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Chronic Stress-Induced Neurotransmitter Plasticity within the Hypothalamic Paraventricular Nucleus

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Abstract

Chronic stress precipitates pronounced enhancement of central stress excitability, marked by sensitization of hypothalamic-pituitary-adrenocortical (HPA) axis responses and increased ACTH secretagogue biosynthesis in the paraventricular nucleus of the hypothalamus (PVN). Chronic stress-induced enhancement of HPA axis excitability predicts increased excitatory and/or decreased inhibitory innervation of the parvocellular PVN. We tested this hypothesis by evaluating chronic variable stress (CVS)-induced changes in total (synaptophysin), glutamatergic (VGluT2), GABAergic (GAD65), and noradrenergic (DBH) terminal immunoreactivity on PVN parvocellular neurons using immunofluorescence confocal microscopy. CVS increased the total PVN bouton immunoreactivity as well as the number of glutamatergic and noradrenergic immunoreactive boutons in apposition to both the CRH-immunoreactive cell bodies and dendrites within the parvocellular PVN. However, the number of GABAergic immunoreactive boutons in the PVN was unchanged. CVS did not alter CRH median eminence immunoreactivity, indicating that CVS does not enhance CRH storage within the median eminence. Taken together, the data are consistent with a role for both glutamate and norepinephrine in chronic stress enhancement of HPA axis excitability. These changes could lead to an enhanced capacity for excitation in these neurons, contributing to chronic stress-induced hyper-reactivity of stress effector systems in the brain.

Keywords

HPA axis; CRH; synaptophysin; glutamate; norepinephrine; GABA

INTRODUCTION

The paraventricular nucleus of the hypothalamus (PVN) is an important site of integration in the regulatory control of the hypothalamo-pituitary-adrenocortical (HPA) axis (Herman et al., 2003). Afferents from limbic, brainstem, and hypothalamic regions converge upon the

medial parvocellular division of the PVN to both precipitate and terminate HPA axis responses to stressors, triggering the neuroendocrine cascade culminating in the release of glucocorticoids (Herman et al., 2003).

The HPA axis can be activated by glutamatergic or noradrenergic afferent stimulation of corticotropin-releasing hormone (CRH) neurons of the PVN. Intraventricular or local glutamate (Makara and Stark, 1975) or norepinephrine (Cole and Sawchenko, 2002; Szafarczyk et al., 1987) infusion stimulate ACTH secretion, corticosterone release, and PVN Fos activation. Local injections of alpha adrenergic and ionotropic glutamate receptor antagonists inhibit stress-induced corticosterone release and PVN Fos induction (Feldman and Weidenfeld, 1997; Itoi et al., 1994; Leibowitz et al., 1989; Ziegler and Herman, 2000). In contrast, PVN CRH neurons are inhibited by GABA inputs. Local blockade of GABA_A receptors with bicuculline methiodide initiates PVN Fos activation in the absence of a stressor (Cole and Sawchenko, 2002), and local application of GABA_A receptor agonists can inhibit stress-induced HPA axis responses (Stotz-Potter et al., 1996). There is evidence for direct innervation of CRH neurons by noradrenergic, glutamatergic, and GABAergic terminals (Liposits et al., 1986; Miklos and Kovacs, 2002; van den Pol, 1991), and all of these transmitters regulate the electrophysiological activity of parvocellular PVN neurons (Boudaba et al., 1997; Boudaba et al., 1996; Daftary et al., 2000). Ionotropic glutamate receptors (Ziegler et al., 2005), alpha-1 adrenergic receptors (Day et al., 1999), and GABA_A receptors (Cullinan, 2000) are all expressed in PVN CRH neurons, providing a means through which these transmitters can control activation and inhibition of HPA axis responses to stress.

Repeated or chronic stress in rodents produces numerous changes in the function and regulation of the HPA axis, including hypersecretion of corticosterone during the circadian trough (Herman et al., 1995a) and facilitated HPA axis responses to novel stressors (Akana et al., 1992). At the level of the PVN, chronic stress upregulates parvocellular PVN CRH and AVP mRNA expression (Herman et al., 1995b; Imaki et al., 1991; Kiss and Aguilera, 1993; Makino et al., 1995). Chronic stress also produces alterations in ionotropic glutamate and GABA_A subunit expression (Cullinan and Wolfe, 2000; Ziegler et al., 2005) that are consistent with enhanced excitability. At the cellular level, chronic stress attenuates mIPSC frequency in the medial parvocellular PVN (Verkuyt et al., 2004). Finally, interleukin 1-beta and amphetamine-induced sensitization of the HPA axis are correlated with reduced dopamine-beta-hydroxylase (DBH) immunoreactivity in the parvocellular PVN (Jansen et al., 2003), suggesting that activity-dependent changes in synaptic actions may be related to altered neurotransmitter innervation.

The current study tests the hypothesis that chronic stress alters neurotransmitter innervation of parvocellular PVN CRH neurons in a manner that favors excitation. To test this hypothesis, we quantified the number of glutamate, norepinephrine, and GABA immunoreactive presynaptic boutons in apposition to CRH neurons within the parvocellular division of the PVN following chronic variable stress (CVS). Our results indicate that exposure to CVS induces striking alterations in excitatory innervation of the parvocellular PVN and support the hypothesis that altered neurotransmitter innervation underlies the enhanced excitatory drive on the HPA axis following chronic stress.

MATERIALS AND METHODS

Subjects

Male Sprague-Dawley rats from Harlan (Indianapolis, IN) weighing 200–225 g upon arrival were group-housed two per cage for the duration of the experiment in clear polycarbonate cages containing granulated corncob bedding, with food and water available *ad libitum*. The colony room was temperature- and humidity-controlled with a 12 h light cycle (lights on 6:00 am; lights off 6:00 pm). Rats acclimated to the colony facility for five weeks prior to experimental manipulations. 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.

Chronic Stress Procedure

Subjects were randomly assigned to either one-week chronic variable stress (n=8) or non-handled control (n=8) groups. The chronic stress protocol consisted of twice-daily (morning and afternoon) exposure to randomly assigned stressors, with occasional overnight stressors, for one week. Morning stressors were conducted between 8:30 am and 10:30 am and afternoon stressors were administered between 2:30 pm and 4:30 pm. Overnight stressors commenced immediately after cessation of the afternoon stressor and ended with the initiation of the following morning's stressor. 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), overnight social isolation (1 rat/cage) and overnight social crowding (6 rats/cage). 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 microtome.

