4.617$; $P = 0.001$; SHAM: 421.89 ± 52.920 pg/ml, WAS: 866.89 ± 80.550 pg/ml) in response to the NES.

Effect of CRF_1 and CRF_2 Receptor Antagonism in the $BNST_{AL}$ on Basal Behaviors

Control SHAM animals were treated with VEH, a selective CRF_1 antagonist, or a selective CRF_2 antagonist. As illustrated in Figure 3a, there was a significant effect of infusion on total time spent exploring the open arm ($F_{(2,\ 14)} = 9.780$; $P < 0.01$). CRF_2 receptor antagonism with $Astressin_2B$ decreased open arm time by 31.7 ± 8.57% ($P < 0.01$). However, CRF_1 receptor antagonist treatment with CP376395 produced no effect on anxiety-like behavior ($P > 0.05$). There was no significant effect of infusion on total distance traveled (Figure 4b; $F_{(2,\ 14)} = 0.3858$; $P = 0.69$). As shown in Figure 3c, we found a significant effect of infusion on the somatic mechanical threshold ($F_{(2,\ 14)} = 102.1$; $P < 0.001$). CRF_2 antagonist treatment with $Astressin_2B$ decreased the threshold by 19.7 ± 1.74 g ($P < 0.001$). There was also a significant effect of antagonist infusion on the VMR to CRD (Figure 3d; $F_{(2,\ 14)} = 58.35$; $P < 0.001$), in addition to a significant effect of distension pressure ($F_{(3,\ 14)} = 415.6$; $P < 0.001$) and interaction ($F_{(2,\ 14)} = 13.58$; $P < 0.001$). Significant decreases in the number of abdominal contractions were seen at 20, 40, and 60 mm Hg for treatment with CP376395 ($P < 0.001$) or $Astressin_2B$ ($P < 0.001$).

The Effect of CRF_1 and CRF_2 Antagonism in the $BNST_{AL}$ on WAS-Induced Behaviors

Animals were exposed to the WAS protocol, and behaviors were evaluated 24 h following the final stressor. Before behavioral assessments, the animals were treated with a selective CRF_1 antagonist, a selective CRF_2 antagonist, or control VEH and the results are illustrated in Figure 4a. Overall there was a significant effect of treatment on total time spent exploring the open arms ($P < 0.001$; $F_{(2,\ 14)} = 12.78$), and *post hoc* analysis revealed a 29.4 ± 6.13% increase in open arm time ($P < 0.001$) with CP376395 infusions and no significant difference of $Astressin_2B$ ($P = 0.69$) compared with VEH control. There was no significant effect of treatment on total distance traveled (Figure 4b; $P = 0.63$; $F_{(2,\ 14)} = 0.4719$). Following WAS, there was a significant main effect of antagonist infusion (Figure 4c; $P < 0.001$; $F_{(2,\ 14)} = 32.86$). Treatment with CP376395 increased the threshold 22.1 ± 3.64 g ($P < 0.001$) compared with VEH; however, infusions of $Astressin_2B$ had no effect ($P = 0.53$). In the visceral sensitivity assay, there

