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Evidence for Involvement of a Limbic-Paraventricular Hypothalamic Inhibitory Network in HPA Axis Adaptations to Repeated Stress

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Abstract

Emotional stressors activate a stereotyped set of limbic forebrain cell groups implicated in constraining stress-induced hypothalamic-pituitary-adrenal (HPA) axis activation by inhibiting hypophysiotropic neurons in the paraventricular hypothalamic nucleus (PVH). We previously identified a circumscribed, anterior, part of the bed nuclei of the stria terminalis (aBST) that houses stress-sensitive, PVH-projecting, GABAergic neurons, as representing a site of convergence of stress-inhibitory influences originating from medial prefrontal and hippocampal cortices. Here we investigated whether exaggerated HPA axis responses associated with chronic variable stress (CVS; daily exposure to different stressors at unpredictable times over 14 d, followed by restraint stress on d 15), and diminished HPA output seen following repeated (14 d) restraint stress exposure, may be associated with differential engagement of the limbic modulatory network. Relative to acutely restrained rats, animals submitted to CVS showed the expected increase (sensitization) in HPA responses, and diminished levels of activation (Fos) of GABAergic neurons and glutamic acid decarboxylase (GAD) mRNA expression in aBST. By contrast, repeated restraint stress produced habituation in HPA responses, and maintained levels of activation of GABAergic neurons and increased GAD expression in aBST. aBST-projecting neurons in limbic sites implicated in HPA axis inhibition tended to show diminished activational responses in both repeated stress paradigms, with the exception of paraventricular thalamic nucleus, whose responsiveness was maintained in repeatedly restrained animals. The results are consistent with the view that differential engagement of HPA-inhibitory mechanisms in aBST may contribute to alterations in HPA axis responses to emotional stress in sensitization and habituation paradigms.

Keywords

chronic stress; bed nuclei of the stria terminalis; GABAergic neurons; HPA axis; medial prefrontal cortex; hippocampus; ventral subiculum; paraventricular nucleus; hypothalamus; AB_90738

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INTRODUCTION

“Emotional” stress is a designation attached to one major subset of animal challenge paradigms that model fear, anxiety and social stress, such as are encountered in everyday life. Stressors of this type engage a seemingly stereotyped set of interconnected cell groups in the limbic forebrain, including aspects of the septum, amygdala, hippocampus and prefrontal cortex (Diorio et al., 1993; Cullinan et al., 1995; Cullinan et al., 1996; Li and Sawchenko, 1998; Dayas et al., 2001), each of which is implicated in modulating hormonal (hypothalamo-pituitary-adrenal, or HPA) responses to stress (Sapolsky et al., 1984; Kovacs and Makara, 1988; Diorio et al., 1993; Weinberg et al., 2010). None of these regions provides an appreciable innervation of neurosecretory neurons in the paraventricular hypothalamic nucleus (PVH) that comprise the final common path for stress-induced HPA axis activation. Instead, these influences appear to be mediated indirectly. We have recently identified a circumscribed, anterior, part of the bed nuclei of the stria terminalis (aBST) that houses stress-sensitive, PVH-projecting, inhibitory (GABAergic) neurons, and serves as a site of convergence of acute (restraint) stress-inhibitory influences imparted by prefrontal (i.e., prelimbic or PL) and hippocampal (ventral subiculum or vSUB) components of the limbic network, at least (Radley et al., 2009; Radley and Sawchenko, 2011).

This aBST region is thus positioned to serve as a clearinghouse that receives, integrates and distributes information for limbic regulation of the stress axis. We have now completed a study that probes the potential involvement of the aBST in adaptations to chronic stress. Secretory responses of the HPA axis tend to decline upon repeated exposure to the same (homotypic) stressor, but over-respond to a novel (heterotypic) challenge (Dallman et al., 1992; Dallman, 1993a; Bhatnagar and Dallman, 1998). These phenomena of habituation and facilitation, respectively, represent adaptations to chronic stress that are highly relevant to understanding and managing the negative health consequences of longer term stress exposure.

Chronic variable stress (CVS; a.k.a., chronic unpredictable stress, chronic mild stress, chronic intermittent stress) is a widely used facilitation paradigm that involves daily exposure of rodents to different stressors at unpredictable times daily typically over a 2-3 week period (Otteweller et al., 1989; Willner, 1997; Grippo et al., 2003). The behavioral, physiological, and endocrine alterations that ensue following CVS exposure are similar to a number of the clinical features in stress-related psychiatric disorders (Willner, 1997). Here we address the question of whether limbic system modulation of PVH output via the aBST may be in position to participate in adaptations of HPA axis responses to repeated stress. We find that the dampening and enhancement of axis responses associated with habituation (repeated restraint) and facilitation (CVS) paradigms, respectively, have correlates in cellular activation/gene expression profiles in the aBST, as well as in a differential propensity to habituate among stress-sensitive cell groups in the limbic forebrain that project to aBST. The aBST is thus positioned to play a pivotal role in adaptations to repeated stress.

MATERIALS AND METHODS

Animals and treatments

Adult male Sprague-Dawley albino rats (275 - 325 g), maintained under standard laboratory conditions, were used in all experiments. All experimental protocols were approved by the Institutional Animal Care and Use Committees of the Salk Institute for Biological Studies and the University of Iowa. CVS involved daily exposure to either two brief or one sustained stressor over 14 d, in semi-randomized order, at unpredictable times of day. Brief stressors included elevated plus maze (5 min), shaker stress (30 min on an orbital shaker at 100 rpm), hypertonic saline injection (i.p.; 1 ml of 1.0 M saline), tail suspension (10 min), forced swim (10 min in RT water) and cold exposure (1 h at 5-7 °C). Sustained stressors included overnight (i.e., 18-24 h) exposure of rats to wet bedding, cage crowding (4 animals/cage), or isolation. Repeated restraint stress was performed in the morning (9:00) in plastic restrainers (Bantree Scientific, Bantree, MA) for 30 min each day over a 14-day period. Controls were handled comparably, but were not restrained. On day 15, animals were subjected to 30 min of restraint (acute restraint, CVS, repeated restraint), and remained in their home cages during restraint and until the prescribed time of perfusion for histology, 2 h after the termination of restraint. A separate group of handled, unstressed controls was included in these experiments to assess the effects of stress paradigms relative to baseline indices.

Retrograde labeling experiments

For retrograde labeling of afferent neurons in the aBST → PVH circuit, unilateral pressure injections of 2% Fluoro-Gold (FG; Fluorochrome LLC; (Schmued and Fallon, 1986) were made into either aBST or PVH in discrete volumes (30-60 nl for PVH: anteroposterior, -0.13 mm; mediolateral, +0.35-0.40 mm; dorsoventral, -7.20 mm from dura; 60-90 nl for aBST: anteroposterior, -0.10 mm; mediolateral, +1.20 mm; dorsoventral, -7.20 mm from dura). The quality of retrograde-labeling following deposits into aBST were verified by comparison with previous experiments employing iontophoretic injections into the same region (Radley et al., 2009). Upon analysis of tracer injection sites in separate series sections prepared for either immunoperoxidase or epifluorescence detection of FG, the most accurate localization of tracer deposits and size was achieved in fluorescence material. Thereafter, tracer placement and size were reconstructed under epifluorescence in separate series of sections (see below). For each regions in which retrograde labeling was analyzed in the present study, the number of retrogradely labeled neurons did not differ significantly as a function of treatment (stress) status (data not shown).

Histology and tissue processing

Rats were anesthetized with chloral hydrate (350 mg/kg, ip) and perfused via the ascending aorta with 100 ml 0.9% saline followed by 900 ml of ice-cold 4% paraformaldehyde in 0.1 M borate buffer, pH 9.5, at a flow rate of 55 ml/min. The brains were removed, postfixed for 3 h, and cryoprotected in 20% sucrose in 0.1 M phosphate buffer overnight at 4°C. Five one-in-five series of 30 µm-thick frozen coronal sections through the entire brain were cut and collected in cryoprotectant solution and stored at -20°C until processing.

