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The role of the posterior bed nucleus of the stria terminalis in modulating hypothalamic-pituitary-adrenocortical axis responsiveness to acute and chronic stress

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Summary

The bed nucleus of the stria terminalis (BST) plays a prominent role in brain integration of acute responses to stressful stimuli. This study tests the hypothesis that the BST plays a complementary role in regulation of physiological changes associated with chronic stress exposure. Male Sprague-Dawley rats received bilateral ibotenate lesions or sham lesions of the posterior medial region of the BST (BSTpm), an area known to be involved in inhibition of HPA axis responses to acute stress. Chronic stress was induced by 14-day exposure to twice daily stressors in an unpredictable sequence (chronic variable stress (CVS)). On the morning after the end of CVS, stressed and non-stressed controls were exposed to a novel restraint stress challenge. As previously documented, CVS caused adrenal hypertrophy, thymic involution, and attenuated body weight gain. None of these endpoints were affected by BSTpm lesions. Chronic stress exposure facilitated plasma corticosterone responses to the novel restraint stress and elevated CRH mRNA. Lesions of the BSTpm increased novel stressor-induced plasma ACTH and corticosterone secretion and enhanced *c-fos* mRNA induction in the paraventricular nucleus of the hypothalamus (PVN). In addition, lesion of the BSTpm resulted in an additive increase in CVS-induced facilitation of corticosterone responses and PVN CRH expression. Collectively these data confirm that the BSTpm inhibits HPA responses to acute stress, but do not strongly support an additional role for this region in limiting HPA axis responses to chronic drive. The data further suggest that acute vs. chronic stress integration are subserved by different brain circuitry.

Keywords

BST; corticotropin releasing hormone; vasopressin; paraventricular nucleus of the hypothalamus; corticosterone; ACTH

Introduction

Activation of the hypothalamic-pituitary-adrenal (HPA) axis is a critical component of the body's stress response. The main output of this axis is the release of glucocorticoids from the adrenal cortex into the circulation, primarily to redistribute energy resources following a real or perceived threat to homeostasis (McEwen and Stellar, 1993). The release of glucocorticoids from the adrenal glands is stimulated by circulating adrenocorticotrophic hormone (ACTH) released from the anterior pituitary. Upstream, ACTH release is driven by

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hypophysiotrophic secretagogues, including corticotropin releasing hormone (CRH) and arginine vasopressin (AVP), which are synthesized and released by neurons in the paraventricular nucleus of the hypothalamus (PVN).

During chronic stress, prolonged or repeated activation of the HPA axis can lead to long-term changes in HPA tone and responsiveness. In particular, chronic stress can lead to potentiated basal ACTH and/or corticosterone secretion, adrenal hypertrophy and elevated PVN CRH and AVP mRNA and protein expression (Hauger et al., 1988; Herman et al., 1995; Kiss and Aguilera, 1993; Ulrich-Lai et al., 2006b). Chronic exposure to homotypic stressors typically engender some degree of HPA axis habituation (Dhabhar et al., 1997; Odio and Brodich, 1989; Pitman et al., 1988; Viau and Sawchenko, 2002). However, despite the presence of an enhanced glucocorticoid feedback signals, HPA responses to new stressors are either maintained or augmented following chronic stress a process known as stress facilitation (Armario et al., 1985; Bhatnagar and Dallman, 1998; Bhatnagar and Vining, 2003; Dallman et al., 1992; Hauger et al., 1990; Kiss and Aguilera, 1993; Ostrander et al., 2006). Additionally, chronic stress is implicated in the dysregulation of glucocorticoid secretion that is associated with many disease states such as depression, post-traumatic stress disorder (PTSD) and other anxiety disorders (see (Gold and Chrousos, 2002)).

There is considerable evidence indicating that HPA axis reactivity to stress is influenced by a number of limbic forebrain regions, including the amygdala, hippocampus and prefrontal cortex (see (Herman et al., 2005)). These limbic regions have little direct input to the medial parvocellular PVN and most likely modulate the release of CRH and AVP through synaptic relay circuits. Anatomical studies indicate that the hippocampus and amygdala mainly relay signals by heavily innervating PVN-projecting basal forebrain and hypothalamic structures. Notably, the bed nucleus of the stria terminalis (BST) is among the main extrahypothalamic regions that receives abundant input from all of the noted limbic regions (Cullinan et al., 1993; Dong et al., 2001b; Dong and Swanson, 2004; Gu et al., 2003; Sawchenko and Swanson, 1983).

