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PAC1 receptor antagonism in the bed nucleus of the stria terminalis (BNST) attenuates the endocrine and behavioral consequences of chronic stress

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Summary

Chronic or repeated stressor exposure can induce a number of maladaptive behavioral and physiological consequences and among limbic structures, the bed nucleus of the stria terminalis (BNST) has been implicated in the integration and interpretation of stress responses. Previous work has demonstrated that chronic variate stress (CVS) exposure in rodents increases BNST pituitary adenylate cyclase activating polypeptide (PACAP, *Adcyap1*) and PAC1 receptor (*Adcyap1r1*) transcript expression, and that acute BNST PACAP injections can stimulate anxiety-like behavior. Here we show that chronic stress increases PACAP expression selectively in the oval nucleus of the dorsolateral BNST in patterns distinct from those for corticotropin releasing hormone (CRH). Among receptor subtypes, BNST PACAP signaling through PAC1 receptors not only heightened anxiety responses as measured by different behavioral parameters but also induced anorexic-like behavior to mimic the consequences of stress. Conversely, chronic inhibition of BNST PACAP signaling by continuous infusion with the PAC1 receptor antagonist PACAP(6-38) during the week of CVS attenuated these stress-induced behavioral responses and changes in weight gain. BNST PACAP signaling stimulated the hypothalamic-pituitary-adrenal (HPA) axis and heightened corticosterone release; further, BNST PACAP(6-38) administration

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blocked corticosterone release in a sensitized stress model. In aggregate with recent associations of PACAP/PAC1 receptor dysregulation with altered stress responses including post-traumatic stress disorder, these data suggest that BNST PACAP/PAC1 receptor signaling mechanisms may coordinate the behavioral and endocrine consequences of stress.

Keywords

pituitary adenylate cyclase activating polypeptide (PACAP); PAC1 receptor; bed nucleus of the stria terminalis (BNST); chronic variate stress; anxiety-related behavior; hypothalamic-pituitary-adrenal (HPA) axis

1. Introduction

Severe or repeated stress places undue challenges on physiological and psychological systems, which typically results in the mobilization of adaptive response systems designed to reestablish homeostatic states. Multiple response systems are engaged following stressor exposure, including the hypothalamic-pituitary-adrenal (HPA) axis, the sympathetic nervous system, and central nervous system (CNS) circuits mediating fear and anxiety-like behavior. The bed nucleus of the stria terminalis (BNST), a region of the extended amygdala, has been argued to mediate all three of these responses to sustained threats (Walker et al., 2003; Herman et al., 2005; Walker and Davis, 2008; Walker et al., 2009; Radley and Sawchenko, 2011). For example, hypothalamic paraventricular nucleus (PVN) activity, which mediates both the endocrine and autonomic response to stressor exposure, is tightly regulated by both anterior and posterior BNST subregions (Choi et al., 2007; Choi et al., 2008a; Radley and Sawchenko, 2011). Moreover, Waddell et al. (2006) and Walker et al. (2003, 2008, 2009) have argued that the BNST mediates responding to long duration threats, and that the BNST may mediate anxiety-like emotional states (Walker et al., 2003; Waddell et al., 2006; Choi et al., 2007; Choi et al., 2008a; Walker and Davis, 2008; Radley and Sawchenko, 2011). Consistent with these observations, the BNST has been implicated in regulating anxious temperament in nonhuman primates (Kalin et al., 2005; Fox et al., 2008; Kalin et al., 2008; Oler et al., 2009) and humans (Straube et al., 2007; Somerville et al., 2010).

Accordingly, altered BNST functioning is likely critical for the etiology of many anxiety disorders associated with stressor exposure (Schulkin et al., 1998; Vyas et al., 2003; Pego et al., 2008; Hammack et al., 2009; Hammack et al., 2010). In support, repeated exposure to stressors or drugs of abuse has been correlated with enhanced BNST neuroplasticity, including increased neuronal peptide expression (Stout et al., 2000; Choi et al., 2006; Hammack et al., 2009), dendritic length and branching (Vyas et al., 2003; Pego et al., 2008), volume (Pego et al., 2008), and synaptic activity (Dumont et al., 2005). These observations suggest that repeated or chronic stressor exposure may enhance BNST function, which may lead to pathological anxiety.

Increasingly, the neuropeptides that mediate BNST signaling during stressor exposure have generated significant interest. In addition to the roles of extra-hypothalamic corticotropin-releasing hormone (CRH) signaling in fear- and anxiety-like behavior (Lee and Davis, 1997; Koob and Heinrichs, 1999; Hammack et al., 2002), pituitary adenylate cyclase activating

polypeptide (PACAP) and its cognate PAC1 receptor have been implicated in mediating responses associated with stress and anxiety. PACAP and PAC1 receptors are selectively upregulated in the BNST following chronic variate stress, and BNST PACAP activation is anxiogenic (Hammack et al., 2009). PACAP and PAC1 receptor null mice also demonstrate reduced anxiety-like behavior (Hashimoto et al., 2001; Otto et al., 2001; Gaszner et al., 2012; Hattori et al., 2012), and the glucocorticoid response in PACAP null animals is altered after stressor exposure (Morita et al., 2006; Hatanaka et al., 2008; Stroth and Eiden, 2010; Tsukiyama et al., 2011; Lehmann et al., 2013). In humans, PACAP dysregulation has recently been associated with a number of psychopathologies including post-traumatic stress disorder (PTSD). Elevated circulating PACAP levels, and PAC1 receptor gene polymorphism and methylation each predicted PTSD symptoms and diagnosis (Ressler et al., 2011; Jovanovic et al., 2013).

However, while accumulating evidence implicate PACAP in the consequences of stressor exposure, the mechanisms and functions of PACAP activities in the BNST are still unclear. In the current work, we examined the intersection of PACAP and CRH in the BNST, the PACAP/VIP receptor subtypes involved in BNST function, and the roles of endogenous BNST PACAP in mediating the stress-induced HPA and behavioral responses. Notably, we demonstrate that blocking BNST PACAP/PAC1 receptor signaling can attenuate the physiological and behavioral consequences of repeated stress exposure which may offer insights for therapeutics.

2. Methods

2.1. Animals

Adult male Sprague-Dawley rats (250 - 275 gm) were obtained from Charles River Laboratories (Wilmington, MA). After delivery, rats were allowed to habituate in their home cages for at least one week before experimentation. Rats were single-housed and maintained on a 12 h light/dark cycle (lights on at 0700 h). Food and water were available ad libitum. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Vermont.