Immunohistochemical Procedures

Brains were cut at 25 µm on a sliding microtome through hypothalamic regions, and the resulting sections were stored in cryoprotectant (0.1 M phosphate buffer, 30% sucrose, 1% polyvinylpyrrolidone, and 30% ethylene glycol) at –20°C until used for immunohistochemistry. Table 1 lists the utilized primary antibodies. The synaptophysin antibody (Zymed laboratories, 18-0130) recognizes a single band of 38 kDa on immunoblot of total homogenate from rat cerebral cortex (Melone et al., 2005). This antibody was designed against amino acids 288–312 of the c-terminus of human synaptophysin using a synthetic peptide fragment with the sequence YGPQGDYQGQGYGPQGAPTSFSNQ (information obtained from Invitrogen). The density of this synaptophysin immunoreactivity labels to a similar density compared to stereologically corrected electron micrographs (Micheva and Beaulieu, 1996). The CRH antiserum (RC70, courtesy of Wylie Vale) recognizes rat CRH by radioimmunoassay (Vale et al., 1983) and fails to label after preabsorption with the peptide (Sawchenko, 1987). The vGluT2 antibody (Synaptic Systems, 135102) recognizes a single 65 kDa band using brain tissue (Zhou et al., 2007) and does not label following preabsorption with the immunizing protein (Graziano et al., 2008). vGluT2 immunoreactivity was specifically localized in regions known to receive projections

from areas with dense vglut2 mRNA expression. It did not correspond with patterns of staining seen using antibodies against VGlut1 (data not shown). The GAD6 antibody (Developmental Studies Hybridoma Bank) recognizes a 59 kDa band by western blot using brain tissue, but not the 63kDa GAD (Chang and Gottlieb, 1988). The antibody also specifically immunoprecipitates GAD65, but not GAD67 (Kaufman et al., 1992). The axonal and perikaryal labeling of the DBH antibody (Chemicon, MAB308) is absent following pre-absorption with a ten-fold excess of bovine adrenal DBH, but not bovine PNMT (Rinaman, 2001).

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). Cryoprotectant was rinsed (5×5 min) in KPBS, the sections were transferred to KPBS + 1.0% H_2O_2 , 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. Sections were incubated overnight at $4^\circ C$ in primary antibody diluted in blocking solution. The following morning sections were rinsed in KPBS (5×5 min) and incubated in biotinylated anti-rabbit secondary antibody (Vector Laboratories, Inc., Burlingame, CA), diluted 1:500 in KPBS + 0.1% BSA for 1 hour at RT. Sections were rinsed in KPBS (5×5 min) and then treated with avidin-biotin complex (ABC, Vector Laboratories, Inc., Burlingame, CA) at 1:1,000 in KPBS + 0.1% BSA for 1 hour at RT. Following this incubation, sections were rinsed again in KPBS (5×5 min) and subsequently incubated in biotin-labeled tyramide (PerkinElmer Life Sciences, Inc., Boston, MA) 1:250 in KPBS with 0.3% H_2O_2 for 10 min at RT. Sections were rinsed in KPBS (5×5 min) and incubated in Cy3-conjugated streptavidin (Jackson ImmunoResearch Labs, West Grove, PA) diluted 1:500 for 30 min at RT on a shaker in the dark. For double-labeling, sections were rinsed (5×5 min) in KPBS and then incubated in the second primary antibody diluted as indicated in KPBS + 0.1% BSA. Following KPBS rinses (5×5 min.), slices were then incubated in Alexa 488-labeled secondary antibody (Molecular Probes, Eugene, OR) diluted 1:500 in KPBS + 0.1% BSA at RT for 30 min, covered. Sections were rinsed five times 5 minutes in KPBS at RT following the final antibody incubation, mounted onto Superfrost Plus slides and coverslipped with Gelvatol. To determine specificity of primary antibodies, control reactions were performed in the absence of one or both primary antibodies.

Image collection and processing

Confocal imaging was performed on a Zeiss 510 Meta microscope system in multichannel mode. All confocal images processed for analysis were collected using a 63x oil immersion lens with a numerical aperture of 1.0; z-step, 0.5 μm ; and image size 1024 x 1024 pixels. Cy3 was excited using the yellow line (568 nm) of the krypton/argon laser to collect images of synaptophysin, while the green line (488 nm) was used to collect images of Alexa 488 labeled neurotransmitters. All image acquisition and quantification was performed by individuals blind to the treatment conditions. Images were consistently collected at but not past the threshold of overexposure to standardize analysis parameters across image stacks. Four z-stacks of the parvocellular division of the PVN (0.5 μm interval; 30 to 40 optical

sections per animal) were collected from each animal at approximately -1.8 mm from bregma (Paxinos and Watson, 1986). For analysis of median eminence CRH content, three z-stacks were taken at the medial portion of the median eminence, using only the Cy3 channel, with the same specifications at approximately -2.8 mm from bregma (Paxinos and Watson, 1986). CRH-labeling of neurons within the PVN was the guideline to determine that z-stacks were taken from the parvocellular division of the PVN as displayed in Figure 1.

All image processing was performed on an IBM compatible computer using Zeiss LSM 510 Image Browser software. Five projections from the middle of each z-stack were created and subsequently quantified for percentage of the field density occupied by synaptophysin immunoreactivity. To produce each projection, z-stacks were subdivided into 5 consecutive images to ensure separation of synaptic boutons. Single projections (first angle = 0, maximum transparency) were generated for each subdivision of the z-stack. Projections were analyzed using the measurement function of Axiovision 4.4 software to obtain the field area percent occupied by the labeled synaptophysin 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. The occupied field area percent across the z-stack was determined by averaging across the projections for each z-stack. 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 (control vs. CVS).

To determine CRH cell size and neurotransmitter innervation of the CRH cells, four CRH-expressing cells were chosen from each z-stack. In order to meet our criteria for selection, each cell had to have 1) definitive CRH immunoreactivity, 2) the total z-plane of the soma visible within the z-stack, 3) a defined nucleus, 4) sufficient separation from other cells to clearly identify neurotransmitter appositions. The cells were identified by slowly scrolling through the z-plane. The first four neurons in the z-stack that satisfied the criteria for analysis were selected by observers blind to treatment condition. In order to quantify the number of cell body neurotransmitter appositions, we counted the total number of immunoreactive boutons in apposition to the soma through the complete z-axis of each of the four cells. These appositions were defined by visualizing absolutely no visible space between the neurotransmitter terminal marker and CRH cell membrane. Figure 2 demonstrates our criteria for the determination of neurotransmitter appositions. To ensure that each bouton was only counted once, the appositions were quantified while scrolling back and forth through all of the optical sections containing a specified quadrant of the immunoreactive cell body. Following the quantification of a given quadrant, we would move clockwise to the next quadrant until all of the appositions were accounted for.