Several lines of evidence mark the BST as a major mediator of the HPA axis responses to stress (Casada and Dafny, 1991; Crane et al., 2003; Dunn, 1987; Feldman et al., 1990; Forray and Gysling, 2004; Gray et al., 1993; Zhu et al., 2001) raising the possibility that the BST may play a role in HPA axis disorders mediated by upstream limbic structures. The BST can be subdivided into a number of cytoarchitecturally distinct subnuclei with distinct afferent input and efferent targets (Swanson, 1998). In particular, the posterior medial area of the BST (BSTpm) which includes the principal nucleus, is known to be a major inhibitor of neuroendocrine responses to acute stress (Choi et al., 2007; Dunn, 1987; Herman et al., 1994). However, the role of the BSTpm in chronic stress regulation remains to be determined and may provide further understanding of how adaptations in limbic BST circuitry may play a role in stress-related disease states. Since our previous work had demonstrated strong inhibition of acute HPA responses by the BSTpm (Choi et al., 2007), the current studies test the hypothesis that the BSTpm is necessary for regulating neuroendocrine and physiological responses to chronic stress. To test this hypothesis, this study uses bilateral ibotenate lesions targeting the PVN-projecting BSTpm to assess the necessity of this region for mediating central and peripheral HPA axis responses to 14 days of chronic variable stress (CVS).

Materials and Methods

Animals

Forty-eight adult male Sprague Dawley rats (275–300g Harlan, Indianapolis, IN) were used for this study. All rats were housed three per cage in conventional shoebox rat cages. Food

and water were available *ad libitum* in a temperature and humidity controlled vivarium, maintained on a 12/12-hr light/dark cycle (lights on at 0600 hr). Upon arrival, all rats acclimated to the animal facility for at least 7 days prior to surgery. Animals were maintained in accordance with the Guide for the Care and Use of Laboratory Animals (NIH, 1996). All animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Cincinnati.

Ibotenate lesions

Rats were anesthetized by intraperitoneal injections of 87 mg/kg ketamine/13 mg/kg xylazine mixture. Preemptive analgesia was administered by subcutaneous injections of 260 µg/kg butorphanol (Torbugesic). Each rat was placed in a Kopf stereotaxic apparatus upon which skulls were exposed and burr holes were drilled at the calculated coordinates. Each rat received bilateral microinjections of ibotenate (0.5 µl per side, 4.0 µg / µl) in sterile phosphate-buffered saline (pH 7.4) or 0.9% sterile saline into the posterior BST (AP −0.8 mm, ML +/−1.3 mm, DV (dura) −6.5 mm), with coordinates calculated from bregma (Paxinos and Watson, 1998). Each microinjection used a 26 gauge 1 µl Hamilton injection syringe, mounted on the stereotaxic apparatus. Injectors were lowered to the DV coordinate over a 1 min period and left in place for 1 min prior to injection. The ibotenate or saline was manually infused over a total of 5 min at a rate of 0.05 µl / 30 s, followed by another 5 min waiting period to allow diffusion and minimize dorsal spread of injection up the needle track. The syringes were raised over a 1 min period. Skull burr holes were sealed with sterile bone wax and the skin closed with wound clips. Animals recovered for at least 7 days post-surgery prior to CVS exposure. Body weights were measured on the day of surgery, on Day 1 of CVS exposure, on Day 8 of CVS exposure, and on Day 15 following restraint stress challenge and just prior to sacrifice.

Chronic variable stress (CVS) protocol

Rats were randomly assigned to 'sham no CVS' (n=12), 'sham CVS' (n=12), 'lesion no CVS' (n = 12) and 'lesion CVS' (n = 12) groups, where 'CVS' rats were exposed to the CVS paradigm while 'no CVS' rats remained in their home cages as non-handled controls. The CVS paradigm consisted of twice daily exposure to alternating stressors along with occasional overnight stressors for 14 consecutive days (Days 1–14). Morning stressors were administered between 0930 hr and 1030 hr while afternoon stressors were conducted between 1430 hr and 1530 hr. Overnight stressors began immediately after cessation of afternoon stressors and ended with initiation of the next day's morning stressor. CVS stressors consisted of hypoxia (30 min in 8% oxygen), cold stress (1 h at 4°C, 2 rats/cage without bedding), rotation stress (1 h at 100 rpm on a platform orbital shaker), warm swim (20 min at 31–33°C); cold swim (10 min at 16–18°C), overnight social isolation (1 rat/cage) and overnight social crowding (6 rats/cage). Stressors were presented in a randomized order with each stressor (except the overnight stressors) represented an equal number of times.