2.2. Surgical procedures

For acute infusion studies, rats were anesthetized with isoflurane vapor and secured in a stereotaxic apparatus. After skin incision, the skull was exposed and four screws were inserted to provide skullcap stability. Two stainless steel cannula (26 gauge, Plastics One, Roanoke, VA) were lowered at a 20 degree angle just above the dorsolateral BNST, from bregma in mm; AP = -0.3, ML = $\pm$ 3.8 and DV = -5.3 from the surface of the dura (Paxinos and Watson, 2009). A skullcap was created with dental cement to hold the cannula in place. One week after cannulation, the rats were acutely infused bilaterally into the BNST with PACAP38, VIP (both from American Peptide Co., Sunnyvale, CA; 0.1 - 1 μ g in 0.5 μ l per side), maxadilan (kind gift from Ethan Lerner, Harvard Medical School/Massachusetts General Hospital) or saline/BSA vehicle. PACAP38 was preferentially used in these studies instead of the alternatively processed PACAP27 from the precursor because of its high (~10- to 100-fold) prevalence in the CNS. In acute receptor antagonist studies, rats were

infused bilaterally with PACAP(6-38) (0.3 µg in 0.5 µl per side). Following infusion (0.5 µl/min) the cannulae were left in place for 1 min before removal. PACAP(6-38) has been well used as an antagonist at PAC1 and VPAC2 receptors. The application of PACAP(6-38) and the PAC1 receptor specific agonist maxadilan in the current studies facilitate the identification of PACAP/PAC1 receptor subtype signaling in BNST function.

Similar surgical procedures were followed for chronic infusions. Once the BNST cannulae (Plastics One, Roanoke, VA; No. 328OP/SPC) and skullcap were in place, the cannulae were attached to a single bifurcation connector (Plastics One, Roanoke, VA; No. 21y) using 3 mm catheter tubing (Alzet, Cupertino, CA; OC 1.14 mm, ID 0.69 mm). The bifurcation connector was attached to 6.7 cm of catheter tubing attached a mini-osmotic pump (Alzet, Cupertino, CA; No. 2002, 200 µl volume, 0.5 µl/h) containing either antagonist PACAP(6-38) (American Peptide Co., Sunnyvale, CA; 75 µM) or vehicle (0.05% BSA in saline). Long term PACAP(6-38) stability at 37 C was assessed separately by daily aliquot removal for intact and amidated peptide radioimmunoassays. Peptide loss was 4% per day; final minipump PACAP(6-38) concentration at the end of the study was approximately 40 µM. All catheters were filled with vehicle to delay antagonist infusion into the BNST before the chronic variate stress procedure. The minipumps were primed according to manufacturer's instructions and secured subcutaneously in the interscapular space. The rats were returned to their home cages for 6 day postsurgical recovery; all rats were monitored for food/water intake and weighed daily.

2.3. Chronic variate stress (CVS)

Following acclimation or postsurgery recovery, each animal was randomly assigned to either a control or chronically stressed group. Control group animals were handled and housed in their home cages until euthanasia. The chronically stressed group of animals underwent a chronic variate stress (CVS) paradigm in which rats were exposed to one of 5 different stressors (oscillation, forced swim, restraint, pedestal standing and footshock) each day for 7 days, as described previously (Hammack et al., 2009; Roman et al., 2012). All animals within the group were exposed to the same order of stressors for the same duration.

2.4. Behavioral testing

Novel object—Behavioral testing was performed one day after the last stressor. Rats were placed individually into a 55 × 55 cm opaque white arena with 50 cm walls (United States Plastics Corp., Lima, OH). A novel object (blue thermos cup) was secured to the center of the chamber and the rats were allowed to move freely for 5 min. Illumination in the center of the chamber was 14 lux. The sessions were captured digitally and the amount of time the rat either spent actively touching the object or approached the object within 3 mm of its nose was tabulated.

Open field—As above, rats were placed individually into a 55 × 55 cm white arena with 50 cm walls with 14 lux illumination in the center square. Time spent in the center 18 × 18 cm square, as well as the outer 9 cm wall perimeter was recorded over a testing period of 8 min.

Elevated plus maze—A plus maze of black painted wood and elevated 74 cm from the floor, consisted of two opposing open and two opposing closed arms (each arm 60 cm long and 9 cm wide) that extended perpendicularly from a central square platform (9 × 9 cm). The length of the closed arms were walled by black opaque panels 30 cm in height. Illumination with a red bulb was at 6 lux in the center of the maze. The animals were individually placed on the central platform facing a closed arm and allowed to freely explore the maze for 7 min. The sessions were captured digitally and the time spent in the open and closed arms, and the total number of crosses into the open arms was scored. In all behavioral tests, the activities were tabulated by observers blinded to the experiment.

2.5. Cannulae verification

Upon completion of behavioral testing, the rats were deeply anesthetized and perfused transcardially with saline followed by 4% paraformaldehyde. The brains were removed, fixed for an additional 24 h, washed and cryoprotected through graded sucrose solutions (30% sucrose final). The brains were blocked and frozen in Tissue-Tek for cryosectioning (30 μ m); all slides were processed for cresyl violet staining and cannulae placement were verified under brightfield microscopy. Rats with cannulae placements in the body of the caudate-putamen or lateral septum were not included in the studies. Tissue damage from acute and chronic infusions, and from vehicle and peptide/antagonist injections were comparable. Dorsolateral BNST infusion spread was verified in select animals by cannulae injection with dextran conjugated Texas Red (Molecular Probes/Invitrogen, Carlsbad, CA; 2.5 mg/ml) at experiment termination and tissue visualization under fluorescent microscopy.

2.6. Hormone assays

For hypothalamic and anterior pituitary hormone measurements, the tissues were extracted in 5 N acetic acid with 0.3 mg/ml phenylmethylsulfonyl fluoride (PMSF) using a ground glass -ground glass homogenizer. An aliquot was removed for total protein assay using the BCA reagent. The homogenates were frozen and thawed three times, and centrifuged at 1000 × g to remove debris; the resulting extracts were lyophilized and subsequently reconstituted in 100 mM sodium phosphate buffer, pH 7.5, containing 1% Triton X-100 and PMSF before assay. Serum for corticosterone assays were prepared as described in Experimental Procedures. Corticosterone ELISA assays (Arbor Assays, Ann Arbor, MI) had a detection limit of 18 pg/ml, and an intra- and interassay variability of 5.7% and 7.9%, respectively. The ACTH assay (MD Bioproducts, St. Paul, MN) demonstrated a detection limit of 0.5 pg/ml, and an intra- and interassay variability of 6.7% and 8.2%, respectively. The CRH assay (Phoenix Pharmaceuticals, Burlingame, CA) had a detection limit of 35 pg/ml, and intra- and interassay variability of 4.6% and 7.1%, respectively.