Hybridization histochemistry

Techniques for probe synthesis, hybridization, and autoradiographic localization of mRNA signal were adapted from (Simmons et al., 1989). *In situ* hybridization was performed using ³⁵S-labeled sense (control) and antisense cRNA probes labeled to similar specific activities encoding corticotropin-releasing factor (CRF) mRNA (1.2 kb; Dr. K. Mayo, Northwestern University), and the 67 kDa isoform of glutamic acid decarboxylase, (GAD67, Dr. A. Tobin, University of California, Los Angeles)(Erlander et al., 1991). Sections were mounted onto poly-L-lysine-coated slides and dried under vacuum overnight. They were postfixed with 10% paraformaldehyde for 30 min at room temperature, digested with 10 µg/ml proteinase K for 15 minutes at 37°C, and acetylated for 10 min. Probes were labeled to specific activities of 1–3 10⁹ dpm/µg and applied to the slides at concentrations of ~10⁷ cpm/ml, overnight at 56°C in a solution containing 50% formamide, 0.3 M NaCl, 10 mM Tris, 1 mM EDTA, 0.05% tRNA, 10 mM dithiothreitol, 1× Denhardt's solution, and 10% dextran sulfate, after which they were treated with 20 µg/ml of ribonuclease A for 30 minutes at 37°C and washed in 15 mM NaCl/1.5 mM sodium citrate with 50% formamide at 70°C. Slides were then dehydrated and exposed to X-ray films (Kodak Biomax MR, Eastman Kodak, Rochester, NY) for 18 hours. They were coated with Kodak NTB-2 liquid emulsion and exposed at 4°C for 10-14 days, as determined by the strength of signal on film. Slides were developed with Kodak D-19 and fixed with Kodak rapid fixer, and lightly counterstained with thionin.

Hormone assays

Separate groups of animals subjected to the stress regimens described above were implanted with indwelling jugular catheters 2 days prior to the first (i.e., acute restraint) or final stress episode (i.e., CVS/ repeated restraint) using previously described procedures (Ericsson et al., 1994). On the morning of day 15, blood samples (250 µl) were taken prior to restraint stress to estimate basal ACTH and corticosterone levels. Additional samples were collected at 0, 30, 60, 90, and 120 min after the termination of restraint. ACTH was measured using a two-site immunoradiometric assay obtained in kit form (DiaSorin, Stillwater, MN), with intra- and interassay coefficients of variation of 3 and 9%, respectively, and a sensitivity of 1.5 pg/ml. Plasma corticosterone was measured without extraction, using an antiserum raised in rabbits against a corticosterone-BSA conjugate, and ¹²⁵I-corticosterone-BSA as tracer (MP Biomedicals, Solon, OH). The sensitivity of the assay was 0.8 µg/dl; intra- and interassay coefficients of variation were 5 and 10%, respectively.

Immunohistochemistry

Localization of Fos protein and other antigens was carried out on free-floating sections by using an avidin-biotin immunoperoxidase staining protocol (Sawchenko et al., 1990). Fos immunolocalization was performed using a primary antiserum raised against a fragment of rat Fos protein (residues 4-17, Radley et al., 2008). Endogenous peroxidase was neutralized by treating tissue for 10 minutes with 0.3% hydrogen peroxide, and sections were incubated with primary antiserum at 4°C for 48 hours in phosphate-buffered saline (PBS) containing 0.3% Triton X-100 and 3% blocking serum. The primary antiserum was localized using

Vectastain Elite (Vector Laboratories, Burlingame, CA) reagents, and the reaction product was developed using a nickel-enhanced glucose oxidase method (Shu et al., 1988).

Characterization of neurons in aBST on the basis of projections to PVH, the GABAergic phenotype and stress-sensitivity involved concurrent labeling for (1) Fos- and FG-immunoreactivity, and (2) Fos protein and GAD 67 mRNA in separate series of sections. This approach was necessitated by our inability to efficiently localize all three markers in a single series (see results section, below). Dual immunolocalization of Fos and FG was carried out by sequentially localizing the antiserum against Fos using a nickel-enhanced diaminobenzidine method (black nuclear reaction product), as above, and then a FG antiserum (Chang et al., 1990), without nickel enhancement (brown cytoplasmic product). A combined immunoperoxidase and isotopic hybridization histochemical localization of Fos immunoreactivity and GAD67 mRNA, respectively, was carried out using a modification of protocols detailed previously (Chan et al., 1993). For these experiments, immunostaining was carried out first, and the two constituent methods were modified to allow efficient dual localization.

Antiserum characterization

The primary antibodies used in this study are listed in Table 1. Fos antiserum was raised in rabbits against an N-terminal synthetic fragment of rat Fos protein (residues 4-17; Radley et al., 2008). Western blotting of extracts from brains of stressed and unmanipulated rats displayed 52 kDa bands. Specificity was further evaluated by direct colabeling for *c-fos* mRNA over a range of challenge conditions, and specific staining in experimental and control tissue was abolished by preadsorbing the antiserum overnight at 4°C with 50 µM of the synthetic peptide immunogen.

Fluorogold antiserum was raised in rabbit against a fluorogold-BSA conjugate (Chang et al., 1990)(AB153; Millipore, Bedford, MA; RRID: AB_90738). Specificity was verified by detection of native fluorescence or immunoperoxidase reaction product only in the brains of animals that received intracerebral injections of the fluorochrome.

Data analysis

Stereological methods were used to quantify the number of Fos-immunoreactive neurons. These analyses were performed using a computer-assisted morphometry system consisting of a photomicroscope equipped with an XYZ computer-controlled motorized stage, MicroFire camera (Optronics, Goleta, CA), Gateway microcomputer, and StereoInvestigator morphometry and stereology software (MBF Biosciences, Williston, VT). For each analysis, boundaries defining the regions of interest were drawn at 25X using an adjacent series of Nissl-stained sections. In regions identified as aBST-projecting (i.e., retrogradely labeled following FG injections in aBST), labeled cells were used as a guide to further aid the delineation of anatomical boundaries. Analyses of Fos immunoreactive cells were carried out on every fifth section, avoiding cells in the outermost plane of focus. Counts were then multiplied by five to estimate the total number of labeled neurons in the defined region of interest. Volume estimates from cross-sectional area measurements were obtained using the Cavalieri method to probe for possible treatment effects on PVH volume, but no reliable

effects were observed. Fos- and GAD-labeled neurons were counted manually in every fifth section. In these analyses, the section thickness was too small relative to the average diameter of labeled neurons in to permit stereologic approaches, and estimates of cell numbers were obtained using the Abercrombie correction (Abercrombie, 1949).

Semiquantitative densitometric analysis of relative levels of CRF and GAD67 mRNA was performed on emulsion-coated slides using ImageJ software. The optical densities of hybridization signals were determined under darkfield illumination at 10X magnification. The hypophysiotropic region of the PVH (i.e., dorsal medial parvicellular subdivision) was defined from Nissl staining pattern (Swanson and Kuypers, 1980) and aligned with corresponding darkfield images of hybridized sections by redirected sampling. aBST was defined from Nissl material using the parcellation scheme of Dong and colleagues (2001). Optical density readings, corrected for background, were taken from sections at regular, 150 μ m, intervals and average values were determined through the extent of cell groups for each animal. Images from CRF and GAD mRNA densitometry were collected using a QImaging EXi Blue camera. Images collected from each analysis were exported first to Adobe PhotoShop (v. 8) for adjustments to optimize/balance contrast and brightness, and then to Canvas (v. 10) for assembly and labeling.

Statistics

Group data from the immunoperoxidase and hybridization histochemical experiments (N = 3-5 per group) were compared using a one-way analysis of variance for treatment status (control, acute restraint, CVS, repeated restraint) followed by *post hoc* pairwise comparisons using Tukey's HSD. Group data from the hormone assays (N = 6-8 per group) were compared using a mixed design analysis of variance with one within- (time) and one between- (stress status) group variable, followed by individual pairwise comparisons as above. Data are expressed as the mean \pm SEM.

RESULTS

HPA axis responses to differing repeated stress regimens

Previous studies have shown that 14 days' exposure to CVS produces a robust enhancement of HPA axis responses to a novel challenge, relative to otherwise naïve controls subjected to the same insult (Ottenweller et al., 1989; Herman et al., 1995). As we have previously addressed the neural substrates for limbic forebrain modulation of HPA axis responses to acute restraint, the use of restraint as the novel challenge (on day 15) in a CVS paradigm allows for direct comparison with the acute stress situation. An additional group of animals subjected to 14 consecutive daily restraint sessions provided a contrast with the effects observed under CVS, as repeated exposure to the same (homotypic) challenge has been widely documented to produce response decrements (i.e., habituation) in HPA axis activation.