Acute restraint stress protocol

All rats received restraint stress between 0830–1030 hr on Day 15, the day after the cessation of CVS exposure. Animals were placed in well-ventilated Plexiglas restraint tubes and a tail clip blood sample (250–300 µl) was immediately collected to determine basal AM plasma ACTH and corticosterone titers. All rats remained in the restrainers for 20 min at which point another blood sample was collected. Animals were then released into their home cages to recover. An additional blood sample was taken at 40 min from the onset of the restraint stress. At 60 min from the onset of restraint, the rats were immediately sacrificed by decapitation and trunk blood was collected. Brains were removed, flash frozen in isopentane on dry ice (−45°C), and stored at −80°C. Adrenal and thymus glands were also collected and weighed.

Radioimmunoassay (RIA)

Plasma corticosterone levels were measured by RIA using ^{125}I RIA kits from MP Biomedicals (Orangeburg, NY); plasma ACTH levels were measured by RIA using an antiserum donated by Dr. William Engeland (University of Minnesota) using ^{125}I -labeled ACTH (Amersham Biosciences, Piscataway, NJ) as tracer (Jasper and Engeland, 1991). For each hormone, all plasma samples were analyzed within the same RIA.

Lesion verification by Nissl stain and NeuN immunohistochemistry

Brains were serially sectioned at 14 μm using a Microm cryostat, mounted onto charged glass slides and stored at -20°C . Lesion sites were verified by both Nissl staining of cells and neuronal nuclei (NeuN) immunolabeling as a neuronal marker. Sections were fixed in 4% paraformaldehyde and Nissl stained with cresyl violet, dehydrated through an ascending ethanol series and xylene, and coverslipped using DPX mountant. For NeuN immunohistochemistry, circumferences of tissue sections were surrounded by a hydrophobic slide marker using Super HT PAP pen (Research Products International). The tissue sections were fixed in 4% paraformaldehyde and extensively washed with 50mM potassium phosphate buffered-saline (KPBS) between each step. Subsequently, sections were incubated in 1% H_2O_2 for 10 min and blocked in incubation solution (4% normal goat serum and 0.3% Triton X-100 in KPBS) for one hour. Sections were incubated overnight with a monoclonal antibody against NeuN (1:1000; Chemicon International, Temecula, CA) with Parafilm coverslips, followed by one hour incubation in biotinylated donkey anti-goat IgG (1:500; Vector Laboratories, Burlingame, CA), and one hour incubation in avidin-horseradish peroxidase complex (1:500; ABC Elite Kit, Vector Laboratories), where each step was in incubation solution. Finally, sections were incubated for 5 min in 0.02% diaminobenzidine (DAB, Sigma-Aldrich, St. Louis, MO) resulting in a brown reaction product. Slides were dehydrated through an ascending ethanol series and xylene, and coverslipped using DPX mountant.

Lesions were identified by the location of the needle track, loss of neurons, and gliosis. Lesions targeting the BSTpm were confirmed as 'hits' when the primary damage included the principal nucleus of the BST, with moderate damage extending into the adjacent interfascicular and transverse nuclei of the BST. BSTpm lesions that were centered outside of the primary target (principal nucleus) were considered missed lesions. Rats with bilateral damage were included in the study, while rats with only partial unilateral lesions and/or missed lesions with damage outside of the BSTpm were removed from the analysis. The final 'n' for experimental groups following lesion verification were: 'Sham No CVS' (n=12), 'Sham CVS' (n=12), 'Lesion No CVS' (n = 8) and 'Lesion CVS' (n = 9).

In situ hybridization

Antisense AVP, CRH, and c-*fos* riboprobes were generated from linearized clones by *in vitro* transcription using ^{35}S labeled UTP and the appropriate RNA polymerase as previously described in detail (Choi et al., 2007). Brain tissue slides were pretreated with 4% paraformaldehyde, acetylated, delipidized in chloroform, and dehydrated through a graded ethanol series. Each riboprobe was diluted (1.0×10^6 cpm / 50 μl buffer) in hybridization buffer (50% formamide, 1X Denhardt's Solution, 10% dextran sulfate, 200 $\mu\text{g/ml}$ fish sperm ssDNA, 100 $\mu\text{g/ml}$ yeast tRNA, and 20 mM DTT), applied to slides of brain sections, coverslipped, and placed in hybridization chambers over blotting paper soaked in 50% formamide and incubated overnight at 55°C . The next day coverslips were removed and slides washed in 2X standard saline citrate (SSC). Slides were subsequently incubated in 100 $\mu\text{g/ml}$ RNase A for 30 min at 37°C , washed numerous times in $0.2 \times \text{SSC}$, once in $0.2 \times \text{SSC}$ for 1 hr at 65°C , and finally dehydrated through a graded ethanol series.