The geometry of CRH neurons did not permit visualization of full dendritic trees. To obtain unbiased estimates of neurotransmitter innervation of CRH dendrites, 8–12 dendrites per z-stack were chosen in a similar manner to the CRH-positive cells. Measured dendrites were all clearly separated from the other cells and dendrites. Dendritic appositions were counted in a similar fashion to soma appositions. Since the number of dendritic appositions is dependent on dendrite length, the length of each dendrite was calculated using the

measurement function on Axiovision 4.4, in order to express the data as the number of appositions per 10 μm of CRH dendrite.

For the analysis of cell size, we estimated cell volume using the unbiased “nucleator” method (Gundersen, 1988). After estimating the radius at a consistent point (the nucleolus), we estimated cellular volume using the equation for the volume of a sphere: $v = 4/3; \pi r^3$. Using Axiovision 4.4, we focused the CRH cell at the level of the nucleolus and measured the distance from the center of the nucleolus to the edge of the cell along a randomly selected angle between 1–90 and again 90, 180, and 270 degrees away from the original angle (Morrow et al., 2005). The radii were averaged for each cell and used to determine cellular volume. Each of these values (cell body appositions, dendritic appositions, and cell volumes) were averaged across the z-stacks taken from each animal and then across the animals by each treatment group (control vs. CVS)

Statistics

Data are reported as mean $\pm$ SEM. All data were analyzed by Student’s t-tests. When necessary, data underwent a log transformation to achieve homogeneity of variance and then reanalyzed. Significance for each experiment was set at $P = 0.05$.

RESULTS

Following the termination of chronic stress, we determined the efficacy of the chronic stress paradigm by assessing alterations in body weight change and thymus weight. As observed previously, CVS attenuated body weight gain [and decreased thymus weights (as a function of its body weight) (Table 2), providing verification of the efficacy of the stress regimen.

Synaptophysin immunoreactivity was used as a marker of presynaptic terminals to determine whether chronic stress increased the number of synaptic inputs to the PVN (Figure 3). The area occupied by immunoreactive synaptic boutons in the medial parvocellular PVN was calculated to quantify transmitter-specific terminal density. The relative density of synaptophysin in the CRH-containing medial parvocellular region of the PVN was higher in CVS than in control animals. This qualitative observation is supported by quantitative data (Figure 3C) demonstrating that CVS exposure increased the area occupied by synaptophysin-labeled terminals within the PVN by about 125% [$t(10) = 3.72$; $P < 0.05$], consistent with neuroplastic alterations in presynaptic inputs to the PVN (Marqueze-Pouey et al., 1991).

Next, we tested whether chronic stress brings upon specific neurotransmitter changes to the CRH cell bodies of the medial parvocellular PVN. Therefore, we predicted that CVS either increased the number of glutamatergic and/or noradrenergic terminals in apposition to the CRH cell bodies or decreased the number of GABAergic terminals. One week exposure elicited marked changes in both glutamatergic [$t(14) = 8.898$, $P < 0.05$] and noradrenergic [$t(14) = 3.553$, $P < 0.05$] terminals in apposition to CRH neurons, as illustrated in Figure 4. In contrast, there was no change in the GABAergic innervation of CRH somata.

It is well known that neuronal somata and dendrites are selectively targeted by afferents from different cell groups. Given that medial parvocellular PVN glutamate and GABA inputs may emanate from multiple sources, we assessed CVS-induced changes in dendritic innervation (Figure 5). In agreement with the results above, CVS increased the number of VGluT2 [$t(14)=2.371$, $P<0.05$] and DBH [$t(14)=2.705$, $P<0.05$] boutons in apposition to CRH-immunoreactive dendrites within the PVN. There were no changes in GAD65 immunoreactive dendritic appositions. These results indicate that CVS alters excitatory innervation of dendrites as well as somata of medial parvocellular CRH neurons.

Magnocellular neurons in the supraoptic nucleus exhibit cellular hypertrophy following dehydration (Hatton and Walters, 1973; Mueller et al., 2005), but it is unknown whether chronic stress similarly affects CRH neuronal size within the PVN. We therefore estimated CRH somatic volume. One week of chronic stress caused a 148% increase in PVN CRH soma volume [$t(14)=4.844$, $P<0.05$], indicating that CVS induces cellular hypertrophy of PVN CRH neurons (Figure 4T).

Due to the observation of CRH cellular hypertrophy following one week of chronic stress, we also investigated whether one week of chronic stress alters CRH fiber density in the median eminence, where the terminals of the PVN CRH cells are located. CVS did not alter the CRH fiber density within the median eminence (Figure 6), indicating that the chronic stress is unlikely to alter the resting pool of releasable CRH in the PVN.

DISCUSSION

The results of this study support the hypothesis that chronic stress produces striking alterations in neurotransmitter innervation of CRH neurons in the parvocellular PVN. Presynaptic innervation of the PVN, as determined by synaptophysin immunoreactivity, was markedly enhanced by prior chronic stress. Further analysis revealed that at least part of this enhanced presynaptic innervation is due to increased glutamatergic and noradrenergic inputs onto somata and dendrites of CRH-expressing cells in the parvocellular PVN following chronic stress. Enhanced excitatory innervation following chronic stress was associated with hypertrophy of parvocellular PVN CRH neurons, but not with changes in CRH stores in fibers located in the median eminence.

Chronic excitation of the HPA axis due to CVS exposure produced a robust increase in the density of synaptophysin, a synaptic vesicle glycoprotein that is an indirect marker of nerve terminal innervation (Masliah and Terry, 1993). Increased density of synaptophysin is suggestive of increased synaptic input, in line with our hypothesis of altered neurotransmitter innervation following chronic stress. These data can be interpreted to indicate that number and/or strength of PVN synaptic input is enhanced post-stress. The current results cannot distinguish among these possibilities. Prior work indicates that chronic restraint stress attenuates synaptophysin protein and mRNA levels in the hippocampus (Cunha et al., 2006; Thome et al., 2001; Xu et al., 2004), a limbic region that exerts negative regulatory control over the HPA axis (Herman et al., 2005). Diminished levels of synaptophysin are suggestive of a loss of synaptic contacts, and electron microscopy studies confirm that chronic stress decreases the total synapse density in CA3 hippocampal subfield

(Sandi et al., 2003; Sousa et al., 2000b), as well as simple asymmetric synapse density in CA3 (Sandi et al., 2003). Collectively, these findings suggest that altered synaptic density occurs in multiple brain regions following stress exposure. Notably, the loss of synapses is associated with functional deficits, which may result in impaired HPA axis inhibition and contribute to the enhancement of synaptic density seen in the PVN (Sousa et al., 2000a; Sousa et al., 2000b).

