Evidence for Involvement of a Limbic Paraventricular Hypothalamic Inhibitory Network in Hypothalamic-Pituitary-Adrenal 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, γ-aminobutyric acid (GABA)-ergic neurons as representing a site of convergence of stress-inhibitory influences originating from medial prefrontal and hippocampal cortices. Here we investigate whether exaggerated HPA axis responses associated with chronic variable stress (CVS; daily exposure to different stressors at unpredictable times over 14 days, followed by restraint stress on day 15) and diminished HPA output seen following repeated (14 days) restraint-stress exposure are associated with differential engagement of the limbic modulatory network. Relative to acutely restrained rats, animals subjected 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 the aBST. By contrast, repeated restraint stress produced habituation in HPA responses, maintained levels of activation of GABAergic neurons, and increased GAD expression in the 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 the paraventricular thalamic nucleus, in which responsiveness was maintained in repeatedly restrained animals. The results are consistent with the view that differential engagement of HPA inhibitory mechanisms in the aBST may contribute to alterations in HPA axis responses to emotional stress in sensitization and habituation paradigms. J. Comp. Neurol. 000:000–000, 2015.

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

"Emotional" stress is a designation attached to one major subset of animal challenge paradigms that models fear, anxiety, and social stress, such as that 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, 1996; Li and Sawchenko, 1998; Dayas et al., 2001), each of which is implicated in modulating hormonal (hypothalamo-pituitary-adrenal [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 comprises the final

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common path for stress-induced HPA axis activation. Instead, these influences appear to be mediated indirectly. We recently identified a circumscribed, anterior part of the bed nuclei of the stria terminalis (aBST) that houses stress-sensitive, PVH-projecting, inhibitory (γ-aminobutyric acid [GABA]-ergic) neurons and serves as a site of convergence of acute (restraint) stress inhibitory influences imparted by prefrontal (i.e., prelimbic [PL]) and hippocampal (ventral subiculum [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 overrespond to a novel (heterotypic) challenge (Dallman et al., 1992, 1993; Bhatnagar and Dallman, 1998). These phenomena of habituation and facilitation 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; also known as *chronic unpredictable stress*, *chronic mild stress*, or *chronic intermittent stress*) is a widely used facilitation paradigm that involves daily exposure of rodents to different stressors at unpredictable times, typically over 2–3 weeks (Ottenweller 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 is 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 the 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 days in semirandomized order at unpredictable times of day. Brief stressors included elevated plus maze (5 minutes), shaker stress (30 minutes on an orbital shaker at 100 rpm), hypertonic saline injection (1 ml 1.0 M saline, i.p.), tail suspension (10 minutes), forced swim (10 minutes in room temperature water), and cold exposure (1 hour at 5–7°C). Sustained stressors included overnight (i.e., 18–24 hour) exposure of rats to wet bedding, cage crowding (four animals/cage), or isolation. Repeated-restraint stress was performed in the morning (9:00 AM) in plastic restrainers (Braintree Scientific, Braintree, MA) for 30 minutes each day over 14 days. Controls were handled comparably but were not restrained. On day 15, animals were subjected to 30 minutes 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 hours 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, Englewood, CO; Schmued and Fallon, 1986) were given into either the aBST or the 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 the aBST was 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 of 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 region 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, i.p.) and perfused via the ascending aorta

with 100 ml 0.9% saline, followed by 900 ml ice-cold 4% paraformaldehyde in 0.1 M borate buffer, pH 9.5, at a flow rate of 55 ml/minute. Brains were removed, postfixed for 3 hours, 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, 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 with ^{35}S-labeled sense (control) and antisense cRNA probes labeled to similar specific activities encoding corticotropin-releasing factor 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 on poly-L-lysine-coated slides and dried under vacuum overnight. They were postfixed with 10% paraformaldehyde for 30 minutes at room temperature, digested with 10 μg/ml proteinase K for 15 minutes at 37°C, and acetylated for 10 minutes. Probes were labeled to specific activities of 1–3 $\times$ 10^9 dpm/μg and applied to the slides at concentrations of $\sim 10^7$ 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$\times$ Denhardt's solution, and 10% dextran sulfate, after which they were treated with 20 μg/ml 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 the film. Slides were developed with Kodak D-19, 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 (i.e., CVS/ repeated restraint) stress episode by following 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 adrenocorticotropic hormone (ACTH) and corticosterone levels. Additional samples were collected 0, 30, 60, 90, and 120 minutes after the termination of restraint. ACTH was measured by a two-site immunoradiometric assay obtained in kit form (Dia-Sorin, 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 with an antiserum raised in rabbits against a corticosterone–bovine serum albumin (BSA) conjugate and ^{125}I-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 with 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 containing 0.3% Triton X-100 and 3% blocking serum. The primary antiserum was localized with Vectastain Elite (Vector Laboratories, Burlingame, CA) reagents, and the reaction product was developed by using a nickel-enhanced glucose oxidase method (Shu et al., 1988).

Characterization of neurons in the aBST on the basis of projections to the PVH, the GABAergic phenotype, and stress sensitivity involved concurrent labeling for 1) Fos and FG immunoreactivity and 2) Fos protein and GAD67 mRNA in separate series of sections. This approach was required because of our inability to localize all three markers efficiently in a single series (see Results). Dual immunolocalization of Fos and FG was carried out by sequentially localizing the antiserum against Fos by using a nickel-enhanced diaminobenzidine method (black nuclear reaction product), as described above, and then an 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 with 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

TABLE 1.
Primary Antibody List

Name	Immunogen	Manufacturer, species, catalog 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 (Billerica, MA), rabbit polyclonal, AB153	1:10,000

an N-terminal synthetic fragment of rat Fos protein (residues 4–17; Radley et al., 2008). Western blots 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.