Image Analysis

Hybridized slides were exposed on Kodak Biomax MR film (5 h for AVP, 7 days for CRH, and 14 days for *c-fos*). Film images of brain sections were captured by a digital video camera. Anatomical brain regions were identified using Swanson (Swanson, 1998), and Paxinos and Watson (Paxinos and Watson, 1998) rat brain atlases. Brain sections were matched for rostrocaudal level between rats for analyses. All brain regions and nuclei were clearly distinguishable by the specificity and intensity of AVP, CRH, and *c-fos* expression patterns. The parvocellular and magnocellular aspects of the PVN were identified using Nissl stained sections from a parallel series of tissue. Semi-quantitative analyses of autoradiograph images were performed using Scion Image (Scion, Frederick, MD) software and hybridization signal was expressed as gray level units. The gray level signal of a hybridized tissue region of interest was corrected by subtracting the gray level signal over a non-hybridized area of tissue (white matter, corpus callosum), and expressed as corrected gray level. ^{14}C standards were also measured using Scion Image, and transferred to Assay Zap (Biosoft and P.L. Taylor) to generate a standard curve to verify that all measured gray levels were on the linear range of the film.

Statistical analysis

Data are expressed as mean $\pm$ standard error of the mean (SEM). To take into account the differences in body weight, adjusted thymus and adrenal gland weights were calculated as organ weight (mg) per 100 g final body weight. AVP, CRH, and *c-fos* mRNA expression were expressed as corrected gray level. Integrated plasma ACTH and corticosterone were calculated as the total area under the curve (AUC) for the hormone time course of the restraint challenge. Organ weights, basal AM plasma hormones, integrated plasma hormones, and *in situ* hybridization data were analyzed by two-way factorial ANOVA (lesion, CVS) and significant main effects were further analyzed by Fisher's Least Significant Difference (LSD) *post hoc* test. Body weight and time courses of plasma ACTH and corticosterone responses to restraint were analyzed by three-way ANOVA (lesion, CVS, time) with repeated measures (time) and significant main effects were further analyzed by Fisher's Least Significant Difference (LSD) *post hoc* tests. Hormone and body weight data were analyzed using GBStat (Dynamic Microsystems Inc. Silver Spring, MD), while organ weight and mRNA data were analyzed using Statview (SAS Institute Inc., Cary, NC). Statistical significance was set at $p < 0.05$. Tests for homogeneity of variance were performed, and when necessary log transformations were used. Detection of outliers was performed using the Dixon-Massey method, and when necessary data were reanalyzed following outlier removal and/or transformations.

Results

Verification of lesions of the posterior medial BST

Bilateral ibotenate lesions targeted the principal nucleus of the BST. Lesion location and extent was verified by NeuN immunolabeling and Nissl stain (Figure 1). Confirmed BSTpm lesions were centered approximately 0.8 mm posterior to bregma according to the Paxinos and Watson atlas. The overall size of the lesions was relatively consistent but was not large enough to destroy the entire principal nucleus, usually sparing the ventral tip, while moderate damage was observed laterally in the interfascicular/transverse nuclei. All confirmed BSTpm lesions had minimal damage laterally into the striatum and spared any rostral damage into the anterior division of the BST. Our previous study (Choi et al., 2007) using similarly-placed lesions indicated extensive loss of GABAergic neurons in the region of the posterior medial BST, further corroborating the overall efficacy of this lesion approach.

Body and Organ Weights

Lesions of the BSTpm did not affect CVS-induced decreases in body weight gain. During the course of the study, analyses of body weight gain (Figure 2) indicate main effects of CVS ($F_{1,167} = 26.28$; $p < 0.05$) and day ($F_{3,167} = 314.61$; $p < 0.05$), and a CVS $\times$ day interaction ($F_{3,167} = 63.41$; $p < 0.05$). However, there was no effect of lesion on body weight gain and there were no other interactions. Post-hoc analyses revealed that all experimental groups gained body weight through the first day of CVS (Day 1) ($p < 0.05$). Upon exposure to chronic stress, 'CVS' groups gained less weight than their respective 'No CVS' groups on CVS Day 8 ($p < 0.05$) and CVS Day 15 ($p < 0.05$).

Similarly, BSTpm lesions did not affect stress induced adrenal hypertrophy or thymic atrophy. Whereas CVS caused increases in adrenal weights (Table 1) ($F_{1,38} = 17.60$; $p < 0.05$) and on adjusted adrenal weight/100 g body weight (Table 1) ($F_{1,38} = 59.64$; $p < 0.05$); there was no effect of lesion on adrenal weights and no lesion $\times$ CVS interaction. Chronic stress exposure also decreased thymus weight regardless of lesion (main effect of CVS on raw thymus weights (Table 1) ($F_{1,38} = 13.83$; $p < 0.05$), however, there was no effect on adjusted thymus weight/100 g body weight (Table 1)).

