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Adrenalectomy Decreases Corticotropin-Releasing Hormone Gene Expression and Increases Noradrenaline and Dopamine Extracellular Levels in the Rat Lateral Bed Nucleus of the Stria Terminalis

Marcos Santibañez,[1] Katia Gysling,[1] and María Inés Forray[1,2]*

[1]Department of Cell and Molecular Biology, Faculty of Biological Sciences, Catholic University of Chile, Santiago, Chile
[2]Department of Pharmacy, Faculty of Chemistry, Catholic University of Chile, Santiago, Chile

The bed nucleus of the stria terminalis (BNST) has a high density of corticotropin-releasing hormone (CRH)-containing neurons that are significantly innervated by noradrenergic and dopaminergic nerve terminals. This limbic structure is involved in the extrahypothalamic response to stress. The purpose of the present work is to study whether the absence of glucocorticoids, induced by a long-term adrenalectomy, regulates CRH gene expression and noradrenaline and dopamine extracellular levels in the rat BNST. The results showed that adrenalectomy decreases CRH mRNA in the dorsal lateral BNST but not in the ventral lateral BNST. Adrenalectomy also decreases CRH-like immunoreactivity both in BNST subnuclei and in the central nucleus of the amygdala. In addition, adrenalectomy significantly increases noradrenaline and dopamine extracellular levels in the lateral BNST. The present results suggest that adrenalectomy regulates CRH gene expression and noradrenaline and dopamine extracellular levels in the BNST in an opposite way. Thus, the present study adds novel evidence further supporting that the BNST and the central nucleus of the amygdala form part of an adrenal steroid-sensitive extrahypothalamic circuit that has been involved in fear and anxiety responses and in clinical syndromes such as melancholic depression, posttraumatic stress disorders, and addiction.

Key words: long-term adrenalectomy; lateral BNST; microdialysis; in situ hybridization; immunoreactivity

The bed nucleus of the stria terminalis (BNST) and the central nucleus of the amygdala (CeA) form part of a complex neural system, the so-called extended amygdala, involved in emotions and in the control of cognitive, autonomic, and behavioral responses to stress and appetite behaviors. Bidirectional projections between the CeA and the dorsal lateral and ventral lateral subnuclei of the BNST have been shown (Alheid and Heimer, 1981; De Olmos et al., 1985; Dong et al., 2001a,b).

The BNST and CeA have a high density of corticotropin-releasing hormone (CRH)-containing neurons (Moga and Gray, 1985; Gray and Magnuson, 1987, 1992; Moga et al., 1989). CRH neurons are located mainly in the dorsal lateral and ventral lateral subnuclei of BNST (Moga and Saper, 1994; Phelix et al., 1994). High levels of circulating glucocorticoids increase CRH mRNA expression in the dorsal lateral BNST (dlBNST) and CeA. The ventral lateral BNST (vlBNST) is less sensitive to glucocorticoids than dlBNST and CeA (Makino et al., 1994a,b). Makino et al. (1999) also showed that psychosocial stress increases CRH mRNA expression in the dlBNST and CeA. Thus, it has been suggested that the lateral BNST and the CeA form part of an adrenal steroid-sensitive extrahypothalamic circuit (Makino et al., 1994a,b; Schulkin et al., 1998).

The BNST and the CeA are innervated by the brainstem A1 and A2 noradrenergic cell groups (Wallace et al., 1989; Riche et al., 1990; Woulfe et al., 1990; Roder and Ciriello, 1994; Delfs et al., 2000; Forray et al., 2000). Nevertheless, different physiological roles between the noradrenergic system of the BNST vs. the CeA have been reported. Noradrenaline (NA) in the lateral BNST regulates stress-induced anxiety responses measured in the elevated plus maze (EPM) but not the reduction of social interaction (SI; Cecchi et al. 2002a). In contrast, NA in the CeA regulates stress-induced anxiety responses reducing the SI but not anxiety responses on EPM (Cecchi et al., 2002b). NA also regu-

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*Correspondence to: Dr. María Inés Forray, Department of Pharmacy, Faculty of Chemistry, Catholic University of Chile, P.O. Box 114-D, Santiago, Chile. E-mail: mforray@bio.puc.cl

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lates adrenocorticotropic hormone (ACTH) plasma levels acting in the BNST, but not in the CeA (Cecchi et al., 2002a,b). Moreover, it has been shown that acute immobilization stress and chronic morphine administration increase NA extracellular levels in the lateral BNST (Pacak et al., 1995; Aston-Jones et al., 1999; Fuentealba et al., 2000; Cecchi et al., 2002a). On the other hand, lateral BNST and CeA are innervated by the ventral tegmental area (VTA) dopaminergic cell group (Phelix et al., 1992; Freedman and Casssell, 1994; Hasue and Shammah-Lagnnado, 2002). This mesocorticolimbic dopaminergic system is involved in the acquisition and expression of emotions and of appetitive behavior related to the rewarding properties of food, sex. and drug of abuse.

The above evidence indicates that both the CRH and the noradrenergic system of the lateral BNST are sensitive to increased circulating levels of glucocorticoids. At present, there is no evidence showing the effect of glucocorticoids on CRH gene expression and extracellular levels of NA and DA in the lateral BNST, under basal conditions. Thus, the aim of the present work was to study the effect of the absence of glucocorticoids, induced by adrenalectomy, on the CRH gene expression and on both NA and DA extracellular levels, in the lateral BNST. For this purpose, nonisotopic in situ hybridization (ISH) and CRH immunohistochemistry were used to study whether the CRH gene expression in the lateral BNST is affected by long-term adrenalectomy. For comparison, the PVN was also studied. In addition, in vivo microdialysis was used to study whether NA and DA extracellular levels in the lateral BNST were also affected by long-term adrenalectomy.

MATERIALS AND METHODS

Experimental Animals

Male Sprague-Dawley rats weighing 210–310 g were used. Rats were maintained on a 14-hr light, 10-hr dark schedule (lights on between 07:00 and 21:00), and food and water were available ad libitum. All experiments were conducted in accordance with institutional (Catholic University of Chile) and international guidelines (NIH guidelines for the care and use of laboratory animals).

Animal Preparation

Adrenalectomy. Male rats weighing 210–250 g were bilaterally adrenalectomized (ADX) via a dorsal approach under xylazine 2% plus ketamine 10% (1:1, 1 ml/kg i.p.) anesthesia. After surgery, isotonic saline (0.9%) was given to ADX rats. Sham-operated rats were subjected to the same procedure, without adrenal extirpation. Water was given to naïve control and sham-operated animals.

Brain tissue fixation for ISH. Rats were deeply anesthetized with chloral hydrate (400 mg/kg ip) and perfused transcardially with physiological saline, followed by 3% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 9.0. Thereafter, brains were removed, cut into blocks, and maintained in paraformaldehyde fixative solution for 30 min. The blocks were transferred and maintained in 30% sucrose in 0.1 M PB during 12 hr. Finally, brain blocks were sectioned into 30-μm coronal slices by means of a vibratome (Vibratome 3000; Technical Products International).

Brain tissue fixation for immunohistochemistry. Rats were deeply anesthetized with chloral hydrate (400 mg/kg) and transcardially perfused with saline (0.9% NaCl), followed by ice-cold 4% paraformaldehyde, 7.5% picric acid in a phosphate-buffered saline solution (PBS; 0.1 M NaH_2PO_4, 0.9% NaCl, pH 7.4). After an overnight postfixation period (using 4% paraformaldehyde, 7.5% picric acid in 0.1 M PBS as a fixative), brains were dehydrated in 30% sucrose solution at 4°C for 48 hr. Thereafter, 30-μm-thick coronal sections were cut at the level of the BNST (0.2–0.6 mm posterior to bregma), CeA (2.0–2.85 mm posterior to bregma), and PVN (1.4–2.12 mm posterior to bregma) on a cryostat (CM 1510; Leica, Heidelberg, Germany).