FG antiserum was raised in rabbit against an FG–BSA conjugate (AB153; Millipore, Bedford, MA; RRID: AB_90738; Chang et al., 1990). 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 with 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 Stereo Investigator morphometry and stereology software (MBF Bioscience, Williston, VT). For each analysis, boundaries defining the regions of interest were drawn at ×25 by using an adjacent series of Nissl-stained sections. In regions identified as aBST projecting (i.e., retrogradely labeled following FG injections in the aBST), labeled cells were used as guides to aid in 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 5 to estimate the total number of labeled neurons in the defined region of interest. Volume estimates from cross-sectional area measurements were obtained by 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 to permit stereologic approaches, and estimates of cell numbers were obtained with the Abercrombie correction (Abercrombie, 1949).

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

Statistical analysis

Group data from the immunoperoxidase and hybridization histochemical experiments ($n = 3–5$ per group) were compared by one-way ANOVA for treatment status (control, acute restraint, CVS, repeated restraint), followed by post hoc pairwise comparisons with Tukey's honestly significant difference. Group data from the hormone assays ($n = 6–8$ per group) were compared by a mixed-design ANOVA with one within (time)- and one between (stress status)-group variable, followed by individual pairwise comparisons as described above. Data are mean ± SEM.

RESULTS

HPA axis responses to differing repeated-stress regimens

Previous studies have shown that a 14-day exposure to CVS produces a robust enhancement of HPA axis

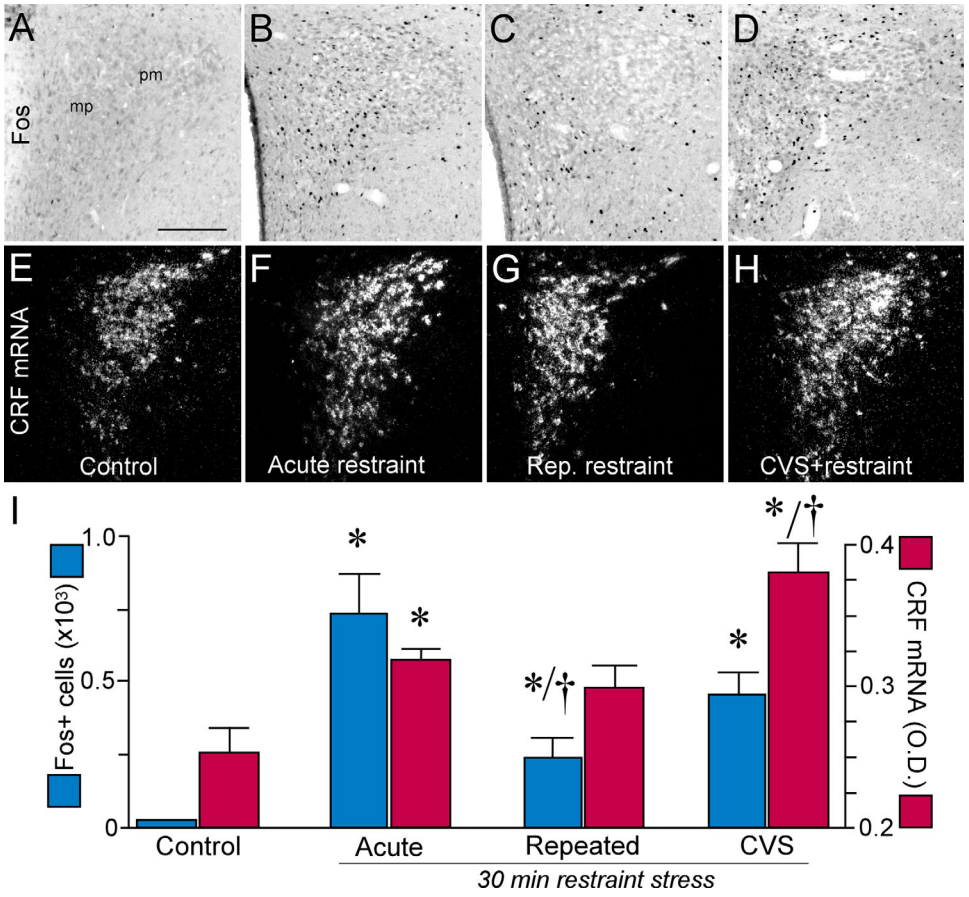

Figure 1. Photomicrographs showing representative examples of Fos immunoreactivity (**A–D**) and CRF mRNA expression (**E–H**) in the PVH as a function of treatment status. mp, Medial parvicellular subdivision; pm, posterior magnocellular subdivision. **I:** Mean ± SEM number of Fos immunoperoxidase-labeled neurons in the 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 minutes of restraint stress. Fourteen days of CVS resulted in augmented CRF mRNA expression in the PVH with exposure 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 the acute-stress group. Control group, n = 4; acute, repeated, and CVS groups, n = 5. *$P < 0.05$ vs. unstressed control group; †$P < 0.05$ vs. acute-stress group. Scale bar = 150 μm.