Plasma ACTH and corticosterone responses to novel restraint challenge

Chronic stress caused slight but significant increases in resting AM plasma ACTH levels (significant main effect of CVS ($F_{1,37} = 13.65$; $p < 0.05$)) that was independent of lesion (Figure 3A). Exposure to a 20-min restraint stress increased plasma ACTH in all experimental groups (Figure 3B) (significant main effects of time ($F_{3,167} = 95.96$; $p < 0.05$) and lesion ($F_{1,167} = 16.45$; $p < 0.05$) as well as a lesion $\times$ time interaction ($F_{3,167} = 16.51$; $p < 0.05$) and CVS $\times$ time interaction ($F_{3,167} = 3.92$; $p < 0.05$)). Post-hoc analyses indicated that the posterior BST lesions potentiated plasma ACTH levels compared to respective sham control levels at the 20-min time point regardless of CVS exposure ($p < 0.05$). Exposure to CVS elevated ACTH levels at the 20-min time point of 'CVS' shams compared to 'No CVS' shams ($p < 0.05$), consistent with HPA axis facilitation (Armario et al., 1985; Bhatnagar and Dallman, 1998; Bhatnagar and Vining, 2003; Dallman et al., 1992; Hauger et al., 1990; Kiss and Aguilera, 1993a; Ostrander et al., 2006). Lesions enhanced the integrated plasma ACTH response to restraint (Figure 3C) ($F_{1,33} = 13.55$; $p < 0.05$) regardless of CVS exposure ($p < 0.05$).

As was the case for ACTH, CVS resulted in elevated basal AM plasma corticosterone levels (Figure 4A) ($F_{1,37} = 26.02$; $p < 0.05$). Elevated basal plasma corticosterone was not affected by lesion. In contrast, plasma corticosterone responses to the 20-min novel restraint (Figure 4B) were affected by lesion ($F_{1,167} = 25.88$; $p < 0.05$) and CVS ($F_{1,167} = 13.66$; $p < 0.05$) and time ($F_{3,167} = 302.19$; $p < 0.05$), with a significant lesion $\times$ time interaction ($F_{3,167} = 9.74$; $p < 0.05$). Post-hoc analyses revealed that posterior BST lesions potentiated plasma corticosterone levels at the 20- and 40-min time points compared to respective sham control levels ($p < 0.05$). Chronic stress also caused elevations in plasma corticosterone at the 40 min time point of lesion rats compared to lesion 'No CVS' rats ($p < 0.05$), suggesting that CVS potentiated corticosterone secretion within the lesion group. For integrated plasma corticosterone levels (Figure 4C), there were significant main effects of lesion ($F_{1,37} = 33.91$; $p < 0.05$) and CVS ($F_{1,37} = 14.90$; $p < 0.05$), but there was no CVS $\times$ lesion interaction. Lesions elevated the integrated plasma corticosterone levels ($p < 0.05$), while CVS further increased integrated corticosterone levels within the lesion group ($p < 0.05$). Together with the time-course data, these data are consistent with an additive effect of lesions on chronic stress facilitation of corticosterone responses.

c-fos, CRH, and AVP mRNA expression in the PVN

Induction of *c-fos* mRNA was assessed as a measure of PVN neuronal activation by the novel restraint stress (Figure 5). Lesion significantly augmented *c-fos* mRNA induction in the PVN ($F_{1,38} = 5.98$; $p < 0.05$). However, there was no effect of CVS and no lesion $\times$ CVS interaction, indicating that *c-fos* mRNA induction was not sensitized by chronic stress in BST lesion rats. In fact, post-hoc analyses revealed that BST lesions potentiated PVN *c-fos* mRNA induction primarily in the absence of CVS exposure ($p < 0.05$).

In contrast, both lesion ($F_{1,38} = 7.76$; $p < 0.05$) and CVS ($F_{1,38} = 9.65$; $p < 0.05$) augmented CRH mRNA expression in the PVN (Figure 6). *Post-hoc* analyses revealed that CVS significantly elevated CRH mRNA expression in the PVN ($p < 0.05$). In addition, lesions of BSTpm caused a differential enhancement of PVN CRH mRNA expression in rats with prior chronic stress exposure ($p < 0.05$).

There were no main effects of lesion or CVS on AVP mRNA expression in the parvocellular PVN (Figure 7), and there was no lesion $\times$ CVS interaction. Similarly, there were no differences among experimental groups in AVP mRNA levels in the magnocellular PVN or in the supraoptic nucleus (data not shown).