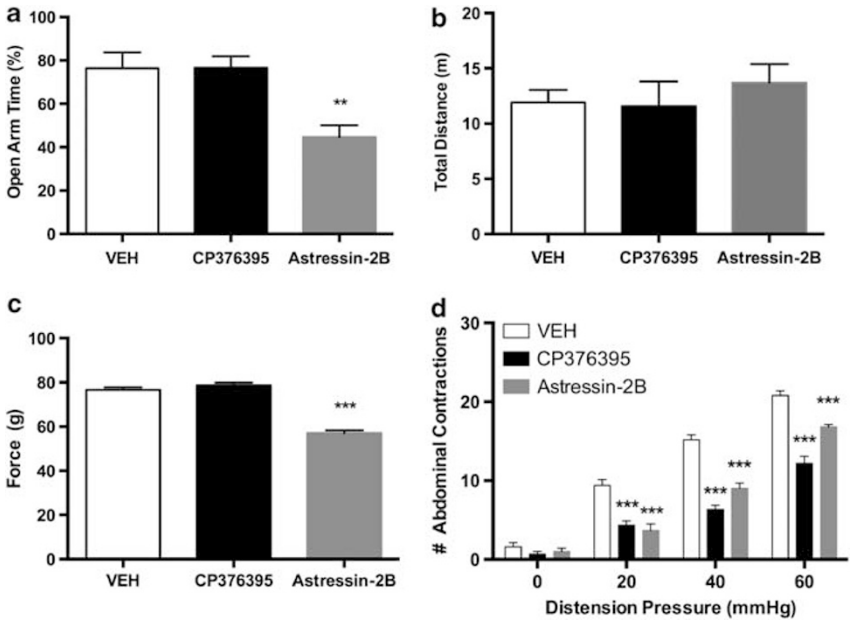

Figure 3 Effect of corticotropin-releasing factor (CRF) receptor antagonists on baseline behavior. Animals implanted with bilateral cannulas localized to the anteriolateral bed nucleus of the stria terminalis ($BNST_{AL}$) received infusions of either vehicle (VEH; normal saline; $n = 5$), the CRF_1 receptor antagonist CP376395 (10 mg/ml; $n = 6$), or the CRF_2 receptor antagonist $Astressin_2B$ (100 μM; $n = 6$). Compared with VEH infusions, treatment with CP376395 had no effect on (a) the percent time spent exploring the open arms, (b) total distance traveled, or (c) somatic mechanical threshold. In contrast, infusion with $Astressin_2B$ decreased open arm exploration without changing total distance traveled, and decreased the somatic mechanical threshold. (d) Both antagonists decreased the visceromotor reflex (VMR) relative to VEH treatment. Data represent mean ± SEM; $^{**}P < 0.01$ and $^{***}P < 0.001$ by one-way analysis of variance (ANOVA) or two-way ANOVA with repeated measures (ANOVA-RM) with Bonferonni *post hoc* analysis.