Microdialysis probe implantation. Rats were deeply anesthetized with chloral hydrate (400 mg/kg ip) and placed in a sterotaxic apparatus (Stoelting, Wood Dale, IL). The skull was exposed, and a small hole was drilled in the area overlying the BNST. A concentric microdialysis probe (2 mm length, 0.24 mm outer diameter; BR-2 Bioanalytical Systems, West Lafayette, IN; or 2 mm length, 0.5 mm outer diameter CMA-11; Carnegie Medicine; Acton, MA) was implanted into the BNST with the following coordinates: 0.3 mm posterior to bregma, 1.5 mm lateral, and 7.6 mm below the dura, according to the atlas of Paxinos and Watson (1987). Body temperature was maintained between 36.5°C and 37.6°C by a thermostatically controlled electric heating pad. Supplemental choral hydrate was given as needed to maintain anesthesia. At the end of each experiment, the animals were killed and brains removed and stored in formalin. Then, sections of 50 μm were obtained by means of a vibratome (Vibratome 3000; Technical Products International) and stained with cresyl violet to verify the probe location.

Nonisotopic ISH

Labeling of oligonucleotide probes. Deoxynucleotide probes (39-mer) synthesized by BIOS-Chile (Santiago, Chile) were used for the ISH experiments. CRH antisense probes were 5′-cagtttcctgttgctgtgagcttgctgagctaactgctc-3′ (CRH-A) and 5′-ttccccaggcggaggaagtattcttcacccatgcggatc-3′ (CRH-1) complementary to nucleotides 680–718 and 343–381 of the CRH mRNA sequence (NM_031019), respectively. One hundred picomoles of CRH-A and CRH-1 probes were 3′ end-labeled by incubation with 55 units of terminal transferase in 20 μl of tailing buffer, 9 nmol of dATP, and 1 nmol of digoxigenin-labeled deoxyuridine-triphosphate (DIG-dUTP).

Immunological detection. Brain coronal sections were rinsed with PBS and then incubated at 60°C in a prehybridization solution containing Denhardt's 4× SSC (0.6 M NaCl and 60 mM sodium citrate, pH 7.0) for 30 min. Tissue sections were then hybridized overnight at 38°C with 10 pmol/ml of CRH-A and CRH-1 DIG-labeled probes in a buffer containing 50% formamide, 1 mg/ml dextran sulfate, and 10 mM dithiotreitol, in 0.06 M Tris, pH 7.5. After hybridization, the tissue slices were rinsed twice with 2× SSC

and once in 1× SSC, for 10 min each at 42°C. For control, similar coronal sections were hybridized in the presence of CRH-A and CRH-1 DIG-labeled probes plus 100× of non-labeled CRH-A and CRH-1 probes.

The presence of DIG labeling in brain tissue sections was detected with anti-DIG antibodies conjugated to alkaline phosphatase (Boehringer Mannheim GmbH Biochemica, Mannheim, Germany) and using NBT and BCIP (Gibco, Gaithersburg, MD) as enzyme substrates. Briefly, tissue sections were initially rinsed with 0.1 M Tris, pH 7.5, and incubated for 90 min with the antibody at room temperature. The sections were then rinsed several times and finally incubated with NBT and BCIP in Tris buffer, pH 9.5, during 12 hr. Finally, the sections were mounted on glass slides with gelatin (0.1%), dried overnight, and observed under light microscopy.

CRH Immunohistochemistry

Free-floating coronal brain sections were placed in 0.3% H_2O_2 and rinsed three times in 0.1 M PBS, followed by 1 hr of incubation in a blocking buffer containing 3% normal goat serum, 0.3% bovine serum albumin (BSA), 0.2% Triton X-100, 0.4% carrageenan (Sigma, St. Louis, MO), and 0.02% sodium azide in 0.1 M PBS. Then, sections were incubated for 18 hr at 4°C with agitation in the presence of a primary polyclonal CRH antibody (kindly provided by Dr. E. Rodriguez, Austral University of Chile) diluted 1/1,000 in a blocking buffer. After being rinsed in 0.1 M PBS, sections were incubated for 1 hr at room temperature with biotinylated goat anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA) diluted 1/1,000 in a blocking buffer. Sections were rinsed in 0.01 M PBS and incubated for 1 hr at room temperature with the avidin-biotin horseradish peroxidase complex (ABC Elite Kit; Vector Laboratories) in PBS. For the development of color reaction, the sections were rinsed in 0.01 M PBS and incubated for 8 min at room temperature, with 0.05% 3.3′-diaminobenzidine (DAB; Sigma) 0.01% H_2O_2 in Tris-saline buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.6), yielding a dark brown staining. Finally, the sections were mounted on glass slides with gelatin (0.1%), dried overnight, and coverslipped for light microscopy analysis.

Analysis of CRH mRNA and Peptide Expression

The CRH mRNA expression was evaluated with an Olympus microscope (Olympus BX51, Melville, NY) equipped with an Olympus digital camera (Camedia C-3030) coupled to a computer (Pentium IV). Positive DIG-labeled cells in the dlBNST, vlBNST, and PVN were counted in coronal sections, at a rostrocaudal level of 0.26 to 0.3 mm posterior to bregma (Paxinos and Watson, 1987). In the case of PVN, positive DIG-labeled cells were counted in coronal sections, at the rostrocaudal level 1.40–2.12 mm posterior to bregma (Paxinos and Watson, 1987). Cells with alkaline phosphatase reaction product originating from DIG-labeled probes were counted in photomicrographs (×20 magnifications) with sample areas of 98.8 μm^2 for each brain area studied; by means of the Image-Pro Plus program (Media Cybernetics). In all experimental animals, three or four slices at the same rostrocaudal level were used to examine the number of DIG-labeled cells in each area studied. Data are presented as mean ± SEM. The number of DIG-labeled cells present in the different experimental groups was compared by a one-way ANOVA, followed by Newman-Keuls post hoc test.

The CRH mRNA signal intensity was also measured in the same mounted brain slices from the vlBNST and PVN as used in the previous analysis. In addition, CRH-like immunoreactivity (CRH-LI) signals were quantified in the dorsal and ventral lateral BNST at 0.26 mm, CeA at 2.00–2.85 mm, and PVN at 1.40–2.12 mm posterior to bregma (Paxinos and Watson, 1987). Brain slices were visualized under a light Nikon microscope (Labophot-2), and images (×20 and ×10 magnifications) were captured with a video camera (Sony CCD-Iris) and digitized with the NIH Image 1.61/ppc program with a computer (Power Macintosh 7600/132). Digitized images were analyzed with the Unscanit program. Cells with alkaline phosphatase reaction product originating from DIG-labeled probes were analyzed in sample areas of 15.7 μm^2 vlBNST and 35.7 μm^2 PVN. In the case of the CRH-like immunoreactivity, the intensity of the labeling was analyzed in sample areas of 109 μm^2 dlBNST, 94 μm^2 vlBNST, 71.95 μm^2 PVN, and 134 μm^2 CeA. The positive signals were quantified by measuring the average pixel intensity/area. The background signal originated from DIG-labeled probes was measured in a nonlabeled lateral area out of the nucleus in all nuclei studied. The background signal originated by CRH-antibody labeling was measured in the anterior comissure for vlBNST and, in the case of dlBNST, CeA, and PVN, in a nonlabeled lateral area out of the nucleus. Background levels obtained in each brain slice were subtracted from the respective values obtained in the dlBNST, vlBNST, CeA, and PVN (Vecchiola et al., 1999). All photographs were obtained with an Olympus digital camera (Camedia C-3030) coupled to an Olympus microscope (Olympus BX51).

Microdialysis

During implantation, the probe was continuously perfused at the rate of 2 μl/min with artificial cerebrospinal fluid (CSF) with a Harvard infusion pump (model 22; Harvard, Dover, MA). The composition of the CSF solution was 120 mM NaCl, 2.4 mM KCl, 1.2 mM $CaCl_2$, 0.9 mM NaH_2PO_4, and 1.4 mM Na_2HPO_4 with 2 μM desipramine (pH 7.4). After a 90-min stabilization period, perfusion sample were collected every 10 min in 4 μl of perchloric acid (0.2 N). Perfusion samples were maintained on ice and subjected to HPLC-EC for determination of NA and DA levels. The in vitro recoveries for NA and DA under these conditions were 7.9% and 7.4%, respectively.

