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Role of Paraventricular Nucleus-projecting Norepinephrine/Epinephrine Neurons in Acute and Chronic Stress

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Abstract

Chronic variable stress (CVS) exposure modifies the paraventricular nucleus of the hypothalamus (PVN) in a manner consistent with enhanced central drive of the hypothalamo-pituitary-adrenocortical axis. Since previous reports suggest that post-stress enhancement of norepinephrine (NE) action contributes to chronic stress regulation at the level of the PVN, we hypothesized that PVN-projecting NE neurons were necessary for the stress facilitatory effects of CVS. Following intra-PVN injection of saporin toxin conjugated to a dopamine- β -hydroxylase (DBH) antibody (DSAP), PVN DBH immunoreactivity was almost completely eliminated, while sparing immunoreactive afferents to other key regions involved in stress integration (e.g. DBH fiber densities were unaffected in the central nucleus of the amygdala). Reductions in DBH-positive fiber density were associated with reduced numbers of DBH-immunoreactive neurons in the nucleus of the solitary tract (NTS) and locus coeruleus (LC). Following two weeks of CVS, DSAP injection did not alter stress-induced adrenal hypertrophy or attenuation of body weight gain, indicating that PVN-projecting NE (and epinephrine (E)) neurons are not essential for these physiological effects of chronic stress. In response to acute restraint stress, PVN-targeted DSAP injection attenuated peak adrenocorticotrophic hormone (ACTH) and corticosterone in controls, but only attenuated peak ACTH in CVS animals, suggesting that enhanced adrenal sensitivity compensated for reduced excitatory drive of the PVN. Our data suggest that PVN-projecting NE/E neurons contribute to the generation of acute stress responses, and are required for HPA axis drive (ACTH release) during chronic stress. However, loss of NE/E drive at the PVN appears to be buffered by compensation at the level of the adrenal.

Keywords

Glucocorticoids; ACTH; corticotropin releasing hormone; saporin; nucleus of the solitary tract

Introduction

Chronic stress causes widespread changes in neuronal structure and function, culminating in behavioral and physiological adaptation or pathology resulting in both stress facilitation and stress habituation. Previous studies suggest that dysregulation of the paraventricular nucleus of the hypothalamus (PVN) contributes to behavioral and physiological alterations caused by chronic stress (Herman *et al.*, 2008). The PVN contains corticotrophin-releasing-hormone (CRH)-expressing medial parvocellular neurons that initiate pituitary adrenocorticotrophic hormone (ACTH) release and subsequent secretion of adrenal glucocorticoids (comprising the hypothalamo-pituitary-adrenocortical (HPA) axis). Chronic stress alters peptide (Imaki *et al.*, 1991; Kiss & Aguilera, 1993; Herman *et al.*, 1995; Makino *et al.*, 1995), receptor (Cullinan & Wolfe, 2000; Ziegler *et al.*, 2005), and electrophysiological (Verkuyl *et al.*, 2004) function in PVN neurons in a manner consistent with enhanced central drive of the HPA axis by stress contributing to stress facilitation. Chronic stress exposure increases the number of pre-synaptic excitatory neurotransmitter boutons in apposition to CRH cell bodies and dendrites (Flak *et al.*, 2009), indicating that chronic stress enhances afferent input of the PVN.

Norepinephrine (NE) plays a prominent role in PVN activation. Norepinephrine terminals directly contact CRH neurons (Liposits *et al.*, 1986; Kitazawa *et al.*, 1987), and the PVN expresses alpha-1 adrenergic receptors (Cummings & Seybold, 1988; Day *et al.*, 1997). Intraventricular or local infusion of NE induces PVN cFos and stimulates the HPA axis (Szafarczyk *et al.*, 1987; Itoi *et al.*, 1994; Cole & Sawchenko, 2002; Khan *et al.*, 2007), whereas local alpha-1 adrenergic receptor antagonists inhibit both stress-induced PVN cFos and corticosterone release (Leibowitz *et al.*, 1989; Itoi *et al.*, 1994), consistent with a functional role of NE in HPA axis activation. Tract tracing studies indicate that mpPVN NE is primarily supplied by neurons of the A2 catecholaminergic cell group, largely confined to the area of the nucleus of the solitary tract (NTS) (Cunningham & Sawchenko, 1988). Knife cuts severing ascending medullary inputs to the PVN reduce PVN dopamine beta-hydroxylase (DBH)-staining, reduce CRH immunoreactivity, and blunt HPA axis responses to stress (Sawchenko, 1988; Li *et al.*, 1996), supporting a role for the NTS in PVN regulation. In addition, recent studies indicate that local ablation of NE/E terminals using saporin (SAP)-DBH antibody (DSAP) conjugates attenuate HPA axis responses to systemic stressors such as glucoprivation (Ritter *et al.*, 2003), IL1-beta (Li *et al.*, 1996), LPS (Bienkowski & Rinaman, 2008), insulin (Khan *et al.*, 2011), saline injection (Khan *et al.*, 2011), and anesthesia (Khan *et al.*, 2011), but not swim stress (Ritter *et al.*, 2003), suggesting that PVN NE/E is necessary for appropriate HPA axis responses to some (but not all) stressors.