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, given that 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 2 hours after the termination of 30 minutes of 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 the 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 compared with the unstressed group ($P < 0.01$ for each; Fig. 1A–D,I). Although CVS produced increments

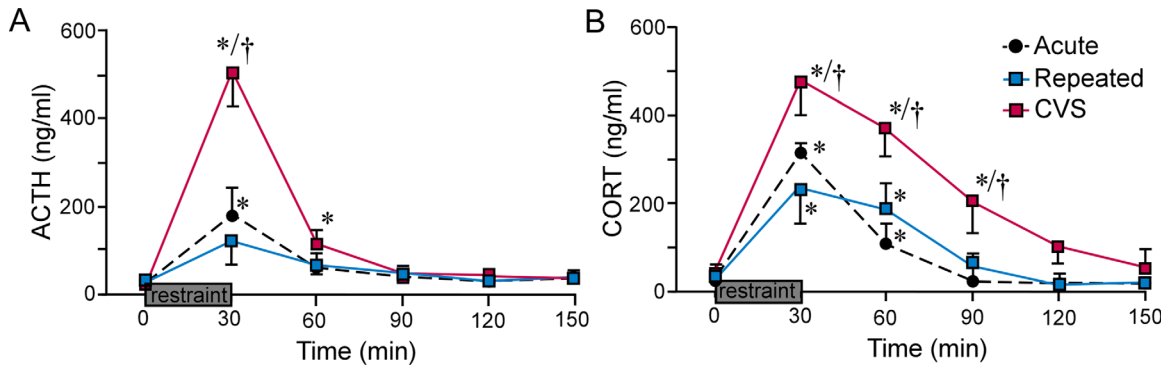

Figure 2. Effects of previous stress exposure on pituitary adrenal responses to a 30-minute restraint challenge. Mean ± SEM plasma ACTH and corticosterone (CORT) levels in control, repeated-restraint, and CVS groups before (0 minutes) and at varying intervals after 30 minutes of exposure to restraint. **A:** Restraint significantly increases plasma levels of ACTH in previously unstressed (acute) animals (at 30 minutes), and a 14-day CVS exposure significantly enhances this effect. In contrast, plasma ACTH in animals previously subjected to daily restraint for the 14 consecutive days prior was not significantly elevated over prestress levels ($P = 0.2$). **B:** Thirty-minute restraint exposure also significantly increased plasma CORT levels in all treatment groups (at 30 and 60 minutes; $P < 0.05$ for each), and resulted in a prolonged increase in CVS animals ($P < 0.05$ at 30, 60, and 90 minutes compared with 0 minutes). The repeated-restraint group shows significantly lower peak values of CORT (at 30 minutes) compared with acute-stress and CVS groups, whereas CVS-treated animals display significantly elevated titers at 60- and 90-minute time points compared with other treatments. Acute, n = 8; repeated, n = 8; CVS, n = 6. *$P < 0.05$ vs. basal (0 min) values within each group; †$P < 0.05$ vs. acutely and repeatedly restrained animals.

in the number of Fos-activated neurons in the PVH comparable to those of acutely restrained animals, repeatedly restrained animals' responses were blunted (by 67%; $P < 0.05$) compared with the acute-restraint group. CRF mRNA expression in the PVH was reliably increased in the acute and the CVS groups following 30 minutes of 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 in the acutely or repeatedly restrained animals (by 20% and 21%, respectively; $P < 0.05$). In contrast, the repeated-restraint group failed to exhibit significant changes in CRF mRNA levels 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 ($F_{2,19} = 5.2$, $P < 0.05$) and 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 prestress levels (0 vs. 30 minutes, $P < 0.05$ for each). CVS-treated animals also showed a more prolonged stress-induced rise in ACTH, which remained significantly elevated above baseline through the 60-minute time point ($P = 0.05$). By contrast, repeatedly restrained animals failed to show any significant increase over basal (prestress) 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 compared with both the acute- and the 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 ($F_{2,19} = 7.4$, $P < 0.01$) and 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 minutes later in all three groups (0 vs. 30 minutes and 0 vs. 60 minutes, respectively, $P < 0.05$ for each); in only the CVS group were corticosterone responses seen to be elevated beyond this, through the 90-minute 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 minutes vs. 60 minutes for the others. Corticosterone titers of repeatedly restrained rats did not differ from those of acutely stressed animals at