We initially surveyed two independent central indices of HPA axis activation at 2 hours after the termination of 30 min restraint in CVS, repeatedly restrained, and control (i.e., acute restraint and unstressed) groups (Fig. 1). This time point is one at which Fos protein

levels are maximally elevated, and is sufficient for observing stress effects on CRF mRNA expression in PVH (Viau and Sawchenko, 2002). Main effects of stress were found on both endpoints (Fos: $F_{3,15} = 10.7$, $p < 0.01$; CRF mRNA: $F_{3,15} = 18.4$; $p < 0.01$). Each group exposed to restraint (acute, repeated, CVS) showed increases in the number of Fos-ir cells in the PVH as compared to the unstressed group ($p < 0.01$ for each; Fig. 1A-D, I). Whereas CVS produced increments in the number of Fos-activated neurons in PVH comparable to those of acutely restrained animals, repeatedly restrained animals' response were blunted (by 67%; $p < 0.05$) as compared with the acute restraint group. CRF mRNA expression in PVH was reliably increased in the acute and CVS groups following 30 min restraint (by 28% and 52%, respectively), relative to unstressed controls ($p < 0.05$ for each; Fig. 1E-I). CVS-induced increments in relative levels of CRF message were significantly greater than those seen the acutely or repeatedly restrained animals (by 20% and 21%, respectively; $p < 0.05$); by contrast, the repeated restraint group failed to exhibit significant changes in CRF mRNA levels, as compared with unstressed controls ($p = 0.5$).

We next compared HPA secretory responses to restraint as a function of prior stress experience (acute, repeated, CVS; Fig. 2). For these experiments, indwelling jugular catheters were implanted on the afternoon of day 13 of CVS/ repeated restraint, after completion of stressor exposure for that day. Mixed design ANOVA of ACTH data (Fig. 2A), with time of blood sampling tested as a within-subjects factor, demonstrated main effects of treatment group ($F_{2,19} = 5.2$; $p < 0.05$), time of blood sampling ($F_{5,19} = 22.8$; $p < 0.01$), and a significant interaction between these variables ($F_{10,19} = 5.6$; $p < 0.01$). Both CVS-treated and acutely-stressed animals displayed significant increases in peak values of plasma ACTH relative to each group's pre-stress levels (0 min vs. 30 min; $p < 0.05$ for each); CVS-treated animals also showed a more prolonged stress-induced rise in ACTH, which remained significantly elevated above baseline through the 60 min time point ($p = 0.05$). By contrast, repeatedly restrained animals failed to show any significant increase over basal (pre-stress) values of ACTH at any of the time points examined. Analysis of between-group differences at individual time points showed a significantly greater increase in peak plasma ACTH levels in CVS-treated animals as compared with both the acute and repeated restraint groups (by 3.8- and 2.6-fold, respectively; $p = 0.05$ for each).

A similar pattern of results was seen in analysis of plasma corticosterone levels (Fig. 2B). Evident here were main effects of treatment group ($F_{2,19} = 7.4$; $p < 0.01$), time of blood sampling ($F_{5,19} = 53.7$; $p < 0.01$), and a significant interaction term ($F_{10,19} = 3.6$; $p < 0.01$). Within-group measures revealed reliable increases in plasma corticosterone immediately after the termination of restraint, and 30 min later, in all three groups (0 min vs. 30 min and 0 vs. 60 min, respectively; $p < 0.05$ for each); in only the CVS group were corticosterone response seen to be elevated beyond this, through the 90 min time point ($p < 0.05$). Although between-group comparisons failed to show a significant difference between peak levels of corticosterone in the CVS and acute restraint groups ($p = 0.1$), the response of the CVS animals was again more long-lasting, being significantly elevated through 90 min, vs. 60 for the others. Corticosterone titers of repeatedly restrained rats did not differ from those of acutely stressed animals at any time point examined (all $p > 0.1$). Collectively, these data are in line with previous reports that CVS and repeated restraint respectively result in

sensitization and habituation of responses to restraint at each level of the HPA axis (Dallman, 1993a; Choi et al., 2008).

Differential engagement of aBST following CVS vs repeated restraint

We have identified PVH-projecting, GABAergic neurons in aBST as providing a gateway for limbic forebrain influences to be exerted on HPA axis responses to acute restraint stress (Radley et al., 2009; Radley and Sawchenko, 2011). We now broach the possibility that such modulatory capabilities of aBST may also be involved in differential adaptations of axis responses to repeated stress, as are seen in CVS and/or repeated restraint paradigms. Retrograde tracer injections (FG) were placed in PVH, and neurons labeled in aBST were assayed for alterations in responsiveness (Fos induction) at 2 hr after a 30 min restraint exposure on the day following regimens of 14 d of repeated restraint, 14 d of CVS, or no prior stress exposure. The original intent was to couple tracing and Fos assays with markers of the GABAergic phenotype, but decrements in the sensitivity of one or more of the constituent methodologies in the triply labeled preparations made quantitative analyses unfeasible, so we were left to examine markers of interest singly or in pairs.

Tracer injections were aimed at the medial parvicellular part of the PVH (Fig. 3; 4A). Only animals bearing appropriately centered deposits, with lesser involvement of potentially confounding cell groups that adjoin the PVH, were included in the analysis. Whereas retrograde tracer placements were not devoid of any spread into aBST-projecting cell groups residing in close proximity to PVH (i.e., anterior hypothalamic nucleus or nucleus reuniens of the thalamus), no differences were noted in the distribution or density of retrograde labeling in aBST resulting from injections that may have differentially involved either of these two potential sources of contamination. In this material, retrograde labeling in the aBST was focused in the fusiform, dorsomedial, and subcommissural subnuclei defined by Dong and colleagues (2001)(Fig. 4B), in line with our prior characterization of GABAergic cell groups that issue projections to the hypophysiotropic zone of the PVH proper (Radley et al., 2009). Rats in each stress group displayed a robust Fos induction in aBST, including within the portion of neurons labeled concurrently for tracer, relative unstressed controls, which did not show constitutive Fos expression in this region ($F_{3,11} = 8.0$; $p < 0.05$; Fig. 4C-F). Fos induction in aBST of both repeatedly stressed groups tended to be reduced both globally and in identified PVH-projecting neurons, relative to acutely restrained controls, but the only statistically reliable effect was seen in retrogradely labeled neurons of the CVS group, which were reduced by 77% ($p < 0.05$).

A compatible set of results was obtained in analyzing material in which immunolocalization of Fos protein was combined with hybridization histochemical detection of mRNA encoding for the 67 kDa isoform of glutamic acid decarboxylase (GAD67; marker for GABAergic neurons) in aBST as a function of stress experience (Fig. 5). Animals in each of the stress groups displayed robust Fos induction in GAD67-expressing neurons as compared with unstressed animals ($F_{3,12} = 8.2$; $p < 0.01$). Of the two repeatedly stressed groups, however, only animals that were exposed to CVS showed a significant decrement (by 45%) in this measure relative to the acute restraint group ($p < 0.05$).

In the preceding experiment, apparent differences were noted in the strength of GAD67 mRNA signal as a function of stress status. This was confirmed in a subsequent densitometric comparison of relative levels of this transcript across treatment conditions ($F_{3,12} = 11.3$; $p < 0.01$; Fig. 6). Compared to the acute restraint group, in which GAD67 mRNA measures were reliably elevated over those of unstressed controls, repeatedly restrained values were further enhanced, and CVS measures significantly reduced ($p < 0.05$ for each), the latter to the levels of basal controls. Together, these findings indicate that the aBST responds differently to an emotional stressor (restraint) in animals with differing repeated stress histories. In CVS-exposed animals, the aBST under-responds to a novel restraint stimulus, while rats with repeated restraint experience exhibit aBST reactivity at least on par with that seen in naïve rats.