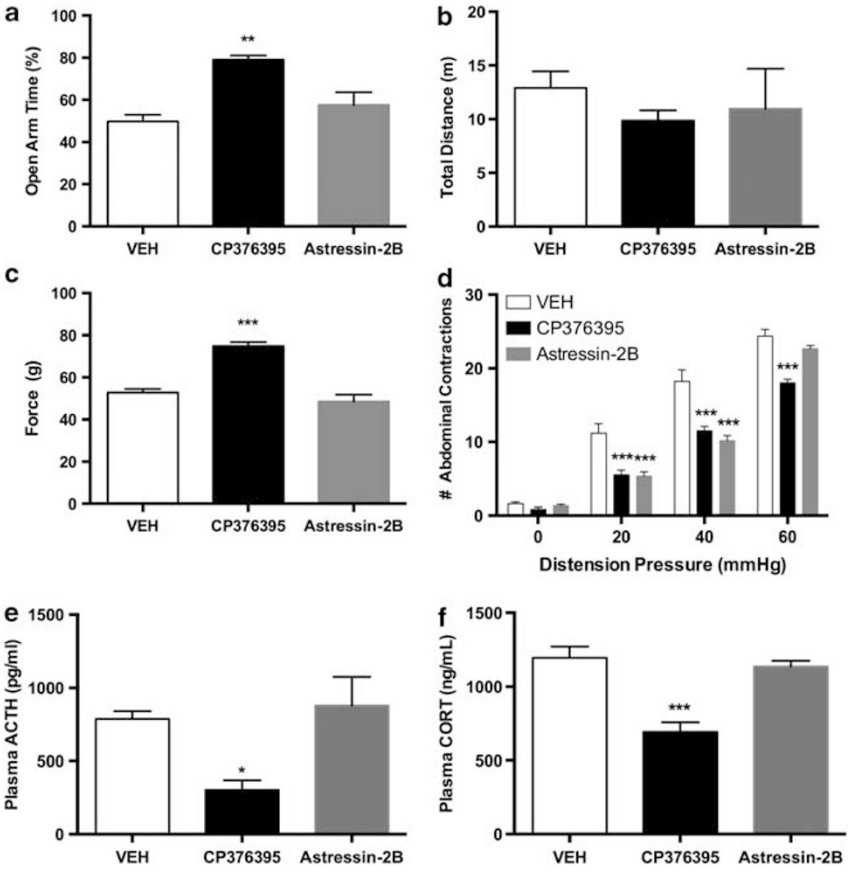

Figure 4 Effect of corticotropin-releasing factor (CRF) receptor antagonist following water avoidance stress (WAS). Animals were implanted with bilateral cannulas localized to the anteriolateral bed nucleus of the stria terminalis ($BNST_{AL}$) and exposed to the WAS protocol. Before behavioral assessment, the animals received infusions of either vehicle (VEH; normal saline; $n=5$), the CRF_1 receptor antagonist CP376395 (10 mg/ml; $n=6$), or the CRF_2 receptor antagonist $Astressin_2B$ (100 μM; $n=6$). Compared with WAS-treated animals with VEH infusions, (a) antagonizing CRF_1 receptors increased percent time spent on the open arm. Blocking CRF_2 had no effect on the time spent exploring the open arm. (b) Neither CRF_1 nor CRF_2 receptor antagonists changed the total distance traveled compared with VEH control. (c) Following WAS, inhibiting CRF_1 receptors in the $BNST_{AL}$ attenuated the decrease in somatic mechanical threshold induced by WAS compared with VEH infusions, but blocking CRF_2 receptors had no effect on threshold. (d) Both CRF_1 and CRF_2 decreased the number of abdominal contractions in response to colorectal distension. At the highest distension pressure, CRF_2 receptor was no longer significantly different from VEH. (e) Serum ACTH and (f) corticosteroid (CORT) output was quantified following 30 min of novel environment stress. Animals exposed to WAS and infused with CP376395 had decreased plasma ACTH and CORT compared with animals exposed to the WAS protocol and infused with VEH or $Astressin_2B$. Data represent mean ± SEM; $^*P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$ by one-way analysis of variance (ANOVA) or two-way ANOVA with repeated measures (ANOVA-RM) with Bonferonni *post hoc* analysis.

was a significant main effect of infusion (Figure 4d; $P<0.001$; $F_{(2,\ 14)}=21.67$), a significant effect of distension pressure ($P<0.001$; $F_{(3,\ 14)}=710.0$), and a significant interaction ($P<0.001$; $F_{(2,\ 14)}=14.12$). Significant differences were seen at 20 and 40 mm Hg for both CP376395 and $Astressin_2B$ infusions, respectively, compared with VEH ($P<0.001$), but at 60 mm Hg only CP376395 had a significant effect ($P<0.001$). Following exposure to NES, HPA responses were assessed (Figure 4e and f). There was a significant effect of infusion on plasma ACTH ($P=0.01$; $F_{(2,\ 14)}=6.312$) and CORT ($P<0.001$; $F_{(2,\ 14)}=19.61$). Animals infused with CRF_1 receptor antagonist had an average CORT concentration that was lesser ($P<0.001$; 691.62 ± 66.507 ng/ml) than animals treated with VEH (1196.1 ± 74.641 ng/ml) or CRF_2 receptor antagonist (1135.3 ± 40.138 ng/ml). Animals infused with a CRF_1 receptor antagonist had significantly lesser ($P=0.04$; 302.13 ± 65.989 pg/ml) serum ACTH than animals treated with CRF_2 receptor antagonist (878.40 ± 196.33 pg/ml) or VEH (798.75 ± 66.845 pg/ml).

The Effect of WAS on CRF Antagonism in the $BNST_{AL}$

To determine whether the WAS procedure affects the outcomes following infusion with the CRF antagonists, the data were pooled between experimental series and analyzed. On the EPM, there was a significant effect of infusion ($P<0.001$; $F_{(2,\ 28)}=14.82$), stress protocol ($P=0.04$; $F_{(1,\ 28)}=4.396$), and significant interaction ($P=0.01$; $F_{(2,\ 28)}=5.101$) on the percent time spent exploring the open arms (Figure 5a). Similarly, there was a significant effect of infusion ($P<0.001$; $F_{(2,\ 28)}=78.26$), stress protocol ($P<0.001$; $F_{(1,\ 28)}=55.40$), and significant interaction ($P<0.001$; $F_{(2,\ 28)}=13.29$) on the somatic mechanical threshold (Figure 5b). When analyzing the VMR at 60 mm Hg, there was a significant effect of infusion ($P<0.001$;