Glutamatergic innervation of the CRH parvocellular PVN neurons, as reflected by VGluT2 immunoreactivity, was markedly increased following exposure to chronic stress. VGluT1 and VGluT2 immunoreactive terminals are accepted markers of presynaptic glutamatergic terminals, given that these trans-membrane proteins specifically transport glutamate into presynaptic vesicles (Moechars et al., 2006). Notably, VGluT2 is the primary form within the parvocellular division of the PVN (Herzog et al., 2001; Ziegler et al., 2002). Enhanced density of VGluT2 innervation following chronic stress likely reflects an increased capacity for glutamatergic stimulation of CRH neurons within the parvocellular division of the PVN. In agreement with the hypothesis, recent work indicates that VGluT2-immunoreactive axons densely innervate all parvocellular divisions of the PVN, with VGluT2 boutons establishing close contacts to all CRH neurons in the medial parvocellular PVN (Wittmann et al., 2005). Moreover, ultrastructural analysis verified that VGluT2 terminals closely appose CRH-ir perikarya and dendrites (Wittmann et al., 2005). While the anatomical origin(s) of the enhanced glutamatergic innervation is unknown, there are several likely hypothalamic and limbic sources, including the suprachiasmatic nucleus, dorsomedial hypothalamus, anterior hypothalamic nucleus, perifornical area, as well as within the PVN (Boudaba et al., 1997; Daftary et al., 1998; Hermes et al., 1996). There is preliminary evidence to suggest that the increased glutamatergic innervation of CRH neurons of the PVN results in an increase in the synaptic excitation of these cells (Franco et al., 2007).

Norepinephrine terminals in apposition to CRH neurons were also increased after CVS. The PVN receives prominent NE/E input from brainstem regions (Cunningham and Sawchenko, 1988; Sawchenko and Swanson, 1982), and NE exerts an excitatory influence on the HPA axis (Plotsky, 1987). Previous studies indicate that sensitized HPA axis responses to immobilization stress after chronic cold stress are attributable to enhanced responsiveness of post-synaptic α 1-adrenergic receptors in the PVN (Ma and Morilak, 2005) rather than enhanced NE release per se. It is possible that enhanced responsiveness may be associated with increased proximity of NE terminals onto CRH-containing somata. Chronic stress may induce any number of structural or functional alterations in norepinephrine neurons that would increase PVN excitation. In fact, electrophysiological evidence points to norepinephrine having an excitatory effect on PVN neurons by enhancing presynaptic glutamate release (Daftary et al., 2000).

It is unclear how changes in excitatory innervation affect the excitability of CRH neurons of the parvocellular PVN. It is logical to predict that the increased number of excitatory synapses would lead to an increased capacity for excitation of the neurons, but this remains to be tested. Nonetheless, it is important to consider that similar increases in excitatory innervation are observed in magnocellular neurons following both lactation (El Majdoubi et al., 1996) and dehydration (Mueller et al., 2005), and these changes are accompanied by an

increase in glutamatergic excitatory synaptic inputs to these cells (Di and Tasker, 2004; Stern et al., 2000).

We found that the GABAergic innervation of the parvocellular division of the PVN was not altered by chronic stress. A prior study reported that chronic intermittent stress for 21 d markedly attenuated basal inhibitory postsynaptic currents in the parvocellular division of the PVN via a putative reduction in the number of functional GABA synapses (Verkuyl et al., 2004). These findings suggest that stress may result in a decrease in physiologically-active synapses without affecting gross synaptic number. In addition, postsynaptic alterations in the PVN GABAergic innervation may also occur, as mRNA transcripts encoding the $\beta 1$ and $\beta 2$ subunits of the GABA_A receptor are diminished in the PVN following CVS (Cullinan and Wolfe, 2000).

Previous studies in the magnocellular system indicate that chronic stimulation affects cell size. For example, magnocellular neurons in the supraoptic nucleus exhibit hypertrophy during lactation (Gies and Theodosis, 1994), and following chronic dehydration (Hatton and Walters, 1973; Mueller et al., 2005), and chronic restraint stress (Miyata et al., 1994), which suggests that cellular hypertrophy may be a common neurobiological adaptation to increased activity. We observed a significant increase in CRH cell volume following chronic stress (48%) that was similar to that reported in the magnocellular system.

Confocal microscopic quantification of presynaptic terminals in apposition to neurons has been used previously to assess chronic dehydration-induced changes to neurotransmitter afferents of SON vasopressin neurons (Mueller et al., 2005). In this previous study, chronic dehydration increased the number of GABA and glutamate terminals in apposition to vasopressin neurons, which replicated previous studies of synaptic plasticity using quantitative electron microscopy (El Majdoubi et al., 1996; Gies and Theodosis, 1994). Thus, while the confocal approach cannot definitively identify synaptic contacts, it provides a reliable estimate of neuroplastic events occurring within CRH-containing cell groups in the PVN.

While these types of morphological changes have been widely documented in hypothalamic magnocellular neurons of both the SON and PVN, the cellular mechanism of these alterations has not yet been elucidated. Importantly, cell adhesion molecules have been linked to hypothalamic neuroplasticity, with PSA-NCAM being the most widely studied. PSA-NCAM is robustly expressed throughout the SON and PVN (Bonfanti et al., 1992) and is regulated during lactation (Soares et al., 2000). In addition, polysialylation of NCAM in the SON is necessary for the lactation- and dehydration-induced increase in synapses onto magnocellular neurons (Theodosis et al., 1999). It remains to be determined whether PSA-NCAM plays a causal role in PVN stress-related plasticity.

We did not observe a CVS-induced change in CRH content in the median eminence. These data suggest that capacity of CRH storage in the median eminence remains stable despite marked enhancement of CRH biosynthesis (Herman et al., 1995b; Imaki et al., 1991; Kiss and Aguilera, 1993; Makino et al., 1995), CRH cell size, and excitatory inputs to CRH neurons. Together, the data are consistent with increased post-stress release of CRH,

although this prediction requires testing. Previous studies also report inconsistent effects of stress on median eminence CRH stores. Chronic stress paradigms have equivocal effects on the quantity of CRH immunoreactivity in the median eminence (Chappell et al., 1986; de Goeij et al., 1991; Inoue et al., 1993). Acute immobilization stress also has inconsistent effects on CRH stores in the median eminence, with some reports indicating decrements in CRH immunoreactivity and others reporting no significant changes (Chappell et al., 1986; Culman et al., 1991; Inoue et al., 1993).