Prior studies suggest that NE/E neurons in the nucleus of the solitary tract (NTS) are recruited during chronic stress (Zhang *et al.*, 2010). Given that the NTS provides the primary NE/E input to the PVN (Cunningham & Sawchenko, 1988), these studies suggest that enhancements in NE/E output may materially contribute to physiological and behavioral dysfunction associated with chronic stress. Thus, this study tested the hypothesis that PVN-projecting NE/E neurons are necessary for chronic stress-induced drive of the HPA axis.

Materials and Methods

Subjects

Male Sprague-Dawley rats from Harlan (Indianapolis, IN) (weighing 250–275 g upon arrival) were singly housed in clear polycarbonate cages containing granulated corncob bedding, with food and water available *ad libitum*. The colony room was temperature- and humidity-controlled, and maintained on a 12 h light cycle (lights on 6:00 am; lights off 6:00 pm). All experimental procedures were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Animals and approved by the University of Cincinnati Institutional Animal Care and Use Committee. Following one week of acclimation to the facilities, pre- and post-surgery body weights and food intake were monitored in order to insure that the animals appropriately recovered from the procedure. Body weight and food intake were also collected prior to chronic stress regimen and additionally every third day until the termination of the experiment.

PVN-targeted DSAP injections

Prior to surgery, the singly housed animals were anaesthetized with a ketamine (90 mg/kg) and xylazine (10 mg/kg) cocktail followed by a pre-emptive analgesic, butorphenol, and antibiotic, gentamycin. Animals received bilateral injections of either the phosphate buffered saline (PBS), the saporin toxin (SAP) (8.82ng/ 200nl, pH 7.4) (PR-01, Advanced Targeting Systems, San Diego, CA), or the saporin toxin conjugated to an antibody directed to DBH (DSAP) (42ng/ 200nl, pH 7.4) (MAB394, Millipore, Temecula, CA), using a Hamilton syringe directed toward the PVN (−1.8 mm from Bregma, +/−0.4 mm from the midline, and −8.2 mm from skull). A preliminary study determined the coordinates used, based on the Paxinos and Watson atlas (Paxinos & Watson, 1986). The saporin-DBH antibody conjugate is endocytosed following binding at the synaptic cleft and transported back to the cell body (Wrenn *et al.*, 1996). In the cell body, the toxin inactivates ribosomes (Ippoliti *et al.*, 1992), leading to cell death within two weeks (Madden *et al.*, 1999). DSAP has previously been reported to specifically ablate NE/E neurons and provide no effects on neighboring neurons (Madden *et al.*, 1999; Ritter *et al.*, 2001; Rinaman, 2003). Unconjugated SAP cannot enter cells and is non-toxic to neurons, serving as the appropriate control for DSAP administration. Over 3 minutes, 200 nl of either PBS, SAP, or DSAP was gradually injected at the targeted site, in a similar manner to previously published work (Ritter *et al.*, 2003; Bienkowski & Rinaman, 2008). Since previously published studies indicate that two weeks is necessary to eliminate PVN NE/E afferents using DSAP, the animals were allowed to recovery from surgery for two weeks.

Chronic Stress Procedure

Prior to chronic stress, the subjects were weighed and food intake recorded, in order to control for pre-experiment metabolic differences that may influence stress reactivity/ responsiveness. The chronic stress protocol consisted of twice-daily (morning and afternoon) exposure to randomly assigned stressors for two weeks. Other than to record weight and food intake, control animals were not disturbed. Morning stressors were conducted between 8:00 am and 11:30 am and afternoon stressors were administered between 1:30 pm and 5:00 pm. Stressors consisted of rotation stress (1 h at 100 rpm on a

platform orbital shaker), warm swim (20 min at 31°C); cold swim (10 min at 18°C), cold room stress (kept in 4°C for one hour) and hypoxia (8% O₂ 92% N₂).

Stress testing

Following 14 days of CVS, blood was collected via tail vein in chilled tubes containing 10ul 100mM EDTA at 0, 30, 60, and 120 minutes following the onset of 30 minutes of restraint stress in a well-ventilated plastic restraint tube. The animals were placed into a plastic restraint tube and blood collected. Following collection, blood was spun for 15 minutes at 6000 rpm at 4° C. Plasma was collected and stored at –20 ° C. A radioimmunoassay kit from MP Biomedicals (Santa Ana, CA) was used to determine plasma corticosterone. Plasma ACTH levels were determined using an antibody provided by Dr. William Engeland as previously described (Choi *et al.*, 2007). Area under curve was calculated using equation for a trapezoid as previously described (Ulrich-Lai *et al.*, 2011a). In the morning following the last afternoon stressor, rats received an overdose of sodium pentobarbital and were perfused with phosphate buffered saline, followed by 4% paraformaldehyde. Brains were post-fixed overnight in 4% paraformaldehyde and transferred to 30% sucrose at 4°C until they were cut on a freezing-stage microtome.