Analysis of the Dialysate

Quantification of NA. Five microliters of the dialysate were injected by means of a Rheodyne injector valve into an HPLC system with the following configuration: a one-piston pump (Shimadzu LC-6A; Kyoto, Japan), a SepStik microbore column [Bionalytical Systems (BAS), West Lafayette, IN], and an amperometric detector (LC4C; BAS). The mobile phase contained 0.1 M NaH_2PO_4, 1.8 mM sodium 1-decanesulfonate, 1.0 mM EDTA, pH 2.3, and it was pumped at a

flow rate of 70 μl/min. The potential of the amperometric detector was set at 650 mV. Under these experimental conditions, retention time for NA was 10 min. Routine limit for NA detection was 0.06 fmol/μl.

Quantification of DA. Five microliters of the dialysate were injected by means of a Rheodyne injector valve into an HPLC system with the following configuration: a dual-piston pump, a SepStik microbore column, and an amperometric detector (LC4C; BAS). The mobil phase used was 100 mM NaH_2PO_4, 1.7 mM 1-octansulfonic acid, and 6% v/v acetonitrile, pH 2.30. Flow rate was 80 μl/min. Oxidation potential was 650 mV. Under these conditions, DA retention time was 6.8 min.

Statistical Analysis

CRH mRNA expression and CRH-LI. The number and intensity of DIG-labeled cells and the intensity of the CRH-LI present in the different experimental groups were analyzed by one-way ANOVA, followed by Newman-Keuls post hoc test. Data are presented as the mean ± SEM of three to six independent experiments.

Microdialysis studies. Data are presented as absolute values, without correcting for recovery; each value corresponds to the mean ± SEM of three to five independent experiments. Data in Figures 4 and 5 were statistically analyzed by two-way repeated-measures ANOVA. Data in the insets in Figures 5 and 6 and Table I were statistically analyzed by one-way ANOVA, followed by Newman-Keuls post hoc test. The statistical analysis was carried out with GraphPad Prism 3.0 (GraphPad Software, San Diego, CA).

Materials

Deoxynucleotide probes were from BIOS-Chile, DIG antibodies conjugated to alkaline phosphatase were from Boehringer Mannheim GmbH Biochemica, and NBT and BCIP were from Gibco. All other reagents used in HIS were of molecular biology grade. NA, DA, desipramine, 1-octansulfonic acid, and sodium 1-decanesulfonate were purchased from Sigma. All other reagents were of analytical grade.

RESULTS

In Situ Expression of CRH mRNA-Positive Cells in Male Rat BNST and PVN

DIG-labeled oligonucleotide probes complementary to CRH mRNA were able to generate positive hybridization signals in the dorsolateral and ventrolateral subnuclei of the BNST and PVN (Fig. 1), in addition to other brain regions, such as cerebral cortex and hippocampus. Control experiments in the presence of ×100 nonlabeled antisense oligonucleotides did not show any significant DIG-labeled cells in all the brain regions studied (data not shown).

Effect of Adrenalectomy on the Number of Cells Expressing CRH mRNA in BNST

Numerous cells expressing CRH mRNA were observed in the BNST of naïve and sham-operated rats. These DIG-labeled cells were grouped mainly in the dorsolateral and ventrolateral aspects of the BNST (Fig. 1). Positive DIG-labeled cells in both BNST nuclei were counted in coronal sections, at a rostrocaudal level from 0.26 to 0.4 mm posterior to bregma (Paxinos and Watson, 1987) in naïve male rats and after 28 days of adrenalectomy or sham operation. A significant decrease in the number of cells expressing CRH mRNA was observed in dlBNST but not in the vlBNST of ADX rats, compared with naïve and sham-operated rats (Figs. 1, 2).

TABLE I. Semiquantitative Analysis of the Intensity of DIG-Labeled Cells Expressing CRH mRNA in the BNST and PVN†

Condition	Intensity of DIG-labeled cells (average pixel intensity/area)	
	vlBNST	PVN
Naïve	9.78 ± 1.52	2.85 ± 0.87
Sham-operated	20.11 ± 10.16	4.59 ± 0.85
ADX	6.80 ± 1.28	25.55 ± 8.47★

†The intensity of the labeled cells was measured in sample areas from each nucleus of the different experimental groups of rats as described in Materials and Methods. Data are mean ± SEM values of three to five independent experiments. A one-way ANOVA showed a significant difference between experimental conditions in all areas studied. vlBNST: $F = 1.70$; $R^2 = 0.33$; $P < 0.249$. PVN: $F = 6.542$; $R^2 = 0.685$; Newman-Keuls multiple comparison *post hoc* test. $P < 0.031$.
★$P < 0.05$ compared with *naïve* and sham-operated rat.

Effect of Adrenalectomy on the Number of Cells Expressing CRH mRNA in PVN

Numerous cells expressing CRH mRNA were observed in PVN of naïve and sham-operated rats (Fig. 1). These cells were located mainly in the magnocellular division of PVN. Positive DIG-labeled cells in the PVN were counted in coronal sections, at the rostrocaudal level from 1.4 to 2.12 mm posterior to bregma (Paxinos and Watson, 1987), in all experimental groups. Twenty-eight days after adrenalectomy, no significant changes in the number of cells expressing CRH mRNA were observed in the PVN of ADX rats compared with naïve and sham-operated rats (Figs. 1, 2).

Effect of Adrenalectomy on the Intensity of the CRH mRNA Labeling in BNST and PVN

To test whether the number of cells expressing CRH mRNA is a fine method for distinguishing the changes in CRH mRNA expression, analysis of intensity of the DIG labeling was performed in the same vlBNST and PVN slices used to study the number of cells expressing the CRH mRNA. The results show no significant changes in the intensity of the DIG labeling in the vlBNST of the ADX rats compared with naïve and sham-operated rats (Table I). In contrast, the analysis of intensity of the DIG labeling shows a significant increase in the CRH mRNA expression in the PVN of ADX