There are some interpretive caveats that bear consideration. First, since we did not include an acute stress group, we cannot definitively conclude that the observed changes are specific to chronic stress. It is possible that a single stressful event can produce rapid changes in synaptic organization. For example, there is some evidence indicating that acute dehydration (24 hours) can alter soma-somatic appositions and enhances dendritic bundling in magnocellular neurons of the supraoptic nucleus (Hatton et al., 1984; Tweedle and Hatton, 1987). Importantly, chronic dehydration greatly augments these changes, as well as also producing double synapses (Tweedle and Hatton, 1984; Tweedle and Hatton, 1987). Whereas there are no data indicating such rapid changes in axo-somatic and axo-dendritic appositions, as seen in the current study, we cannot definitively preclude this possibility. Second, our approach did not allow us to quantify neurotransmitter appositions within the full dendritic tree of CRH neurons, as 1) full arborizations could not be clearly demonstrated within the 30 μ m sections used in this study and 2) we cannot assume that CRH immunoreactivity completely ‘fills’ parvocellular dendrites. Thus, it is possible that we missed changes in synaptic organization occurring on distal dendrites.

In summary, our data are consistent with a marked neuroplasticity of excitatory neurotransmitter innervation of PVN CRH neurons induced by chronic stress. These results are consistent with abundant data documenting maintenance and, indeed, exacerbation of HPA axis responses following chronic stress, despite the existence of an enhanced feedback signal (i.e., elevated glucocorticoids). Together, the data suggest that a chronic activation of the HPA axis induces adaptations in PVN CRH neurons that allow the system to maintain the capacity to mount stress responses despite its prior stress history. Inappropriate regulation of this neuroplastic mechanism may contribute to glucocorticoid hypersecretion (and perhaps hyposecretion) seen in stress-related disease states and brain aging.

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LITERATURE CITED

- Akana SF, Dallman MF, Bradbury MJ, Scribner KA, Strack AM, Walker CD. Feedback and facilitation in the adrenocortical system: unmasking facilitation by partial inhibition of the glucocorticoid response to prior stress. *Endocrinology*. 1992; 131(1):57–68. [PubMed: 1319329]
- Bonfanti L, Olive S, Poulain DA, Theodosis DT. Mapping of the distribution of polysialylated neural cell adhesion molecule throughout the central nervous system of the adult rat: an immunohistochemical study. *Neuroscience*. 1992; 49(2):419–436. [PubMed: 1436474]

- Boudaba C, Schrader LA, Tasker JG. Physiological evidence for local excitatory synaptic circuits in the rat hypothalamus. *J Neurophysiol.* 1997; 77(6):3396–3400. [PubMed: 9212285]
- Boudaba C, Szabo K, Tasker JG. Physiological mapping of local inhibitory inputs to the hypothalamic paraventricular nucleus. *J Neurosci.* 1996; 16(22):7151–7160. [PubMed: 8929424]
- Chang YC, Gottlieb DI. Characterization of the proteins purified with monoclonal antibodies to glutamic acid decarboxylase. *J Neurosci.* 1988; 8(6):2123–2130. [PubMed: 3385490]
- Chappell PB, Smith MA, Kilts CD, Bissette G, Ritchie J, Anderson C, Nemeroff CB. Alterations in corticotropin-releasing factor-like immunoreactivity in discrete rat brain regions after acute and chronic stress. *J Neurosci.* 1986; 6(10):2908–2914. [PubMed: 3020187]
- Cole RL, Sawchenko PE. Neurotransmitter regulation of cellular activation and neuropeptide gene expression in the paraventricular nucleus of the hypothalamus. *J Neurosci.* 2002; 22(3):959–969. [PubMed: 11826124]
- Cullinan WE. GABA(A) receptor subunit expression within hypophysiotropic CRH neurons: a dual hybridization histochemical study. *J Comp Neurol.* 2000; 419(3):344–351. [PubMed: 10723009]
- Cullinan WE, Wolfe TJ. Chronic stress regulates levels of mRNA transcripts encoding beta subunits of the GABA(A) receptor in the rat stress axis. *Brain Res.* 2000; 887(1):118–124. [PubMed: 11134596]
- Culman J, Kopin IJ, Saavedra JM. Regulation of corticotropin-releasing hormone and pituitary-adrenocortical response during acute and repeated stress in the rat. *Endocr Regul.* 1991; 25(3):151–158. [PubMed: 1662551]
- Cunha GM, Canas PM, Oliveira CR, Cunha RA. Increased density and synapto-protective effect of adenosine A(2A) receptors upon sub-chronic restraint stress. *Neuroscience.* 2006; 141(4):1775–1781. [PubMed: 16797134]
- Cunningham ET Jr, Sawchenko PE. Anatomical specificity of noradrenergic inputs to the paraventricular and supraoptic nuclei of the rat hypothalamus. *J Comp Neurol.* 1988; 274(1):60–76. [PubMed: 2458397]
- Daftary SS, Boudaba C, Szabo K, Tasker JG. Noradrenergic excitation of magnocellular neurons in the rat hypothalamic paraventricular nucleus via intranuclear glutamatergic circuits. *J Neurosci.* 1998; 18(24):10619–10628. [PubMed: 9852597]
- Daftary SS, Boudaba C, Tasker JG. Noradrenergic regulation of parvocellular neurons in the rat hypothalamic paraventricular nucleus. *Neuroscience.* 2000; 96(4):743–751. [PubMed: 10727792]
- Day HE, Campeau S, Watson SJ Jr, Akil H. Expression of alpha(1b) adrenoceptor mRNA in corticotropin-releasing hormone-containing cells of the rat hypothalamus and its regulation by corticosterone. *J Neurosci.* 1999; 19(22):10098–10106. [PubMed: 10559417]
- de Goeij DC, Kvetnansky R, Whitnall MH, Jezova D, Berkenbosch F, Tilders FJ. Repeated stress-induced activation of corticotropin-releasing factor neurons enhances vasopressin stores and colocalization with corticotropin-releasing factor in the median eminence of rats. *Neuroendocrinology.* 1991; 53(2):150–159. [PubMed: 1849619]
- Di S, Tasker JG. Dehydration-induced synaptic plasticity in magnocellular neurons of the hypothalamic supraoptic nucleus. *Endocrinology.* 2004; 145(11):5141–5149. [PubMed: 15297447]
- El Majdoubi M, Poulain DA, Theodosis DT. The glutamatergic innervation of oxytocin- and vasopressin-secreting neurons in the rat supraoptic nucleus and its contribution to lactation-induced synaptic plasticity. *Eur J Neurosci.* 1996; 8(7):1377–1389. [PubMed: 8758945]
- Feldman S, Weidenfeld J. Hypothalamic mechanisms mediating glutamate effects on the hypothalamo-pituitary-adrenocortical axis. *J Neural Transm.* 1997; 104(6–7):633–642. [PubMed: 9444563]
- Gies U, Theodosis DT. Synaptic plasticity in the rat supraoptic nucleus during lactation involves GABA innervation and oxytocin neurons: a quantitative immunocytochemical analysis. *J Neurosci.* 1994; 14(5 Pt 1):2861–2869. [PubMed: 8182444]
- Graziano A, Liu XB, Murray KD, Jones EG. Vesicular glutamate transporters define two sets of glutamatergic afferents to the somatosensory thalamus and two thalamocortical projections in the mouse. *J Comp Neurol.* 2008; 507(2):1258–1276. [PubMed: 18181146]
- Gundersen HJ. The nucleator. *J Microsc.* 1988; 151(Pt 1):3–21. [PubMed: 3193456]