Body Composition analysis

The carcasses of the animals were placed into a Plexiglas tube and inserted into an EchoMRI whole body composition analyzer system (Echo Medical Systems, Houston, TX). The EchoMRI provides estimations of fat and lean mass (Table 1) (Flak *et al.*, 2011).

Immunohistochemistry

Brains were coronally sectioned at 35 µm on a sliding freezing-stage microtome throughout the brain in a 1 in 12 series stored in cryoprotectant (0.1 M phosphate buffer, 30% sucrose, 1% polyvinylpyrrolidone, and 30% ethylene glycol) at –20°C. Sections were transferred from cryoprotectant to 50 mM potassium phosphate buffered saline (KPBS; 40 mM potassium phosphate dibasic, 10 mM potassium phosphate monobasic, and 0.9% sodium chloride) at room temperature (RT). The sections were then transferred to KPBS + 1.0% H₂O₂, and incubated for 10 minutes at room temperature (RT). Sections were then washed (5 × 5 min) in KPBS at RT, and placed in blocking solution (50 mM KPBS, 0.1% bovine serum albumin (BSA), and 0.2% Triton X-100) for 1 hour at RT. To assess DBH staining and extent of DBH innervations of the PVN, one series of sections was incubated overnight at 4°C in rabbit anti-CRH (rc-70, courtesy of Wylie Vale) diluted 1:2500 and mouse anti-DBH (MAB308, Millipore; Temecula, CA) at 1:2500. An additional series was used to assess PVN terminal density, using rabbit anti-synaptophysin (18–0130, Zymed Laboratories, San Francisco, CA) 1:300 and guinea pig anti-vesicular glutamate transporter 2 (vGluT2) (AN2251, Millipore) 1:1500 in blocking solution. A third series was solely incubated with cFos antibody (SC-52, Santa Cruz Biotechnology, Santa Cruz, CA) 1:10,000. All antibodies are widely used and have well-documented specificity, as noted by inclusion on the Journal of Comparative Neurology Antibody Database of validated antibodies (http://onlinelibrary.wiley.com/journal/10.1002/%28ISSN%291096-9861/homepage/jcn_antibody_database.htm). The following morning, sections were rinsed in KPBS (5 × 5

min) and (CRH/DBH and cFos sections) incubated in biotinylated anti-rabbit secondary antibody (Vector Laboratories, Inc., Burlingame, CA) and (CRH/DBH sections) Cy5 goat anti-mouse secondary antibody (Jackson Immuno Research; West Grove, PA) or (synaptophysin/vGluT2) Cy3 goat anti-rabbit (Jackson Immuno Research) and Alexa 488 donkey anti-guinea pig (Molecular Probes, Eugene, Oregon) diluted 1:500 in KPBS + 0.1% BSA for 1 hour at RT. CRH/DBH and cFos-stained sections were rinsed in KPBS (5 × 5 min) and then treated with avidin-biotin complex (ABC, Vector Laboratories, Inc.) at 1:1,000 in KPBS + 0.1% BSA for 1 hour at RT. Following this incubation, *CRH/DBH* sections were rinsed again in KPBS (5 × 5 min) and incubated with Cy3 Streptavidin (Jackson Immuno Research). cFos sections were rinsed again in KPBS (5 × 5 min) followed by reaction with .02% diaminobenzidine/ .09% hydrogen peroxide. All sections were rinsed following the final incubation/reaction (5 × 5min) in KPBS and coverslipped in Fluka Mounting Medium (Sigma Aldrich; St. Louis, MO).

Fiber Density

For each region, two sets of z-stack images on each side were collected for image analysis at the lowest possible magnification to both distinguish immunoreactivity from background and contain the whole region within a single image (40x for PVN, 20x for SON, 10x for CeA, and 5x for Posterior Cingulate Gyrus). DBH fiber density was assessed in the PVN, SON, CeA, and Posterior Cingulate Gyrus. Synaptophysin and VgluT2 were assessed only in the PVN. For the PVN, z-stacks were collected in the region of the mpPVN containing dense CRH-immunoreactivity, as previously reported (Flak *et al.*, 2009). All image processing was performed on an IBM compatible computer using Zeiss LSM 510 Image Browser software. Images were collected 0.5 micrometers apart. For every five consecutive images, a projection was compiled. To produce each projection, z-stacks were subdivided into five consecutive images to ensure separation of synaptic boutons. Single projections (first angle 0, maximum transparency) were generated for each subdivision of the z-stack. Only the five middle projections were selected to undergo analyses, in order to ensure there was no bias toward intensity of staining or potentially damaged sections. Projections were analyzed using the measurement function of Axiovision 4.4 software to obtain the field area percent occupied by the labeled immunoreactivity within each projection. The threshold for pixel inclusion was obtained by analysis of several random projection images and was held constant for all images analyzed. For each animal the occupied field area percent was determined by averaging across the z-stacks taken from that animal. Finally, the field area percent was averaged across animals by treatment group (DSAP control, DSAP CVS, SAP control, SAP CVS).