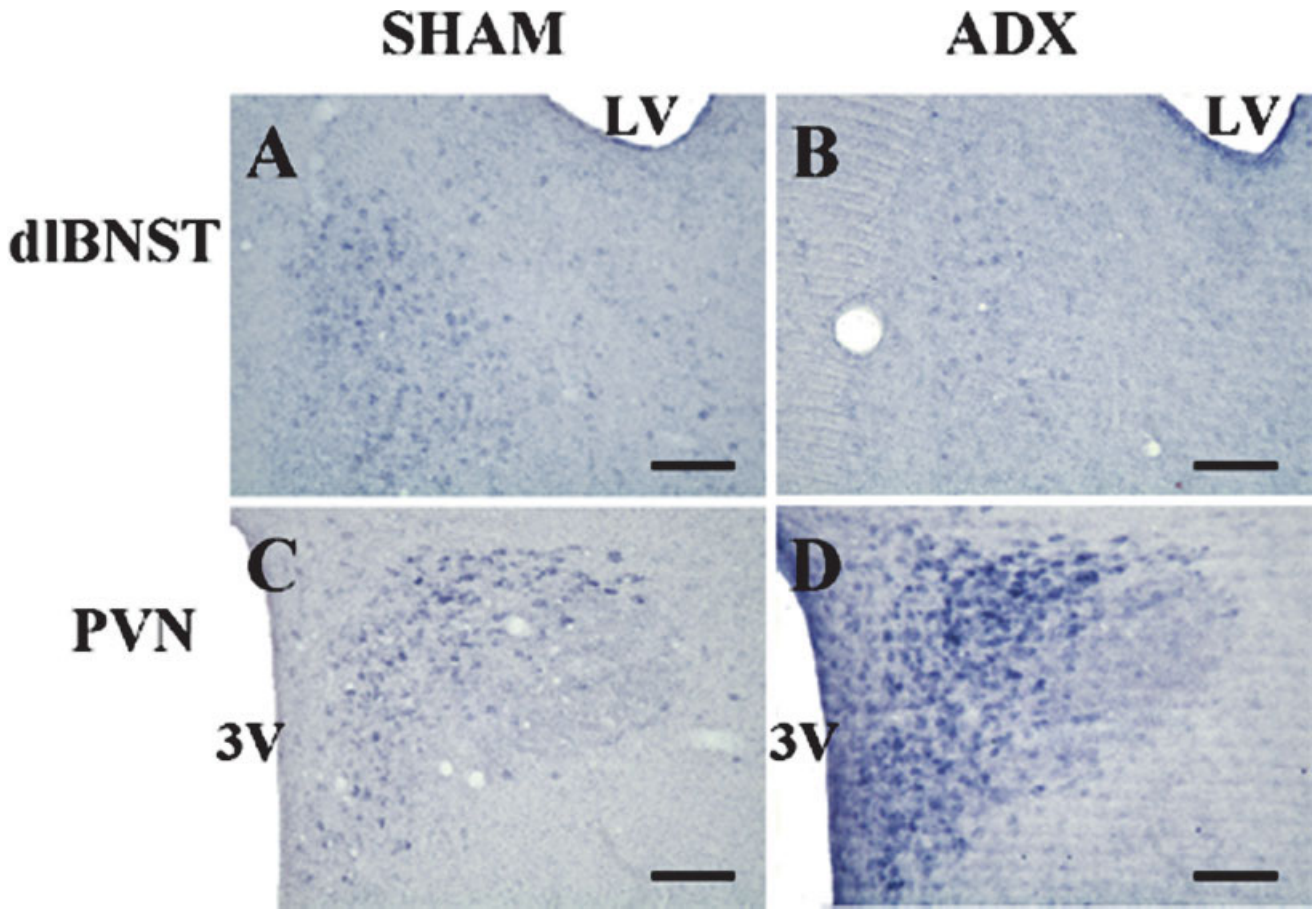

Fig. 1. Expression of CRH mRNA in the dorsal lateral BNST and PVN of male rats (A–D). Coronal brain slices hybridized with the DIG-labeled probes complementary to CRH mRNA (CRH-1 and CRH-A), as described in Materials and Methods. **A:** dlBNST of a sham-operated rat. **B:** dlBNST of an ADX rat. **C:** PVN of a sham-operated rat. **D:** PVN of an ADX rat. LV, lateral ventricle; 3V, third ventricle. Scale bars = 100 μm. Figure can be viewed in color online via www.interscience.wiley.com.

rats compared with naïve and sham-operated rats (Table I). In the case of the dlBNST, the low intensity of DIG labeling observed in ADX rats did not allow to discriminate between label background signals.

Immunocytochemistry of CRH in BNST and PVN

The CRH antibody generated positive immunoreactive signals with the known pattern of CRH expression. Positive immunolabeling was observed in the dorsolateral and ventrolateral subnuclei of the BNST, CeA, and PVN (Fig. 3), in addition to other brain regions, such as cerebral cortex and hippocampus. Control experiments in the absence of CRH antibody did not show significant immunoreactive signals in any of the brain regions studied. In addition, the possible cross-reaction of the antibody with urocortin, a closely related peptide of the CRH family, was evaluated at the level of the Edinger Westphal nucleus, which presents the highest expression of urocortin. No significant labeling was observed (data not shown).

Effect of Adrenalectomy on the CRH Immunoreactivity in BNST and CeA

Positive CRH-LI was observed in both the BNST and the CeA of naïve and sham-operated rats. This CRH-LI labeling was grouped mainly in the dorsal lateral and ventral lateral aspects of the BNST (Fig. 3) and the CeA. The intensity of the positive CRH-LI labeling of both BNST nuclei and the CeA was measured in coronal sections at a rostrocaudal level from 0.26 to 0.4 mm and in the CeA from 2.00 to 2.85 mm posterior to bregma (Paxinos and Watson, 1987) in naïve, sham-operated, and ADX rats. Significant decreases in the intensity of CRH-LI labeling were observed in both dorsolateral and ventrolateral BNST and the CeA of the ADX rats compared with naïve and sham-operated rats (Fig. 4).

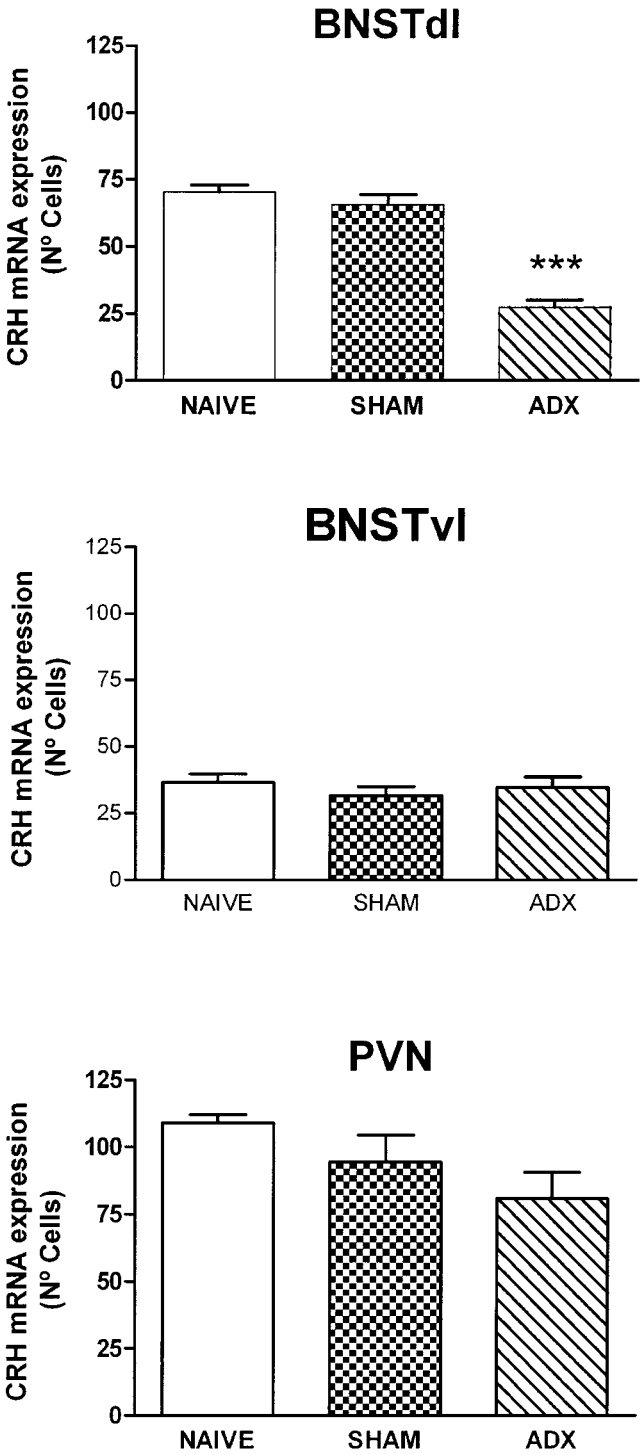

Fig. 2. Quantification of cells expressing CRH mRNA in the dorsal lateral and ventral lateral BNST and the PVN of male rats. Labeled cells were counted in sample areas from each nucleus of the different experimental groups of rats as described in Materials and Methods. Data are mean ± SEM values of three independent experiments. An one-way ANOVA showed a significant difference between experimental conditions in all areas studied. dlBNST: $F = 63.58$; $P < 0.0001$. vlBNST: $F = 0.429$; $P < 0.6671$. PVN: $F = 2.93$; $P < 0.129$. Newman-Keuls multiple-comparisons post hoc test gave $^{***}P < 0.001$ compared with naïve and sham-operated rats in the dlBNST.