- Hatton GI, Perlmutter LS, Salm AK, Tweedle CD. Dynamic neuronal-glial interactions in hypothalamus and pituitary: implications for control of hormone synthesis and release. *Peptides*. 1984; 5(Suppl 1):121–138. [PubMed: 6384946]
- Hatton GI, Walters JK. Induced multiple nucleoli, nucleolar margination, and cell size changes in supraoptic neurons during dehydration and rehydration in the rat. *Brain Res*. 1973; 59:137–154. [PubMed: 4747747]
- Herman JP, Adams D, Prewitt C. Regulatory changes in neuroendocrine stress-integrative circuitry produced by a variable stress paradigm. *Neuroendocrinology*. 1995a; 61(2):180–190. [PubMed: 7753337]
- Herman JP, Adams D, Prewitt C. Regulatory changes in neuroendocrine stress-integrative circuitry produced by a variable stress paradigm. *Neuroendocrinology*. 1995b; 61(2):180–190. [PubMed: 7753337]
- Herman JP, Figueiredo H, Mueller NK, Ulrich-Lai Y, Ostrander MM, Choi DC, Cullinan WE. Central mechanisms of stress integration: hierarchical circuitry controlling hypothalamo-pituitary-adrenocortical responsiveness. *Front Neuroendocrinol*. 2003; 24(3):151–180. [PubMed: 14596810]
- Herman JP, Ostrander MM, Mueller NK, Figueiredo H. Limbic system mechanisms of stress regulation: hypothalamo-pituitary-adrenocortical axis. *Prog Neuropsychopharmacol Biol Psychiatry*. 2005; 29(8):1201–1213. [PubMed: 16271821]
- Hermes ML, Coderre EM, Buijs RM, Renaud LP. GABA and glutamate mediate rapid neurotransmission from suprachiasmatic nucleus to hypothalamic paraventricular nucleus in rat. *J Physiol*. 1996; 496 (Pt 3):749–757. [PubMed: 8930841]
- Herzog E, Bellenchi GC, Gras C, Bernard V, Ravassard P, Bedet C, Gasnier B, Giros B, El Mestikawy S. The existence of a second vesicular glutamate transporter specifies subpopulations of glutamatergic neurons. *J Neurosci*. 2001; 21(22):RC181. [PubMed: 11698619]
- Imaki T, Nahan JL, Rivier C, Sawchenko PE, Vale W. Differential regulation of corticotropin-releasing factor mRNA in rat brain regions by glucocorticoids and stress. *J Neurosci*. 1991; 11(3):585–599. [PubMed: 2002354]
- Inoue T, Koyama T, Muraki A, Yamashita I. Effects of single and repeated immobilization stress on corticotropin-releasing factor concentrations in discrete rat brain regions. *Prog Neuropsychopharmacol Biol Psychiatry*. 1993; 17(1):161–170. [PubMed: 8416601]
- Itoi K, Suda T, Tozawa F, Dobashi I, Ohmori N, Sakai Y, Abe K, Demura H. Microinjection of norepinephrine into the paraventricular nucleus of the hypothalamus stimulates corticotropin-releasing factor gene expression in conscious rats. *Endocrinology*. 1994; 135(5):2177–2182. [PubMed: 7956940]
- Jansen AS, Schmidt ED, Voorn P, Tilders FJ. Substance induced plasticity in noradrenergic innervation of the paraventricular hypothalamic nucleus. *Eur J Neurosci*. 2003; 17(2):298–306. [PubMed: 12542666]
- Kaufman DL, Erlander MG, Clare-Salzler M, Atkinson MA, Maclaren NK, Tobin AJ. Autoimmunity to two forms of glutamate decarboxylase in insulin-dependent diabetes mellitus. *J Clin Invest*. 1992; 89(1):283–292. [PubMed: 1370298]
- Kiss A, Aguilera G. Regulation of the hypothalamic pituitary adrenal axis during chronic stress: responses to repeated intraperitoneal hypertonic saline injection. *Brain Res*. 1993; 630(1–2):262–270. [PubMed: 8118692]
- Leibowitz SF, Diaz S, Tempel D. Norepinephrine in the paraventricular nucleus stimulates corticosterone release. *Brain Res*. 1989; 496(1–2):219–227. [PubMed: 2804631]
- Liposits Z, Sherman D, Phelix C, Paull WK. A combined light and electron microscopic immunocytochemical method for the simultaneous localization of multiple tissue antigens. Tyrosine hydroxylase immunoreactive innervation of corticotropin releasing factor synthesizing neurons in the paraventricular nucleus of the rat. *Histochemistry*. 1986; 85(2):95–106. [PubMed: 2875047]
- Ma S, Morilak DA. Chronic intermittent cold stress sensitises the hypothalamic-pituitary-adrenal response to a novel acute stress by enhancing noradrenergic influence in the rat paraventricular nucleus. *J Neuroendocrinol*. 2005; 17(11):761–769. [PubMed: 16219005]