2.7. Immunocytochemistry

Rats were anesthetized with pentobarbital 24 h after the last stressor (day 8) and perfused transcardially with heparinized saline followed by 4% paraformaldehyde. The brains were quickly removed, postfixed and protected in 30% sucrose for cryosectioning (30 μ m). For BNST staining, tissue sections from control and stressed animals were matched using the anterior commissure and fornix as landmarks.

All immunocytochemistry reagents were chosen to allow PACAP and CRH colocalization studies. Brain cryosections were permeabilized with 0.3% triton X-100, treated with 1% BSA blocking solution and incubated in a mouse monoclonal PACAP antibody (1:10; Jens Hannibal, Bispebjerg Hospital, Copenhagen, Denmark) and/or a rabbit affinity-purified CRH antibody (1:100; Phoenix Pharmaceuticals, Burlingame, CA) 48 hours at 4 C. The monoclonal antibody is directed to the N-terminus of PACAP and does not discriminate between PACAP27 and PACAP38; PACAP localizations were performed using tyramide amplification as previously described (Hannibal, 2002). After primary PACAP antibody incubation and washings, the cryosections were incubated in biotinylated horse anti-mouse IgG (1:200; Vector Laboratories, Burlingame, CA) for 2 hours. The sections were washed, treated with streptavidin horseradish peroxidase (HRP; 1:250) for 30 min, washed again and subsequently incubated in a tyramide-biotin reagent (1:100; Perkin Elmer, Boston, MA) for 10 min. After extensive buffer rinses, the PACAP immunoreactivity in the sections was localized using avidin-conjugated Cy2 (1:200; Jackson ImmunoResearch, West Grove, PA). For CRH localizations, the cryosections were incubated in Cy3-conjugated donkey anti-rabbit IgG (1:250; Jackson ImmunoResearch, West Grove, PA). In some studies, the distribution of the PACAP immunocytochemical staining patterns was identified using the avidin-biotin-complex (ABC; Vector Laboratories, Burlingame, CA) to identify sites of low PACAP expression. Following tyramide amplification, the sections were incubated with 1:200 ABC solution (in place of avidin-Cy2) for 2 h, washed and processed using diaminobenzidine (DAB) and hydrogen peroxide as substrates. To obviate fluorochrome signal occlusion in these dual localization studies, the cryosections with CRH localizations using Cy3 were imaged first; the sections were subsequently returned to the immunocytochemical process for PACAP staining using DAB. To compare CRH (Cy3) and PACAP (DAB) staining patterns on the same tissue sections, images of the PACAP DAB patterns were digitally extracted and superimposed onto the CRH immunofluorescence image with the aid of CNS structures as landmarks. All antibody staining was blocked with their respective blocking peptides. Omission of any one of the processing reagents also failed to produce signals.

2.8. Transcript analyses

For PACAP transcript analysis by *in situ hybridization*, control and chronically stressed rats were anesthetized and euthanized by decapitation. The brains were rapidly removed and frozen; cryosections (20 μ m) were mounted onto Superfrost Plus slides and stored at -80 C before processing. As before, the cryosections were fixed in 4% paraformaldehyde, acetylated and chloroform treated before probe hybridization (approximately 500 ng/slide) in a humidified chamber at 45 C for 24 h (Brandenburg et al., 1997; Braas and May, 1999). Sense and antisense PACAP cRNAs were transcribed from linearized plasmids using biotin-labeled UTP. After high stringency washes in 20 $\times$ SSC, and 0.2 $\times$ SSC, and RNase A digestion of residual probe, the cryosections were incubated in streptavidin-HRP for tyramide-biotin amplification. The sections were finally incubated with ABC complex for signal detection with DAB.

2.9 Behavioral experimental procedures

Experiment 1 - Effects of BNST PACAP38 infusions on stress-related

responses—Adult male rats were cannulated for BNST infusions as described in Surgical procedures (Section 2.2). The rats were handled daily for habituation and after 6 day postsurgery recovery, the rats were randomly assigned to vehicle or PACAP groups ($n = 5$ per group). On experimental day, the rats were weighed for baseline measures and bilaterally injected with vehicle or PACAP38 (indicated dose in $0.5 \mu\text{l}$ per side, random order). The injection needle was left in place for 1 min after which the rats were returned to their home cages for 30 min before behavior testing on the elevated plus maze. The rats were allowed to freely roam the maze for 7 min and all data were captured digitally. Separate groups of rats were prepared similarly for weight change measurements. The rats were weighed, injected with PACAP38 and returned to their home cages. At the same time the following day, the rats were re-weighed to assess weight change over 24 h; food and water consumption were also measured. All behavior and weight change measures in this and subsequent experiments were performed between 0900 and 1130 h.

Experiment 2 - BNST PACAP receptor subtypes mediating PACAP responses

—Adult male rats were surgically prepared for BNST cannulation and peptide infusions as described in Surgical procedures (Section 2.2). The rats were handled daily and after postsurgery recovery, the rats were randomly assign to the different treatment groups. On day of experiment, the rats were weighed and injected bilaterally with vehicle ($n = 9$), maxadilan ($n = 8$) or VIP ($n = 6$) at the indicated dose ($0.5 \mu\text{l}$ per side) in random sequence. The injection needle was left in place for 1 min; the rats were returned to their home cages for 30 min before open field or elevated plus maze tests. The rats freely explored the arena or maze and all movements were tracked. A separate set of rats was used investigate the potential effects of maxadilan and VIP on weight change and feeding. After baseline weight measurement and peptide infusions, the rats were returned to their home cages; both food and water were measured. At the same time the following day, the rats were weighed again to assess 24 h weight change and food/water consumption was determined.

Experiment 3 - Effects of BNST PACAP receptor antagonism on stress-related

responses—Adult male rats for this set of experiments were handled and weighed daily after arrival at the animal care facility. After acclimation the rats were prepared for bilateral BNST cannulation using procedures described in Methods (Section 2.2). Briefly, following implantation, the cannulae were secured with dental cement and each cannula was attached to the ends of a bifurcation connector with 3 mm of catheter tubing. The bifurcation connector in turn was attached to a 6.7 cm length of tubing attached to a mini osmotic pump containing vehicle or PACAP(6-38) PAC1 receptor antagonist. All of the catheter tubing was filled with vehicle to delay antagonist infusion in the BNST during the postsurgery recovery period. Given the flow rate of the mini osmotic pump, the content of the mini pump was calculated to reach the BNST on the first day of the CVS exposure. The mini pump (200 μl reservoir) was selected to continuously deliver the contents during the entire experimental period. The rats were then randomly assigned to one of 4 groups: (1) control no stress - vehicle, $n = 8$; (2) control no stress - antagonist, $n = 8$; (3) stress - vehicle, $n = 9$; and (4) stress - antagonist, $n = 9$). The stress groups received the 7 day CVS paradigm as

described. Non stressed rats were handled and returned to home cages. The day after the last CVS challenge (24 h post CVS), the rats in random sequence were placed on an elevated plus maze for behavior testing; this was followed by novel object tests the following day (48 h post CVS).