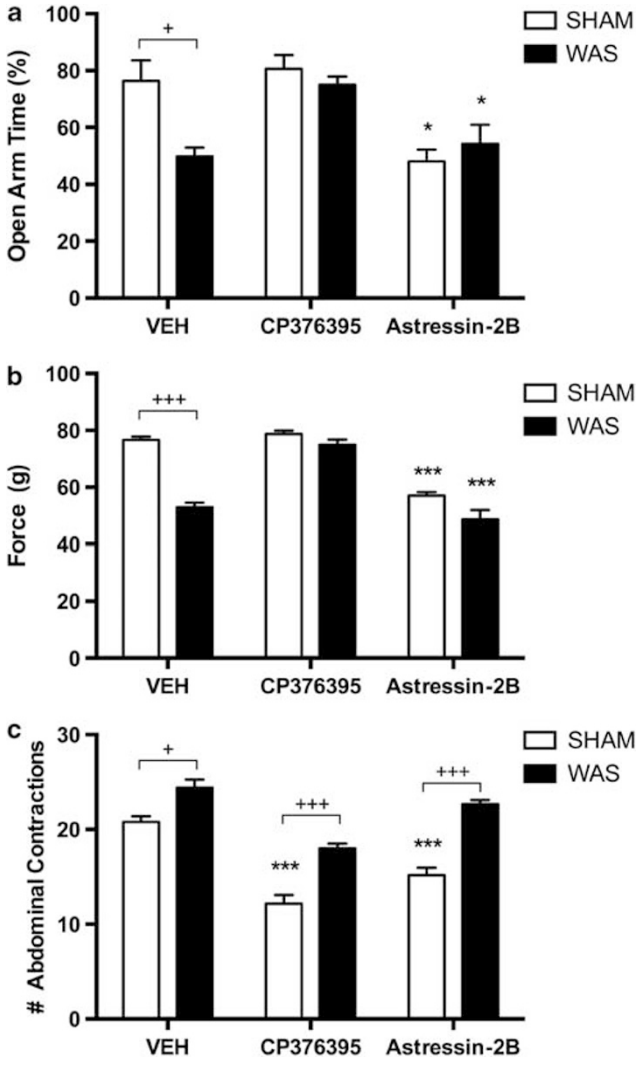

Figure 5 Effect of water avoidance stress (WAS) on corticotropin-releasing factor (CRF) receptor antagonism. The data for animals exposed to the SHAM protocol and animals exposed to the WAS protocol were compared. Overall, there were significant effects of infusion, stress protocol, and significant interaction between the two for anxiety-like behavior on the (a) percent of time spent exploring the open arm of the EPM, (b) somatic mechanical threshold, and (c) the visceromotor reflex (VMR) to colorectal distension (CRD) analyzed at 60 mm Hg (see text for details). Data represent mean ± SEM, $^{*}P<0.05$, and $^{***}P<0.001$ compared with SHAM + vehicle (VEH) infusion, and $^{+}P<0.05$ and $^{+++}P<0.001$ compared with WAS + indicated infusion by two-way analysis of variance (ANOVA) with Tukey's *post hoc* analysis.

$F_{(2,\ 28)}=54.85$), stress protocol ($P<0.001$; $F_{(1,\ 28)}=95.28$), and significant interaction ($P=0.03$; $F_{(2,\ 28)}=3.679$) on the number of abdominal contractions in response to CRD (Figure 5c).

The Effect of CRF_1 and CRF_2 Antagonism into the $BNST_{AL}$ on WAS-Induced ASR and PPI