Effect of Adrenalectomy on the CRH Immunoreactivity in PVN

Positive CRH-LI labeling was observed in the PVN of naïve and sham-operated rats. This CRH-LI was located mainly in the magnocellular division of PVN (Fig. 3). The intensity of positive CRH-LI labeling of the PVN was measured in coronal sections, at a rostrocaudal level from 1.4 to 2.12 mm posterior to bregma (Paxinos and Watson, 1987) in naïve, sham-operated and ADX rats. There was a significant increase in the intensity of CRH-LI labeling in the PVN of the ADX rats compared with naïve and sham-operated rats (Figs. 3, 4).

Effect of Adrenalectomy on NA and DA Extracellular Levels in Lateral BNST

For all microdialysis experiment, CSF in the presence of 2 μM desipramine was perfused through a microdialysis probe located in the lateral BNST. After a stabilization period of 90 min, nine samples of 10 min were collected from each rat, and NA and DA extracellular levels were measured in each sample. As shown in Figure 5, the time course of NA extracellular levels in the lateral BNST showed that adrenalectomy induces a significant increase in NA extracellular levels in comparison with naïve and sham-operated rats. The inset in Figure 5 shows the basal NA extracellular levels under the three experimental conditions. No significant difference was observed between naïve (5.09 ± 0.88 fmol/μl) and sham-operated (5.81 ± 1.04 fmol/μl) rats. In contrast, ADX rats show a significant increase in the NA basal extracellular levels (9.96 ± 0.63 fmol/μlk). The one-way ANOVA analysis revealed a significant difference between experimental conditions ($F = 8.85$, $R^2 = 0.6389$, $P < 0.0061$). Newman-Keuls multiple-comparisons post hoc test gave a significance of $P < 0.01$ compared with naïve and sham-operated rats.

In the case of DA, the analysis of the time course of DA extracellular levels in the lateral BNST also showed that ADX induces a significant increase in DA extracellular levels in comparison with naïve or sham-operated rats (Fig. 6). The inset in Figure 6, shows the basal DA extracellular levels. One-way ANOVA analysis showed a significant difference between experimental conditions ($F = 11.9$, $R^2 = 0.726$, $P < 0.003$). No significant difference between naïve (0.68 ± 0.19 fmol/μl) and sham-operated (2.44 ± 0.76 fmol/μl) rats was observed. In addition, ADX (4.07 ± 0.83 fmol/μl) rats showed a significant increase in comparison with both sham-operated ($P < 0.05$) and naïve ($P < 0.01$) rats (Newman-Keuls multiple-comparisons post hoc test).

Effect of Adrenalectomy on Body Weight

The body weight of the rats was controlled during the 28 days in all experimental groups as an index of ADX (Gosselin and Cabanaca, 1997). As shown in

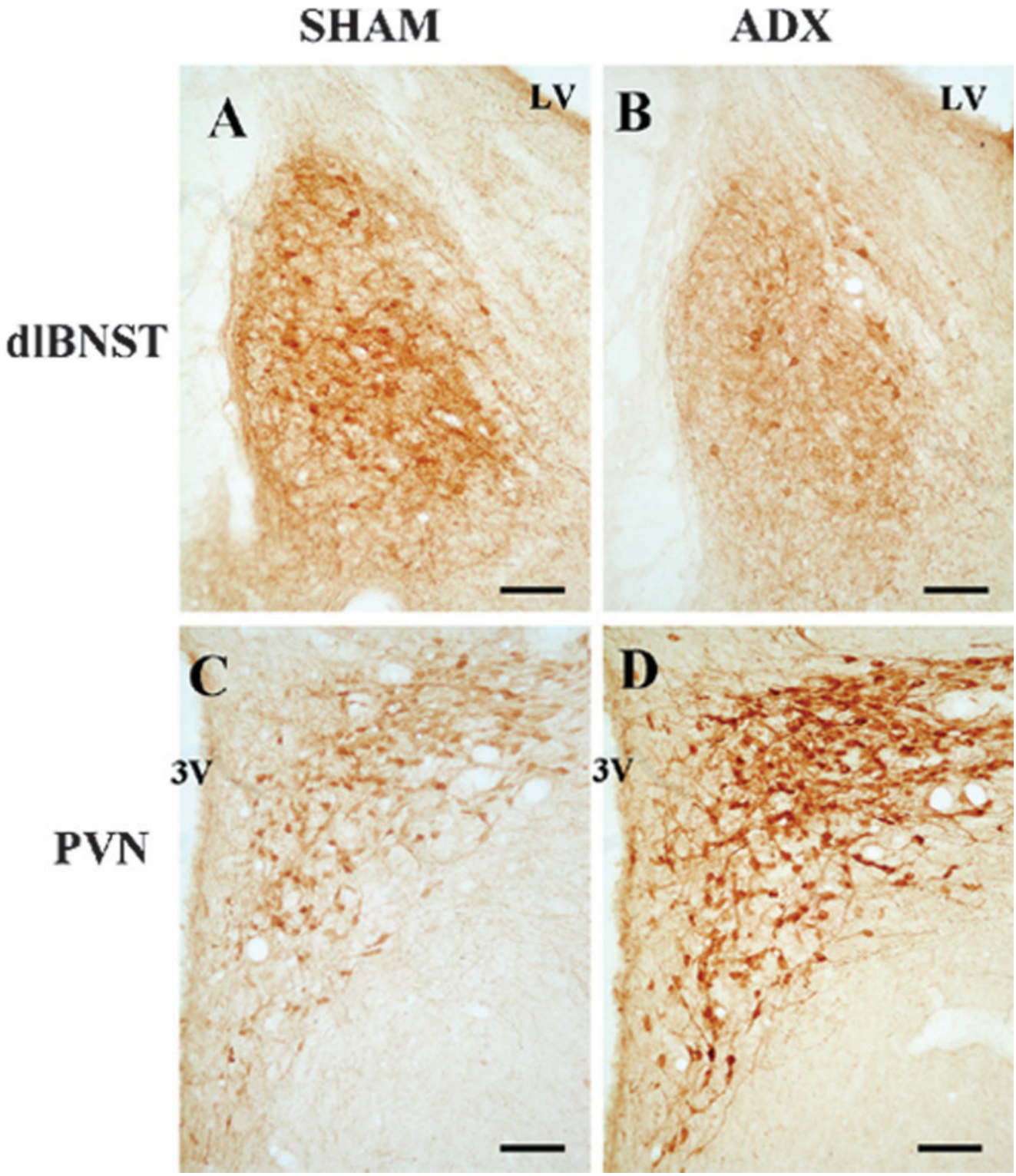

Fig. 3. CRH-like immunoreactivity in the dlBNST and PVN of male rats (A–D). Coronal brain slices with the CRH-LI labeling, as described in Materials and Methods. **A:** dlBNST of a sham-operated rat. **B:** dlBNST of an ADX rat. **C:** PVN of a sham-operated rat. **D:** PVN of an ADX rat. LV, lateral ventricle; 3V, third ventricle. Scale bars = 100 μm.