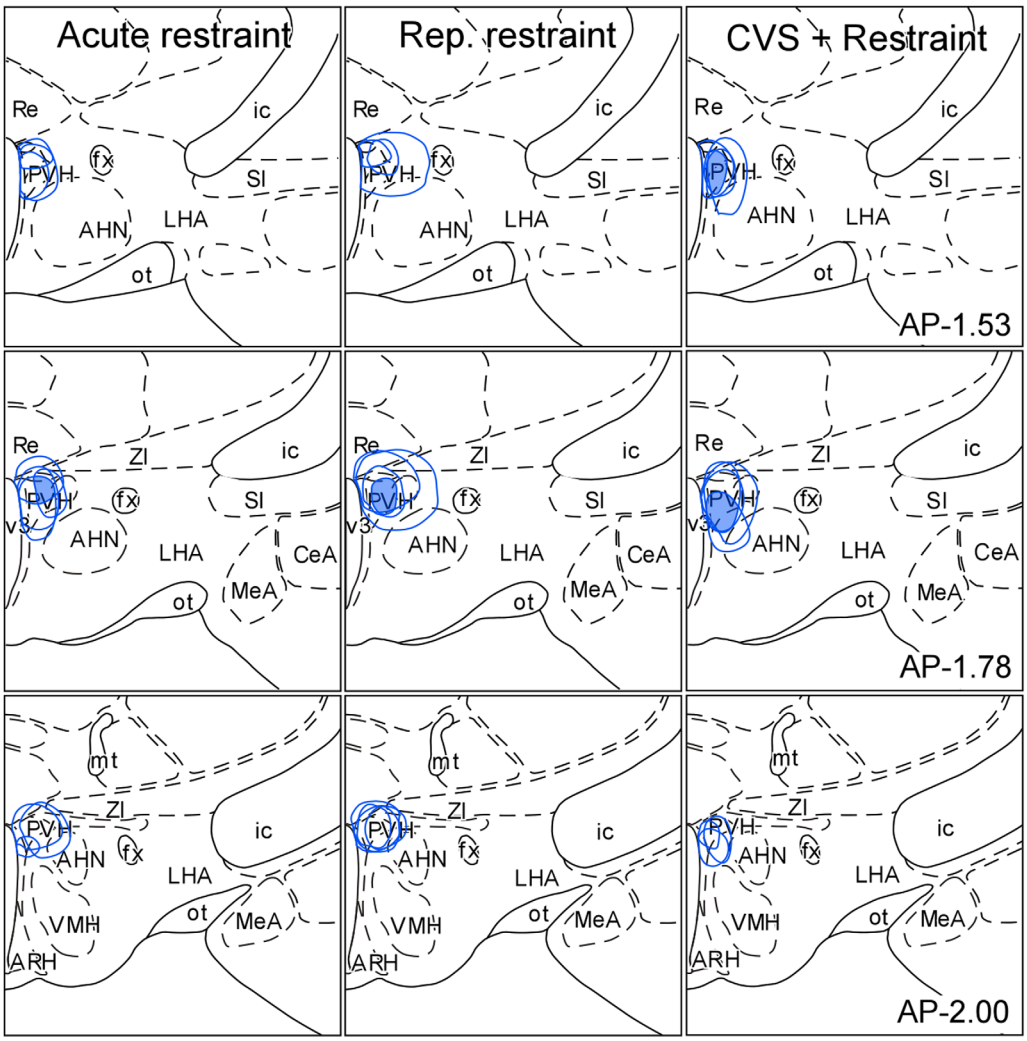

Figure 3. Reconstructions of FG tracer injection placements in the PVH in acute-stress, repeated-stress, and CVS groups (not shown are reconstructions in unstressed controls; n = 3–4/group). Shaded regions indicate areas of overlap common to all tracer injections. Atlas plates are adapted from Swanson (1992); distance in millimeters relative to bregma is indicated. AHN, anterior hypothalamic nucleus; ARH, arcuate nucleus hypothalamus; CeA, central nucleus amygdala; 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.

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, 1993; Choi et al., 2008).

Differential engagement of the aBST following CVS vs. repeated restraint

We have identified PVH-projecting, GABAergic neurons in the 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 the aBST might 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 the PVH, and neurons labeled in the aBST were assayed for alterations in responsiveness (Fos induction) 2 hours after a 30-minute restraint exposure on the day following regimens of 14 days of repeated restraint, 14 days 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

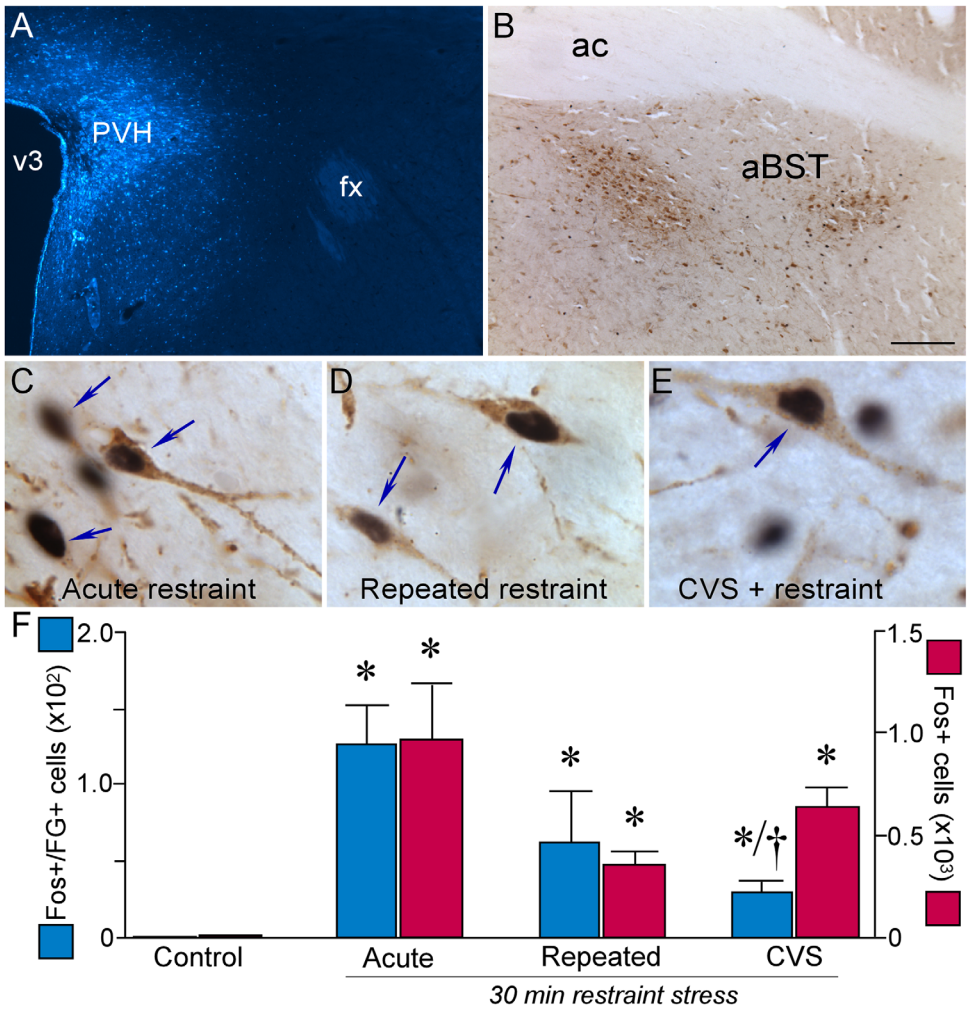

Figure 4. **A,B**: Epifluorescence photomicrograph illustrates an FG tracer deposit centered within the PVH following stereotaxic injection (A) and a brightfield image depicting the distribution of immunoperoxidase-labeled FG neurons (brown reaction product) following a tracer injection in the PVH (B). **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 show mean ± SEM for Fos + FG (blue bars) and Fos only (red bars). $*P < 0.05$ vs. unstressed controls; $\dagger P < 0.05$ vs. acute stress group. Control, n = 3; acute, n = 4; repeated, n = 4; CVS, n = 4. Scale bar (in B) = 150 μm in A,B; 15 μm in C–E.