Experiment 4 - Effects of BNST PACAP infusions on endocrine stress

hormones—Adult male rats were cannulated bilaterally for BNST infusions as described above. Following postsurgery recovery, PACAP was infused into the BNST (1 µg/0.5 µl; n = 5) as described above; control animals received vehicle (n = 5). The rats were returned to their home cages for 50 min before euthanasia by decapitation. Trunk blood was collected and serum prepared for corticosterone assays. Anterior pituitary and hypothalamus tissues were dissected and frozen immediately on dry ice. All blood and tissue harvest procedures were performed between 0900 and 1000 h. For peptide assays, the tissues were homogenized in acetic acid and prepared as described in Methods (Section 2.6).

Experiment 5 - Effects of BNST PACAP receptor antagonism in a sensitized stress model

—Adult male rats were prepared for bilateral BNST cannulation as described above. Following recovery, the animals were divided into their respective groups: (1) control - vehicle, n = 7; (2) control - antagonist, n = 6; (3) stress - vehicle, n = 7; and (4) stress - antagonist, n = 8. All of the animals in the stress groups were exposed to the 7 day CVS paradigm for stress sensitization; the control rats were handled and returned to their home cages. Twenty four hours after the last stressor, all animals received their respective vehicle or the PAC1 receptor antagonist infusions 15 min before placement into an open arena as a novel environmental stressor. For the control groups, the open arena represented an acute stressor; the open arena represented a novel stressor to the already sensitized CVS animal groups. The animals freely explored the arena for 15 min and returned to their home cages for 15 min before tail blood collection (approx. 300 µl; between 0900 - 1000 h) for corticosterone measurements. A separate group of cannulated adult male (n = 7) rats was prepared and handled identically but did not receive any stressor for baseline corticosterone measurements.

2.10. Statistics

Quantitative image analyses were performed using NIH ImageJ (Schneider et al., 2012). After background thresholding, the images were examined by pixel analyses over uniform circumscribed areas for changes in immunoreactivity (staining pixel number/area); staining intensity was not used as a measure. Statistical Student's t-tests and one-way/two-way ANOVAs with post hoc Newman-Keuls, Tukey's or Holm-Sidak multiple pairwise comparisons were performed using SigmaPlot/SigmaStat (Systat Software, San Jose, CA). Weight change data were analyzed by repeated measures ANOVA; in Experiment 3, a linear mixed model was fitted to the data using the MIXED procedure of the SAS System for Windows version 9.2 (SAS Institute Inc, Cary, NC). For comparisons between the stress/vehicle group and the other three groups, the combination of the control/stress and vehicle/PACAP(6-38) groups was analyzed as a single four-level factor. Analysis was restricted to the stress-induced period from days 8 through 14. A linear mixed model approach was used to perform a repeated measures ANOVA, with group as a fixed factor, day as the repeated

measure, and baseline weight as a covariate. An auto-regressive covariance structure was assumed for the repeated measure. All values represent the mean change $\pm$ SEM. $p < 0.05$ was considered significant.

3. Results

3.1. Chronic variate stress induces BNST oval nucleus PACAP expression

Among limbic structures, we have shown previously that repeated variate stress selectively increases dorsolateral BNST PACAP transcript expression (Hammack et al., 2009). The BNST is a complex structure that can be parceled into more than 30 subdivisions based on neurotransmitter/neuropeptide distribution, cytoarchitecture and afferent/efferent projections (Dong et al., 2001). As our previous transcript analyses by quantitative polymerase chain reaction (QPCR) could not discriminate the BNST subdivisions exhibiting chronic stress-induced PACAP expression, we used immunocytochemical and *in situ* hybridization approaches to better identify the subregions of BNST PACAP signaling and its distribution patterns compared to CRH. Unlike previous studies, we wanted to assess endogenous tissue peptide levels and patterns and hence did not pretreat the rats with colchicine as this stressor procedure alone can induce peptide expression. In the coronal cryosections examined, (bregma -0.25 thru -0.35; Figure 1), PACAP immunoreactivity in the dorsolateral division of the BNST was evident in fiber varicosities and terminals in the oval nucleus (Figure 1E). As in previous studies (Piggins et al., 1996; Kozicz et al., 1998; Hannibal, 2002) few cell bodies were identified which may have reflected the thin cytoplasm circumscribing the small BNST neurons (15 - 20 μ m). In contrast to patterns for PACAP, CRH fiber immunoreactivity in control tissues was more pervasive throughout the oval nucleus (Figure 1B) (Ju et al., 1989; Day et al., 1999).

Compared to control non-stressed animals, chronic variate stress selectively increased PACAP immunoreactivity in the oval nucleus more than 4-fold compared to the control group (Figure 1D, $t = -7.17$; $p < 0.002$; compare Figure 1E and 1F) in good agreement with previous QPCR results (Hammack et al., 2009). The PACAP immunocytochemical and QPCR data were further corroborated by *in situ* hybridization studies (Figure 1H - 1I). While PACAP mRNA expression was apparent in only a few somal profiles in the oval nucleus of control animals, the number of PACAP expressing neurons was increased significantly after chronic stress (Figure 1G, $t = -8.35$; $p < 0.001$). BNST CRH immunoreactivity by contrast was not altered by stress (Figure 1D) which correlated with QPCR data failing to detect CRH transcript changes following chronic variate stress (Roman, unpublished observations).

Despite CRH and PACAP expression in BNST oval nucleus, dual labeling studies suggested that the two peptides were not colocalized within the same fiber elements (Figure 2), which appeared consistent with the differential regulation of the two peptidergic systems. CRH fiber staining was not uniform across the oval nucleus and interestingly, PACAP immunoreactivity was heaviest in regions where CRH staining appeared sparse. This difference was especially evident in the stressed tissues where PACAP expression was induced (Figure 2E - 2F). The apparent dichotomy of the two peptide systems was evident even under enhanced DAB procedures to obviate differences in staining sensitivities. These

results suggested that CRH and PACAP represent separate peptidergic systems with potentially different targets and roles in BNST neurocircuits. Lower levels of PACAP immunoreactivity were also observed in the dorsomedial and ventrolateral BNST of control and stressed animals (not shown).