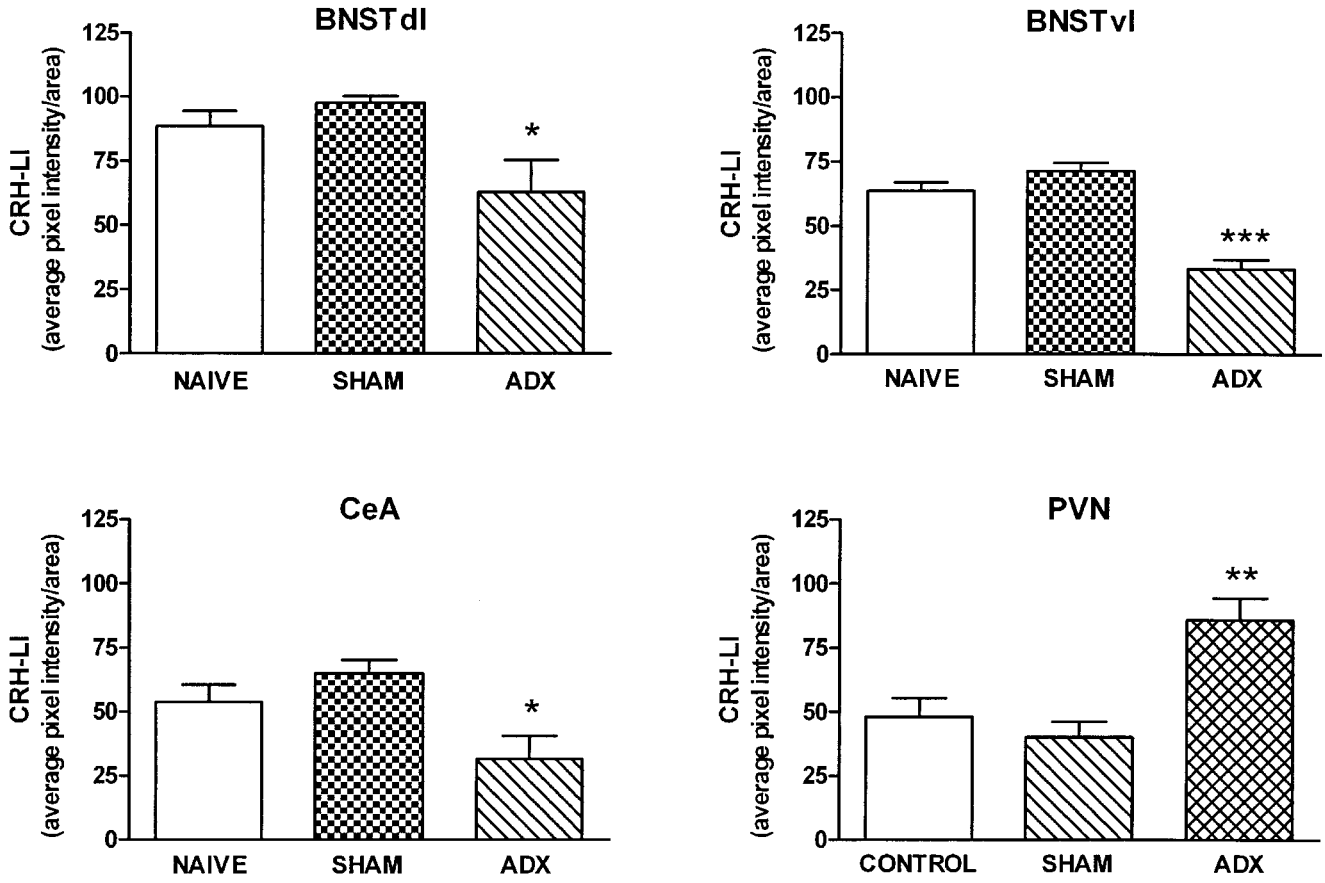

Fig. 4. Semiquantitative analysis of the intensity of CRH-LI in the BNST, CeA, and PVN. CRH-LI was measured in sample areas from each nucleus of the different experimental groups of rats as described in Materials and Methods. Data are mean ± SEM values of three independent experiments. A one-way ANOVA showed a significant difference between experimental conditions in all areas studied. dlBNST: $F = 5.57$, $P < 0.017$. vlBNST: $F = 33.23$, $P < 0.0001$. CeA: $F = 5.32$, $P < 0.026$. PVN: $F = 11.22$, $P < 0.0056$. Newman-Keuls multiple comparison post hoc test gave $^{*}P < 0.05$ compared with naïve and sham-operated rats in the dlBNST and CeA, $^{**}P < 0.01$ compared with naïve and sham-operated rats in the PVN. $^{***}P < 0.001$ compared with naïve and sham-operated rats in the vlBNST.

Table II, ADX rats showed a significant decrease in body weight in comparison with sham-operated rats ($P < 0.002$). Direct observation of the absence of the adrenal gland further confirmed the total extirpation of the adrenal glands.

DISCUSSION

The present study shows that, in the absence of glucocorticoids induced by adrenalectomy, the CRH gene expressions in dlBNST and PVN are differentially regulated and that adrenalectomy increases both NA and DA extracellular levels in the BNST. First, the in situ expression of CRH mRNA and CRH-LI was similar in the BNST subnuclei studied and PVN in naïve and sham-operated animals. Second, adrenalectomy induced a significant decrease in the in situ expression of CRH mRNA in the dlBNST but not in the vlBNST and a significant increase in PVN. Third, adrenalectomy induced a significant decrease of CRH-LI in dlBNST, vlBNST, and CeA and a significant increase in the PVN. Fourth, the results also show that these changes in CRH expression occur together with an increase in NA and DA extracellular levels in the lateral BNST.

In Situ Expression of CRH mRNA in BNST and PVN

In the present work, numerous CRH mRNA-expressing cells in the BNST and PVN were observed. These results corroborate previous findings showing that BNST contains two populations of CRH neurons, dorsolateral and ventrolateral groups (Moga et al., 1989; Phelix and Paull, 1990; Moga and Saper, 1994). Moreover, the nonisotopic ISH used in the present study allowed us to analyze the number of cells expressing CRH mRNA and the intensity of DIG labeling. Long-term adrenalectomy decreased the number of DIG-labeled cells as well as the intensity of the DIG labeling in the dlBNST, but not in the vlBNST. In the case of the PVN, no changes in the number of DIG-labeled cells and an increase in the intensity of the DIG labeling were observed, suggesting that all CRH mRNA-containing cells in PVN were expressing measurable levels

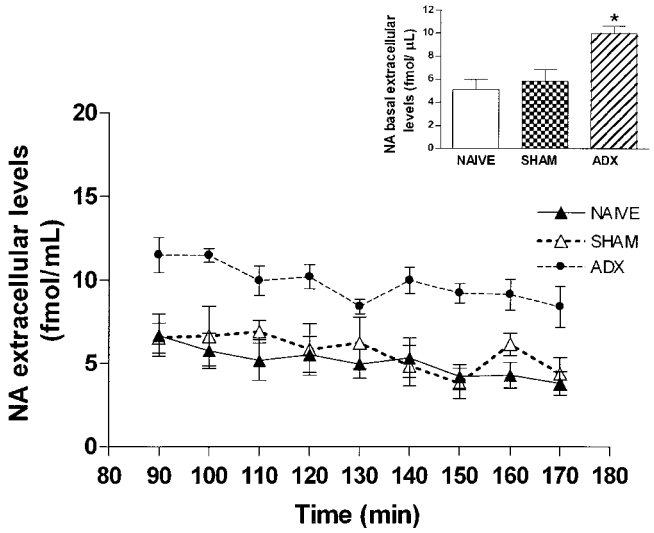

Fig. 5. Time course of NA extracellular levels in the lateral BNST of ADX rats. Rats were ADX (circles) or sham-operated (open triangle) 28 days before the microdialysis experiments. Naïve (solid triangles) rats correspond to intact control animals. In vivo microdialysis with the rat under general anesthesia was performed on day 28 after sham operation or adrenalectomy as described in Materials and Methods. Data are the mean ± SEM of four or five independent experiments. A two-way repeated-measures ANOVA showed a significant effect of adrenalectomy ($F = 68.42$, $P < 0.0001$ and $F = 104.6$, $P < 0.0001$ compared with sham-operated and naïve rats, respectively). The time and interaction are considered nonsignificant. The **inset** shows the mean ± SEM of the basal extracellular levels of NA of four or five independent experiments. $^{*}P < 0.05$ compared with sham-operated and naïve rats.