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 (Figs. 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 the 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 the aBST resulting from injections that might 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) and 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 the aBST, including within the portion of neurons labeled concurrently for tracer, relative to 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 the aBST of

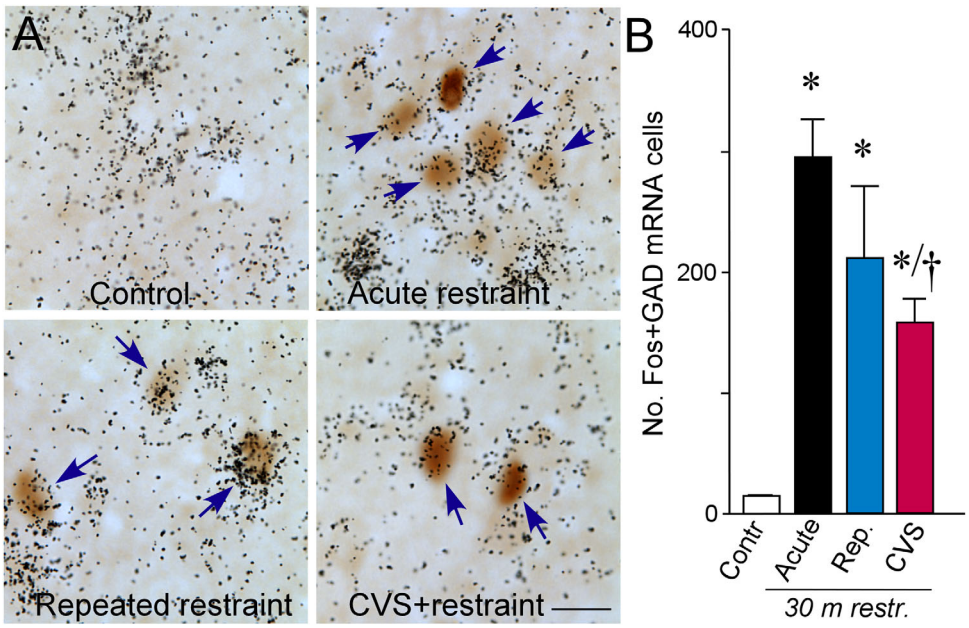

Figure 5. **A**: Photomicrographs show representative examples of concurrent labeling for Fos (brown) with GAD67 mRNA (black grains) in doubly labeled cells (arrows) in the aBST (notably within the dorsomedial and fusiform subdivisions). We have previously shown that these cell groups provide a source of GABAergic innervation of CRF-expressing neurons in the PVH (Radley et al., 2009; Radley and Sawchenko, 2011). All animals subjected to 30 minutes of restraint stress showed increases in colabeled GAD^+ and Fos^+ cells in the aBST compared with unstressed controls, which were void of any Fos expression in this region. CVS resulted in significant decrements in doubly labeled cells 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 colabeled for Fos and GAD67 mRNA in aBST treatment groups. $^*P < 0.05$ vs. unstressed controls; $\dagger P < 0.05$ vs. acute-stress group. Control, acute, and repeated groups, n = 4; CVS group, n = 5. Scale bar = 15 μm.

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% ($P < 0.05$).

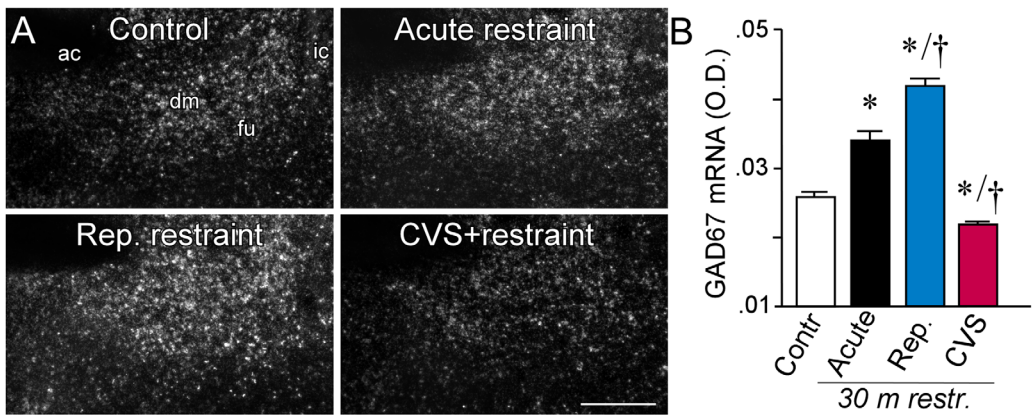

Figure 6. **A**: Representative darkfield photomicrographs show GAD67 mRNA expression in the aBST in all four treatment groups. ac, Anterior commissure; dm dorsomedial subdivision; fu, fusiform subdivision; sc, subcommissural subdivision. **B:** Mean ± SEM relative optical densities for GAD67 mRNA in the aBST of treatment groups. Whereas acute stress elevated GAD expression, repeated restraint produced further enhancements in GAD expression in the aBST. Conversely, GAD expression was significantly decreased following CVS compared with all other treatment groups. $^*P < 0.05$ vs. unstressed controls; $\dagger P < 0.05$ vs. acute-stress group. Control, acute, and repeated groups, n = 4; CVS, n = 5. Scale bar = 150 μm.