The effects of WAS on ASR and PPI are shown in Supplementary Figure 2S. There was a significant effect of startle intensity ($P<0.001$; $F_{(3,\ 30)}=134.1$), a significant effect of WAS ($P<0.001$; $F_{(1,\ 10)}=36.73$), and significant interaction ($P<0.001$; $F_{(3,\ 30)}=10.75$) on ASR (Supplementary Figure 2SA). There was also a significant effect of WAS ($P<0.001$; $F_{(1,\ 10)}=370.1$), a significant effect of prepulse intensity ($P<0.001$, $F_{(2,\ 20)}=12.18$), and interaction ($P=0.06$; $F_{(2,\ 20)}=3.198$) on PPI (Supplementary Figure 2SB). A separate group of animals exposed to the WAS protocol and treated with a selective CRF_1 antagonist, a selective CRF_2 antagonist, or VEH showed significant differences in the acoustic startle amplitudes (Figure 5a). There was a significant effect of startle intensity ($P<0.001$; $F_{(3,\ 36)}=77.97$), a significant effect of infusion ($P<0.001$; $F_{(2,\ 12)}=13.31$), and significant interaction ($P<0.001$; $F_{(6,\ 36)}=9.207$). Treatment with either CP376395 or $Astressin_2B$ significantly decreased ($P<0.001$) the startle amplitude 419 ± 47.2 and 232 ± 47.2 mV, respectively, compared with VEH control. In addition, in the PPI paradigm (Figure 5b), there was a significant effect of infusion ($P<0.001$; $F_{(2,\ 12)}=16.26$), with CP376395 infusions decreasing the %PPI ~18.5%, 22.9%, and 30.77%, respectively, but no significant effect of prepulse intensity ($P=0.65$, $F_{(2,\ 24)}=0.4346$), or interaction ($P=0.18$; $F_{(4,\ 24)}=1.726$), and there was no significant difference in percent habituation (Figure 6c; $P=0.59$; $F_{(2,\ 12)}=0.5479$).

DISCUSSION

The BNST acts as a conduit between the amygdala and the HPA axis, and, through tonic regulation of the PVN (Herman *et al*, 1994; Herman *et al*, 2002), mediates both the physiological (Choi *et al*, 2007) and the behavioral-affective response to stress (Cecchi *et al*, 2002; Davis and Shi, 1999). The aim of the present investigation was to explore the mechanisms mediating the sequalae of behavioral and endocrine phenotypes induced by chronic intermittent psychological stress. Moreover, these experiments are the first to delineate the role of the CRF receptors of the $BNST_{AL}$ in modulating anxiety and pain following repeated psychological stress.

The Effects of WAS on the HPA Function

The psychological and psychosomatic impact of repeated exposure to stress includes deterioration of mental and physiological functions, which can result from failure to habituate to a repetition of the same stressor (McEwen, 1998; McEwen and Gianaros, 2010). In the present study, we confirmed that the F-344 rat strain fails to habituate to stress, as serum CORT and ACTH levels remain high after repeated exposure to WAS, similar to that previously reported (Dhabhar *et al*, 1997). The potentiated HPA response and subsequent stress-induced psychological and physiological conditions are postulated to be due to hyperexcitability of limbic circuitry (Rosen and Schulkin, 1998), and result from repeated exposure to CRF activation (Rainnie *et al*, 2004). Interestingly, impairment of the HPA axis in F-344 rats was reported to involve CRF (Sternberg *et al*, 1992), which is supported by the present findings in the $BNST_{AL}$.

The Effect of WAS on CRF Mechanisms in the $BNST_{AL}$

In contrast to the numerous studies implicating the involvement of CRF mechanisms in the amygdala—reviewed by