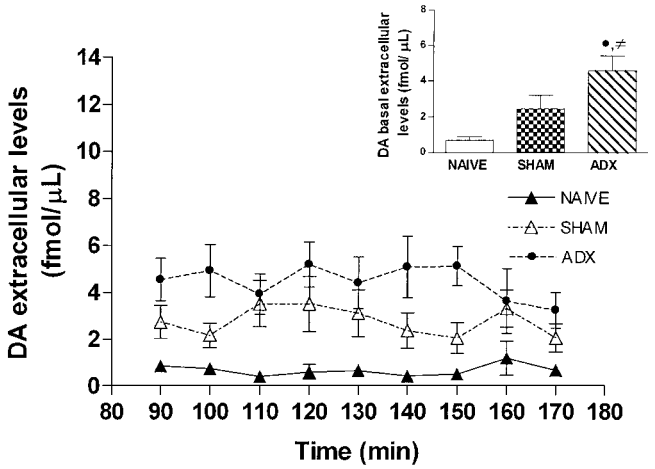

Fig. 6. Time course of DA extracellular levels in the lateral BNST of ADX rats. Rats were ADX (circles) or sham-operated (open triangles) 28 days before the microdialysis experiments. Naïve (solid triangles) rats correspond to intact control animals. In vivo microdialysis with the rat under general anesthesia was performed on day 28 after sham operation or adrenalectomy as described in Materials and Methods. Data are the mean ± SEM of four or five independent experiments. A two-way repeated-measures ANOVA showed a significant effect of adrenalectomy ($F = 11.37$, $P < 0.0017$ and $F = 45.06$, $P < 0.0001$ compared with sham-operated and naïve rats, respectively). The time and interaction are considered nonsignificant. The **inset** shows the mean ± SEM of the basal extracellular levels of DA of three or four independent experiments. $^{\bullet}P < 0.01$ compared with naïve rats. $^{\neq}P < 0.05$ compared with sham-operated rats.

of CRH mRNA under basal conditions. In this regard, it has been proposed that CRH mRNA levels in the PVN of unstressed animals are at maximal levels and that increases of corticosterone induced by diurnal surge or stress are sufficient to decrease these levels (Swanson and Simmons, 1989).

Immunohistochemistry of CRH in BNST, CeA, and PVN

In the present study, numerous cells and nerve fibers showing CRH-LI were observed in the dlBNST, CeA, and PVN. The present results corroborate previous findings showing CRH-containing neurons and nerve fibers in the lateral BNST (Moga et al., 1989; Phelix and Paull, 1990), CeA (Moga and Gray, 1985), and PVN (Olschowka et al., 1982a,b). Principally, CRH-containing nerve terminals were observed in the vlBNST.

Effect of Adrenalectomy on CRH Gene Expression in BNST and PVN

The present results showed that, after long-term adrenalectomy, the number of cells expressing CRH mRNA and the labeling intensity significantly decreases in the dlBNST. A previous study using an isotopic ISH and short-term adrenalectomy (7 or 14 days) showed that adrenalectomy did not affect CRH mRNA levels in the dlBNST (Makino et al., 1994b; Watts and Sanchez-Watts, 1995). These apparent differences may be due to the different ISH methods and/or the time of adrenalectomy used. In the case of the vlBNST, the results showed that long-term adrenalectomy does not affect the CRH mRNA levels of expression in the vlBNST in agreement with previous findings showing that short-term adrenalectomy does not affect the CRH mRNA levels in the vlBNST (Makino et al., 1994b; Watts and Sanchez-Watts, 1995), suggesting that CRH expression in the vlBNST is not regulated by, or at least is less responsive to, circulating glucocorticoids. This is further supported by the fact that only constant high levels of corticosteroids increase CRH mRNA expression in the vlBNST (Makino et al., 1994b). Both nonisotopic and isotopic ISH show similar effects on CRH mRNA expression in the vlBNST and PVN, suggesting that changes in CRH mRNA expression in the dlBNST require a longer period of adrenalectomy to be observed. Further studies are necessary to address this issue.

In addition, the present results showed CRH-LI decreases in the dlBNST and vlBNST after long-term adrenalectomy. The results also showed that adrenalectomy decreases CRH-LI in the CeA. In this regard, it

TABLE II. Body Weight of Sham-Operated and ADX Rats†

Body weight (g)	Initial	Final
Sham	202.28 ± 3.66	372.50 ± 12.76
ADX	198.62 ± 2.12	317.10 ± 8.26★

†The initial body weight represents the values before sham operation or adrenalectomy. Final body weight represents the values on day 28 after sham operation or adrenalectomy. The values correspond to the mean ± SEM (n = 7–18).
★$P < 0.002$ compared with sham-operated rats.

has been described that CRH-containing nerve fibers from the CeA project into the lateral division of the BNST (Sakanaka et al., 1986). Thus, it is tempting to suggest that the observed decrease in CRH-LI in the lateral BNST may be due to a decrease in CRH-containing fibers arising from the CeA. Accordingly, a decrease in the number of CRH mRNA expressing cells of the CeA 5 days after adrenalectomy has been shown. This decrease was prevented by glucocorticoids (Palkovits et al., 1998).

The present findings showing that long-term adrenalectomy increases the levels of CRH mRNA expression in PVN CRH neurons are in agreement with previous evidence, obtained by isotopic ISH CRH mRNA detection. This evidence shows that, after short-term adrenalectomy, the levels of CRH mRNA expression in the PVN are increased (Young et al., 1986; Beyer et al., 1988; Swanson and Simmons, 1989; Imaki et al., 1991; Makino et al., 1994a; Watts and Sanchez-Watts, 1995; Herman and Morrison, 1996). Moreover, the present results also showed that long-term adrenalectomy increases CRH-LI in the PVN, corroborating previous evidence obtained by immunoradiographic CRH peptide detection (Herman and Morrison, 1996). The results further support the hypothesis that CRH expression in the PVN is negatively regulated by circulating glucocorticoids (Beyer et al., 1988; Imaki et al., 1991; Watts and Sanchez-Watts, 1995). Taken together, the above-described results indicate that long-term adrenalectomy regulates CRH expression in the dlBNST and PVN in opposite ways, suggesting that these brain nuclei have an opposite response to circulating corticosteroids.

Effect of the Adrenalectomy on NA and DA Extracellular Levels in Lateral BNST

The present results showed a significant increase in NA extracellular levels in the lateral BNST after long-term adrenalectomy in agreement with previous evidence showing that adrenalectomy increases NA extracellular levels in the PVN (Pacak et al., 1993a). In this regard, it has been shown that tyrosine hydroxylation increases in A1 and A2 noradrenergic cells 10 days after adrenalectomy and that this effect is also prevented by glucocorticoid treatment (Lachuer et al., 1992). The above evidence is further supported by the fact that most noradrenergic neurons of groups A1–A7 show glucocorticoid receptor immunoreactivity (Härfstrand et al., 1986). It is noteworthy that the ventral region of the BNST is innervated mainly from brainstem noradrenergic cell groups A1 and A2, which also innervate the PVN (Riche et al., 1990; Woulfe et al., 1990; Roder and Ciriello, 1994; Forray et al., 2000).