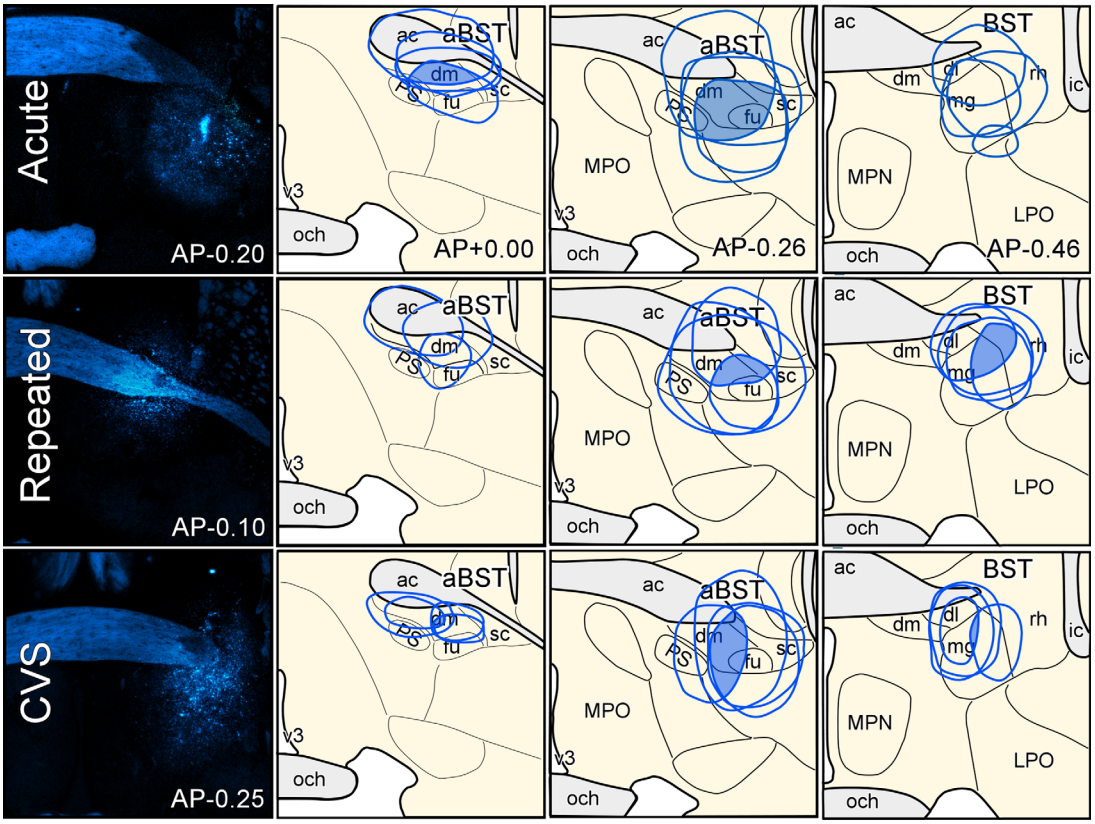

Figure 7. Reconstructions of FG tracer injection placements in the aBST in acute-stress, repeated-stress, and CVS rats (top, middle, and bottom rows, respectively). Epifluorescence photomicrographs (left column) depict examples of FG tracer deposits, whereas the shaded regions in the diagrams indicate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

A compatible set of results was obtained by analyzing material in which immunolocalization of Fos protein was combined with hybridization histochemical detection of mRNA encoding for GAD67 (marker for GABAergic neurons) in the aBST as a function of stress experience (Fig. 5). Animals in each of the stress groups displayed robust Fos induction in GAD67-expressing neurons compared with unstressed animals ($F_{3,12} = 8.2$, $P < 0.01$). Among 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 with 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 were 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 underresponds to a novel restraint stimulus, whereas 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 has been shown to

TABLE 2.

Effect of Different Stress Regimens on Functional Activation in Select Limbic Forebrain Regions[1]

	Number of Fos-labeled nuclei			
Cell groups	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*

[1]Values are mean ± SEM for counts made within each region. Statistical comparisons were made by employing a one-way ANOVA, followed by post hoc pairwise comparisons with Tukey's honestly significant difference.

*$P < 0.05$ vs. unstressed control group.

†$P < 0.05$ vs. acutely stressed animals.

provide any appreciable innervation of the 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 the aBST serves 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 contributes to altered aBST GABAergic influences on PVH and corresponding HPA axis adaptations to repeated restraint and/or CVS. This was investigated by placing discrete retrograde tracer (FG) injections in the 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. As displayed 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 (for pacellation/terminology see Dong et al., 2001). 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 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 a 30-minute restraint as a function of stress experience were noted overall (Table 2) and in aBST-projecting neurons (Fig. 8) in each of these regions (PL: $F_{3,12} = 25.9$; lateral septum: $F_{3,12} = 7.0$; vSUB: $F_{3,12} = 25.8$; PVT: $F_{3,12} = 13.9$; all $P < 0.01$). Activational profiles in aBST afferent neurons identified 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: $F_{3,12} = 7.7$, $P < 0.01$) and found that both responded similarly to the cell group as a whole. Such differential propensities of aBST afferents to habituate to repeated restraint vs. CVS (i.e., PVT vs. PL, vSUB,