Repeated stress effects on aBST afferents from limbic forebrain

A network of interconnected cell groups in the limbic forebrain has been previously implicated in modulating HPA axis responses to acute emotional stressors (for reviews, see Van de Kar and Blair, 1999; Ulrich-Lai and Herman, 2009). An important feature of this modulation is that none of these regions have been shown to provide any appreciable innervation of PVH, suggesting a more complex, or indirect interaction with the stress axis. Our previous work shows that aBST GABAergic neurons form a disynaptic circuit interceding for stress-inhibitory influences of PL and vSUB on HPA activation (Radley et al., 2009; Radley and Sawchenko, 2011; Radley, 2012). These data raise the possibility that aBST may serve as a neural hub imparting inhibitory influences from other portions of the limbic forebrain (i.e., septum, PVT, amygdala), and importantly, that altered activation in these upstream cell groups may contribute to altered aBST GABAergic influences on PVH and corresponding HPA axis adaptations to repeated restraint and/or CVS. This was interrogated by placing discrete retrograde tracer (FG) injections in aBST, and examining for alterations in Fos induction in identified aBST-projecting neurons in cell groups implicated in HPA modulation, as a function of stress condition. In Figure 7, reconstructions of tracer deposits demonstrate areas of overlap common to all FG injections, and the maximal extent of diffusion of tracer deposits for each animal. Areas of overlap common to all placements judged to be appropriate for inclusion in the analysis encompassed aspects of the dorsomedial and fusiform subnuclei of BST just ventral to the anterior commissure. The maximal extent of diffusion involved portions of the parastrial nucleus (medially), the subcommissural and magnocellular nuclei (caudally) and/or the anterior commissure (dorsally) of the BST (see Dong et al., 2001, for pacellation/terminology). Although spread of the tracer to involve aspects of the anterior commissure represented a potential source of spurious retrograde labeling, we failed to detect such on either side of the brain in olfactory structures (e.g., olfactory bulb and tubercle, anterior olfactory nucleus) whose axons cross the midline via this fiber tract. This is consistent with the weight of evidence that under minimally invasive delivery conditions (pressure, iontophoresis), FG is at least relatively resistant to uptake and transport by axons-of-passage (Schmued and Fallon, 1986; Cullinan et al., 1993; Radley and Sawchenko, 2011).

Tracer injections yielded moderate to dense retrograde labeling in cell groups implicated in the inhibitory control of the stress axis, including the lateral septum, PL, vSUB and the

paraventricular nucleus of the thalamus (PVT). In unstressed control animals, these regions were conspicuously lacking in Fos-immunoreactivity, with the exception of PVT, which is known to display low-level constitutive Fos expression (Herdegen et al., 1995). Significant differences in activational responses to 30 min restraint as a function of stress experience were noted overall (Table 2), and aBST-projecting neurons (Fig. 8) in each of these regions (PL: $F_{3,12} = 25.9$; lateral septum: $F_{3,12} = 7.0$, $p < 0.01$; vSUB: $F_{3,12} = 25.8$; PVT: $F_{3,12} = 13.9$, all $p < 0.01$). Activational profiles in identified aBST afferent neurons in each cell group followed the general trend seen in both repeatedly stressed groups toward diminished numbers of neurons displaying restraint-induced Fos-ir, relative to acutely stressed rats ($p < 0.05$ for each; Fig. 8B), with one exception. In the PVT of animals subjected to repeated restraint, Fos responses to the final restraint session were maintained, that is, they did not differ significantly from those of the acutely stressed group (431 ± 25 and 448 ± 139 , respectively; $p = 0.5$), contrasting with the significant reduction seen in CVS. Because of prior data implicating a distinct, posterior, region of the PVT in adaptations to repeated stress (Bhatnagar et al., 2002; Jaferi et al., 2003), we also analyzed data from the anterior and posterior PVT separately (anterior PVT: $F_{3,12} = 14.3$, $p < 0.01$; posterior PVT: $F_{3,12} = 7.7$, $p < 0.01$), finding that both responded similarly to the cell group as a whole.

Such differential propensities of aBST afferents to habituate to repeated restraint versus CVS (i.e., PVT versus PL, vSUB and lateral septum) could underlie the differential adaptations of aBST GABAergic neurons and the HPA axis seen under these two distinct repeated stress paradigms.

DISCUSSION

Here we addressed the question of how the activity of nodes in a limbic network implicated in modulating HPA axis responses to acute emotional stresses may be altered under differing repeated stress regimens, to determine whether and how that network may take part in mediating differential neuroendocrine adaptations to those regimens. Fourteen days' exposure to CVS led to significant increases both in PVH and adrenocortical output in response to restraint on day 15 as compared with animals subjected acutely to the same challenge. Augmentation of HPA output in CVS animals was accompanied by a reduction of activational responses in PVH-projecting and GABAergic neurons in aBST, as well as decreases in overall GAD 67 mRNA expression in this region. By contrast, habituated HPA axis responses in the repeated restraint group were associated with an increased expression of GAD 67 mRNA and functional activation in PVH-projecting and GABAergic neurons in aBST. In view of evidence we have provided to support a role for aBST in integrating limbic modulatory influences on HPA axis responses to acute emotional stress (Radley et al., 2009; Radley and Sawchenko, 2011), these current findings justify consideration of an involvement of aBST in differential adaptations of the stress axis to repeated stress paradigms associated with sensitization (CVS) versus habituation (repeated restraint).

Further, in surveying key cell groups in an extended limbic forebrain circuitry that may underlie differential effects of the two repeated stress regimens, we found a pervasive tendency of identified aBST afferents from the septal region, vSUB, PL, and PVT to habituate under CVS conditions, which is consistent with a model of facilitation of axis

responses by disinhibition (i.e., inhibiting an inhibitory relay in aBST). By contrast, we found that repeated restraint resulted in habituation of all but one of the aBST afferents listed above, namely the PVT, a cell group strongly implicated in adaptations to repeated stress (Bhatnagar and Dallman, 1998; Bhatnagar et al., 2002; Jaferi and Bhatnagar, 2006). Overall, our findings outline a network in which GABAergic neurons of the aBST may serve as a neural hub for stress-modulatory influences from the limbic forebrain, whereby inhibition or disinhibition of PVH-projecting components contributes to habituation or sensitization, respectively, of HPA axis adaptations to repeated stress (Fig. 9).

Methodological Considerations

Two technical-methodological issues that bear on the interpretation of the present findings warrant additional consideration. One has to do with our heavy reliance on Fos protein induction as an index of cellular activation under longer-term, complex stimulation conditions, when there is evidence to suggest that other factors, notably FosB (e.g., Perrotti et al., 2004) may be more suitable makers of chronic activational effects. First, we would note that the stress models employed here are not chronic, in the sense of being continuously applied over a substantial time period (typically days), but rather involve intermittent exposure to discrete episodes. This is an important distinction, as we are not asking Fos to register the effect of a chronic treatment; its ability to inhibit its own expression for a period of a few hours after acute induction (Morgan and Curran, 1990; Brown and Sawchenko, 1997) makes it ill-equipped to do so. Instead, we are asking it to indicate how the response to discrete events varies with the amount and nature of prior stress experience, as attested by its capacity to be acutely induced in response to a range of signaling mechanisms. Perhaps the strongest argument we can offer for the relevance of Fos as a marker in this context is that its restraint-induced expression in the hypothalamus of animals with vastly differing stress histories (no stress, prior repeated restraint, prior repeated variable stress) varied in tandem with the effects of that final restraint episode on an independent cellular marker (CRF mRNA), and, critically, with stress hormone secretion, the PVH-dependent endpoints whose regulation we ultimately aimed to clarify.

We considered including immunohistochemical analyses of additional transcription factor markers, including FosB, which shows a more gradual increase and persistent expression in some brain regions under repeated stress paradigms (Melia et al., 1994; Perrotti et al., 2004). However, the appeal of this candidate for examining long-term alterations in HPA-modulatory pathways in response to repeated challenges is offset by technical limitations.

FosB is a truncated form of FosB, and the two forms exhibit some degree of overlap in their temporal expression patterns, while spatial resolution is limited by the fact that immunohistochemical approaches are unable to distinguish the full-length proteins from splice variants (for discussion, see Perrotti et al., 2004). Furthermore, evidence from immunoblotting methods that can accurately differentiate Fos family proteins according to molecular weight, fail to reveal any frank differences in forebrain regional expression patterns of FosB as a function of repeated restraint or CVS (Perrotti et al., 2004). While

FosB may well provide an index of enduring changes in cellular function as a result of experience, it is clearly not a useful marker for purposes of the present study. This was confirmed by surveying (Radley, unpublished) FosB expression following repeated

restraint and CVS exposure (i.e., omitting the restraint challenge on the final day of stress). Analysis using several different antibodies for FosB reported in other published studies failed to demonstrate any convincing degree of regional or treatment specificity for this protein. Western blot experiments comparing extracts from forebrain regions of interest (e.g., prefrontal cortex) resulted in the recognition of at least 4 molecular weight bands that migrated at positions corresponding to FosB, FosB, and several other unknown proteins, probably in the Fos family.