3.2. Effects of BNST PACAP38 infusions mimic stress-related behavioral responses

We have shown previously that acute BNST PACAP infusions produce anxiogenic responses as measured by baseline startle (Hammack et al., 2009). To further demonstrate that BNST PACAP signaling can mimic stress-related responses, we bilaterally cannulated adult male rats for targeted BNST PACAP38 (1 μ g/0.5 μ l) or vehicle infusions (Experiment 1). Similar to previous results, BNST PACAP38 infusion was anxiogenic on the elevated-plus maze as measured by open arm time (Figure 3A, $df = 8$, $t = 3.3$, $p = 0.01$). These responses did not reflect changes in locomotion, as locomotor activity, measured by the total number of arm entries, was not different between treatments (Figure 3B, $df = 8$, $t = 0.53$, $p = 0.61$). Further, BNST PACAP signaling was also anxiogenic from open field and novelty tests (see below).

In rats, the stress-induced anxiety-like responses are accompanied by anorexia and consequent weight loss. In the current paradigm, for example, chronic stress reliably decreased weight gain approximately 50% over the experimental period in male or female rats (data not shown). To examine BNST PACAP abilities to simulate stress-mediated anorexia, the rats (separate from those used in the behavior studies) were cannulated as before, and following recovery, the groups were weighed, infused into the BNST with PACAP38 or vehicle, and returned to their home cages for weight change and food/water intake assessments 24 h later (Experiment 1). BNST PACAP infusions produced pronounced weight loss (Figure 3C, $df = 8$, $t = 8.53$, $p < 0.001$) in agreement with previous BNST studies (Ciccocioppo et al., 2003; Roman et al., 2012; Yamashita et al., 2013; Kocho-Schellenberg et al., 2014). The rats typically lost approximately 5 - 8% of their body weight which appeared to reflect in part a decrease in food intake and an increase in thermogenesis (Hawke et al., 2009). The anorexic effects of PACAP were dose-dependent; one-way ANOVA revealed a significant effect of dose ($F(3,32) = 6.21$, $p = 0.002$), and post hoc Tukey's multiple comparison tests revealed a significant effect at both 0.5 and 1 μ g PACAP38 concentrations. These BNST PACAP-induced weight changes appeared transient as feeding and weight gain returned 48 - 72 h after the initial treatment.

3.3. BNST PACAP PAC1R activation induces stress-induced anxiety-like responses

The effects of PACAP peptides can be mediated by 3 receptor subtypes; the PAC1 receptors are selective for PACAP peptides while the VPAC1/VPAC2 receptors demonstrate near equal high affinity for PACAP and VIP. Hence to assess the roles of BNST PAC1 vs VPAC receptor signaling in anxiety-related behaviors, the effects of PAC1 receptor specific agonist maxadilan (Moro and Lerner, 1997), and VPAC receptor ligand VIP were compared (Experiment 2). As previous studies demonstrated that 0.5 μ g BNST PACAP infusions per side produced approximate half-maximal responses in behavior and weight change, comparable doses were used. In open field tests, BNST infusions with the PAC1 receptor agonist maxadilan elicited anxiogenic responses, reflected by decreased rat exploratory

times in the center of a brightly lit field and increased thigmotaxis (arena perimeter run time; Figure 4A and 4B), compared to VIP or vehicle controls (one-way ANOVA; center time, $F(2,20) = 4.484$, $p = 0.024$; perimeter time, $F(2,20) = 4.894$, $p = 0.018$). Post-hoc Bonferroni tests revealed significant differences between maxadilan and vehicle groups ($p < 0.05$). A similar anxiety-like response was observed on elevated plus maze tests in which maxadilan decreased open arm time 47% compared to vehicle treated controls ($t(7) = 3.22$, $p = 0.018$). In separate experiments, maxadilan similarly elicited a decrease in body weight ($F(2,23) = 7.21$, $p = 0.008$) and food intake ($F(2,23) = 9.00$, $p = 0.004$; Figure 4C and 4D). The maxadilan responses contrasted with those for VIP which failed to elicit anxiety-like behaviors in open field or elevated plus maze tests, and feeding or weight change responses. Given the very modest VIP effects and robust responses to maxadilan, these results in aggregate demonstrate that the PACAP-induced anxiety-like behaviors are mediated by PAC1 receptor activation.

3.4. BNST PAC1 receptor antagonist infusions block the anxiogenic effects of chronic stress

To determine whether BNST PACAP receptor signaling mediates the behavioral consequences of repeated stress, we used osmotic minipumps attached to bilateral intracerebral cannulae to chronically infuse the BNST with PACAP(6-38), a PAC1 receptor antagonist, during the 7 day repeated variate stress procedure (Experiment 3, Figure 5A). As before, animal weight and food intake were monitored daily during the entire duration of the experiment, and behavior assessed on elevated plus-maze and novelty tests 24 - 48 h after the last stressor. After cannulae placement and postsurgical recovery, the control nonstressed animals with vehicle infusions steadily increased their body weight (Figure 5B, filled circles); nonstressed animals receiving PACAP(6-38) gained weight similarly (Figure 5B; closed circles) demonstrating that there were no apparent effects of the antagonist alone on weight. By contrast, in agreement with prior studies of stress-induced anorexia (Krahn et al., 1990; Martí et al., 1994; Martí and Armario, 1997), chronic variate stress over the last week of study attenuated weight gain in the vehicle-treated rats (Figure 5B; closed triangles). However, continuous BNST PACAP(6-38) infusions during the chronic stress period blocked the stress-induced changes in weight gain (Figure 5B; open triangles). Unlike the plateau in weight change observed in the stress/vehicle group, stressed rats receiving PACAP(6-38) gained weight at rates comparable to those of nonstressed controls.

The repeated measures ANOVA showed a significant overall effect of day ($F(6,186) = 14.95$, $p < 0.001$). The day $\times$ group interaction was also significant ($F(18,186) = 2.75$, $p < 0.001$), indicating that the change in body weight over the 7 day stress period was not similar in all groups. In addition, the baseline weight covariate was significant ($F(1,30) = 63.7$, $p < 0.001$), while the main effect of group was not significant ($F(3,30) = 1.95$, $p = 0.14$).

Simple effect tests of group differences on each day showed that the four groups differed in mean body weight (after adjusting for the baseline weight of each animal) on days 12 through 14 (day 12, $F(3,186) = 3.46$, $p = 0.017$; day 13, $F(3,186) = 4.37$, $p = 0.005$; day 14, $F(3,186) = 4.55$, $p = 0.004$) Planned contrasts revealed a significant difference between the

stress/vehicle group and the average of the three remaining groups on experimental days 12 through 14 (day 12, $F(1,186) = 9.20$, $p = 0.003$; day 13, $F(1,186) = 12.55$, $p = 0.001$; day 14, $F(1,186) = 11.20$, $p = 0.001$), but there was no significant difference on day 11 ($F(1,186) = 3.31$, $p = 0.071$). Hence, chronic BNST PACAP antagonism via PACAP(6-38) infusions attenuated the effect of CVS on weight gain.