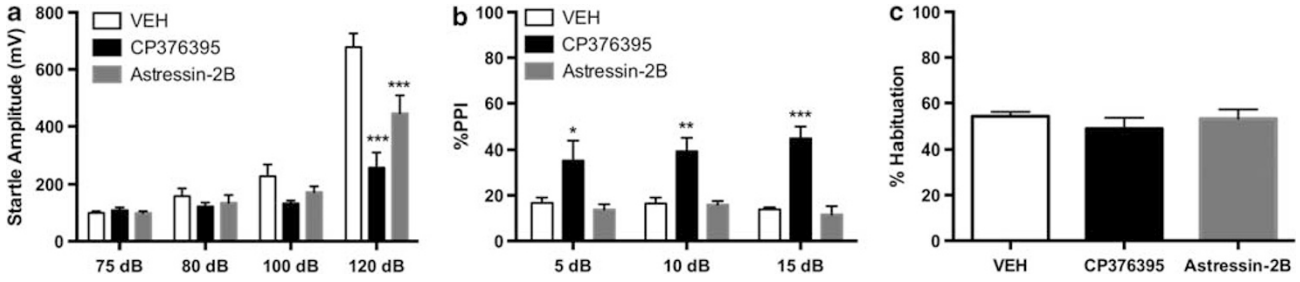

Figure 6 Effect of corticotropin-releasing factor (CRF) receptor antagonists on acoustic startle reflex (ASR) and prepulse inhibition (PPI). Animals were implanted with bilateral cannulas localized to the anteriolateral bed nucleus of the stria terminalis ($BNST_{AL}$) and exposed to the water avoidance stress (WAS) protocol. Before behavioral assessment, the animals received infusions of either vehicle (VEH; normal saline; $n=5$), the CRF_1 receptor antagonist CP376395 (10 mg/ml; $n=5$), or the CRF_2 receptor antagonist $Astressin_2B$ (100 μM; $n=5$). Following WAS, animals infused with CRF_1 receptor antagonists had (a) a decrease in startle amplitude and (b) increased prepulse inhibition compared with animals infused with VEH. Animals infused with CRF_2 receptor antagonists also had a decrease in startle amplitude, but to a lesser extent, and no change in prepulse inhibition. (c) Neither treatment had any effect on percent habituation. Data represent mean ± SEM, $^*P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$ by one-way analysis of variance (ANOVA) or two-way ANOVA with repeated measures (ANOVA-RM) with Bonferonni *post hoc* analysis.

(Bonaz and Bernstein, 2013; Myers and Greenwood-Van Meerveld, 2007)—less is known about the role of CRF in the BNST. Therefore, in the present study we aimed to investigate the role of the CRF system in the $BNST_{AL}$ in precipitating exaggerated anxiety and nociception following chronic intermittent psychological stress. In response to our WAS protocol, we found an increase in CRF mRNA in the $BNST_{AL}$ similar to our previous studies and others (Makino *et al*, 1994; Tran *et al*, 2012c; Watts and Sanchez-Watts, 1995). These data suggest that much like the amygdala, CRF content in the $BNST_{AL}$ facilitates the behavioral responses to a challenge.

In subsequent studies, the importance of CRF expression in the amygdala with regard to the observed behaviors was confirmed using site-specific genetic manipulation of CRF (Regev *et al*, 2012). However, recent findings using similar CRF overexpression techniques in the BNST suggested that regulation of stress-induced anxiety in this brain region may depend more on the CRF receptors (Sink *et al*, 2013). Thus, our following experiments focused on delineating the function of the CRF receptors, which were disproportionally upregulated after WAS.

The Function of the CRF Receptors in the $BNST_{AL}$ May be Pathway-Dependent

Chronic stress and CRF dysregulation in the BNST have been linked to many traits commonly associated with the amygdala dysfunction including fear (Walker *et al*, 2003), anxiety (Ventura-Silva *et al*, 2012), anhedonia (Stout *et al*, 2000), addiction (Erb *et al*, 2001), and the emotional component of visceral and somatic pain (Deyama *et al*, 2007; Tran *et al*, 2012c). Our investigation focused primarily on anxiety and pain behaviors induced by a chronic repeated psychological stressor. Using specific CRF_1 and CRF_2 receptor antagonists, we found circumstantial evidence for both converging and opposing receptor functions, dependent on the behavior under investigation. Although our results support previous reports of CRF receptor function on the anxiety-like behavior in the EPM (Sahuque *et al*, 2006; Sink *et al*, 2013) and modulation of somatic pain processing (Ji and Neugebauer, 2008), the observed changes in visceral sensitivity contradicted previous studies using peripherally restricted CRF_1 and CRF_2 receptor antagonists (Greenwood-Van Meerveld *et al*, 2005; Nijsen *et al*, 2005). This suggested that the effects of CRF_1 and CRF_2 receptor activation, at least on visceral sensitivity, may differ depending on the localization of the receptors.

CRF Modulation of the ASR and PPI Highlights Potential Receptor Configuration

Although experimental evidence suggests differences in the central processing of somatic and visceral pain, the information outlining the divergence in the pathways is limited (Zhou *et al*, 2010). However, further studies on the relationship between CRF receptor function and well-established pathways may lead to key insights into the pain circuitry. Therefore, we focused our investigation on anxiety-related behaviors, which exhibit both complementary and opposing CRF receptor mechanisms in unconditioned and conditioned paradigms (Lee and Davis, 1997). Specifically, we examined the ASR, which is sensitive to anxiety levels, anatomically well defined, and involves CRF activation in the BNST (Davis *et al*, 1997). The acoustic startle paradigm is also clinically relevant as it is heightened in patients with IBS (Naliboff *et al*, 2008), enhanced by abdominal stimuli (Hubbard *et al*, 2011), and linked to increased activation of the extended amygdala (Naliboff *et al*, 2006).