- Makara GB, Stark E. Effect of intraventricular glutamate on ACTH release. *Neuroendocrinology*. 1975; 18(2):213–216. [PubMed: 170555]
- Makino S, Smith MA, Gold PW. Increased expression of corticotropin-releasing hormone and vasopressin messenger ribonucleic acid (mRNA) in the hypothalamic paraventricular nucleus during repeated stress: association with reduction in glucocorticoid receptor mRNA levels. *Endocrinology*. 1995; 136(8):3299–3309. [PubMed: 7628364]
- Marqueze-Pouey B, Wisden W, Malosio ML, Betz H. Differential expression of synaptophysin and synaptoporin mRNAs in the postnatal rat central nervous system. *J Neurosci*. 1991; 11(11):3388–3397. [PubMed: 1941089]
- Masliah E, Terry R. The role of synaptic proteins in the pathogenesis of disorders of the central nervous system. *Brain Pathol*. 1993; 3(1):77–85. [PubMed: 8269086]
- Melone M, Burette A, Weinberg RJ. Light microscopic identification and immunocytochemical characterization of glutamatergic synapses in brain sections. *J Comp Neurol*. 2005; 492(4):495–509. [PubMed: 16228991]
- Micheva KD, Beaulieu C. Quantitative aspects of synaptogenesis in the rat barrel field cortex with special reference to GABA circuitry. *J Comp Neurol*. 1996; 373(3):340–354. [PubMed: 8889932]
- Miklos IH, Kovacs KJ. GABAergic innervation of corticotropin-releasing hormone (CRH)-secreting parvocellular neurons and its plasticity as demonstrated by quantitative immunoelectron microscopy. *Neuroscience*. 2002; 113(3):581–592. [PubMed: 12150778]
- Miyata S, Nakashima T, Kiyohara T. Structural dynamics of neural plasticity in the supraoptic nucleus of the rat hypothalamus during dehydration and rehydration. *Brain Res Bull*. 1994; 34(3):169–175. [PubMed: 8055345]
- Moechars D, Weston MC, Leo S, Callaerts-Vegh Z, Goris I, Daneels G, Buist A, Cik M, van der Spek P, Kass S, Meert T, D'Hooge R, Rosenmund C, Hampson RM. Vesicular glutamate transporter VGLUT2 expression levels control quantal size and neuropathic pain. *J Neurosci*. 2006; 26(46):12055–12066. [PubMed: 17108179]
- Morrow BA, Redmond DE Jr, Roth RH, Elsworth JD. Development of A9/A10 dopamine neurons during the second and third trimesters in the African green monkey. *J Comp Neurol*. 2005; 488(2):215–223. [PubMed: 15924344]
- Mueller NK, Di S, Paden CM, Herman JP. Activity-dependent modulation of neurotransmitter innervation to vasopressin neurons of the supraoptic nucleus. *Endocrinology*. 2005; 146(1):348–354. [PubMed: 15388644]
- Paxinos, G.; Watson, C. *The Rat Brain in Stereotaxic Coordinates*. New York: Academic Press; 1986.
- Plotsky PM. Facilitation of immunoreactive corticotropin-releasing factor secretion into the hypophysial-portal circulation after activation of catecholaminergic pathways or central norepinephrine injection. *Endocrinology*. 1987; 121(3):924–930. [PubMed: 3497798]
- Rinaman L. Postnatal development of catecholamine inputs to the paraventricular nucleus of the hypothalamus in rats. *J Comp Neurol*. 2001; 438(4):411–422. [PubMed: 11559897]
- Sandi C, Davies HA, Cordero MI, Rodriguez JJ, Popov VI, Stewart MG. Rapid reversal of stress induced loss of synapses in CA3 of rat hippocampus following water maze training. *Eur J Neurosci*. 2003; 17(11):2447–2456. [PubMed: 12814376]
- Sawchenko PE. Evidence for differential regulation of corticotropin-releasing factor and vasopressin immunoreactivities in parvocellular neurosecretory and autonomic-related projections of the paraventricular nucleus. *Brain Res*. 1987; 437(2):253–263. [PubMed: 3325130]
- Sawchenko PE, Swanson LW. The organization of noradrenergic pathways from the brainstem to the paraventricular and supraoptic nuclei in the rat. *Brain Res*. 1982; 257(3):275–325. [PubMed: 6756545]
- Soares S, von Boxberg Y, Ravaille-Veron M, Vincent JD, Nothias F. Morphofunctional plasticity in the adult hypothalamus induces regulation of polysialic acid-neural cell adhesion molecule through changing activity and expression levels of polysialyltransferases. *J Neurosci*. 2000; 20(7):2551–2557. [PubMed: 10729335]
- Sousa N, Lukoyanov NV, Madeira MD, Almeida OF, Paula-Barbosa MM. Erratum to “Reorganization of the morphology of hippocampal neurites and synapses after stress-induced damage correlates with behavioral improvement”. *Neuroscience*. 2000a; 101(2):483. [PubMed: 11074170]

- Sousa N, Lukoyanov NV, Madeira MD, Almeida OF, Paula-Barbosa MM. Reorganization of the morphology of hippocampal neurites and synapses after stress-induced damage correlates with behavioral improvement. *Neuroscience*. 2000b; 97(2):253–266. [PubMed: 10799757]
- Stern JE, Hestrin S, Armstrong WE. Enhanced neurotransmitter release at glutamatergic synapses on oxytocin neurones during lactation in the rat. *J Physiol*. 2000; 526(Pt 1):109–114. [PubMed: 10878104]
- Stotz-Potter EH, Morin SM, DiMicco JA. Effect of microinjection of muscimol into the dorsomedial or paraventricular hypothalamic nucleus on air stress-induced neuroendocrine and cardiovascular changes in rats. *Brain Res*. 1996; 742(1–2):219–224. [PubMed: 9117398]
- Szafarczyk A, Malaval F, Laurent A, Gibaud R, Assenmacher I. Further evidence for a central stimulatory action of catecholamines on adrenocorticotropin release in the rat. *Endocrinology*. 1987; 121(3):883–892. [PubMed: 3040380]
- Theodosis DT, Bonhomme R, Vitiello S, Rougon G, Poulain DA. Cell surface expression of polysialic acid on NCAM is a prerequisite for activity-dependent morphological neuronal and glial plasticity. *J Neurosci*. 1999; 19(23):10228–10236. [PubMed: 10575020]
- Theodosis DT, Poulain DA. Evidence for structural plasticity in the supraoptic nucleus of the rat hypothalamus in relation to gestation and lactation. *Neuroscience*. 1984; 11(1):183–193. [PubMed: 6709185]
- Theodosis DT, Poulain DA, Vincent JD. Possible morphological bases for synchronisation of neuronal firing in the rat supraoptic nucleus during lactation. *Neuroscience*. 1981; 6(5):919–929. [PubMed: 7242921]
- Thome J, Pesold B, Baader M, Hu M, Gewirtz JC, Duman RS, Henn FA. Stress differentially regulates synaptophysin and synaptotagmin expression in hippocampus. *Biol Psychiatry*. 2001; 50(10):809–812. [PubMed: 11720700]
- Tweedle CD, Hatton GI. Synapse formation and disappearance in adult rat supraoptic nucleus during different hydration states. *Brain Res*. 1984; 309(2):373–376. [PubMed: 6541076]
- Tweedle CD, Hatton GI. Morphological adaptability at neurosecretory axonal endings on the neurovascular contact zone of the rat neurohypophysis. *Neuroscience*. 1987; 20(1):241–246. [PubMed: 3561763]
- Vale W, Vaughan J, Yamamoto G, Bruhn T, Douglas C, Dalton D, Rivier C, Rivier J. Assay of corticotropin releasing factor. *Methods Enzymol*. 1983; 103:565–577. [PubMed: 6321896]
- van den Pol AN. Glutamate and aspartate immunoreactivity in hypothalamic presynaptic axons. *J Neurosci*. 1991; 11(7):2087–2101. [PubMed: 1676727]
- Verkuyt JM, Hemby SE, Joels M. Chronic stress attenuates GABAergic inhibition and alters gene expression of parvocellular neurons in rat hypothalamus. *Eur J Neurosci*. 2004; 20(6):1665–1673. [PubMed: 15355334]
- Wittmann G, Lechan RM, Liposits Z, Fekete C. Glutamatergic innervation of corticotropin-releasing hormone- and thyrotropin-releasing hormone-synthesizing neurons in the hypothalamic paraventricular nucleus of the rat. *Brain Res*. 2005; 1039(1–2):53–62. [PubMed: 15781046]
- Xu H, He J, Richardson JS, Li XM. The response of synaptophysin and microtubule-associated protein 1 to restraint stress in rat hippocampus and its modulation by venlafaxine. *J Neurochem*. 2004; 91(6):1380–1388. [PubMed: 15584914]
- Zhou J, Nannapaneni N, Shore S. Vesicular glutamate transporters 1 and 2 are differentially associated with auditory nerve and spinal trigeminal inputs to the cochlear nucleus. *J Comp Neurol*. 2007; 500(4):777–787. [PubMed: 17154258]
- Ziegler DR, Cullinan WE, Herman JP. Distribution of vesicular glutamate transporter mRNA in rat hypothalamus. *J Comp Neurol*. 2002; 448(3):217–229. [PubMed: 12115705]
- Ziegler DR, Cullinan WE, Herman JP. Organization and regulation of paraventricular nucleus glutamate signaling systems: N-methyl-D-aspartate receptors. *J Comp Neurol*. 2005; 484(1):43–56. [PubMed: 15717303]
- Ziegler DR, Herman JP. Local integration of glutamate signaling in the hypothalamic paraventricular region: regulation of glucocorticoid stress responses. *Endocrinology*. 2000; 141(12):4801–4804. [PubMed: 11108297]