On the elevated plus maze, ANOVA revealed a significant effect of chronic PACAP antagonism (Figure 5C; $F(1,30) = 4.95$, $p = 0.034$), a near significant effect of stress ($F(1,30) = 2.98$, $p = 0.066$); notably a planned contrast revealed an effect of stress in vehicle treated rats ($df = 30$, $t = 2.42$, $p = 0.022$) but no interaction between PACAP(6-38) and repeated stress ($F(1,30) = 0.45$, $p = 0.63$). Similarly on the novel object test, ANOVA revealed a significant effect of chronic PACAP antagonism (Figure 5D; $F(1,30) = 4.57$, $p = 0.041$, a significant effect of stress ($F(1,30) = 18.15$, $p < 0.001$) but no interaction between antagonist and stress ($F(1,30) = 0.48$, $p = 0.49$). Planned contrast did reveal a significant difference between stress/vehicle and stress/PACAP(6-38) treatment ($df = 30$, $t = 2.06$, $p = 0.047$). Therefore, for both elevated plus maze and novelty tests, BNST PACAP(6-38) infusions were capable of reducing anxiety-like responses in both stressed and non-stressed rats. These studies demonstrate the abilities for PACAP(6-38) to block BNST PACAP behavior signaling.

3.5. PACAP and PACAP signaling have endocrine roles in chronic stress

In parallel with stress-related behavioral activities, the BNST also engages hypothalamic endocrine stress responses via direct or indirect pathways. As PACAP regulates hypothalamic activities and stressed PACAP null mice demonstrate blunted corticosterone responses, the abilities for BNST PACAP signaling to engage HPA activation were also examined. For these studies (Experiment 4), PACAP was injected acutely into the BNST and after 50 min, trunk blood was collected for corticosterone measurements to approximate previous temporal response studies. Hypothalamic and anterior pituitary tissues were also collected concurrently for HPA stress hormone measurements. Under this paradigm, acute BNST PACAP administration increased serum corticosterone levels approximately 3 - fold ($df = 8$, $t = 3.8$, $p = 0.005$). These corticosterone changes did not reflect errant ventricular injections and diffusion to the hypothalamus since direct lateral ventricular PACAP infusions ($0.1 - 1 \mu\text{g}/0.5 \mu\text{l}$) failed to elicit either corticosterone responses or animal weight change (Lezak et al., 2014). The very small injection volumes likely contributed to differences with previous PACAP ICV injection results (Agarwal et al., 2005). To assess whether the corticosterone responses reflected other upstream changes in the HPA pathway, anterior pituitary ACTH levels were examined. From the same BNST PACAP-infused animals, there was a small but significant decrease in anterior pituitary ACTH content ($df = 8$, $t = 2.333$; $p = 0.048$) which appeared consistent with heightened corticotrope ACTH release. However, under these temporal parameters, changes in hypothalamic CRH content were not apparent ($df = 8$, $t = 1.2$, $p = 0.257$) which may have reflected homeostatic maintenance CRH production following stimulated secretion.

As in the behavioral studies, we similarly asked whether BNST PACAP(6-38) infusions might influence PAC1 receptor signaling in BNST/hypothalamic circuits and blunt HPA

endocrine stress responses. Since our previous and ongoing studies have suggested that PACAP has larger roles in chronic than acute stress mechanisms (Stroth and Eiden, 2010; Lezak et al., 2014), we chose a well used stress-sensitized HPA activation model for these studies (Experiment 5). Comparable to our previous experimental designs, separate sets of adult male rats were bilaterally cannulated for BNST infusions and half of the animals were subjected to the chronic variate stress paradigm for 7 days. Then 24 h after the last stressor, the non-stressed and chronically stressed animals received a single BNST PACAP(6-38) or vehicle injection 15 min prior to a new acute environment stressor (CVS + acute) for subsequent corticosterone assays. Compared to non-stressed controls, corticosterone levels in animals with acute stress alone were elevated approximately 3-fold and in agreement with previous work (Stroth and Eiden, 2010), acute BNST PACAP(6-38) injections prior to acute stress had no apparent effects on corticosterone responses. In animals with HPA sensitization by chronic variate stress, subsequent acute stress exposure significantly increased blood corticosterone to levels greater than those following acute stress alone (CVS + acute vs acute alone). Notably, acute PACAP(6-38) injections into the BNST blunted the sensitization-induced corticosterone responses (Figure 6). In animals exposed to acute environmental stressor, two-way ANOVA revealed a significant interaction between prior stress treatment and PACAP(6-38) injection ($F(1,23) = 4.847$, $p = 0.037$) and post-hoc Bonferroni test revealed a significant effect of stress in the vehicle-treated group as well as a significant effect of PACAP(6-38) treatment in the chronically stressed groups. Again, these results were consistent with previous observations and in aggregate, suggested that inhibition of BNST PACAP/PAC1 receptor signaling has broad effects in attenuating both chronic stress-induced anxiety-related behaviors and hormonal responses.

4. Discussion

We have shown recently that BNST PACAP signaling elicits stress-related responses. Chronic variate stress selectively increased PACAP and PAC1 receptor transcript expression in the dorsolateral BNST, and acute BNST PACAP38 infusions produced anxiogenic and anorexic responses analogous to those described for CRH (Hammack et al., 2009). Like CRH, PACAP is highly expressed in the hypothalamus and associated limbic circuits (Ju et al., 1989; Piggins et al., 1996; Day et al., 1999; Hannibal, 2002), and can regulate anterior pituitary ACTH release to affect HPA function during stress. Notably, consistent with their roles in stress-related activities, PACAP and PAC1 receptor knockout mice display diminished anxiety-related phenotypes (Timpl et al., 1998; Hashimoto et al., 2001; Otto et al., 2001; Hattori et al., 2012) and impaired stress-induced HPA responses (Stroth and Eiden, 2010; Tsukiyama et al., 2011; Lehmann et al., 2013). Given the many parallels between PACAP and CRH, understanding the functional relationships between the two peptidergic systems is important in delineating behavior neurocircuits. Although PACAP and CRH can be colocalized in a small population of hypothalamic neurons, several studies have suggested a dichotomy in PACAP and CRH systems (Kozicz et al., 1997; Shioda et al., 1997; Agarwal et al., 2005). Similarly, our duallabeling studies demonstrate that BNST PACAP and CRH immunoreactivities are not colocalized together in fiber varicosities and terminals in the oval nucleus. The BNST contains short-axon interneurons interconnected with long-distance projection neurons within shell and core domains; hence the PACAP and

CRH neurons may represent separate but integrated elements in the tripartite organization of the BNST (Larriva-Sahd, 2006).