Cell Counts

The number of DBH-immunoreactive neurons were counted within the rostral (−13.7mm Bregma), middle (−14.0 mm Bregma), and caudal (−14.3 mm Bregma) NTS. Two images were collected on each side, and immunoreactive neurons counted by hand by an observer blind to treatment. The locus coeruleus (LC) is an extremely cell dense region, and its high concentration of DBH makes it extremely difficult to separate neurons from each other. Thus, we quantified the area of the LC containing DBH immunoreactivity as an indirect method of cell loss. Numbers of cFos immunoreactive neurons within the PVN were

determined using Zeiss Axiovision 4.8 software. Four images per animal were collected and subsequently analyzed. Both a threshold grey level and minimum pixel size were determined by using a random subset of images per region. The particle counting algorithm in Axiovision 4.8 was used to determine number of immunoreactive nuclei within the defined region of interest.

Statistical Analysis

Data are expressed as mean $\pm$ standard error. P was set at .05. Outliers were determined if the value exceeded both 1.96 times the standard deviation and 1.5 times the interquartile range (McClave, 1994). All data tested for the presence of outliers prior to statistical analyses. If data exceeded these limits, the individual datum was removed from the analysis. Factorial data were analyzed using two-way ANOVA with Fisher's LSD post-hoc test, with PVN-targeted injection (DSAP and SAP) and stress (CVS and Control) as between-subject factors. Hormone response data were analyzed by three-way repeated measures ANOVA with Fisher's LSD post-hoc test. PVN-targeted injection (DSAP and SAP), stress (CVS and Control) and time (0, 30, 60, 120 minutes) were between-subject factors. In order to insure homogeneity of variance, datasets that failed the Levene Median test for homogeneity of the variance underwent log transformation and were then re-analyzed. Planned comparisons were performed to assess the effect of stress within PVN-injection (DSAP vs. SAP) and PVN-injection within stress condition (CVS vs. control). Since specific hypothesis tests were identified *a priori*, the planned comparisons were performed regardless of the outcome of the omnibus ANOVA (Maxwell & Delaney, 1989). One and two way ANOVAs were conducted using Sigma Stat (Systat Software, San Jose, California) and three way repeated measure ANOVAs, using GB stat (Dynamic Microsystems, Inc., Silver Springs, MD) with p set at .05.

Results

We microinjected DSAP or SAP into the PVN using similar parameters to previously reported studies (Ritter *et al.*, 2003; Bienkowski & Rinaman, 2008), and observed massive loss of PVN DBH immunoreactivity (Figure 1A–B). This qualitative observation was supported by quantitative fiber density analysis that found a main effect of DSAP reducing PVN DBH-positive fiber density { $F(1,47)=159.514, p<.001$ } (Figure 1C). PVN-targeted injection of DSAP resulted in the specific removal of catecholaminergic fibers, but was not observed throughout the whole brain. For instance, DBH-positive fiber density was not altered within the central nucleus of the amygdala (Figure 1D–F). However, we did observe reductions in both supraoptic nucleus (SON) { $F(1,41)=27.498, p<.001$ } (Figure 2A–C) and posterior cingulate gyrus { $F(1,42)=107.071, p<.001$ } (Figure 2D–F) DBH-positive fiber density, indicating that the effects of PVN-targeted DSAP injection were not solely confined to the PVN (Ritter *et al.*, 2001). Specificity of DSAP was not expected, as NE/E neurons from the brainstem project throughout the hypothalamus and amygdala (Sawchenko & Swanson, 1981; Cunningham & Sawchenko, 1988; Cunningham *et al.*, 1990; Delfs *et al.*, 1998) and PVN-targeted DSAP injection has previously been reported to induce widespread fiber loss (Ritter *et al.*, 2001). PVN DBH-fiber density reductions were accompanied by reduced numbers of DBH-immunoreactive neurons in the NTS (Figure 3A–C) (rostral

{ $F(1,42)=8.653, p<.001$ }, middle { $F(1,42)=35.683, p<.001$ }, and caudal { $F(1,42)=50.516, p<.001$ } divisions) and locus coeruleus (LC) { $F(1,41)=35.611, p<.001$ } (Figure 3D), in accord with tract tracing studies showing that both the NTS and LC project to portions of the parvocellular PVN (Cunningham & Sawchenko, 1988). Visual inspection also verified cell loss in the A1 region (data not shown), but due to the diffuse distribution of cells in this area, counts were not performed. In support of previous studies demonstrating an inability of SAP to enter cells (Madden *et al.*, 1999; Ritter *et al.*, 2001; Rinaman, 2003; Ritter *et al.*, 2003), fiber densities and neuron counts did not differ between SAP and PBS-treated animals (data not shown), indicating there was not a cytotoxic effect of SAP.