A second technical issue of concern in the present study has to do with the fact that group sizes in anatomically guided experiments (i.e., those involving subjects that received tracer injections) are small, which would limit confidence, particularly in negative (non-significant) comparisons. The limitations that this imposes may be offset, to a degree at least, by the fact that carrying out the study in anatomically identified neurons facilitates detection of effects in subpopulations of interest that might otherwise be diluted by considering complex cell groups as a whole. In addition, for the first two phases of the study, the use of multiple indices of the behavior of individual cell groups (e.g., Fos-ir, CRF mRNA, ACTH secretion for hypophysiotropic PVH neurons) bolsters confidence in convergent findings.

Concern over the interpretation of non-significant findings in studies with small Ns is more noteworthy in our final experiment, where we attach potential importance to a non-significant difference between the number of Fos-positive PVT neurons that project to the BST under acute versus repeated restraint conditions (see discussion below), which contrasted with highly reliable differences in this comparison in three other identified BST afferent populations. Calculating statistical power offers no help in this instance, as it is generally agreed among statisticians that *post hoc* power determinations are neither meaningful nor valid (see e.g., Hoenig and Heisey, 2001). As an alternative, we used an equivalence testing procedure, TOST (two one-sided tests; Walker and Nowacki, 2011), to test the joint null hypothesis that the difference between the mean counts of doubly labeled cells in the PVT from groups exposed to acute versus repeated restraint falls within a specified range of equivalence, which we defined as the average percent difference between the means from the other three cell groups examined (PL, septum, ventral HF) under these same stress conditions. The results supported equivalence of the acute-repeated comparison of PVT data, leading us to conclude (conservatively) that the PVT responded differently than the other three cell groups to acute versus repeated restraint.

Notwithstanding the considerations detailed above, the heavy reliance on Fos as an activation marker and the interpretation of negative outcomes of underpowered experiments remain valid concerns. It will remain for tests of the principal interpretations/hypotheses to emerge from the present study, which posit roles for the aBST and its inputs from limbic forebrain in adaptations of HPA axis responses to repeated stress, to judge the ultimate value of these approaches.

Relation to previous studies

The tendency for HPA responses to diminish with repeated exposure to the same (homotypic) stressor is a common (Mason, 1972; Keim and Sigg, 1976; Pollard et al., 1976),

though not universal (Hennessy and Levine, 1977; Kant et al., 1983), finding. Habituation may be viewed as an adaptive phenomenon, which serves to protect against adverse catabolic and immunosuppressive effects of sustained elevations in circulating glucocorticoids. When evident, habituation is not generally attributable to an exhaustion of response capacity, since repeated exposure may sensitize, or facilitate, HPA secretory responses to a novel (heterotypic) insult (see e.g., Dallman et al., 1992; Dallman, 1993b). Decrements in immediate-early gene induction with repeated exposure to restraint or immobilization have been described in the PVH, as well as several extrahypothalamic cell groups, leading to the general belief that habituation is likely to be mediated by decreased neuronal activity in facilitatory afferents (Lachuer et al., 1994; Melia et al., 1994; Umemoto et al., 1994; Chen and Herbert, 1995b; cf. Campeau et al., 2002). Conversely, several laboratories have identified cell groups/circuitries whose responses to a novel stressor are *enhanced* in facilitation paradigms, as potential candidates for mediating increased HPA axis responses under such (sensitization) conditions (e.g., Bhatnagar and Dallman, 1998; Ma et al., 2008). One major inference to be drawn from the present findings is that the aBST is in a position to participate in mediating both kinds of adaptations.

The role of aBST in adaptations to repeated stress

Although several limbic forebrain sites are capable of inhibiting the HPA axis during acute emotional stress (Sapolsky et al., 1984; Kovacs and Makara, 1988; Herman et al., 1989; Diorio et al., 1993; Weinberg et al., 2010), none of these provides a substantial direct innervation of the PVH. Combined pathway tracing and immediate-early gene mapping studies have identified candidate cell groups that could serve as disynaptic relays to link forebrain regulators and the PVH, and suggests a complex network of higher-order structures interconnected with PVH in a parallel or multisynaptic manner (Cullinan et al., 1993; Roland and Sawchenko, 1993; Van de Kar and Blair, 1999; Ulrich-Lai and Herman, 2009). However, recent evidence lends support for at least two limbic cortical regions, PL and vSUB, that impart inhibitory influences over acute restraint-induced HPA output by converging on a discrete target, the aBST, that in turn inhibits the PVH (Radley et al., 2009; Radley and Sawchenko, 2011). Our observation of decreased functional activation throughout this network under HPA-sensitizing conditions highlights the need for studies to directly assess whether these perturbations drive increases in circulating glucocorticoids resulting from repeated stress exposure.

Previous reports have demonstrated increases in GAD mRNA expression and functional activation of GAD-expressing neurons afferent to PVH following acute stress exposure (Bowers et al., 1998; Bali et al., 2005). In agreement with these studies, we observed increases in GAD67 mRNA expression in aBST following acute restraint that were paralleled by enhanced functional activation of PVH-projecting GABAergic neurons, and corresponding decreases in both of these indices following CVS exposure. Increased GAD67 expression has been associated with enhanced inhibition, and disrupted GAD67 expression is associated with impaired inhibitory mechanisms (Asada et al., 1997; Lewis et al., 2005; Kobori and Dash, 2006). These findings are consistent with the idea that activation of GABAergic afferents to PVH restrain HPA activation during acute stress, and that following CVS, dampened activation/ GAD expression is associated with a diminished

inhibitory capacity. We also observed increased GAD67 mRNA expression in aBST during HPA axis habituation, lending further support to the idea of a functional link between alterations in GAD expression in PVH-projecting inhibitory cell groups and the differential modulation of HPA output (Bowers et al., 1998; Bali et al., 2005). However, this general interpretation may be complicated by the fact that repeatedly restrained animals failed to display reliable enhancement of Fos-immunoreactivity in PVH-projecting GABAergic neurons in aBST (Fig. 4, 5), leaving questions as to whether changes in the inhibitory capacity of proximate mediators of the stress axis may be sufficient to impart activational influences from upstream regions, such as PVT.

The BST is a limbic forebrain structure that is intimately associated with aspects of the amygdala, and projects in turn to hypothalamic and brainstem target areas that mediate many autonomic and behavioral responses to aversive or threatening stimuli. While earlier behavioral studies tended to treat BST as an homogeneous entity, recent work has defined cytoarchitectonic subdivisions of BST that are associated with distinct types of adaptive behavioral and autonomic responses (Jennings et al., 2013; Kim et al., 2013). BST influences on HPA responses may be differentiated in the rostrocaudal dimension, and more recent evidence suggests that the anterior aspect, alone, may harbor co-mingled excitatory and inhibitory controls over the stress axis (Dunn, 1987; Cecchi et al., 2002; Choi et al., 2007; Radley et al., 2009). On one hand, non-selective lesions to aBST mildly attenuate, while, selective ablation of GABAergic cell groups in this region augment, HPA axis responses to a single acute restraint episode (Choi et al., 2007; Radley et al., 2009). Evidence also supports a role for a distinction in aBST modulation of the stress axis as a function of stressor duration. Excitotoxin lesions of aBST were found to further enhance HPA activation to a novel restraint challenge following CVS exposure (Choi et al., 2008), which suggests a restraining influence on the stress axis even under conditions of HPA axis sensitization. Our finding that the CVS + restraint group showed decreased functional activation in both PVH-projecting and GABAergic neurons in aBST is consistent with the possibility that this cell group generally constrains HPA activation following prolonged stress exposure.

Extended circuitry

The most thorough and systematic analysis of the CNS substrates that might underpin adaptations to repeated stress was initiated by Bhatnagar and Dallman (1998) and developed by Bhatnagar and colleagues (Bhatnagar et al., 2000; Bhatnagar et al., 2002; Jaferi and Bhatnagar, 2006; Grissom and Bhatnagar, 2009). The initial studies identified the posterior paraventricular nucleus of the thalamus (pPVT) at the core of an interconnected series of cell groups, including the lateral parabrachial and several amygdaloid nuclei that are known to project to the PVH, and PVH itself, that displayed increased Fos staining in a facilitation model (i.e., 30 min restraint on day 7, following 6 daily cold stress exposures). Lesions of pPVT enhanced ACTH secretory responses to restraint in previously cold-stressed, but not naïve, rats. The subsequent demonstration that pPVT ablation disrupted habituation of HPA axis activity under conditions of repeated restraint stress, again without affecting responses to acute restraint, has fostered a general championing of the pPVT as a pivotal structure in

“stress memory,” that is, in effecting alterations in axis output as a consequence of prior stress experience.