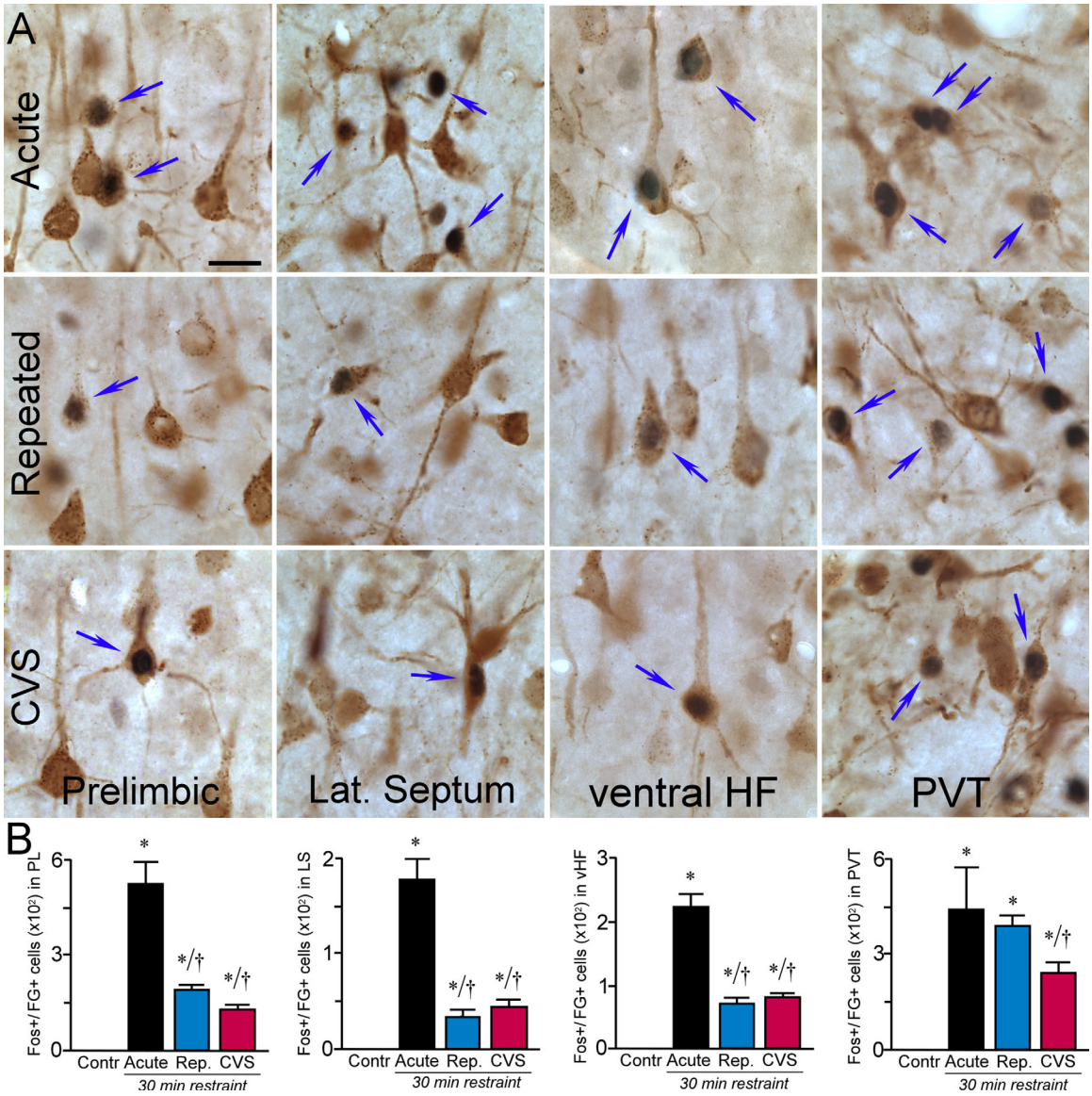

Figure 8. **A**: Photomicrographs show representative examples of dual immunoperoxidase staining of FG and Fos (arrows) at high magnification in treatment groups (rows). This analysis reveals that many of the limbic forebrain regions (columns) previously shown to regulate emotional stress-induced HPA activation negatively are both aBST projecting and functionally activated in response to acute stress. 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 PVT, which remained elevated. HF, hippocampal formation. **B:** Mean ± SEM numbers of Fos + FG colabeling for each of the regions analyzed. $*P < 0.05$ vs. unstressed controls; $\dagger P < 0.05$ vs. acute-stress group. Control, acute, and repeated groups, n = 4; CVS, n = 5. Scale bar = 20 μm.

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

This study addresses 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. A 14-day exposure to CVS led to significant increases both in PVH and in adrenocortical output in response to restraint on day 15 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 the aBST as well as decreases in overall GAD67 mRNA expression in this region. By contrast, habituated HPA axis responses in the repeated-restraint group were associated with an increased expression of GAD67 mRNA and functional activation in PVH-projecting and GABAergic neurons in the aBST. In view of the evidence that we have provided to support a role for the aBST in integrating limbic modulatory influences on HPA axis responses to acute emotional stress (Radley et al., 2009; Radley and Sawchenko, 2011), the current findings justify consideration of an involvement of the aBST in differential adaptations of the stress axis to repeated-stress paradigms associated with sensitization (CVS) vs. habituation (repeated restraint).

Furthermore, 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 the aBST). In 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 might 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).