Following two weeks of recovery from surgery, half of the animals were subjected to chronic variable stress (CVS). Although thymus weight were not different, CVS induced adrenal hypertrophy { $F(1,44)=30.885, p<.001$ } and reductions in body weight gain { $F(1,44)=188.335, p<.001$ } accompanied by losses in adipose { $F(1,43)=19.681, p<.001$ } as well as lean mass { $F(1,45)=21.215, p<.001$ } (Table 1). There was not an interactive effect within these measures, indicating that PVN-projecting NE/E neurons do not play a prominent role in chronic stress regulation of body and organ weight.

Following two weeks CVS exposure, all animals were acutely restrained and blood collected in response to a novel stressor (assessment of stress sensitization or facilitation). As expected, restraint elevated plasma ACTH and corticosterone in all groups, with a return to baseline within two hours after the onset of stress (Figure 4). There was a significant stress X injection X time effect on corticosterone { $F(3,44)=2.95, p=.03$ }, but only an injection X time effect on ACTH { $F(3,43)=9.02, p<.001$ }. DSAP ($p<.001$), but not SAP injected animals, displayed chronic stress-induced elevations in basal glucocorticoids (Figure 4B), suggesting PVN-projecting NE/E neurons normally limit HPA axis hyper-secretion following chronic stress. PVN-targeted DSAP injection blunted peak ACTH ($p<.01$) and corticosterone ($p<.001$) levels in control animals, but only attenuated peak ACTH ($p<.01$) levels in CVS animals (Figure 4A), suggesting that chronic stress enhanced adrenal sensitivity to ACTH. In support of these data, CVS exposure elevated plasma corticosterone/log plasma ACTH levels, previously used an indirect measure of adrenal sensitivity (Ulrich-Lai & Engeland, 2002), in animals with PVN-targeted DSAP injection ($p<.01$), but not SAP injection (Figure 4E). PVN-targeted DSAP injection also reduced 60 minute plasma corticosterone levels in both CVS ($p<.01$) and control ($p<.01$) animals (Figure 4B), suggesting that PVN NE/E attenuates glucocorticoid negative feedback. In support of dissociated ACTH and corticosterone levels between DSAP CVS and DSAP controls, ACTH area under the curve was reduced in CVS animals ($p=.04$) (Figure 4C), while corticosterone area under the curve was reduced in controls ($p<.01$) relative to their SAP-CVS counterparts (Figure 4D). Since PVN-targeted DSAP injection has not been shown to regulate basal PVN CRH mRNA (Ritter *et al.*, 2003), we expect that the ablation of PVN-projecting NE/E neurons would either 1) reduce CRH stores within the median eminence or 2) attenuate the drive of the PVN in response to restraint. However, neither CRH median eminence fiber density nor PVN cFos induction, as previously reported (Schiltz & Sawchenko, 2007), differed between groups, suggesting that PVN-targeted DSAP injection may alter central drive of the HPA axis in a subtle manner (Table 2).

Since chronic stress induced increases in both PVN density of synaptophysin staining and the number of direct excitatory contacts in apposition to PVN CRH neurons (Flak *et al.*, 2009), we hypothesized that PVN-projecting noradrenergic neurons would be necessary for the induction of chronic stress PVN neurotransmitter plasticity. Following fiber density analyses, there was a significant stress X injection effect on both PVN synaptophysin immunoreactivity { $F(1,45)=10.691, p<.001$ } and vGluT2 immunoreactivity { $F(1,42)=7.045, p<.01$ }. As previously shown (Flak *et al.*, 2009; Carvalho-Netto *et al.*, 2011), chronic stress increased the density of synaptophysin staining in the PVN, but this effect was abolished by PVN-targeted DSAP injection (Figure 5A), indicating that PVN projecting NE/E neurons are necessary for the induction of this effect following chronic stress. Similar to synaptophysin staining, previously reported (Flak *et al.*, 2009) CVS-induced elevation in the density of vGluT2 immunoreactivity was abolished following PVN-targeted DSAP injection (Figure 5B), suggesting that CVS-induced increases in excitatory PVN input is reduced following PVN-targeted DSAP injection. Importantly, these results also indicate that there was neither a compensatory increase in glutamatergic innervation of the PVN nor a significant removal of non-NE/E presynaptic PVN innervation following DSAP lesions.