Discussion

Consistent with our previous work, this study indicates that the BSTpm, which includes the principal nucleus of the BST, plays a major role in the inhibition of HPA axis reactivity to an acute stress challenge. The data replicate our previous findings that lesions of the posterior medial BST region enhance stress-induced PVN activation of *c-fos* mRNA and increase plasma ACTH and corticosterone responses to restraint stress (Choi et al., 2007). In addition, lesions of the posterior medial regions of the BST increase peak and integrated plasma corticosterone responses to novel restraint and enhance PVN CRH mRNA expression in chronically-stressed rats, lending further support to the conclusion that the BSTpm is necessary for normal inhibition of acute stress responses (Choi et al., 2007).

Exposure to CVS elevated peak and total plasma corticosterone response to novel restraint only in lesion rats. Whereas a trend was observed in the sham-lesion CVS group, post-hoc analysis did not reveal significant increases in corticosterone relative to respective non-CVS controls at any time point. Nonetheless, it should be noted that the results remain consistent with stress facilitation in this group, as previous studies note that maintained responsiveness reflects the ability to overcome the cumulative impact of chronic stress-induced increases in glucocorticoid exposure (Dallman et al., 1992). The magnitude of the facilitation effect in BSTpm lesion groups appears to be a summation of the effects of lesion and CVS, being additive rather than synergistic in nature. Thus, enhanced facilitation may be due to a lesion-induced increase in acute stress responding superimposed atop increases consequent to CVS, rather than being a separate product of chronic drive on central stress circuits.

Chronic stress exposure also elevated plasma ACTH levels in the sham rats, consistent with previous studies of chronic stress facilitation of responses to novel stressors (Dallman et al., 1992; Hauger et al., 1990). However, in contrast to the corticosterone data, CVS exposure did not further elevate the ACTH response in rats with lesions. The failure to observe facilitation of the ACTH response in lesion CVS rats is somewhat at odds with the corticosterone data. The disagreement between the plasma ACTH and corticosterone data raises the possibility that CVS is able to modulate pathways regulating the sensitivity of the adrenal to stress-induced pulses of ACTH. This possibility is supported by documented dissociations between the plasma ACTH and corticosterone responses in other chronic stress paradigms (Armario et al., 1985; Ostrander et al., 2006). There is evidence that the circadian

corticosterone peak is mediated by increased sympathetic drive at the level of the adrenal (Ulrich-Lai et al., 2006a). Thus, it is possible that interruption of a general stress inhibitory circuit may enhance adrenal responsiveness via descending neural pathways modulating adrenocortical activation (Ulrich-Lai et al., 2006a). Alternatively, since the kinetics of the ACTH response are much more rapid than that for corticosterone, it is possible that sampling time points for ACTH were not optimal for detection of response sensitization. Overall, these data suggest that CVS may differentially affect stress responses at the level of the anterior pituitary vs. adrenal cortex.

Central HPA axis responses to novel restraint were also assessed using *c-fos* mRNA expression as an indirect marker of neuronal activation in the PVN (Cullinan et al., 1995; Imaki et al., 1992). *c-fos* mRNA is robustly induced in the PVN in response to restraint, with peak induction occurring near 30–60 min following stress onset (Cullinan et al., 1995; Figueiredo et al., 2003; Imaki et al., 1992). Importantly, *c-fos* mRNA is absent in the PVN in unstressed animals (Cullinan et al., 1995), reflecting the linkage between *c-fos* gene induction and neural stimulation. Consistent with our previous data (Choi et al., 2007), acute stress-induced *c-fos* mRNA in the PVN was significantly increased by lesions of the BSTpm only in rats with no prior exposure to CVS. However, the lesion-induced increase in PVN activation was absent in the CVS-exposed rats, suggesting an attenuation of *c-fos* mRNA induction by CVS. Regardless, there was no significant main effect of CVS exposure on *c-fos* induction in the PVN, which is in contrast with previous findings where habituation of PVN *c-fos* activation to restraint is observed in models of repeated restraint stress (Melia et al., 1994; Stamp and Herbert, 1999; Umemoto et al., 1994), and on occasion following CVS (Ulrich-lai et al 2007). This discrepancy may be associated with variations in time point sampling of *c-fos* mRNA or Fos protein, and may be related to response duration rather than peak level of Fos induction.