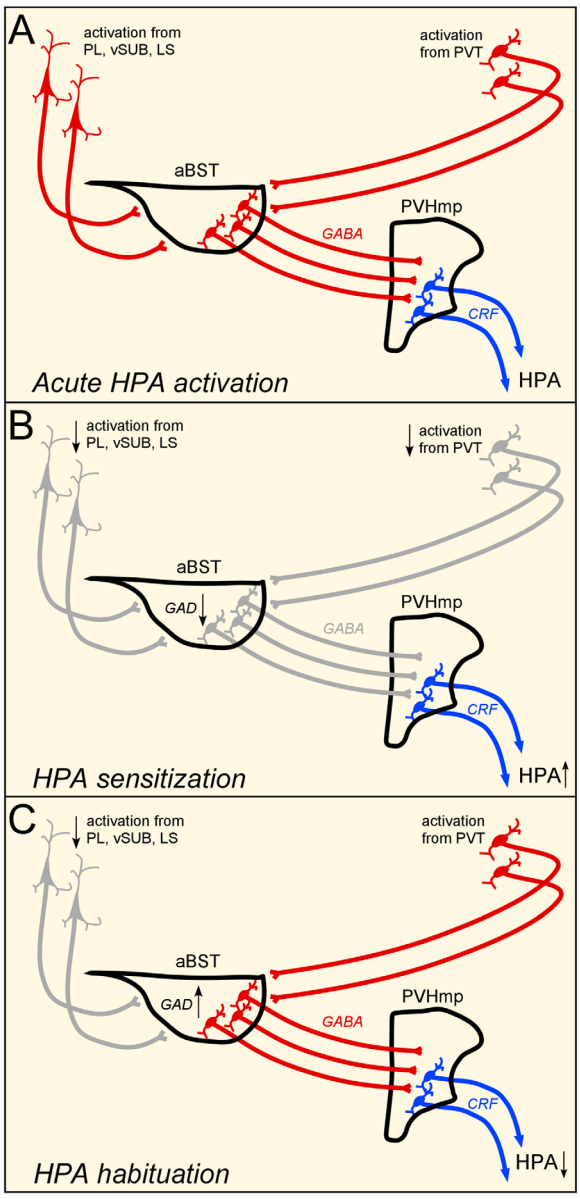

Figure 9. Diagrams illustrate the proposed circuitry that may provide for modulation of HPA-inhibitory influences under repeated-stress conditions. These data support activation in these pathways (highlighted in red) and changes in HPA output as a function of various stress regimens. Whereas GABAergic neurons in the aBST impart restraining influences from the limbic forebrain on parvicellular PVH neurons that control HPA output during acute emotional stressors (**A**), dampened activational responses in this network (gray; **B**) and decreases in GAD expression in the aBST following CVS 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 the aBST may be associated with HPA axis habituation following repeated-restraint stress (**C**). Although functional decrements in the 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.

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 ΔFosB (see, e.g., Perrotti et al., 2004), may be more suitable makers of chronic activational effects. First, we 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 because we are not using 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 using 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) varies 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 ΔFosB, 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. ΔFosB is a truncated form of FosB, and the two forms exhibit some degree of overlap in their temporal expression patterns, although spatial resolution is limited by the fact that immunohistochemical approaches are unable to distinguish 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 ΔFosB as a function of repeated restraint or CVS (Perrotti et al., 2004). Although ΔFosB 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) ΔFosB expression following repeated restraint and CVS exposure (i.e., omitting the restraint challenge on the final day of stress). Analyses with several different antibodies for ΔFosB 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 four molecular weight bands that migrated at positions corresponding to FosB, ΔFosB, 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 could limit confidence, particularly in negative (nonsignificant) 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 nonsignificant findings in studies with small group sizes is more noteworthy in our final experiment, in which we attach potential importance to a nonsignificant difference between the number of Fos-positive PVT neurons that project to the BST under acute- vs. 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 because there is general agreement 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, 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 vs. repeated restraint falls within a specified range of equivalence, which we defined as the average percentage difference between the mean from the other three cell groups examined (PL, septum, ventral HF) under these same stress conditions. The results supported equivalence of the acute vs. repeated comparison of PVT data, leading us to conclude (conservatively) that the PVT responded differently from the other three cell groups to acute vs. 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 to 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 that serves to protect against adverse catabolic and immunosuppressive effects of sustained elevations in circulating glucocorticoids. When evident, habituation is generally not attributable to an exhaustion of response capacity, given that repeated exposure may sensitize or facilitate HPA secretory responses to a novel (heterotypic) insult (see, e.g., Dallman et al., 1992; Dallman, 1993). Decrements in immediate-early gene induction with repeated exposure to restraint or immobilization have been described for 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 (see, 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.

Role of the 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 suggest a complex network of higher-order structures interconnected with the 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 requirement for studies to assess directly whether these perturbations drive increases in circulating glucocorticoids resulting from repeated-stress exposure.

Previous studies have demonstrated increases in GAD mRNA expression and functional activation of GAD-expressing neurons afferent to the 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 the 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 the 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 the 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-ir in PVH-projecting GABAergic neurons in the aBST (Figs. 4, 5), leaving questions concerning whether changes in the inhibitory capacity of proximate mediators of the stress axis are 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. Whereas earlier behavioral studies tended to treat the BST as a homogeneous entity, recent work has defined cytoarchitectonic subdivisions of the 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 harbors comingled excitatory and inhibitory controls over the stress axis (Dunn, 1987; Cecchi et al., 2002; Choi et al., 2007; Radley et al., 2009). On the one hand, nonselective lesions to the aBST mildly attenuate,

whereas 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 the aBST were found to enhance HPA activation further in response to a novel restraint challenge following CVS exposure (Choi et al., 2008), suggesting 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 the 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, 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 the PVH itself that displayed increased Fos staining in a facilitation model (i.e., 30-minute restraint on day 7 following six 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. First, 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 vs. 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 the 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 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 the aBST, the PVT is interconnected with other upstream components of the limbic PVH-inhibitory network, including the ventral subiculum, the amygdala, and most prominently the medial prefrontal cortex (mPFC; Moga et al., 1995; Heidbreder and Groenewegen, 2003; Li and Kirouac, 2012). This raises the possibility that the PVT serves 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 required to evaluate whether and/or how such factors map onto the network outlined here.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

ROLE OF AUTHORS

Research design and performance: JJR. Data analysis: JJR. Writing of the article: JJR, PES.

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