In addition to enhanced anxiety-like behaviors such as increased startle, decreased elevated plus maze open arm entries, decreased open field crossing and novel object avoidance, direct BNST PACAP injections elicited profound anorexic responses. The anorexigenic effects were consistent with previous PACAP feeding studies using systemic or ICV PACAP administrations which reliably decreased rodent food intake resulting in diminished weight gain (Morley et al., 1992; Chance et al., 1995; Mizuno et al., 1998; Tachibana et al., 2003; Hawke et al., 2009; Mounien et al., 2009). But in contrast to previous work implicating PACAP regulation of hypothalamic arcuate nucleus POMC/MC4R signaling in feeding, our studies demonstrate that direct BNST PACAP injections can also produce dramatic acute anorexic responses to accompany the behavioral and neuroendocrine consequences of stress. The regulation of feeding and satiety resides prominently in the lateral and ventromedial hypothalamus, and recent studies have demonstrated that direct dorsolateral BNST projections to the lateral hypothalamus orchestrates feeding behaviors (Jennings et al., 2013). The roles of BNST PACAP within this circuit are unclear but may include activation of lateral hypothalamic glutamergic neurons through long BNST projections or inhibition of intra-BNST GABAergic pathways. Alternatively, the dorsolateral BNST has dense projections to the rhomboid, fusiform, anteromedial and anteroventral BNST which in turn heavily innervate the hypothalamus (Dong and Swanson, 2006a; Dong and Swanson, 2006b) to potentially impact feeding via PACAP signaling.

PACAP binds to several PACAP/VIP receptor subtypes and our characterizations implicated BNST PAC1 receptor signaling in these stress-related responses. BNST infusions of maxadilan, a 61-amino acid sand fly salivary gland peptide and the only PAC1 receptor-specific ligand identified to date, fully recapitulated the PACAP-induced anxiety-like and anorexic responses. While these results alone did not preclude PACAP-mediated VPAC receptor signaling, parallel infusion experiments with VIP to activate preferentially VPAC1/VPAC2 receptors either failed or demonstrated far less efficacy in initiating behavioral responses. These results were in accord with other studies implicating PAC1 and not VPAC receptors in feeding behavior in rodents (Mounien et al., 2009).

But to further these observations and establish endogenous BNST PACAP/PAC1 receptor signaling relevancy in mediating chronic stress-induced anxiety-like and anorexic behaviors, we chronically infused PACAP(6-38) into the BNST during the week-long stress paradigm. While chronic variate stress dramatically diminished plus-maze open arm entry times and novelty interaction scores in rats receiving vehicle infusions, chronic BNST PACAP(6-38) infusions during the stress period, by contrast, blocked or blunted the stress-induced anxietylike responses. Statistical data analyses of the behavioral responses, however, also suggested that PACAP(6-38) had a generalized anxiolytic response as PACAP(6-38) reduced anxiety-like behavior in non-stressed animals. In parallel with its anxiolytic effects, chronic PACAP(6-38) infusions blocked stress-induced anorexia. During the experimental period, repeated stress reliably diminished rodent weight gain compared to nonstressed animals, which was again blocked with PACAP(6-38) administration. The stress-induced changes in weight gain was reflected by reduced food intake although increased PACAP

signaling has also been described to alter body metabolism to facilitate the weight loss (Hawke et al., 2009). The abilities for PACAP(6-38) to block the anxiogenic and anorexic responses appear similar to those for CRH9-41, D-PheCRH and nonpeptide antagonists at the CRH receptor (Smagin et al., 1999; Griebel et al., 2002;). Although heterologous studies demonstrated abilities for CRH receptor antagonist to block PACAP-induced stress responses, supporting PACAP signaling upstream of CRH (Tachibana et al., 2003; Maruyama et al., 2006), other studies have also shown that two peptidergic systems may also be divergent in anorexic responses (Dore et al., 2013) to suggest multiple pathways to feeding centers.

BNST subregions can differentially regulate HPA endocrine function (Choi et al., 2007; Choi et al., 2008a; Choi et al., 2008b) and our data demonstrating BNST PACAP-stimulated increases in blood corticosterone levels suggest mechanisms by which emotional challenges can engage canonical endocrine stress responses. Consistent with these observations, studies with PACAP knockout mice have demonstrated impaired HPA responses following exposure to emotional than physical stressors (Tsukiyama et al., 2011). The BNST PACAP-induced increases in corticosterone levels were similar to those observed after stress and as adrenal cortical zona fasciculata cells do not express PAC1 and VPAC receptors (Mazzocchi et al., 2002a; Mazzocchi et al., 2002b), the corticosterone responses did not reflect circulating PACAP activation of cortical cells after infusion, but adrenal responses following ACTH secretion. In accord, anterior pituitary ACTH content was decreased following acute BNST PACAP injection, which likely reflected enhanced corticotrope hormone release preceding the corticosterone response.

But to further test BNST PACAP participation in HPA stress hormone activation, PACAP(6-38) was again administered into BNST to assess its abilities to attenuate stress-induced corticosterone responses. As previous work implicated prominent roles for PACAP in maintaining corticosterone responses in chronic than acute stress (Stroth and Eiden, 2010), a stress-sensitized HPA model was included for these studies. In this paradigm, the rats were exposed to the chronic variate stress for stress-pathway sensitization which resulted in heightened HPA-axis responses to subsequent novel challenges (Vernikos et al., 1982; Gagliano et al., 2008). Accordingly, after chronic variate stress exposure, the rats received a single BNST PACAP(6-38) injection prior to open field exposure, which in the current context represented an unprotected novel environmental stressor not previously presented. The sensitized animal group demonstrated a heightened corticosterone release after open field challenge and BNST PACAP(6-38) infusions in this paradigm blunted the stress-sensitized release of corticosterone. Together with the abilities for BNST PACAP infusions to stimulate corticosterone release, these results implicated roles for BNST PACAP in HPA regulation and signaling especially during chronic stress. In contrast to chronic stress responses, PACAP(6-38) had no apparent effects in blunting corticosterone levels after acute stress alone which was in agreement with PACAP knockout studies demonstrating impaired HPA responses following chronic but not acute emotional stressors (Stroth and Eiden, 2010).