Aspects of the present findings are compatible with this model. For one, the differential propensity for a relevant (aBST-projecting) PVT subpopulation to habituate under repeated stress paradigms, in which restraint is presented as a final homotypic versus heterotypic challenge, is consistent with a unique role for this thalamic cell group in adaptations to chronic stress. Second, the prior finding that lesions of pPVT *increase* HPA secretory output in a facilitation paradigm (Bhatnagar et al., 1998), is indicative of an inhibitory role in HPA control, in line with our identification of a GABAergic population in the aBST as providing a gateway for convergent limbic forebrain influences on the central limb of the axis (Radley and Sawchenko, 2011).

In addition to issuing prominent inputs to aBST, PVT is interconnected with other upstream components of the limbic-PVH inhibitory network, including the ventral subiculum, amygdala, and, most prominently, mPFC (Moga et al., 1995; Heidbreder and Groenewegen, 2003; Li and Kirouac, 2012). This raises the possibility that PVT may serve as a primary interface between pathways conveying stress-related information to the limbic forebrain (Bubser and Deutch, 1999; Otake et al., 2002), and may indicate a more prominent role for this thalamic region in modulating adaptive responses to prolonged challenges by interceding for higher-order cognitive processing systems such as mPFC. Some progress has been made describing the synaptic mechanisms underlying habituation of the HPA axis following repeated stress (e.g., Levy and Tasker, 2012), although their relationship to the upstream neural substrates that influence the PVH have yet to be sorted out. Activation of forebrain arginine vasopressin 1A receptors and endocannabinoids have been implicated as mediators of HPA axis adaptations following repeated stress exposure (Chen and Herbert, 1995a; Hill et al., 2010; Gray et al., 2014), and further experimental work is needed to evaluate whether and how such factors may map onto the network outlined here.

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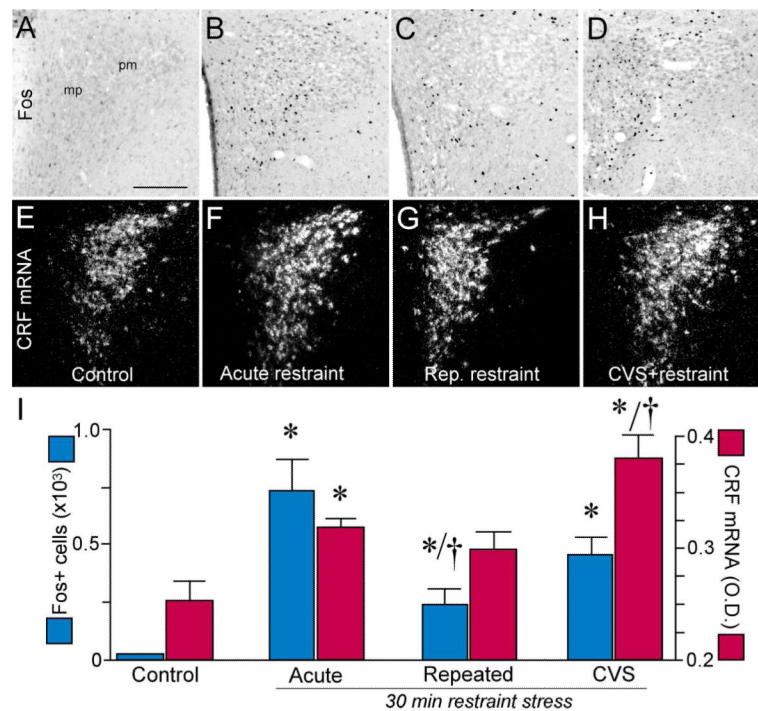


Figure 1.

Photomicrographs show representative examples of Fos immunoreactivity (A-D) and CRF mRNA (E-H) expression in PVH as a function of treatment status. mp, medial parvicellular subdivision; pm, posterior magnocellular subdivision. I: Mean + SEM number of Fos immunoperoxidase-labeled neurons in PVH (blue bars) and relative levels of CRF mRNA expression (red bars) in treatment groups. Both previously unstressed and CVS groups show significant increases in central measures of restraint-induced HPA activation following 30 min restraint stress. 14 d of CVS resulted in augmented CRF mRNA expression in PVH when exposed to this novel stressor, whereas animals exposed to daily restraint throughout this interval did not manifest increases in CRF mRNA relative to unstressed controls. Fos expression also showed significant decreases in the repeated restraint relative to acute stress group. *, $p < 0.05$, differs significantly from the unstressed control group; †, $p < 0.05$, differs significantly from the acute stress group. $N = 4$ control; $N = 5$ for acute, repeated and CVS groups. Scale bar: 150 μm (applies to all).

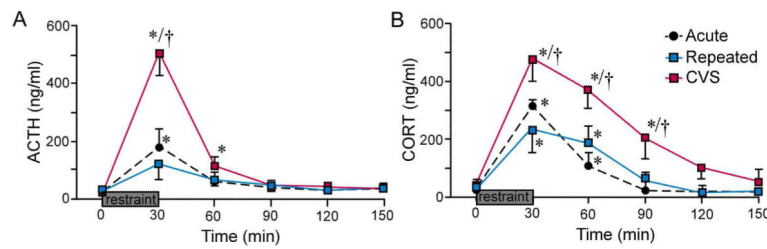


Figure 2.

Effects of previous stress exposure on pituitary adrenal responses to 30 min restraint challenge. Mean \pm SEM plasma ACTH and corticosterone (CORT) levels in control, repeated restraint, and CVS groups before (0 min) and at varying intervals after 30 min of exposure to restraint. **A:** Restraint significantly increased plasma levels of ACTH in previously unstressed (acute) animals (at 30 min), and 14 d CVS exposure significantly enhanced this effect. In contrast, plasma ACTH in animals previously subjected to daily restraint for the 14 consecutive days prior was not significantly elevated over pre-stress levels ($p = 0.2$). **B:** 30 min restraint exposure also significantly increased plasma CORT levels in all treatment groups (at 30 and 60 min. $p < 0.05$ for each), and resulted in a prolonged increase in CVS animals ($p < 0.05$ at 30, 60, and 90 min compared with 0 min). Notably, the repeated restraint group showed significantly lower peak values of CORT (at 30 min) as compared with acute stress and CVS groups, whereas CVS-treated animals displayed significantly elevated titers at 60 and 90 min time points as compared with other treatments. *, $p < 0.05$, differs significantly from basal (0 min) values within each group; †, $p < 0.05$, differs significantly from acutely and repeatedly restrained animals. $N = 8$ acute; $N = 8$ repeated; $N = 6$ CVS.

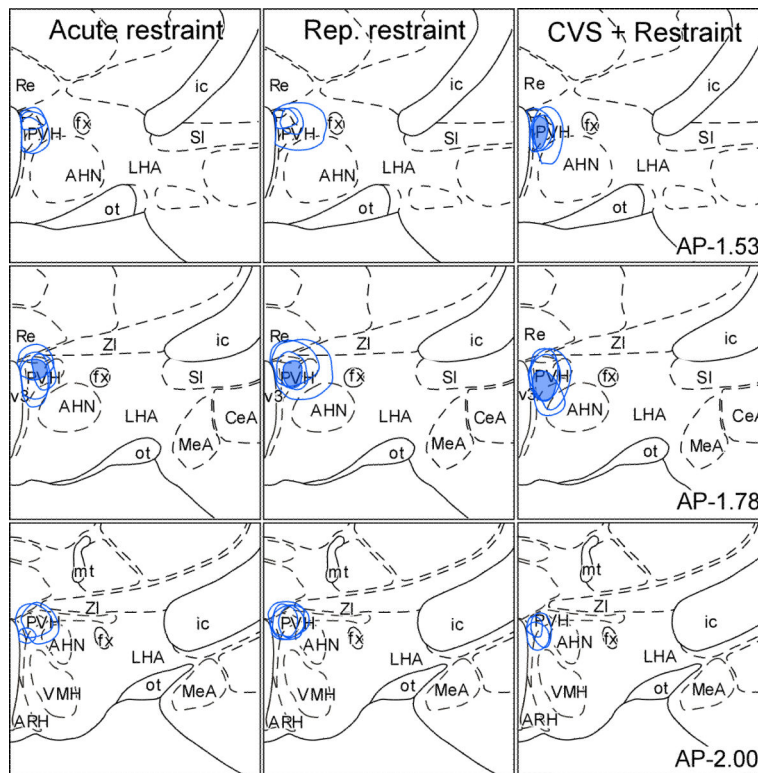


Figure 3.