Based on our previous work investigating the effects of posterior medial BST lesions on acute stress, we predicted that a loss of HPA axis inhibition would generate greater HPA activity following each stress exposure during the two weeks of CVS, thereby resulting in exacerbated pathologies of chronic stress. In this study CVS exposure did evoke reduced body weight gain, adrenal enlargement, and thymic involution, as previously observed with this paradigm (Herman et al., 1995; Prewitt and Herman, 1997; Ulrich-Lai et al., 2006b). In addition, CVS exposure enhanced resting AM ACTH and corticosterone levels. However, none of these indices of chronic stress were affected by lesions of the BSTpm, suggesting that lesion rats were able to successfully adapt to the chronic stress regimen. Thus, while the BSTpm may regulate the magnitude of HPA responses to an acute stress challenge, they do not appear to be responsible for long-term adaptations to chronic stress.

It remains to be determined whether adaptations to chronic stress are regulated by individual executive regions or by multiple inputs from many areas of the brain. Possible regulators of chronic stress adaptation include regions such as the locus coeruleus, which exhibits enhanced norepinephrine sympathetic capacity following chronic stress (Pardon et al., 2003; Ziegler et al., 1999), and the posterior paraventricular thalamus, which appears to be responsible for chronic stress habituation of the HPA axis (Bhatnagar et al., 2000). In addition, there are data to support the existence of a general, CRH-driven network that can modulate responsiveness to repeated stimuli (Dallman et al., 2003).

Previous studies in our laboratory have also demonstrated that large lesions targeting the posterior region of the BST increase CRH mRNA expression in the PVN (Choi et al., 2007; Herman et al., 1994). The present study replicated the observed increases in PVN CRH mRNA expression following BSTpm lesion. In addition, CVS exposure augmented CRH mRNA only in lesion rats. These results are consistent with an additive effect of lesion and

stress on production of CRH which may be relevant to the observed enhancement of corticosterone responses to chronic stress. The overall increase in expression of CRH mRNA is consistent with the enhanced response capacity seen in the posterior medial BST lesion group, and suggests that this enhancement in CRH expression is related to dynamic (i.e., stress-related HPA activation) rather than static (i.e., increases in steady-state corticosterone) HPA axis activity.

Previous work from our group demonstrated that BSTpm lesions also enhance AVP mRNA in the parvocellular PVN (Choi et al., 2007). However, changes in parvocellular AVP mRNA did not reach statistical significance in the current study. This discrepancy may be related to the difference in post-lesion survival time in the two studies (due to the additional 2 week stress period), or possibly to the increased variance associated with the inclusion of additional experimental groups in the design. In combination, the data suggest that changes in parvocellular AVP mRNA may not be a robust or enduring effect of BSTpm ablation.

Determinations of CRH and AVP mRNA were made in animals 60 min after initiation of restraint. Previous work from our group and others suggests that stress-induced elevations in CRH and AVP mRNA occur at relatively long post-stimulus latencies (> 60 minutes) following onset of restraint, for AVP, 90 min (Herman, 1995), and for CRH, 4 hours (Ma et al., 1997). Thus, our measures are highly likely to reflect baseline CRH and AVP mRNA expression.

The posterior medial BST receives input from numerous HPA axis-regulating limbic structures including the ventral subiculum, medial prefrontal cortex, and medial nucleus of the amygdala (Cullinan et al., 1993; Dong et al., 2001a; Dong and Swanson, 2004; Gu et al., 2003). The BSTpm is positioned to integrate emotional information from the basolateral amygdala and/or central nucleus of the amygdala into the stress response via the anterior lateral BST nuclei, which is known to regulate fear and anxiety-like behavior (see (Walker et al., 2003)). However it is unclear whether the posterior medial BST specifically regulates fear and anxiety responses. The excitatory effects of posterior medial BST lesions on HPA axis responses to acute stress are similar to those seen following lesions of the ventral subiculum (Herman et al., 1998) and medial prefrontal cortex (Figueiredo et al., 2003), raising the possibility that the BST may relay information from these structures to the PVN. In the case of the ventral subiculum, anatomical interconnections with the PVN via this portion of the BST have been previously established (Cullinan et al., 1993). However, it is important to note that the role of cortical and hippocampal regions in chronic stress regulation have yet to be established, thus it is unclear whether a partial role of the BST on HPA axis facilitation is mediated by these structures.

In conclusion, the BSTpm is an important inhibitor of stress responsivity to novel stimuli. However, the BSTpm does not appear to be responsible for changes in steady-state HPA activity or HPA axis facilitation that occur following chronic stress. The data suggest that acute vs. chronic response pathways are likely subserved by distinct brain circuits. The position of the BST at the interface of behavioral and neuroendocrine integration, suggests that this region may be critical for interpretation of stimuli relevant to stress-related disease states such as depression and PTSD. Therefore, the posterior medial BST is likely to be a major component of the brain circuitry involved in both normal and pathological stress adaptation.