Discussion

Collectively, our data suggest that PVN-projecting NE/E neurons mediate ACTH responses to novel stressors, both acutely and following exposure to chronic stress. However DSAP injection did not affect corticosterone responses in chronically stress animals, suggesting that removal of NE/E may enhance adrenal sensitivity to ACTH, thereby compensating for the reduced central drive at the level of the PVN. Damage to PVN-projecting NE/E neurons did not block chronic stress-induced alterations in organ or body weight, consistent with the notion that the cumulative glucocorticoid exposure in DSAP CVS and SAP CVS was similar. Together, the data suggest a dual role for central NE/E systems in regulation of the HPA axis during chronic stress: promoting central HPA axis activation and attenuating corticosteroid synthesis at the level of the adrenal cortex. These results suggest that PVN-projecting NE/E neurons are critical for central regulation of PVN responses to chronic, as well as acute stress.

Previous work indicates that NE/E NTS neurons are recruited by both acute and chronic stress (Cullinan *et al.*, 1995; Teppema *et al.*, 1997; Zhang *et al.*, 2010) and are involved in HPA axis responding to glucoprivation (Ritter *et al.*, 2003), consistent with a role in both short- and long-term responses. Our ACTH data are consistent with central HPA drive by NE/E at the PVN, given reduced peak release following stress in DSAP animals. However, our results indicate that the absence of NE/E neurons alters hormone release likely due to modifications in adrenal function, effectively negating central reduction of ACTH release and causing mild hypersecretion of corticosteroids in the morning in CVS rats. Increases in adrenal sensitivity suggest that, if anything, loss of PVN NE may result in enhanced sympathetic drive. It is known that sympathetic drive of adrenal ACTH sensitivity underlies the diurnal corticosterone rhythm (Ulrich-Lai & Engeland, 2002), but this could also be due to changes in melanocortin receptor expression in the adrenal due to ACTH hyposecretion. The observed CVS-induced increase in basal corticosterone, in the absence of increased

ACTH, suggests that a similar mechanism may be engaged during stress. Tracing studies indicate that NTS neurons that project to the hypothalamus are separate from those that project to the ventrolateral medulla or spinal cord, which trigger sympathetic activation (Ritter *et al.*, 2001). Differential projections likely account for the sparing of some 50% of DBH-positive neurons in the NTS. If preserved, NTS projections to the preautonomic areas, such as the ventrolateral medulla, may be sufficient to trigger chronic stress-induced changes in autonomic function (Grippe *et al.*, 2002), which may contribute to HPA axis hyperactivity via autonomic-HPA axis cross-talk both centrally and peripherally with sympathetic innervation of the adrenal gland (Ulrich-Lai & Engeland, 2002; Ulrich-Lai *et al.*, 2006a). In line with this argument, it is known that the NTS provides feedback inhibition of the rostral ventrolateral medulla (RVLM) as part of the baroreceptor reflex (Dampney *et al.*, 2003). Given that the PVN contains a substantial population of parasympathetic preautonomic neurons (Buijs *et al.*, 2003), it is possible that ascending NE neurons may play a similar role in regulation of parasympathetic reactivity at the PVN, resulting in a loss of normal 'inhibition' of sympathoadrenal responses within the context of chronic stress. Loss of this inhibitory signal may remove a brake on RVLM neurons during stress exposure, resulting in an enhanced autonomic response that may contribute to adrenal drive during CVS and in the context of stress exposure. Direct assessment of autonomic function will be required to test this hypothesis.

Previous studies from our lab have reported glucocorticoid-independent increases in NTS tyrosine-hydroxylase (TH) mRNA following CVS (Zhang *et al.*, 2010), suggesting an enhancement of NTS NE/E output to the PVN by unpredictable stress. Given that DSAP lesions do not block enhanced corticosterone responses to novel stressors in CVS animals, this general enhancement of NE/E biosynthetic capacity does not appear to be obligatory for chronic stress-induced HPA axis sensitization (at least at the level of corticosterone release). Thus, other transmitter systems (e.g., glutamate) likely control chronic stress sensitization of the HPA axis, either working independently or in concert with NE/E. However, we should note that 50%, at most, of the catecholaminergic NTS neurons were removed by PVN-targeted DSAP injection, raising the possibility that hyper-responsiveness of remaining NTS NE/E neurons may culminate in HPA facilitation via indirect effects on PVN activation.