In sum, chronic stress and the prolonged activation of emotional circuits have been associated with the development in anxiety-related disorders, depression and related

behavioral abnormalities. Together with previous work, the current studies suggest that PACAP is one of the central regulators of stress responses and the identification of PACAP/PAC1 receptor signaling in this process suggests a potential therapeutic target to attenuate the maladaptive physiological and behavioral consequences of chronic stress.

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Figure 1.

BNST PACAP expression in the oval nucleus is increased after chronic stress. Fixed cryostat sections from control and chronically stressed rats were processed for PACAP or CRH immunoreactivity as described in Methods. The red square in the brain coronal section schematic (A) denotes the area examined for immunocytochemical labeling. The oval nucleus of the BNST was positively identified by CRH immunoreactivity (B, C) using Cy3 as fluorochrome. When adjacent sections were immunocytochemically processed for PACAP using DAB as substrate, dark predominantly fiber staining was identified over the same oval nucleus area (E, F). Unlike the staining patterns for CRH, BNST PACAP immunoreactivity was increased in chronically stressed animals (F) compared to controls (E). From image analyses, the increase in PACAP immunoreactivity above threshold was more than 4-fold in stressed animals compared to untreated animals; CRH immunoreactivity was not changed between the two animal groups (D). Right BNST for control and stressed animals are shown. $n = 4$ animals/group; Data represent mean $\pm$ SEM. *, significantly different from control at $p < 0.05$. Scale bars (B, C, E, F), 100 μ m. The increase in BNST PACAP expression was corroborated by *in situ* hybridization studies using biotin labeled probes (H - I); the number of BNST neuronal soma with PACAP labeling was increased in stressed animals compared to controls (G). $n = 3$, data represent mean $\pm$ SEM. *,

significantly different from control at $p < 0.05$. Scale bar (H, I), 50 μm . All data representative of 3 independent experiments.

Figure 2.

PACAP- and CRH-immunoreactive staining patterns are distinct in the BNST. Tissue sections through the BNST were prepared for dual PACAP and CRH immunocytochemistry to assess whether the two peptidergic systems are colocalized. For both control (A - C) and stressed (D - F) animals, the BNST staining patterns for PACAP (Cy2, green) appeared separate from those for CRH (Cy3, red). For both control and stressed animals, PACAP and CRH immunoreactivity appeared predominantly punctate in fiber varicosities or terminals; few cell bodies were labeled. This was more apparent in the PACAP/CRH merged micrographs (C and F), especially in tissues from the stressed animal groups. Scale bar, 100 μ m. Representative data from 3 independent experiments.

Figure 3.

Acute BNST PACAP infusions elicits anxiogenic responses. Adult male rats with BNST cannulae implants received acute bilateral PACAP38 infusions for elevated plus maze tests as described in Methods. Consistent with previous baseline startle data, PACAP treated animals demonstrated enhanced anxiety-like behavior as reflected by decreased open arm times compared to vehicle-treated controls (CTL; A). Animal locomotion as measured by total arm entries was not changed between groups (B). Twenty-four hours after injection, the PACAP-treated animals also showed significant weight loss (approximately 8% of body weight) compared to vehicle control (C). $n = 5$ per group. Data represent mean $\pm$ SEM; *, significantly different at $p < 0.01$.

Figure 4.

BNST PACAP anxiogenic signaling is mediated by PAC1 receptors. Adult rats were cannulated for BNST injections as described in Methods. Following postsurgical recovery, PAC1 receptor-selective agonist maxadilan (Max) or VPAC receptor ligand VIP were injected bilaterally into the BNST for anxiety-like behavior as measured by arena open field (A) and perimeter running times (B) compared vehicle (Veh) treated rats. Maxadilan decreased open field exploration in a brightly lit environment which was accompanied by an increase in perimeter time. In separate experiments, maxadilan induced weight loss 1 day after bilateral BNST peptide injections, measured as weight change from previous day in grams (C); food intake, measured as grams per 100 grams of animal body weight, was also decreased over the same period (D). By contrast, VIP injections failed to produce significant changes in open field behavior tests or weight change compared to control. The data implicated roles for PAC1 and not VPAC receptors in PACAP-induced anxiety-like behaviors. Open field/perimeter recording time was 8 min; $n = 4 - 10$ rats per group. Data represent mean $\pm$ SEM. *, significantly different from control at $p < 0.05$.

Figure 5.

PACAP receptor antagonist blocks chronic variate stress-induced behavioral responses. Adult male rats were surgically cannulated for chronic BNST vehicle or PACAP(6-38) osmotic minipump infusions as described in Methods. Following post-surgical recovery, half of the rats in the vehicle control and PACAP(6-38) infusion groups were exposed to the 7 day chronic variate stress paradigm before behavioral testing (A, experimental protocol). All rats were weighed on a fixed daily schedule. After a transient post-surgical weight loss, nonstressed rats receiving vehicle (closed circles) or PACAP(6-38) (open circles) gained weight steadily during the course of the experiment (B). Stressed rats receiving vehicle BNST infusions (filled triangles) failed to gain weight; stressed rats receiving PACAP(6-38) infusions (open triangles) by contrast gained weight comparable to controls (B). $n = 8 - 9$ per group; data represent mean percent weight change from experimental start (Day 0) $\pm$ SEM. *, different from control and PACAP(6-38) groups at $p < 0.001$. At the end of chronic variate stress, the different groups were also evaluated on elevated plus maze (C) and novel object tests (D). *, significantly different in planned contrasts at $p < 0.05$. As described in text, chronic infusion with PACAP receptor antagonist was anxiolytic and attenuated stress-induced responses in both behavioral tests.

Figure 6.

Acute BNST infusion with PACAP(6-38) blunts corticosterone release in a sensitized stress model. Following BNST cannulae implantation, one group of adult male rats was exposed to the chronic variate stress paradigm for sensitization. Twenty-four hours after the last stressor, the non-stressed and chronic variate stressed rats were injected with either vehicle or PACAP(6-38) and exposed to an acute novel stress. The acute stressor increased corticosterone responses in all animals compared to a non-stressed, vehicle-treated control group (Veh CTL) kept in home cages. Animals previously exposed to chronic variate stress displayed a potentiated corticosterone response that was significantly higher than other groups. Treatment with BNST PACAP(6-38) prior to the acute stressor exposure blocked the increase in corticosterone response in the chronically stressed animal group, but had no apparent effect on rats exposed to acute environment stress alone. These data coincide with other studies suggesting that BNST PACAP signaling participates in the enhanced corticosterone responses following chronic rather than acute stressor exposures. $n = 6 - 8$ rats per group; data represent mean $\pm$ SEM, *, different from vehicle control at $p < 0.001$. +, different from treatment groups at $p < 0.02$.