Reconstructions of FG tracer injection placements in PVH in acute, repeated, and CVS groups (not shown are reconstructions in unstressed controls; N = 3-4/ group). The shaded regions in the diagrams indicate areas of overlap common to all tracer injections. Atlas plates are adapted from Swanson (1992); distance in millimeters relative to bregma is indicated. AHN, Anterior hypothalamic nucleus; ARH, arcuate nucleus hypothalamus; fx, fornix; ic, internal capsule; LHA, lateral hypothalamic nucleus; MeA, medial nucleus amygdala; ot, optic tract; Re, nucleus reunions; SI, substantia innominata; v3, third ventricle; VMH, ventromedial nucleus hypothalamus; ZI, zona incerta.

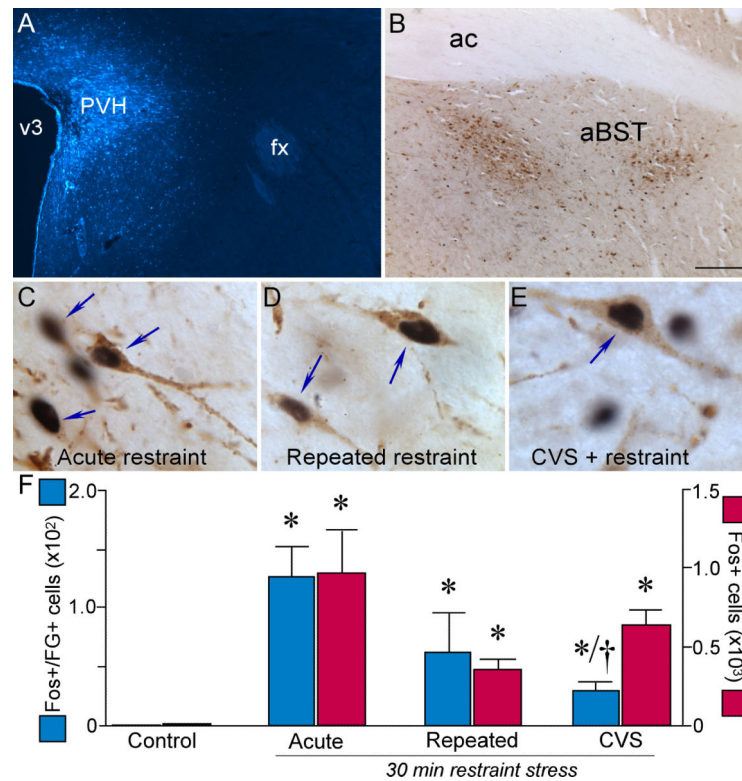


Figure 4.

A-B: Epifluorescence photomicrograph of an illustrating a FG tracer deposit centered within PVH following stereotaxic injection (*A*), and a brightfield image depicting the distribution of immunoperoxidase labeled FG neurons (brown reaction product) following a tracer injection in PVH (*B*). *C-E:* Higher magnification images show representative examples of dual immunoperoxidase staining of Fos and FG (arrows) as a function of treatment condition. Whereas each stress challenge led to the induction of Fos (black reaction product) activation within PVH-projecting neurons (brown), CVS-treated animals displayed an abrogated response. *F:* Histograms showing mean + SEM for Fos plus FG (blue bars) and Fos only (red bars). *, $p < 0.05$, differs significantly from unstressed controls; †, $p < 0.05$, differs significantly from the acute stress group. $N = 3$ control; $N = 4$ acute; $N = 4$ repeated, $N = 4$ CVS. Scale bar (in *B*): 150 μm , top row; 15 μm , middle row.

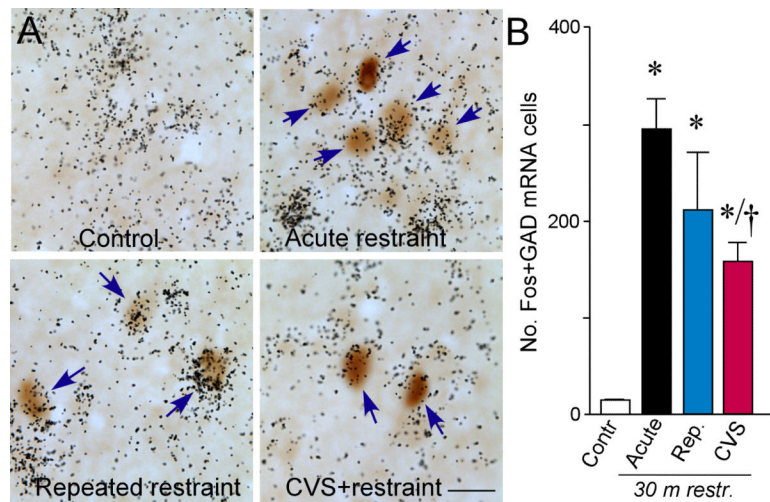


Figure 5.

A: Photomicrographs show representative examples of concurrent labeling for Fos (brown) with GAD67 mRNA (black grains) in doubly-labeled cells (arrows) in aBST (notably within the dorsomedial and fusiform subdivisions). We have previously shown that these cell groups provide a source of GABAergic innervation of CRF-expressing neurons in PVH (Radley et al., 2009; Radley and Sawchenko, 2011). All animals subjected to 30 min of restraint stress showed increases in colabeled GAD+ and Fos+ cells in aBST as compared to unstressed controls, which were void of any Fos expression in this region. CVS resulted in significant decrements in doubly-labeled cells as compared with acutely stressed animals, consistent with the idea that the disinhibition of this pathway may underlie HPA axis sensitization under CVS conditions. **B:** Mean + SEM number of neurons co-labeled for Fos and GAD67 mRNA in aBST in treatment groups. *, $p < 0.05$, differs significantly from unstressed controls; †, $p < 0.05$, differs significantly from the acute stress group. $N = 4$ control, acute, and repeated groups; $N = 5$ CVS. Scale bar: 15 μm (applies to all).

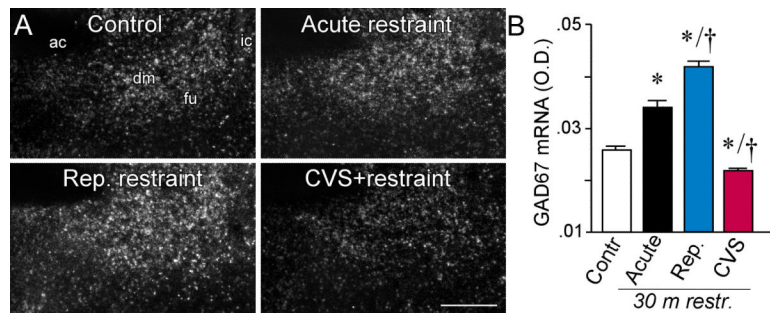


Figure 6.

A: Representative darkfield photomicrographs show GAD 67 mRNA expression in aBST in all four treatment groups. ac, anterior commissure; dm dorsomedial subdivision; fu, fusiform subdivision; sc, subcommissural subdivision. **B:** Mean + SEM relative optical densities for GAD67 mRNA in aBST of treatment groups. Whereas acute stress elevated, repeated restraint produced further enhancements in, GAD expression in aBST. Conversely, GAD expression was significantly decreased following CVS compared to all other treatment groups. *, $p < 0.05$, differs significantly from unstressed controls; †, $p < 0.05$, differs significantly from the acute stress group. $N = 4$ control, acute, and repeated groups; $N = 5$ CVS. Scale bar: 150 μm (applies to all).