Our results indicate that PVN-targeted DSAP injection reduce the number of DBH-immunoreactive neurons in the LC, as well as the NTS. While tract tracing studies have revealed that the NTS supplies the majority of the medial mpPVN with NE (Sawchenko & Swanson, 1982; Cunningham & Sawchenko, 1988), this is not the sole projection location of these neurons. For example, NTS NE neurons also project to the SON (Sawchenko & Swanson, 1982; Cunningham & Sawchenko, 1988), another area where we observed a reduction in DBH-positive fiber density. In addition, loss of DBH-positive cells was also noted within the LC. The LC sends projections to the extreme medial component of the parvocellular PVN and the periventricular zone, largely targeting dopamine-, somatostatin-, and thyrotrophin-releasing-hormone-expressing neurons (Sawchenko & Swanson, 1982). Given the considerable amount of LC NE loss in DSAP animals, there may be a large number of LC neurons that project to the PVN. In addition to PVN CRH neurons, these additional parvocellular PVN projecting axons likely took up DSAP, leading to LC NE cell

loss. Axons of LC NE neurons collateralize extensively throughout the brain, which may account for loss of immunoreactive terminals in regions outside the PVN (e.g., the posterior cingulate gyrus (Fallon & Loughlin, 1982; Loughlin *et al.*, 1982)). However, previous studies have suggested that the LC is not responsive to unpredictable stress. Unlike chronic social stress (Watanabe *et al.*, 1995) and repeated restraint (Mamalaki *et al.*, 1992), LC TH content does not change (Ziegler *et al.*, 1999), suggesting that if anything, the LC may be involved in stress habituation rather than facilitation. However, LC NE loss may regulate acute stress responding. Ablation of the LC NE attenuates HPA axis responses to acute stress (Ziegler *et al.*, 1999), suggesting that HPA axis blunting due to PVN NE/E loss may be mediated by the LC, as well as (or perhaps instead of) the NTS.

The DSAP lesion method destroys neurons that can internalize dopamine beta-hydroxylase. In the PVN, both NE and E terminals take up the DSAP conjugate, and thus the lesion encompasses both cell populations. The PVN is innervated by E-containing terminals, many of which originate in the nucleus of the solitary tract (Mezey *et al.*, 1984; Cunningham *et al.*, 1990). Specific, separate roles for NE and E in HPA axis regulation have not been extensively documented. Nonetheless, it is important to acknowledge that DSAP lesions encompass both catecholaminergic populations, given the possibility that the different receptor affinities of NE and E for PVN alpha vs. beta adrenergic receptors (Szafarczyk *et al.*, 1987) may prove relevant to local stress integration.

The CVS data also suggest that chronic stress is driven by circuits that are independent of PVN NE/E. It is well known that stress responses, particularly those of a psychogenic nature, are regulated by forebrain limbic regions (Jankord & Herman, 2008). Limbic stress excitation is thought to be a disinhibitory process mediated by removal of a tonic 'inhibitory brake' on the PVN (Herman *et al.*, 2005). One proposed mechanism involves amygdala output nuclei (e.g., medial and central amygdaloid nuclei) sending GABAergic projections to GABAergic neurons (e.g., in the bed nucleus of the stria terminalis) that in turn innervate the PVN. These circuits may not be affected by DSAP injections in the PVN (e.g., central amygdaloid NE/E is not reduced in injected animals), and thus may be free to promote chronic stress-induced HPA axis overdrive and weight loss despite reduced hypothalamic NE/E.

Previous work indicates that chronic stress increases the number of synapses in the PVN, prominently including increases in NE/E and glutamate appositions onto CRH neurons (Flak *et al.*, 2009). Our data indicate that DSAP lesions effectively block CVS-induced changes in both synaptophysin and vGluT2 staining, suggesting that increases in PVN synapses are either due to co-localized NE/E and glutamate or reduced NE/E 'drive' of PVN-projecting glutamate neurons. Reduced glutamate innervation of the PVN may contribute to reductions in stress-induced ACTH release, and support a role for increased glutamate, as well as NE/E in hyperdrive of the HPA axis by chronic stress. Given that NE/E projections to the PVN have multiple collateral targets, it would not be surprising to see reduced drive of targeted PVN-projecting vGluT2 containing neurons, located in regions such as the posterior hypothalamus, ventromedial hypothalamus, dorsomedial hypothalamus, and medial preoptic area (Ulrich-Lai *et al.*, 2011b).

While DSAP lesions blocked CVS-induced changes in parvocellular PVN innervation, DSAP treatment increased terminal density within this same region in control animals, indicating that PVN NE/E boutons are replaced by new connections. These added connections likely emanate from axons terminating on the same cell or neighboring cells within the region of DSAP action. However, density of vGluT2 immunoreactivity did not change, indicating that these added boutons do not express vGluT2. The added boutons could still emanate from vGluT2-expressing neurons, but release dense-core vesicles or other non-vGluT2 factors co-expressed from the neuron. Regardless of the mechanism, the modest changes in synaptophysin staining likely do not play a role in basal physiologic regulation (e.g. body weight, body composition, organ weight), but may play a role, albeit an unlikely one, in the reported changes in glucocorticoid responses.