Acute immobilization stress increases NA release and turnover in the PVN, whereas chronic immobilization stress decreases NA basal levels without affecting immobilization-induced NA release in the PVN (Pacak et al., 1992). Adrenalectomy also increases immobilization-induced NA release and turnover in the PVN, effects that are prevented by glucocorticoids (Pacak et al., 1993a; Vetrugno et al., 1993). Thus, these results suggest that immobilization stress stimulates hypothalamic-pituitary-adrenal (HPA) axis activity in part by increasing NA release in the PVN and that high circulating glucocorticoid levels exert a negative feedback control to limit the duration of the stress-induced activation of brainstem noradrenergic projections to the PVN, thus restraining the CRH response. Acute immobilization stress also increases NA release and turnover in the lateral BNST (Pacak et al., 1995; Cecchi et al., 2002a). Furthermore, the infusion of a specific α_1-adrenergic antagonist in the lateral BNST decreases stress-induced ACTH plasma levels (Cecchi et al., 2002a). It has also been shown that acute immobilization stress increases NA release and turnover in the CeA (Beaulieu et al., 1987; Pacak et al., 1993b), whereas chronic immobilization stress decreases both basal and immobilization-induced NA release in the CeA (Pacak et al., 1993b). Beaulieu et al. (1987) also showed that the bilateral lesion of the CeA decreases NA turnover in the PVN and BNST of both unstressed and stressed animals and inhibits ACTH secretion (Beaulieu et al., 1987). The CeA lesion also decreases CRH-LI in the ME (Beaulieu et al., 1989). Moreover, the bilateral lesion of the CeA decreases immune challenge-induced *c-fos* expression in the PVN CRH and oxytocin neurons, in the ventral BNST neurons and in the brainstem A1 and A2 noradrenergic neurons (Xu et al., 1999). These results suggest that the brainstem A1 and A2 noradrenergic neurons, through the noradrenergic terminals of the PVN and the ventral BNST, are involved in the excitatory effect exerted by the CeA on the HPA axis. Thus, stress-induced activation of the brainstem noradrenergic cell groups A1 and A2 stimulates HPA axis activity at the levels of the PVN and BNST and mediates the excitatory effects of the CeA on the HPA axis activity. Moreover, the high circulating glucocorticoid levels induced by stress exert a negative feedback control limiting the duration of the stress-induced activation of brainstem noradrenergic projections to the PVN, BNST, and CeA.

The present results also showed that long-term adrenalectomy increases DA extracellular levels in the lateral BNST. In contrast, it has been shown that adrenalectomy decreases both basal and morphine-induced release of DA in the nucleus accumbens (Piazza et al., 1996), suggesting a differential effect of glucocorticoids within dopaminergic terminals originating from the VTA. In addition, Barrot et al. (2000) showed that these

decreases of basal, morphine-induced, and cocaine-induced release of DA are observed in the shell, but not in the core of nucleus accumbens. Glucocorticoid treatment prevented all of the above effects of adrenalectomy, suggesting that they are glucocorticoid specific (Piazza et al., 1996; Barrot et al., 2000). Moreover, acute immobilization stress decreases dopaminergic activity in the CeA and VTA, in addition to other brain regions studied, such as the ventromedial nucleus of the hypothalamus (Beaulieu et al., 1987). In addition, bilateral lesion of the CeA of unstressed animals decreases BNST dopaminergic turnover (Beaulieu et al., 1987), suggesting a CeA-mediated activation of BNST dopaminergic nerve terminals under basal conditions. It has also been shown that chronic stress decreases DA extracellular levels in the nucleus accumbens shell and medial prefrontal cortex but not in the striatum (Scheggi et al., 2002), suggesting a heterogeneous influence of glucocorticoids within central dopaminergic projections. Present evidence suggests that glucocorticoids affect neither TH mRNA expression in the substantia nigra (SN) or VTA (Smith et al., 1991) nor TH activity in the nucleus accumbens and striatum (Lindley et al., 1999). There is evidence only of an inhibitory effect of glucocorticoids on the basal DA metabolism, without changing DA turnover (Lindley et al., 1999). Thus, these results suggest that glucocorticoids may control dopaminergic neurons activity and DA extracellular levels indirectly through the activation of other glucocorticoid-sensitive nondopaminergic neurons (Piazza et al., 1996). The differences observed here between dlBNST and nucleus accumbens in response to adrenalectomy further support the idea that glucocorticoids control extracellular levels of DA through nondopaminergic mechanisms in the different dopaminergic terminal regions. The available evidence suggests that stress inhibits HPA axis activity in part decreasing the dopaminergic activity in the CeA. In addition, the CEA mediates the activation of dopaminergic projections to the BNST, further supporting the hypothesis that glucocorticoids control dopaminergic neuronal activity and DA extracellular levels through the activation of nondopaminergic neurons.

Anatomical evidence has shown that noradrenergic and dopaminergic terminals make synaptic contacts with dendrites of CRH-containing neurons in PVN, vlBNST, and dlBNST, respectively (Liposits and Paull, 1989; Phelix et al., 1994). The available evidence regarding the role of the ascending brainstem noradrenergic pathway to the PVN on CRH expression suggests that lesion of this pathway decreases CRH-LI, without affecting CRH mRNA expression (Swanson and Simmons, 1989), indicating that brainstem noradrenergic inputs to the PVN influence the CRH peptide levels. Moreover, microinjections of NA into PVN increase first the ACTH plasma levels, which returned to the basal level after 90 min, and later the expression of PVN CRH mRNA, suggesting that the increase of NA levels in the PVN stimulates both CRH release and CRH synthesis (Itoi et al., 1994, 1999). It has been shown that acute immobilization stress induces both NA and CRH release in the CeA (Pacak et al., 1993a; Hand et al., 2002). Moreover, acute immobilization stress induces CRH mRNA expression and *c-fos* expression in neurons of the lateral aspects of the CeA; however, *c-fos*-expressing neurons do not correspond to CRH-synthesizing neurons (Kalin et al., 1994; Palkovits, 2000), In addition, adrenalectomy decreases CRH gene expression, and high doses of corticosterone increase CRH mRNA expression in the CeA (Makino et al., 1994a; Palkovits et al., 1998). Thus, the evidence does not support a direct role of NA in CRH expression in the CeA. In addition, it has been shown that stress-induced activation of the ascending brainstem noradrenergic pathway mediated by the CeA increases the release of NA in the BNST (Beaulieu et al., 1987; Pacak et al., 1995; Cecchi et al., 2002a). Moreover, adrenalectomy decreases CRH gene expression in the dlBNST (present study), and high doses of corticosterone and psychosocial stress increase CRH mRNA expression in the dlBNST (Makino et al., 1994a, 1999). However, CRH expression in the vlBNST is not affected by adrenalectomy or psychosocial stress but is increased by high doses of corticosterone (Makino et al., 1994a, 1999). Thus, the available evidence does not support a direct role of NA in CRH expression in the BNST. This evidence does not exclude a possible interaction between NA and CRH released during stress response in both the CeA and the BNST (for review see Forray and Gysling, 2004). This possibility is further supported by the fact that both NA and CRH in the CeA and BNST participate in fear and anxiety responses to stress and have been involved in stress-induced psychiatric disorders (Lee and Davis, 1997; Schulkin et al., 1998; Cecchi et al., 2002b). With regard to the role of the dopaminergic pathway to the CeA and BNST in CRH expression, it has been shown that a 6-hydroxydopamine lesion in the medial forebrain bundle, in the presence of desipramine to avoid lesioning noradrenergic fibers, decreases CRH mRNA expression in the dlBNST and CeA (Day et al., 2002). The present results showing that adrenalectomy increases DA extracellular levels and decreases CRH expression in the lateral BNST, together with the evidence that adrenalectomy also decreases CRH expression in the CeA (Palkovits et al., 1998), do not support an stimulatory effect of DA on CRH expression. Further studies are needed to address this apparent contradiction.

CONCLUSIONS

The present studies indicate that, in the lateral BNST, CRH gene expression is negatively regulated, whereas NA and DA extracellular levels are positively regulated, in the absence of glucocorticoids induced by long-term adrenalectomy, suggesting that, under basal conditions, glucocorticoids exert a positive regulation on CRH expression and a negative regulation on NA and DA extracellular levels in the BNST. Glucocorticoids regulate CRH gene expression in the dlBNST and PVN

in opposite ways. Adrenalectomy decreases CRH-LI expression in the CeA. Thus, the present study adds novel evidence further supporting the idea that the CeA and lateral BNST form part of an adrenal steroid-sensitive extrahypothalamic circuit. This extrahypothalamic circuit has been involved in fear and anxiety responses and in clinical syndromes such as melancholic depression, posttraumatic stress disorders, and addiction. All these syndromes have been associated with changes in CRH expression, noradrenergic activity, and glucocorticoid plasma levels (Lee and Davis, 1997; Schulkin et al., 1998; Young et al., 2002; Walker et al., 2003). Of special interest is the participation of CRH and NA in BNST functioning and its relevance in the therapeutic approach for these brain disorders.

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