Figure 1. Images demonstrating location of Z-stacks taken within the medial parvocellular PVN
Figure 1A is an image taken at -1.8mm bregma using a 10x objective. The white scale bar represents 1mm. A Cy3 fluorophore is attached to the CRH antibody. The box represents the approximate location of where z-stacks were taken, in the area with the highest CRH cellular density. Figure 1B is a representative projection of ten optical sections taken $0.5\text{ }\mu\text{m}$ apart using a confocal microscope with a 63x objective. The white scale bar represents $20\text{ }\mu\text{m}$.

Figure 2. Demonstration of neurotransmitter apposition quantification

This figure contains a single optical section taken from a confocal z-stack. In this case, DBH is in green and CRH in red. The solid white arrows within the Figure demonstrate boutons that we defined as appositions. However, boutons pointed out by the dashed white arrows did not meet our criteria for an apposition. The white scale bar represents 10 μm .

Figure 3. Effects of CVS on densities of synaptic terminals

Images are taken from the PVN of control rats (A) and rats treated with CVS for one week (B), and represent the projections of 5 optical sections 0.5 μm apart using a 63x objective. The white scale bar at the bottom of each image represents 20 μm . Chronic stress exposure markedly increased the number of synaptic inputs (synaptophysin) within the PVN (C). Data are expressed as mean percentage of control $\pm$ SEM; $n = 8$ for Control, $n = 8$ for CVS; * = CVS is significantly different from control at $P < 0.05$.

Figure 4. Effect of CVS on somatic neurotransmitter appositions on CRH neurons

Series of four optical sections 0.5 μm apart showing VGLUT2-labeled (A–D; green), DBH-labeled (K–N; green), and GAD-labeled (F–I; green) immunoreactive presynaptic terminals colocalized with CRH-labeled neurons (magenta) in the parvocellular portion of the PVN can be seen in this figure. The white scale bar at the bottom of each image represents 10 μm . Quantitative results are illustrated in E, J, O, and T. Chronic stress increased the number of glutamatergic and noradrenergic inputs to CRH neurons of the parvocellular PVN, but had no effect on GABAergic innervation. Chronic stress also caused an increase in the cell volume of CRH-expressing cells, measured at the nucleolus (4T). Neurotransmitter apposition and CRH estimated volume data are expressed as the average number of appositions per cell $\pm$ SEM; $n = 8$ for Control, $n = 8$ for CVS; * = CVS is significantly different from control at $P < 0.05$.

Figure 5. Increase in terminal appositions to PVN CRH dendrites following chronic stress
 Series of four optical sections 0.5 μm apart for VGLUT2-labeled (4A–D; green), DBH-labeled (4K–N; green), and GAD-labeled (4F–I; green) immunoreactive presynaptic terminals apposed to CRH-labeled dendrites (magenta) in the medial parvocellular PVN are exhibited in this figure. The white scale bar at the bottom of each image denotes 10 μm . Chronic stress increased the number of glutamatergic and noradrenergic appositions on CRH dendrites in the parvocellular PVN, but had no effect on the GABAergic innervation. Neurotransmitter apposition data are expressed as the average number of appositions per cell $\pm$ SEM; $n = 8$ for Control, $n = 8$ for CVS; * = CVS is significantly different from control at $P < 0.05$.

Figure 6. CRH fiber density within the median eminence is not altered by chronic stress

Figure 6A illustrates the approximate area of the median eminence where confocal z-stacks were compiled from. One week of CVS had no effect on the density of CRH

immunostaining in the median eminence, expressed as the average mean percentage of control $\pm$ SEM; $n = 8$ for Control and CVS rats (6C). The white scale bar in 6A represents 1 mm, and it represents 20 μ m in 6B.

Table 1

Primary Antibodies used

Antigen	Immunogen	Manufacturer, species, type, catalog number	Dilution used
Synaptophysin	C-terminus of human synaptophysin (288–312)	Zymed laboratories (San Francisco, CA), rabbit antisera, 18-0130	1:300
CRH	human/rat CRF(1–41)	Wylie Vale, rabbit antisera	1:25,000
Vesicular glutamate transporter 2	GST-fusion protein containing amino acid residues 510–582 of rat VGluT2/DNPI	Synaptic Systems, rabbit polyclonal, #135402	1:1,500
GAD-6	Adult rat glutamic acid decarboxylase purified	Developmental Studies Hybridoma Bank (David Gottlieb), mouse monoclonal antibody,	1:100
DBH	Purified bovine DBH	Chemicon, mouse monoclonal, MAB308	1:2,500

Table 2

Body and thymus weight

Treatment Group	body weight change (g) (from original weight)	Thymus weight (% body weight)
Control (n=8)	29.34 ± 1.2	12.5 ± 0.85
CVS (n=8)	4.67 ± 0.88 [*]	10.5 ± 0.30 [*]

Data are expressed as mean ± SEM.

^{*} CVS is significantly different from control at P = 0.05.