In conclusion, our data support a role for PVN NE/E innervations in the regulation of central responses to both acute and chronic stress. However, it is clear from our data that ablating these neurons is not sufficient to block genesis of somatic and endocrine sequelae of chronic stress, indicating that other regions play a central or perhaps complementary role in generation of stress pathologies. Our work also identifies the adrenal cortex as an important site of chronic stress compensation, adding to a growing literature implicating adrenal sensitivity as a defining feature of glucocorticoid dyshomeostasis in disease (see (Bornstein *et al.*, 2008)).

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Figure 1. PVN and CeA Fiber Densities. PVN-targeted DSAP injection qualitatively and quantitatively reduced DBH-immunoreactivity within the PVN relative to saporin treated animal (A–C), but not in the Central Nucleus of the Amygdala (D–F), indicating targeted removal of PVN-projecting NE/E neurons. The scale bar refers to 100 μ m. * denotes group different from corresponding control group.

Figure 2.

SON and Posterior Cingulate Gyrus Fiber Densities. PVN-targeted DSAP injection did not solely reduce PVN DBH-positive fiber density. PVN-targeted DSAP injection qualitatively and quantitatively reduced DBH-fiber density in the supraoptic nucleus (A–C) (SON) and the posterior cingulate gyrus (E–F). The scale bar refers to 100 μ m. * denotes group different from corresponding control group.

Figure 3.

NTS and LC DBH-immunoreactive cell loss. Reductions in forebrain DBH-positive fiber density were accompanied with loss of DBH-immunoreactive neurones in the NTS (rostral, medial, and caudal) (AC) and LC (D). * denotes group different from corresponding control group.

Figure 4.

HPA axis responses to restraint. PVN- targeted DSAP injection blunted 60 minute corticosterone levels, indicating that noradrenaline hastens the recovery to basal glucocorticoid levels following stress (B). In addition, PVN- targeted DSAP injection elevated basal corticosterone levels in CVS animals, which indicate that noradrenaline attenuates chronic stress- induced hypercortisolemia (B). PVN- targeted DSAP injection also attenuated peak levels of ACTH (A) and corticosterone in control animals (B), but only ACTH in CVS animals (A). This dissociation between CVS and control DSAP animals is associated with a difference in corticosterone/log ACTH (F), an indirect method for determining adrenal sensitivity. The data suggest that PVN noradrenaline activates central drive of the HPA axis, but is overcome by adrenal compensation to yield no difference in peak glucocorticoid levels. * denotes group different from corresponding control group.

Figure 5. PVN bouton density. PVN- targeted DSAP injection eliminated the chronic stress enhancement in PVN bouton density in the PVN, indicating that PVN-projecting noradrenergic neurons are necessary for chronic stress alteration of PVN innervations. Additionally, these results demonstrate that there is not a compensatory elevation in glutamatergic contacts following noradrenergic removal. * denotes group different from corresponding control group.

Table 1**Organ and Body Weight Measures**

	SAP Control	SAP CVS	DSAP Control	DSAP CVS
Adrenal Weight (normalised to body weight)	15.4 ± 0.5	18.1 ± 0.5*	15.5 ± 0.3	17.6 ± 0.5*
Thymus Weight (normalised to body weight)	87.5 ± 5.0	91.3 ± 5.0	87.5 ± 3.7	84.7 ± 3.8
Body Weight Gain (grams)	23.4 ± 2.2	-6.3 ± 1.4*	22.5 ± 1.8	-3.8 ± 2.1*
Lean Mass (grams)	216.5 ± 5.3	190.1 ± 2.3*	211.8 ± 5.0	199.5 ± 2.3*
Adipose Mass (grams)	39.6 ± 1.9	34.3 ± 1.4*	38.3 ± 1.0	32.8 ± 0.7*
CRH fibre density (%area immunoreactive)	21.3 ± 1.1	21.5 ± 0.8	20.5 ± 1.7	23.6 ± 1.4

CVS exposure induced adrenal hypertrophy and attenuated body weight gain through reductions in both lean and adipose mass. However, there was not an interactive effect of injection X stress.

* denotes significant main effect of stress.

Table 2

PVN cFos and CRH fibre density in Acutely Restrained Animals

	SAP Control	SAP CVS	DSAP Control	DSAP CVS
CRH fibre density (%area immunoreactive)	21.3 ± 1.1	21.5 ± 0.8	20.5 ± 1.7	23.6 ± 1.4
PVN cFos (#immunoreactive nuclei)	145.8 ± 15.7	151.4 ± 14.0	147.3 ± 10.6	125.3 ± 11.3

Despite there being an effect of PVN-targeted DSAP injection attenuating peak levels of ACTH in response to restraint, there was no effect of DSAP on CRH fibre density and PVN cFos induction.