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Figure 1.

Brain tissue sections were immunolabeled for NeuN for analysis of the extent of lesions. Sham injection sites (A) targeting the posterior medial bed nucleus of the stria terminalis (BSTpm) did not cause significant neuronal cell loss, as noted by abundant NeuN staining in the region. In contrast, BSTpm lesions (B) typically damaged the vast majority of the BSTpm. Note that some staining was preserved in the ventral region of the BSTpm. Abbreviations: sm, stria medularis; V3, third ventricle. Scale bars = 300 μ m. Schematic diagram (C) illustrates the extent of the spread of damage of the smallest (black shaded area) to the largest lesions (cross-hatched shaded area) confirmed as hits with primary damage of the BSTpm. Image adapted from Swanson's Brain Maps: Structure of the Rat Brain, 2nd Edition.

Figure 2.

Exposure to chronic variable stress (CVS) started on Day 1, and significantly decreased percent body weight gain on Days 8 and 15 regardless of lesions of the BSTpm. Data are presented as mean $\pm$ SEM, $n=8-12$ per group. * = $p < 0.05$ versus respective 'No CVS'.

Figure 3.

Exposure to CVS elevated basal AM plasma ACTH levels (A). Exposure to CVS also elevated the plasma ACTH response to acute restraint stress at 20 min following onset of restraint only in sham rats (B). Lesions of the BSTpm potentiated the plasma ACTH response to acute restraint stress at 20 min following onset of restraint regardless of CVS exposure, and at 40 min following onset of restraint only in rats with no exposure to CVS (B). Lesions of the BSTpm elevated the integrated plasma ACTH response regardless of CVS exposure (C). Data are presented as mean $\pm$ SEM, $n = 8-12$. * $p < 0.05$ versus respective 'No CVS', # $p < 0.05$ versus respective 'Sham'. Abbreviations: AUC, area under the curve.

Figure 4.

Exposure to CVS elevated basal AM plasma corticosterone levels (A). Lesions of the BSTpm potentiated plasma corticosterone response to restraint at 20 min and 40 min following onset of stress, regardless of CVS exposure (B). Exposure to CVS elevated the plasma corticosterone response at 40 min following onset of restraint only in rats with lesions. Lesions of the BSTpm enhanced the integrated plasma corticosterone response regardless of CVS exposure (C). Additionally, exposure to CVS elevated the integrated plasma corticosterone response only in rats with lesions (C). Data are presented as mean $\pm$ SEM, $n = 8-12$. * $p < 0.05$ versus respective 'No CVS', # $p < 0.05$ versus respective 'Sham'. Abbreviations: AUC, area under the curve.

Figure 5.

Semi-quantitative analysis revealed that BSTpm lesions elevated *c-fos* mRNA expression in the paraventricular nucleus of the hypothalamus (PVN) only in rats with no exposure to CVS. Data are shown as mean $\pm$ SEM, $n = 8-12$. # $p < 0.05$ versus respective 'Sham'.

Figure 6.

Semi-quantitative analysis revealed that exposure to CVS elevated corticotropin releasing hormone (CRH) mRNA expression in the PVN regardless of lesions of the BSTpm. Lesions of the BSTpm elevated CRH mRNA expression in the PVN only in rats exposed to CVS. Data are shown as mean $\pm$ SEM, $n = 8-12$. * $p < 0.05$ versus respective 'No CVS', # $p < 0.05$ versus respective 'Sham'. Abbreviations: PVNmp, medial parvocellular PVN.

Figure 7.

Semi-quantitative analyses revealed that neither exposure to CVS or lesions affected arginine vasopressin (AVP) mRNA expression in the PVN. Data are shown as mean $\pm$ SEM, $n = 8-12$. Abbreviations: PVNmp, medial parvocellular PVN.

Table 1

Adrenal and Thymus Weights Following BSTpm Lesions and Chronic Variable Stress

	Sham No CVS	Lesion No CVS	Sham CVS	Lesion CVS
Raw Adrenal (mg)	44.4 ± 1.4	45.2 ± 1.2	51.3 ± 1.9 *	51.9 ± 1.9 *
Adjusted Adrenal (mg / 100g BW)	12.5 ± 0.3	12.7 ± 0.4	15.7 ± 0.5 *	16.6 ± 0.6 *
Raw Thymus (mg)	371 ± 20	397 ± 25	317 ± 11 *	312 ± 14 *
Adjusted Thymus (mg / 100g BW)	104 ± 5.7	112 ± 6.7	97 ± 2.9	100 ± 4.8

Data are presented as mean ± SEM, n= 8–12 per group.

*
p < 0.05 versus respective No CVS.