In the present study, the effects of CRF_1 and CRF_2 receptor antagonism on ASR and PPI were not examined at baseline, limiting the interpretation of the effects following WAS to what has been reported in the literature. Those previous studies have suggested increased ASR and attenuation of the PPI following CRF injections (Conti, 2005; Conti *et al*, 2002). Moreover, when the global roles of CRF_1 and CRF_2 receptors were investigated, early findings indicated similar functions in facilitating ASR, but opposing effects on the PPI (Risbrough *et al*, 2004). The data from the present study using specific infusions into the $BNST_{AL}$ following WAS support these findings and further indicate that the primary acoustic startle pathway receives complementary input from

the CRF receptors in the $BNST_{AL}$ while the PPI pathway receives opposing input.

Anatomical Model

Contrasting modulation of different pathways is possible in the $BNST_{AL}$ because of the presence of at least three different cell types determined by the different expression patterns of intrinsic membrane currents in the $BNST_{AL}$ (Hammack *et al*, 2007). Evidence suggests that CRF_1 and CRF_2 receptors are disproportionately distributed on the various cell types in the $BNST_{AL}$ (Dabrowska *et al*, 2011). As the $BNST_{AL}$ integrates both excitatory and inhibitory emotional and nociceptive signals from multiple brain regions (Myers *et al*, 2013; Radley and Sawchenko, 2011), CRF_1 and CRF_2 receptors can both facilitate and attenuate output behavior depending on the localization of the receptors. For example, Fu and Neugebauer (2008) reported that inhibition of CRF_2 receptors with $Astressin_2B$ facilitates glutamatergic synaptic transmission in the amygdala by inhibiting GABAergic transmission. Although the $BNST_{AL}$ is a distinctly different brain region, it similarly comprises a CRF-modulated GABAergic/glutamatergic network, and thus disinhibition following CRF_2 receptors antagonism, such as in the present study, was previously predicted by anatomical models because of the distinct localization of CRF_2 receptors relative to CRF_1 receptors (Dabrowska *et al*, 2011). Moreover, evidence suggests that the outputs from the $BNST_{AL}$, such as to the PVN, are facilitated by activation of CRF_1 receptors and inhibited by reciprocal projections to the BNST via CRF_2 receptors. The behaviors observed in the present study follow this trend, with the exception of the acoustic startle and visceromotor reflexes. However, to our knowledge, there is no evidence indicating that the acoustic startle or visceral pain afferents relay through the BNST.

Visceral pain signals from the thalamus project to the visceromotor pattern network in the medial PVN and then directly to the NTS (Thompson and Swanson, 2003), whereas acoustic startle information projects to the cochlear nucleus and then to the nucleus reticularis pontis caudalis (PnC; Lee *et al*, 1996). On the basis of the results from the current study, we postulate that the $BNST_{AL}$ may modulate these pathways through afferents to brainstem nuclei, which are facilitated by CRF_2 receptors. In support, previous studies have demonstrated that the acoustic startle response is enhanced by fear and anxiety via projections to the PnC (Koch and Schnitzler, 1997). An interesting caveat to note is that our gene expression data following WAS support the facilitation of both ancillary and diametrical roles of the CRF receptors. In pathways where CRF_1 and CRF_2 have complementary roles, our experiments show increased expression of both receptors following WAS, resulting in a net excitatory effect. Pathways in which CRF_1 and CRF_2 have opposing roles on behavior will still have a net excitatory effect on behavior due to the nearly fourfold increase in CRF_1 receptors over the twofold increase observed for CRF_2 receptors.

In summary, our results indicate that the functional role of CRF_1 and CRF_2 receptors varies depending upon the physiological or behavioral responses under investigation. Although our findings in the $BNST_{AL}$ suggest that the observed effects are likely due to differences in the localization of the CRF receptors, further anatomical studies are necessary in order to provide conclusive evidence. Overall, our results provide substantial insight into the CRF receptor-associated mechanisms that likely underpin stress-induced pathologies such as IBS, and are important when considering CRF as a potential therapeutic target.

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Supplementary Information accompanies the paper on the Neuropsychopharmacology website (http://www.nature.com/npp)