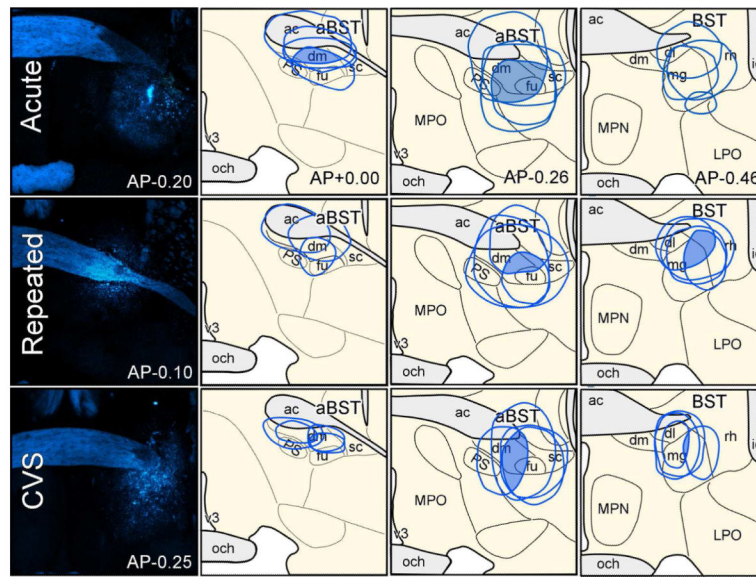


Figure 7. Reconstructions of Fluoro-Gold (FG) tracer injection placements in aBST in acute, repeated, and CVS rats (top, middle, and bottom rows, respectively). Epifluorescence photomicrographs (left column) depict examples of FG tracer deposits, whereas the shaded regions in the diagrams indicate areas of overlap common to all tracer injections, and their approximate extent of diffusion into adjacent-lying structures. Not shown is a fourth group of unstressed controls were also included in these experiments. ac, anterior commissure; dl, dorsolateral subdivision of aBST; dm, dorsomedial subdivision of aBST; fu, fusiform subdivision of aBST; ic, internal capsule; LPO, lateral preoptic area; mg, magnocellular subdivision of posterior BST (pBST); MPN, median preoptic nucleus; MPO, medial preoptic area; och, optic chiasm; PS, parastrial nucleus; rh, rhomboid subdivision of pBST; v3, third ventricle.

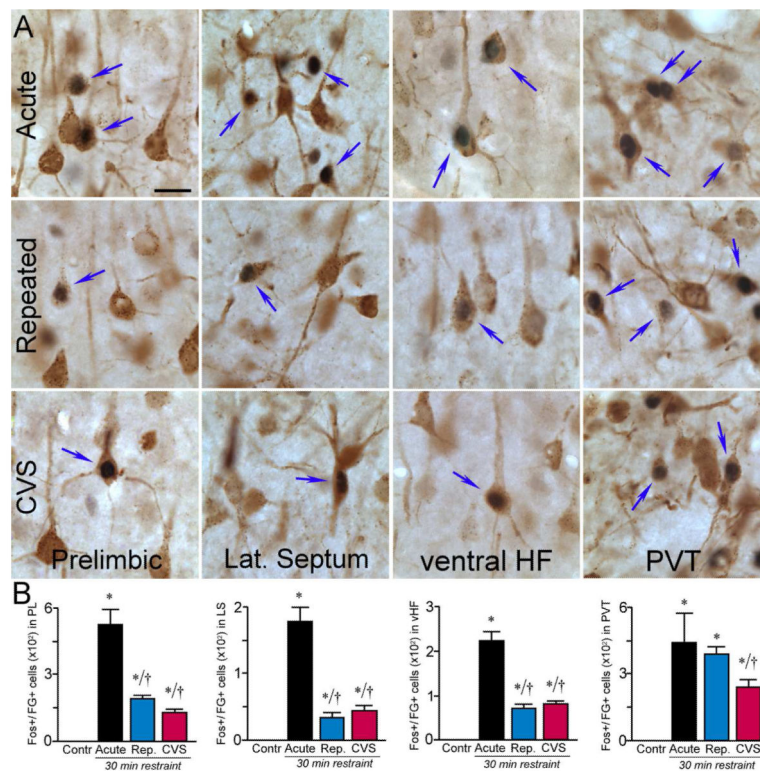


Figure 8.

A: Photomicrographs show representative examples of dual immunoperoxidase staining of FG and Fos (arrows) at high magnification in treatment groups (rows). This analysis reveals that many of the limbic forebrain regions (columns) previously shown to negatively regulate emotional stress-induced HPA activation are both aBST-projecting and functionally activated in response to acute stress. Notably, all of these regions underwent significant decreases in dual colocalization for Fos and FG following CVS. Many of these regions also showed similar decrements following repeated restraint stress, except for the posterior thalamic nucleus (PVT), which remained elevated. HF, hippocampal formation. **B:** Mean + SEM numbers of Fos+FG colabeling for each of the regions analyzed. *, $p < 0.05$, differs significantly from unstressed controls; †, $p < 0.05$, differs significantly from the acute stress group. $N = 4$ control, acute, and repeated groups; $N = 5$ CVS. Scale bar: 20 μm (applies to all).

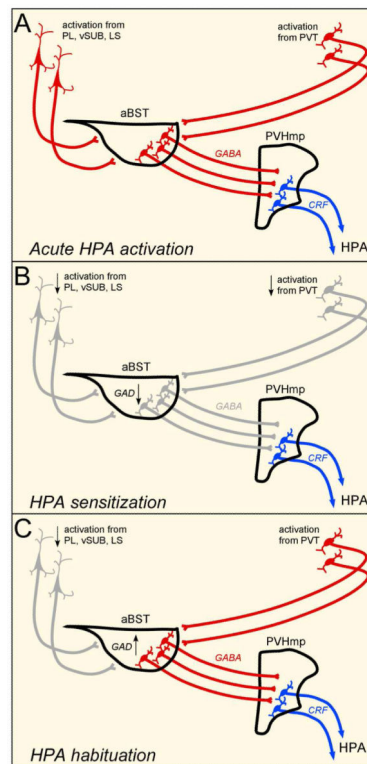


Figure 9.

Diagrams illustrating the proposed circuitry that may provide for modulation of HPA-inhibitory influences under repeated stress conditions. These data support activation in these pathways (highlighted in red) and changes in HPA output as a function of various stress regimens. Whereas GABAergic neurons in aBST impart restraining influences from the limbic forebrain on parvocellular PVH neurons that control HPA output during acute emotional stressors (A), dampened activation responses in this network (gray), and decreases in GAD expression in aBST, following CVS (B) are associated with HPA axis sensitization. By contrast, activation of aBST-projecting neurons from PVT, maintained levels of activation of GABAergic neurons, and increased GAD expression in aBST, may be associated with HPA axis habituation following repeated restraint stress (C). Whereas functional decrements in aBST and related circuits correspond well with HPA axis sensitization following CVS, they do not as easily explain the pattern of results observed under habituation.

Table 1

Primary antibody list

Name, RRID	Immunogen	Manufacturer, species, cat. no.	Concentrations used
anti-Fos	Rat Fos protein, residues 4-17	Dr. Paul Sawchenko, Salk Institute, rabbit polyclonal, N/A	1: 10,000 – 1:20,000
anti-Fluoro-Gold	Fluoro-Gold-BSA conjugate	Millipore, rabbit polyclonal, AB153	1: 10,000

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Table 2

Effect of different stress regimens on functional activation in select limbic forebrain regions

Cell Groups	No. Fos-labeled nuclei			
	Control	Acute Restr.	Repeated Restr.	CVS + Restr.
Prelimbic cortex	255 ± 97	2,308 ± 242 [*]	801 ± 241 ^{*†}	1,976 ± 172 [*]
Lateral septum	nil.	105 ± 43 [*]	58 ± 13 [*]	83 ± 53 [*]
Ventral hippocampal formation	nil.	253 ± 36 [*]	235 ± 58 [*]	105 ± 24 ^{*†}
Paraventricular thalamus (PVT)	120 ± 11	1,183 ± 106 [*]	690 ± 134 ^{*†}	848 ± 47 ^{*†}
PVT, anterior division	58 ± 21	783 ± 103 [*]	398 ± 82 ^{*†}	533 ± 50 ^{*†}
PVT, posterior division	63 ± 23	400 ± 35 [*]	293 ± 55 [*]	315 ± 16 [*]

Values represent mean ± s.e.m. for counts made within each region. Statistical comparisons were made employing a one-way analysis of variance, followed by post-hoc pairwise comparisons using Tukey HSD.

^{*} $p < 0.05$ relative to the unstressed control group;

[†] $p < 0.05$ relative to acutely stressed animals.